The ESC Textbook of Vascular Biology

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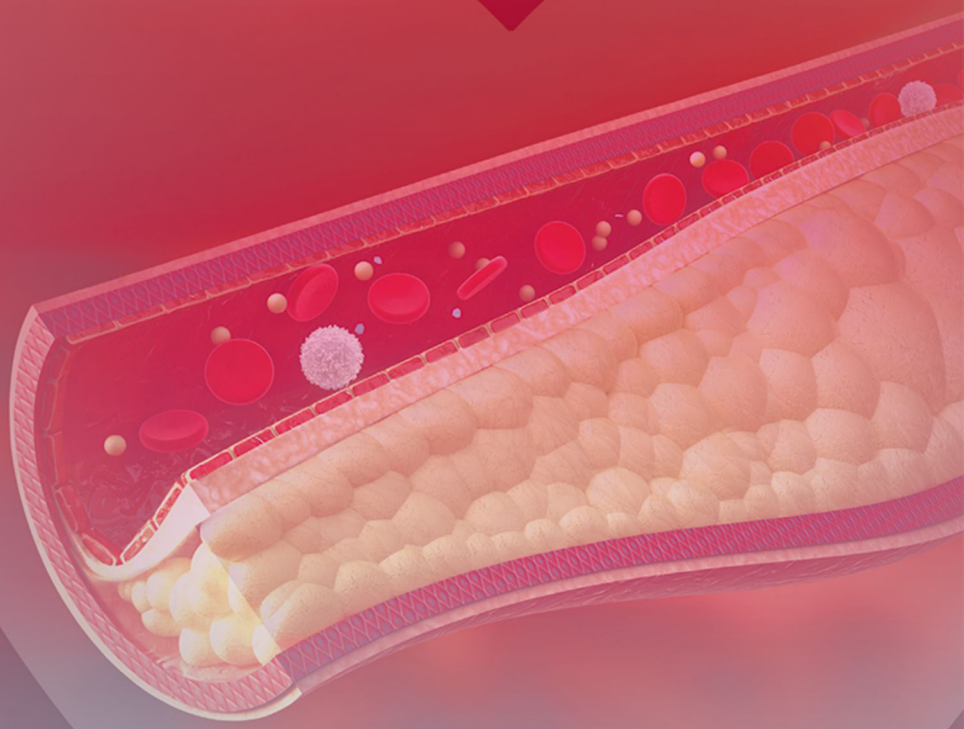

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Foreword

The legacy and prospects of vascular biology

The discovery of circulation

Blood vessels have been known for centuries, but only William Harvey put them in the right order. Indeed, in his seminal work *Exercitatio anatomica de motu cordis et sanguinis in animalibus* published in 1628 (1), he described the motion of the heart and blood in a completely novel manner. For the first time he proposed, and provided supporting data for, the circulatory nature of the blood in the human body. He distinguished arteries and veins based on their function and structure. He had no proof yet of their connective structures—the microcirculation—but he demonstrated that blood injected into arteries shows up in the corresponding veins. Also, he further demonstrated that the blood circulated under pulsatile pressure and that the amount of blood was finite. It took more than 100 years until blood pressure and its changes in systole and diastole was directly measured (2): Stephen Hales performed this crucial experiment in 1733 in a conscious horse using a glass cannula inserted into the femoral artery—an experiment that would not pass any review board today, but made history.

However, how the circulation might be regulated remained unclear for many centuries after Harvey's work, but over time the sympathetic nervous system, the adrenal glands and the role of the kidneys, the renin angiotensin system and, eventually, natriuretic peptides were discovered. Today we do have a reasonable understanding of cardiovascular regulation and the role of the vasculature in this context, although unknown mediators are continuously being discovered.

Atherosclerosis

The role of blood vessels in disease processes was unknown for centuries. The first description of abnormal blood vessels in a patient with coronary disease was provided by Edward Jenner, who later introduced pox vaccination: on 16 October 1793, the then well-known surgeon John Hunter succumbed to a sudden death during an angina attack triggered by a dispute over a controversial issue in the board of St. Georges Hospital. Edward Jenner immediately performed an autopsy on his colleague and concluded '*I found no material disease of the heart, except that the coronary artery appeared thickened*' (3). He was not aware that he had thereby first described coronary atherosclerosis in a patient with a fatal myocardial infarction, a term later used by Rudolf Virchow (1821–1902), the leading pathologist of the 19th century, who said 'Atherosclerosis is a chronic inflammation induced by cholesterol' (4). It took more than a century to prove this bold hypothesis. At first a seminal experiment by Nikolay Nikolaevich Anichkov substantiated the cholesterol hypothesis. Anitschkov (who won the Stalin and not the Nobel Prize, since he worked in Russia during the Soviet era) proved that atherosclerotic plaques can be induced in the rabbit aorta by a high fat diet (5)—one of the first contributions to vascular biology!

Translation of the cholesterol hypothesis

It is the vision of vascular biology, a term that only evolved during recent decades, to stimulate translational research from bench to bedside (Fig. P.1)—obviously this road was at times very bumpy, but eventually opened new avenues for patient care (6). In a sense, this is what the *Framingham Heart Study* did (initiated by the then National Heart Institute in the United States). Indeed, the *Framingham Heart Study* confirmed Anichkov's observations of rabbits in humans,

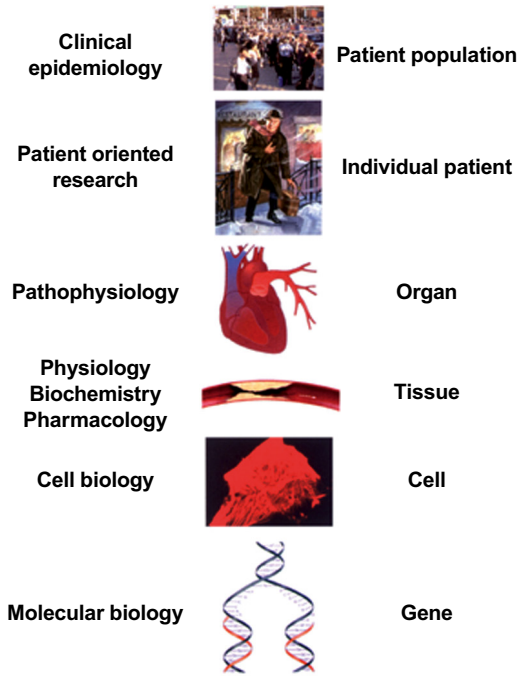

Fig. P.1 The translational nature of vascular biology.

and established that cholesterol, together with blood pressure and diabetes, as the prime cardiovascular risk factors accounting tor myocardial infarction, stroke and premature death (7).

As is typical for modern science, this in turn stimulated vascular biologists to elucidate the mechanisms involved in atherosclerosis. While Michael S. Brown and Joseph L. Goldstein characterized the regulation of lipid metabolisms and LDL-receptors and recieved the Nobel Prize for their discoveries in 1985 (8). Others, such as Russel Ross, described the role of growth factors in atherosclerosis (9) and Paul M. Vanhoutte and his fellows (10) delineated the role of the endothelium in cardiovascular disease. The discovery of inflammatory cells in atherosclerotic plaques by Göran Hansson (11) and Peter Libby (12), as well as that of inflammatory markers in patients with coronary disease, revived Rudolf Virchow's hypothesis and stimulated vascular biology as a research field immensely. Daniel Steinberg provided an important link by showing that particularly oxidized LDL-cholesterol was the culprit as an antigen and initiator of inflammation (13)—as predicted by Virchow a century ago.

The blood vessel on fire

C-reactive protein (CRP), currently widely used as a readout of inflammation, was already discovered in 1930 by William Tillett and Thomas Francis at Rockefeller University (14). Oswald Avery and Maclyn McCarty described CRP as an 'acute-phase reactant' that was increased in the serum of patients suffering from a spectrum of inflammatory stimuli. In 1943 Gunnar Löfström, from the State Bacteriologic Laboratory in Stockholm, for the first time suggested that CRP might be linked to atherothrombosis—a visionary thought that attracted little attention of his colleagues. In the mid-1950s, Irving Kroop and others reported that CRP concentrations are indeed increased after a myocardial infarction. In the mid-1980s, John Volanakis, Mark Pepys, Irving Kushner, identified CRP as a hepatically-derived, nonglycosylated, circulating pentraxin composed of 5 identical subunits arranged with pentameric symmetry.

Despite these early observations, interest in CRP did not re-emerge until the 1980s when Frederick de Beer, Brad Berk, and Wayne Alexander described increased CRP concentrations among patients with coronary artery disease. Attilio Maseri and coworkers then found increased levels of CRP in patients with unstable angina and linked its concentrations to clinical outcome (15). The breakthrough came in 1997 with the publication of a prospective evaluation of CRP in the *Physicians Health Study* in which baseline CRP concentrations were higher among those who subsequently went on to have myocardial infarction or stroke than among those who did not (16). The Jupiter Trial, focusing on the effects of rosuvastatin in patients with elevated CRP, further suggested that anti-inflammatory effects of statins might contribute to the vascular protective effects of the drugs (17).

Inflammasome and interleukins

Science moved again in both directions: from bench to bedside and back again. At first, these clinical data stimulated basic research: soon the role of inflammasomes and that of the interleukin-1β and interleukin-6 pathway were characterized in mouse models and later also in patients with coronary artery disease and acute coronary syndromes (18,19). However, proof is still lacking that these pathways—similar to experimental models—is also causally related to coronary artery disease and acute coronary syndromes and their clinical course in patients. To that end the CANTOS trial is currently testing the protective effects of the interleikin-1β antagonist canakinumab in patients with a past acute coronary syndromes (20). Similarly, the CIRS trial (21) is evaluating the effects of low-dose methotrexate in patients with coronary artery disease. Here again the translation of knowledge from basic to clinical science led to crucial discoveries and hopefully soon to novel therapeutic strategies.

The renin angiotensin system

In parallel with these discoveries, the regulation of blood pressure and its impact on the vasculature has been characterized. Here, the seminal experiment has been performed by Robert Tigerstedt (22) in 1898 when he injected renal extracts in the intact rabbit and observed a marked increase in blood pressure. He called the proposed mediator 'renin'. When Eduardo Braun Menendez discovered angiotensin II in 1939 (23) and, a few years earlier, Harry Goldblatt had demonstrated that a clamp to a renal artery would produce hypertension in dogs (24), an important blood pressure regulatory system was being characterized. Sir John Vane, Nobel Prize Laureate in 1982, showed in the late 1960s that angiotensin I was activated in the pulmonary circulation into angiotensin II by the proposed angiotensin converting enzyme on the surface of endothelail cells that was later biochemically and structurally characterized (25). Miguel Ondetti, Bernard Rubin, and David Cushman, working at Squibb laboratories, eventually discovered captopril in 1977, the first ACE-inhibitor in its class (26), and John Laragh and his team in New York confirmed its clinical use as a blood pressure remedy (27).

Soon it became clear that the renin angiotensin system was not only a circulating endocrine regulator but, as proposed by Victor Dzau (28), a paracrine system within the vessel wall contributing to oxidative stress via NADPH oxidase and to endothelial dysfunction and structural vascular changes typical of hypertension and atherosclerosis alike. The clinical importance of these experimental findings was later confirmed in the HOPE trial with ramipril and thereafter in several following trials with other ACE-inhibitors (29).

The heart as an endocrine organ

In the 1980s, Alfonso de Bold in Canada performed—as had Robert Tigerstedt a century earlier—a simple experiment when he injected homogenized atrial tissue in an intact animal and produced natriuresis (30). The discovery of natriuretic peptides as the natural antagonists of the renin angiotensin system further advanced our understanding of cardiovascular control. Indeed, these peptides are released in atrial and myocardial tissue in response to physical stimuli and have important effects in the vasculature and the kidney as they induce vasodilation, inhibit the renin angiotensin system and cause natriuresis. Importantly, these discoveries were translated to the clinical level where natriuretic peptides, in particular brain natriuretic peptides, became useful biomarkers. Finally, with the introduction of angiotensin receptor antagonists/neprelysin inhibitors, or ARNIs, modulation of plasma levels of natriuretic peptides became an important therapeutic strategy in heart failure (31) and, possibly, will soon be the case in hypertension as well.

The sympathetic nervous system

The vasculature is not only regulated by circulating hormones and local factors derived from the endothelium and vascular smooth muscle cells, but it is also innervated by sympathetic and other fibres that, importantly, regulate vascular tone and structure. Of note, particularly for short-term changes in posture and adaptations of the circulation to increased demand (i.e. during exercise), the sympathetic nervous system is of utmost importance.

While paravertebral ganglia have already been noted in ancient times by Galen and his followers, their function as a relay station of nerve traffic within the body was only discovered in the 20th century. Notably, the sympathetic nervous system is closely connected with local and circulating regulatory systems: for instance angiotensin II and epinephrine enhance synaptic neurotransmission, while acetylcholine reduces it. Finally, the primary neurotransmitter noradrenaline itself limits its own release via activation of presynaptic α2-receptor. Importantly, sympathetic fibers innervate the kidney vasculature and regulate renal blood flow and, via β-receptors, enhance renin secretion in juxtaglomerular cells. Thus, all these regulatory systems are tightly interconnected to allow optimal regulation of the cardiovascular system under resting conditions and during exercise.

Genetics

Gregor Mendel (1822–84) was a monk living in the Austro-Hungarian Empire in the 19th century and until he discovered the fundamental laws of inheritance, genetics did not exist. Neglected by his contemporaries, his seminal experiments became only known at the beginning of the 20th century. Later deoxyribonucleic acid, or DNA, was recognized as the carrier of genetic information, and its helical structure was described by James Watson and Francis Crick in 1953 (32).

Soon these discoveries were applied to biological research and, recently, increasingly so in vascular biology. Although most forms of cardiovascular disease are polygenetic in nature with a strong environmental influence, genetics in particular helped in animal research to delineate mechanisms of disease using transgenic and knockout models to study hypertension (33), its impact on blood vessels (34), as well as to study atherosclerosis (35). As it turned out, with the exception of monogenetic diseases such as

cardiomyopathies or channelopathies, the contribution of genetics in atherosclerosis and its clinical sequelae such as myocardial infarction and stroke is complex and strongly modulated by environmental factors (36,37). However, Mendelian randomization studies have helped to delineate genes involved in cardiovascular conditions (38). A major success story is the discovery of mutations in the PCSK9 gene that led to the characterization of this protein in the regulation of LDL-receptors and, in turn, atherosclerosis and eventually to the development of PCSK9 inhibitors (39).

Also, we have learnt that gene expression is highly regulated by transcription factors binding to the promoter region of distinct genes. These in turn are activated by specific signal transduction pathways linked to surface receptors. Recently, non-coding RNAs have been discovered that profoundly modulate gene expression (40). A vast number of microRNAs with an array of effects under physiological conditions and in disease states have been described and indeed specific signatures of them might become useful biomarkers at the clinical level (41) and possibly even as therapeutic tools or targets.

Vascular biology—a success story

Thus, over recent decades vascular biology has contributed immensely to the understanding of cardiovascular function in health and disease. Notably, research went in both directions: from bench to bedside and from the bedside to the bench (Fig. P.1). Indeed, vascular biologists have stimulated clinical scientists to perform studies and trials in patients and results of clinical studies have stimulated research at the bench side.

The publication of the current *ESC Textbook of Vascular Biology* (edited by Robert Krams and Magnus Bäck) is timely, since it comes at a moment at which vascular biology as a science has fulfilled its promise. Indeed, it has shown that it can provide insights into the molecular mechanisms of vascular disease and that such findings can be translated to the clinical level to the benefit of cardiovascular patients. The editors and the authors should be congratulated for such an excellent textbook which, I am sure, will stimulate the next generation of vascular biologists and established investigators alike. And indeed, this is truly needed as many secrets of vascular biology wait to be discovered.

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Preface

A few years ago, the Working Group on Atherosclerosis and Vascular Biology decided that a good European project would be the coordination of a new European Society of Cardiology supported vascular biology textbook. This idea coincided with the high expectation of a unified Europe and the knowledge that an ESC-supported vascular textbook was missing. Furthermore, the vascular biology and atherosclerosis research community in Europe is active and thriving, and can be considered world-leading. As a consequence, all ingredients for an excellent European vascular biology text book were present and the working group decided to initiate this large, but important project. This preface describes in larger detail the philosophy behind this book.

In 2012, when the ESC turned their interest towards basic science, the working group Vascular Biology and Atherosclerosis of the European Society of Cardiology started discussions on publishing joint papers as road maps for trainees and young scientists. During those discussions the idea of writing a textbook was suggested by the editors and the entire working group realized the power of the idea: a first ESC-supported vascular biology text book was coherent to the ideals of a unified Europe and within the realm of the ESC. It was initially discussed in detail whether it was a textbook for undergraduates in the universities or for postgraduates, pre-clinic and clinic. We decided to supply the information for all interested, including undergraduates and postgraduates, as this would appeal more to the potential authors. To organize the textbook we decided for two major editors, and multiple section editors. The carefully selected section editors are experts of the section they coordinate and as such evaluated the quality of each individual chapter in their section. Their work has, therefore, been of tremendous value to the quality of this book. The two editors subsequently read all chapters to provide a second step of QC, and finally the publisher supplied professional support to homogenize the book. Through all these QC steps we think we have created an excellent textbook.

We were able to get the support of the top European vascular biologists. As a consequence, this book offers a compendium of topics written by the best scientists in Europe on topics very relevant for the field. Due to their skills, the chapters are not only a source for young scientists and students, but also offer interesting reading for experts in the field. Some of the invited authors laudably chose to introduce young scientists as co-authors but guaranteed their support and their hard work, and their knowledge is why this book has reached such a high quality.

The dissemination of the authors' vast knowledge and expertise in a single volume will make *The ESC Textbook of Vascular Biology* a useful companion for undergraduates in medicine and biology, but also for young scientists and early career staff. It has also been our aim to provide a comprehensive reference work for cardiologists and other clinical specialties dealing with vascular diseases and vascular imaging. Indeed, we emphasize the importance of vascular biology for the understanding of both physiological and pathophysiological processes in the vascular wall, and for accomplishing future endeavours in medical research.

It has been a pleasure for us working with such an excellent team of section editors, whose hard work should be especially acknowledged. We are also grateful for the support we received for this book project from the European Society of Cardiology and, in particular, the Working Group on Atherosclerosis and Vascular Biology. Finally, this book would not have been possible without the commitment and hard work of the contributing authors, who we thank from the bottom of our hearts.

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Abbreviations

AAV	antibody-associated vasculitis
ACE	angiotensin-converting enzyme
AMPK	5'AMP-activated protein kinase
ANP	atrial natriuretic peptide
BBB	blood–brain barrier
BMP4	bone morphogenetic protein 4
C	compliance
ChIP	chromatin immunoprecipitation
COUP-TFII	Chicken ovalbumin upstream promoter-transcription factor II
COXs	cyclo-oxygenases
ECM	extracellular matrix
EDHFs	endothelium-derived hyperpolarization factors
EETs	epoxyeicosatrienoic acids
eNOS	endothelial NO synthase
FGF-2	fibroblast growth factor 2
FH	familial hypercholesterolaemia
FSI	fluid structure interaction
GAGs	glycosaminoglycans
ICAM-1	intercellular cell-adhesion molecule 1
IDO	indoleamine dioxygenase
IL	interleukins
ILT	intraluminal thrombus
LAM	leukocyte adhesion molecules
LDL	low-density lipoprotein
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
M-CSF	macrophage-colony stimulating factor
MLC	myosin light chain
MLU	medial laminar units
MMPs	matrix metalloproteinases
MRI	magnetic resonance imaging
NAFLD	non-alcoholic fatty liver disease
NFkB	nuclear factor kappa B
NOD	nuclear oligomerization domain
PAMP	pathogen-associated molecular patterns
PCI	percutaneous coronary intervention
PDGF	platelet-derived growth factors
PET	positron emission tomography
PIV	particle image velocimetry
PRRs	pattern recognition receptors
pVAT	perivascular adipose tissue
PVAT	perivascular adipose tissue
RAAS	renin–angiotensin–aldosterone system
ROS	reactive oxygen species
SDF	sidestream-dark field flowmetry
shh	sonic hedgehog
SMA	smooth muscle actin
SMC	smooth muscle cells
SM-MHCs	smooth muscle myosin heavy chains
SVR	systemic vascular resistance
TCFA	thin-cap fibroatheroma
TFPI	tissue factor pathway
TGF-β	transforming growth factor β
TLRs	Toll-like receptors
TNF-α	tumour necrosis factor-α
UTS	ultimate tensile strength
VCAM-1	vascular cell-adhesion molecule 1
VEGF-A	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VLDL	very low-density lipoprotein
VSMC	vascular smooth muscle cell
WSS	wall shear stress

SECTION I

Foundation of the vascular wall

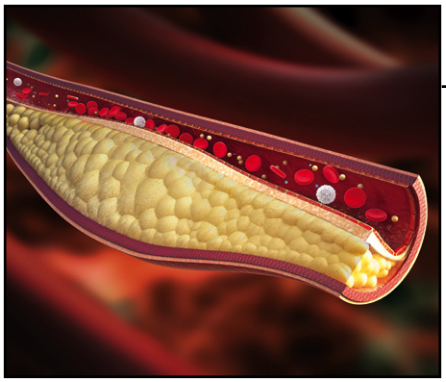
Section introduction

Paul Evans

The survival and function of cells relies on a continuous supply of nutrients, metabolites, and gases, and the expulsion of toxic materials. The transfer of molecules from the environment to the cell is a challenge in metazoans where diffusion provides insufficient molecular transport in multicellular tissues. Because of this, the emergence of multicellular animals was closely paralleled with the evolution of sophisticated vascular systems. The purpose of this section is to introduce the reader to the fundamental properties and functions of the mammalian vascular system, thereby laying foundations that can be developed in later chapters.

In Chapter 1, the architecture of blood vessels (including arteries, veins, and capillaries) is described and the properties of their constituent parts (vascular, endothelial, and smooth muscle cells) are also outlined. This description of vascular form leads on to Chapter 2, which describes the physiological properties of blood vessels. This chapter includes the mechanisms that regulate blood pressure and flow, and the fundamental principles that underlie the exchange of materials between the vasculature and the tissues that it serves. In addition to regulation through physiological systems, blood vessels are also strongly influenced by their physical environment. This topic is introduced in Chapter 3, which summarizes the factors that control mechanical loading of the vessel wall and its effects on vascular biology. This chapter also includes a description of the interaction of flowing blood with the vessel wall and the effects of this on endothelial cell behaviour. One of the endothelial functions that is particularly sensitive to fluid mechanics is the ability to recruit immune cells, a subject that is discussed in Chapter 4. Here, the routes that immune cells follow as they migrate into the vascular wall and the mechanisms that control these processes are described. Moreover, the role of immune cells and inflammation in the initiation and progression of atherosclerosis (a disease of arteries) is discussed. The final chapter in this foundation section focuses on the use of animal models in vascular biology research. It provides an appraisal of several mouse, rat, rabbit, and pig models of atherosclerosis and other vascular diseases, including a description of the strengths and caveats of each model.

Together, this collection of chapters will equip the reader for the subsequent sections of this textbook by providing an extensive overview of the physical properties, anatomy, cell biology, physiology, and immunology of the vessel wall.



CHAPTER 1

Structure and cell biology of the vessel wall

Bibi S. van Thiel, Ingrid van der Pluijm, Roland Kanaar, A.H. Jan Danser, and Jeroen Essers

Content

Introduction

A healthy heart pumps about 6,000–8,000 litres of blood around the body each day. Blood is carried through the body via blood vessels. The blood vessels form a closed system that begins and ends at the heart. In mammals, blood circulates through two separate circuits: the pulmonary circuit and the systemic circuit (➔ Fig. 1.1).

- Pulmonary circuit: the right ventricle of the heart pumps blood into the lungs, where waste gases are exchanged for oxygen, after which the blood is transported back to the left atrium of the heart.
- Systemic circuit: the left ventricle pumps oxygenated blood to all tissues and organs of the body via the aorta, after which deoxygenated blood is transported back to the right atrium of the heart.

➔ Figure 1.1 gives a simplified overview of the blood flow through the body, where deoxygenated blood is depicted in blue and oxygenated blood is depicted in red. Note that somewhat counter-intuitively, deoxygenated blood does not refer to blood without oxygen. Rather, it refers to a lower oxygenation grade than that of oxygenated blood because a certain amount of oxygen has been delivered to tissues. As a result, deoxygenated blood still contains about 75% of oxygen compared to oxygenated blood.

A well-functioning cardiovascular system is essential for all vertebrates. The blood vessels are a conduit for a variety of molecules, such as nutrients, oxygen, and waste products, to and from all parts of the body. Blood vessels have several main functions:

1. Distribution of blood containing nutrients (e.g. glucose and amino acids), oxygen (O_2), water, and hormones to all the tissues and organs of the body.
2. Removal of metabolic waste products and carbon dioxide (CO_2) from the tissues to the excretory organs and the lungs, respectively.
3. Regulation of blood pressure.
4. Maintenance of constant body temperature (thermoregulation).

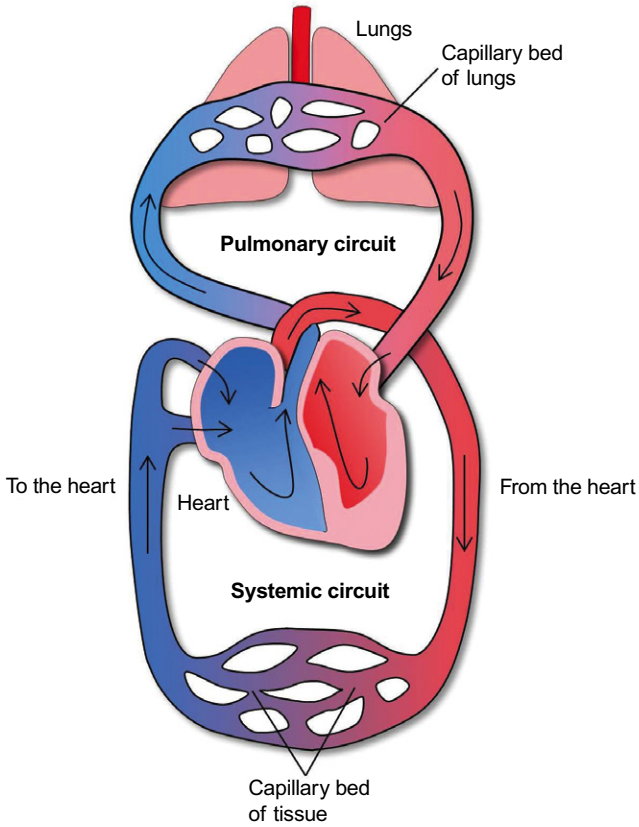


Fig. 1.1 Schematic overview of the cardiovascular circulatory system. Note that arteries and oxygenated blood are depicted in red and veins and deoxygenated blood are depicted in blue.

Distribution of nutrients, gases, and removal of waste products

The primary function of blood vessels is to transport blood around the body, thereby supplying organs with the necessary O_2 and nutrients. At the same time, the vessels remove waste products and CO_2 to be processed or removed from the body.

Regulation of blood pressure

Blood vessels control blood pressure by changing the diameter of the vessel through either constriction (vasoconstriction) or dilation (vasodilation). Variations in blood pressure occur in various parts of the circulation depending on the diameter of the vessel (see ➔ Chapter 3).

Maintenance of constant body temperature (thermoregulation)

Blood vessels help maintain a stable body temperature by controlling the blood flow to the surface area of the skin. To prevent overheating, blood vessels near the surface of the skin can dilate, allowing excessive heat of the blood to be released to the surroundings. In contrast, blood vessels near the skin's surface can constrict, reducing heat loss through the skin when needed under cold circumstances.

Structure of the vessel wall

Blood vessels need to be well-constructed, as they have to withstand the pressure of circulating blood through the body every day. The vessel wall is arranged in three distinct layers, termed tunica: an inner layer (tunica intima), a middle layer (tunica media), and an outer layer (tunica adventitia) (➔ Fig. 1.2). These layers mainly contain endothelial cells, vascular smooth muscle cells, and extracellular matrix, including collagen and elastic fibres.

Tunica intima

The tunica intima ('inner coat') is the innermost layer of a blood vessel. In healthy vessels, it consists of a thin single layer of endothelial cells, which are in direct contact with the blood in the lumen, as well as a subendothelial layer made up mostly by connective tissue. The single layer of endothelial cells, called endothelium, has a smooth surface that minimizes the friction of the blood as it moves through the lumen. The endothelium plays a role in vascular permeability, inflammation, coagulation, and vascular tone, which refers to the maximal degree of contraction by vascular smooth muscle cell relative to its maximally dilated state. The subendothelial layer, also called the basal lamina, provides a physical support base for the endothelial cells and flexibility of the vessel for stretching and recoil. Moreover, it guides cell and molecular movement during tissue repair of the vessel wall. The tunica intima is the thinnest layer of the blood vessel and minimally contributes to the thickness of the vessel wall. In arteries and arterioles, the outer margin of the tunica intima is separated from the surrounding tunica media by the *internal elastic membrane*, a thick layer of elastic fibres. The internal elastic membrane provides structure and elasticity to the vessel and allows diffusion of materials through the tunica intima to the tunica media. Microscopically, the lumen and the tunica intima of an artery appear wavy because of the partial constriction of the vascular smooth muscle cells in the tunica media, the middle layer of the blood vessel, whereas the tunica intima of a vein appears smooth.

Tunica media

The middle layer, tunica media, is considered to be the muscular layer of the blood vessel as it primarily contains circularly arranged smooth muscle fibres together with

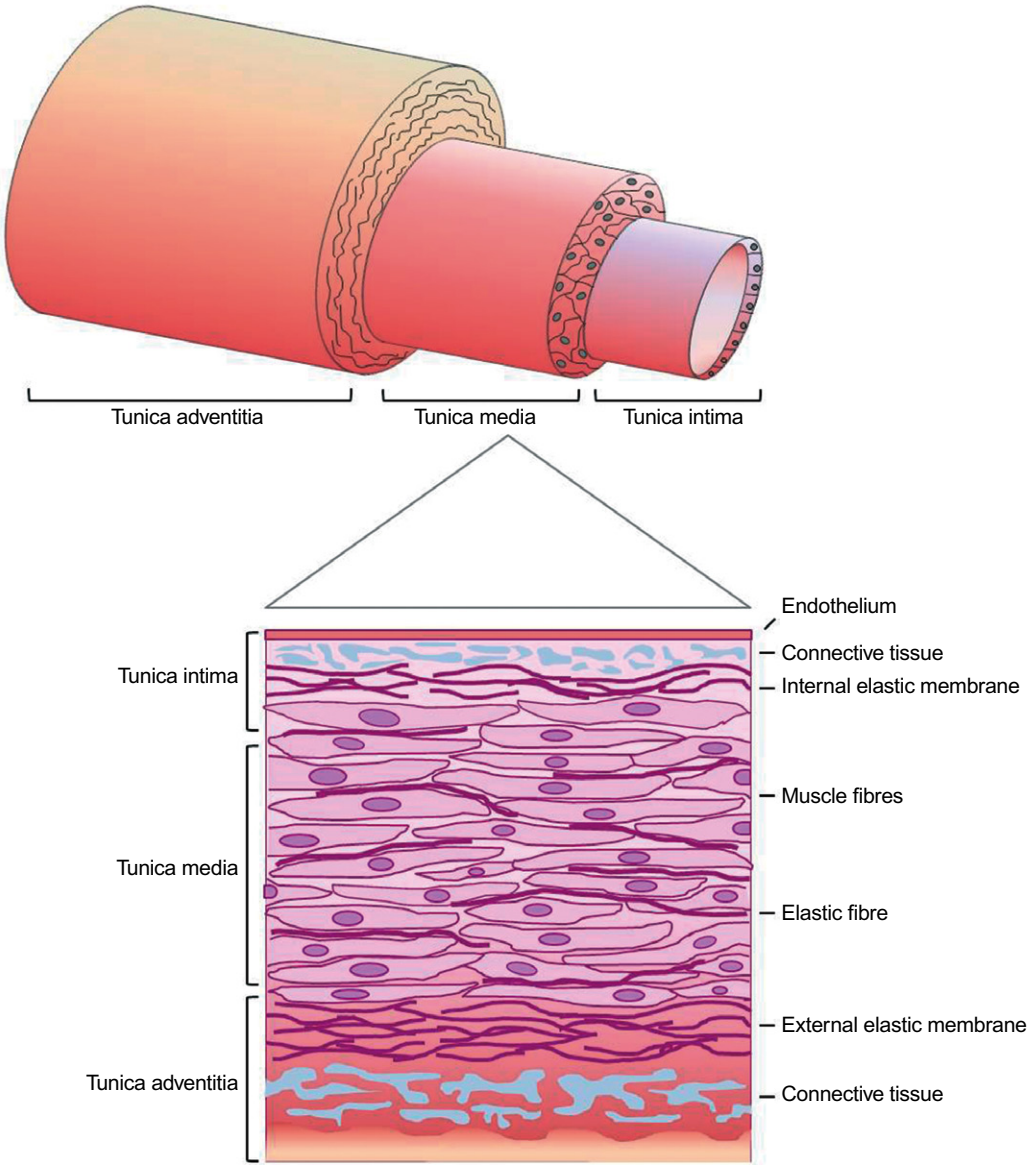


Fig. 1.2 General structure of the vessel wall, showing the tunica intima, tunica media, and tunica adventitia, and a close-up depicting the different structures within these layers.

extracellular matrix, mostly elastin sheets. It is often the thickest layer of the arterial wall and much thicker in arteries than in veins. The tunica media provides structural support as well as vasoreactivity (the ability of blood vessels to contract or to relax in response to stimuli) and elasticity to the blood vessel. The primary role of the vascular smooth muscle cells is to regulate the diameter of the vessel lumen. Concerning blood pressure regulation, the vascular smooth muscle cells in the tunica media can either contract causing vasoconstriction or relax causing vasodilation. During vasoconstriction, the lumen of the vessel narrows, leading to an increase in blood pressure, whereas vasodilation widens the lumen allowing blood pressure to drop. Both vasoconstriction and vasodilation are partially regulated by nerves (nervi vasorum). The tunica media is separated from the tunica adventitia by a dense elastic lamina called the *external elastic membrane*. Under the microscope, these laminae appear as wavy lines. This structure is usually not apparent in small arteries and veins.

Tunica adventitia

The tunica adventitia (also known as tunica externa) is the outermost layer of the vessel wall, surrounding the tunica media. The adventitia is predominantly made up by extracellular matrix (collagen and elastic fibres), nutrient vessels (vasa vasorum), and autonomic nerves (nervi vasorum). Fibroblasts and numerous macrophages are also present in

this layer. The tunica adventitia is often the thickest layer in veins, sometimes even thicker than the tunica media in larger arteries. The tunica adventitia helps to anchor the vessel to the surrounding tissue and provides strength to the vessels as it protects them from overexpansion.

Vasa vasorum

Characteristic of the adventitial layer is the presence of small blood vessels, called the vasa vasorum. The vasa vasorum supplies blood and nourishment to the tunica adventitia and outer parts of the tunica media, as these layers are too thick to be nourished merely by diffusion from blood in the lumen, and removes 'waste' products. Because of the thick and muscular walls of the arteries, the vasa vasorum are more frequent in the wall of arteries than in the wall of veins.

Components of the vascular wall

The vascular wall is composed of many cell types and constituents that influence the diameter and functional control of the vessel wall. Several main cell types include endothelial cells, vascular smooth muscle cells, and immune cells (➔ Fig. 1.3). Interaction between these cell types allows the vessel to adapt to alterations in pressure and various physical stimuli by either dilation or contraction.

Endothelium

Vascular endothelial cells lining the entire circulatory system, from the heart and arteries to the small capillary beds, are in direct contact with blood. They form a single-cell layer (monolayer) called the endothelium, which has been estimated to cover a surface area of more than 1,000 m^2 in humans. The morphological shape of endothelial cells varies across the circulatory system (1). In large arteries, endothelial cells are aligned and elongated in the direction of the blood flow, whereas in regions of disturbed flow, e.g. near bifurcations, endothelial cells are more round and do not align in a specific direction. Varying among the vascular tree, endothelial cells are between 0.2 and 2.0 µm thick and 1 to 20 µm long. They are joined together by tight junctions, which restrict the transportation of large molecules across the endothelium. Endothelial cells are active contributors to a variety of vessel-related activities, including permeability, vascular tone, and haemostasis.

Vascular endothelial cells have several important functions (➔ Box 1.1).

The endothelium has a strategic position in the vessel wall, right between the circulating blood and the vascular smooth muscle cell. From this position, the endothelium plays a vital role in controlling vascular function, as it is able to respond to mechanical and hormonal signals and receive information from cellular constituents of the vessel wall. Endothelial cells are highly dynamic as they need to interpret changes in blood composition and mechanical changes, and respond properly to several stimuli, either physical or chemical, by producing a variety of factors that contribute to the control of vascular tone, vascular inflammation, cellular adhesion, haemostasis, and coagulation. For instance, the endothelium serves as a semi-permeable barrier, restricting and controlling the movement of fluids, molecules, and cells across the

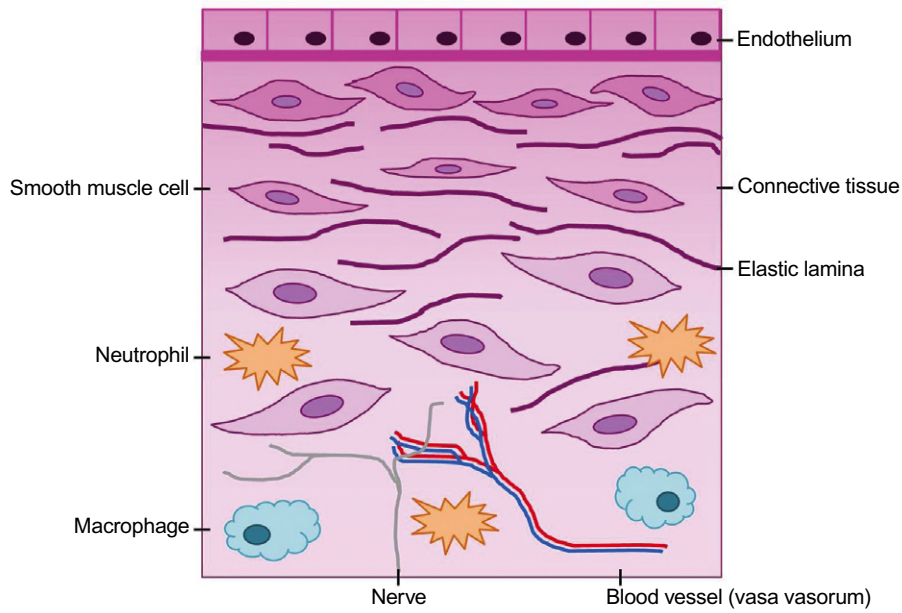


Fig. 1.3 Summary of the major components of the vessel wall.

Box 1.1 Vascular endothelial cell function

1. Providing a semi-permeable barrier between the vessel lumen, containing the blood, and the surrounding tissues. Selective material, electrolytes, macromolecules, fluid, and cells can pass through the barrier entering or leaving the bloodstream.
2. Regulating vascular tone, by secreting vasoactive substances that stimulate the smooth muscle cells of the tunica media to relax or contract, thus widening or narrowing the vessel.
3. Modulating cellular adhesion and inflammation of the vasculature, as endothelial cells regulate lymphocyte and leucocyte adhesion and transendothelial migration, from the bloodstream across the barrier into the vessel wall, by expression of surface adhesion molecules.
4. Modulating haemostasis and coagulation. Under normal conditions, endothelial cells express a wide variety of non-thrombogenic factors that maintain blood fluidity and help prevent inappropriate blood clotting.
5. Involved in the formation of new blood vessels (angiogenesis). Angiogenesis is in part regulated by the endothelial cell, which is important in wound healing

blood-vessel wall. This movement across the endothelial lining can occur via different mechanisms, either through the endothelial cells (transcellular) or by passing the junction between two adjacent endothelial cells (paracellular). The permeability of the barrier can be altered in response to specific stimuli that act on endothelial cells. Also, endothelial cells themselves can secrete different vasoactive substances that influence the activity of the underlying vascular smooth muscle cell, and thereby the contractile state of the vessel. For instance, endothelial cells can secrete nitric oxide, which causes the vascular smooth muscle cells to relax, consequently leading to vasodilation. Moreover, endothelial cells tightly regulate the expression of adhesion molecules on their surface. These adhesion molecules not only modulate cell migration but are also important in response to local injury, as platelets and other inflammatory cells are recruited to the site of damage in need of defence or repair. Furthermore, endothelial cells are required to maintain blood fluidity and prevent thrombus formation. They bind and display tissue factors that have anti-coagulant properties, thereby preventing the initiation of coagulation. More information on endothelial cells can be found in ➔ Chapter 6.

Injury and dysfunction of the endothelium, directly or indirectly, play a vital role in the initiation and development of most human vascular diseases. Endothelial dysfunction not only leads to an imbalance between vasoconstriction and vasodilation, but also causes coagulation disorders and can be involved in the malignant growth of tumours. It is also involved in numerous other physiological and pathological conditions such as hypertension, septic shock, diabetes, and hypercholesterolaemia. Moreover, endothelial dysfunction is seen as the initial step in the atherosclerotic process. More information about atherosclerotic disease can be found in ➔ Chapters 10 to 14.

Vascular smooth muscle cells

Vascular smooth muscle cells are the most prominent cell type of an artery and, depending on the size of the artery, may comprise several layers. Vascular smooth muscle cells are typically 2 to 5 μm in diameter, and vary from 100 to 500 μm in length. Yet, as the vascular smooth muscle cells can either relax or contract, their actual length depends on the physiological conditions and functioning of the cell. The vascular smooth muscle cells exert different functions, which translates into two different phenotypes of the vascular smooth muscle: contractile or synthetic. The contractile smooth muscle cells are long, spindle-shaped cells that contain a single, centrally positioned, elongated nucleus, whereas the synthetic vascular smooth muscle cells are less elongated and have a more cobblestone-type morphology. Each smooth muscle cell is enclosed by a variable amount of extracellular matrix, containing collagen, elastin, and various proteoglycans. Smooth muscle cells are arranged in different orientations, either circumferentially or helically, along the longitudinal axis of the vessel. Smooth muscle cells are connected to each other by tight- and gap-junctions. These junctions permit the transfer of signalling molecules between cells and increase the tensile strength of the medial layer, allowing the control of the diameter of the vessel. Smooth muscle cells are normally quiescent cells that do not divide. However, damage to the vessel wall can drive the smooth muscle cells into a proliferative state in which they will divide and migrate.

In the artery, almost all smooth muscle cells are present in the tunica media. The primary function of smooth muscle cells is to regulate the diameter of the vessel lumen, as it directly controls vessel tone and regulates blood pressure by either contraction or relaxation. In the small arteries (less than 300 μm in diameter) and veins, contraction of the vascular smooth muscle cell is responsible for the regional distribution of the blood flow, as it gives a reduction in lumen diameter and thereby increases vascular resistance, leading to a higher blood pressure. Vasoconstriction in the larger arteries has a different haemodynamic effect and mostly affects the stiffness (compliance) of the blood vessel, increasing the impedance to move blood through the artery.

Regulation of the vascular diameter by activation/deactivation of vascular smooth muscle cells is primarily under control by the autonomic nerves in the adventitial layer that act on specific receptors present on the outside of vascular smooth muscle cell. Yet, other locally produced and blood-borne factors can also act directly on the vascular smooth muscle cell and thus play an important role in its function. Smooth muscle cell contraction can be initiated by electrical, chemical, or mechanical stimuli. Contractility of smooth muscle cells is controlled by actin and myosin filaments of the cytoskeleton, which make up a substantial portion of the cytoplasm of smooth muscle cells. Besides contractility, vascular smooth muscle cells also perform other functions such as migration, proliferation, proinflammatory and secretory responses that become progressively important during vessel remodelling, injury, and disease. For example, the smooth muscle cells produce a variety of extracellular matrix components, including collagen and elastin. Activated vascular smooth muscle cells also secrete matrix metalloproteinases (MMPs), which facilitate extracellular matrix remodelling. The ratio between vascular smooth muscle cells and the amount of extracellular matrix determines the overall mechanical properties and structural integrity of the vessel. The phenotype of the vascular smooth muscle cell can range from contractile to synthetic. These two phenotypes of smooth muscle cells not only differ in morphology but also in the expression levels of different genes, and their proliferative and migratory properties. Contractile smooth muscle cells are most often quiescent, whereas synthetic smooth muscle cells have a high proliferation and migratory rate. The vascular smooth muscle cells can switch between these two phenotypes in response to changes in environmental cues; therefore, these cells are not only important in short-term regulation of the lumen diameter, but also in long-term adaptation of the vessel through structural remodelling.

There is clear evidence that vascular smooth muscle cells are involved in the pathogenesis of several vascular diseases, including atherosclerosis, restenosis, hypertension, asthma, and vascular aneurysms. Upon vascular injury, the smooth muscle cells undergo a phenotypic switch from contractile to synthetic, which most often includes increased proliferation, migration to the site of damage, and increased excretion of extracellular matrix proteins. These characteristics play an important role in vascular repair; however, when occurring in high degrees, it predisposes the cell to acquire characteristics that contribute to the development of vascular diseases. The most acknowledged disease, in which smooth muscle cells play a key role, is atherosclerosis. However, the precise role of smooth muscle cells in atherosclerotic disease progression most likely depends on disease stage, as smooth muscle cells play a disadvantageous role in lesion development and progression, whereas they have a beneficial role in stabilizing the fibrous cap and consequently prevent plaque rupture. A detailed description of smooth muscle cells is found in ➔ Chapter 7.

Pericytes

Pericytes are the contractile cells of the capillaries and venules, whereas vascular smooth muscle cells are the contractile cells of other blood vessels (arteries, arterioles, and veins). The size and morphology of pericytes greatly depends on location and type of vessel, and may be irregular in a single vessel. Generally, they have an elongated shape with a prominent round nucleus, and are surrounded by basal lamina material that is continuous with the basement membrane of the tunica intima. These pericytes are wrapped around the endothelial cells on the luminal side of the basement membrane. Pericytes have been associated with regulating capillary blood flow and stabilization of microvessels.

Cytoskeleton proteins

Cytoskeletal proteins are structural elements surrounding the cell membrane and are important to maintain cellular shape and integrity. These proteins play an active role in the interaction between blood vessels and the surrounding environment. Key cytoskeleton proteins include actin filaments, microtubules, and intermediate filaments. Actin filaments are important in cell–cell control and cell–matrix interactions, as they can bind to plasma membrane proteins. Actin filaments surrounding the endothelial cell are, therefore, involved in fluid and molecule exchange between the tissue and the circulating blood, by tightly regulating the vascular barrier. Furthermore, actin filaments are involved in cell motility, particularly in the contraction of the vessel wall, through association with the motor protein myosin. As such, when vascular smooth muscle cells become activated, the cytoskeleton proteins actin and myosin rapidly reorganize, creating membrane-bound, parallel-organized units termed 'stress fibres'. In this complex, myosin slides along the actin filaments, which produces an increased intracellular tension leading to contraction of the cell. The microtubules not only support the cellular structure, but also are involved in cell division, as they facilitate the formation of spindles during mitosis. Intermediate filaments appear to have a structural role in maintaining cellular integrity but might also provide an anchoring for contractile proteins.

Vascular extracellular matrix

The extracellular matrix is a highly organized network of proteins containing collagen and elastin fibres and a loose network of proteoglycans. Foremost, the extracellular matrix provides structural support and elasticity to the vessel wall, keeping cells in place and allowing adaptation of the vessel wall to high blood pressure. Endothelial cells of the tunica intima, vascular smooth muscle cells of the tunica media, and fibroblasts of the tunica adventitia, all produce extracellular matrix proteins that have a different function in each tunica. The extracellular matrix proteins of the tunica intima make up the subendothelial basement membrane, which provides flexibility of vessels for stretching and recoil, whereas the extracellular matrix of the tunica media is responsible for strength and stretch of the vessel wall, as well as for transmission of muscle contraction. The tunica adventitia is made up principally by extracellular matrix, and contains a limited number of cells. This layer adds further strength to the vessel wall. Additionally, the extracellular matrix provides specific informational cues to vascular cells, thereby regulating cellular adhesion, proliferation, differentiation, and migration.

Collagen

Physical properties of the blood vessel wall largely depend on collagen fibres. These collagen fibres provide a supporting framework that anchors smooth muscle cells in place. When internal pressures are high, the collagen network becomes rigid, limiting elasticity of the vessel wall. Collagen types I, III, and IV are present in the adventitia, tunica media, and basement membranes, respectively. Veins tend to have higher collagen content than arteries. Once blood vessels start to lose their collagen, tiny ruptures can occur in the vessel wall.

Elastin

Elastin provides vessels with the ability to stretch and recoil in response to haemodynamic forces resulting from alterations in blood pressure. Many elastin molecules are cross-linked and connected to each other and other molecules, including microfibrils, fibulins, and collagen, to form an elastic fibre. These elastic fibres allow the vessels to expand during the contractile phase of the heart and then recoil during the filling phase of the heart, keeping the blood flowing forward. These elastic fibres are mainly found in the tunica media of arteries, where smooth muscle cells and collagen fibres are present between these elastic layers. Depletion of elastin is often due to destruction rather than reduced production.

Integrity of the extracellular matrix is essential to maintain both the physical and biological properties of the vessel, as changes in the extracellular matrix affect the local environment that vascular cells are embedded in. As a result, cellular adhesion, proliferation, migration, differentiation, and gene expression of several vascular cells will be affected. Moreover, disruption and/or deletion of these extracellular matrix proteins have deleterious effects on the structure and function of vessels, as it contributes to weakening of the vessel wall. Maintaining a proper balance between the different matrix components depends on new synthesis of matrix proteins and on matrix degradation by enzymes, such as MMPs. An imbalance of extracellular matrix proteins in favour of matrix degradation is, for instance, seen in vascular diseases like aneurysms, where it leads to vessel wall rupture, and atherosclerosis, where it is involved in plaque destabilization.

Infiltrating immune cells

Endothelial cells and vascular smooth muscle cells are able to produce a variety of immune and inflammatory mediators, such as tumour necrosis factor-α (TNF-α), interleukins (IL), and platelet-derived growth factors (PDGF). These factors stimulate migration of immune cells and inflammatory cells from the blood into the tissue. As a result, different immune cells can be found in the vessel wall, including macrophages and lymphocytes. For instance, activated endothelial cells can express adhesion molecules that allow mononuclear leucocytes, such as monocytes and T cells, to attach to the endothelium and penetrate into the tunica intima. Penetration of these cells into the tunica media by endothelial cells is seen as one of the early steps in the development of atherosclerotic lesions and is described in detail in ➔ Chapter 4.

Types of blood vessels

Blood vessels are found throughout the body and can be categorized by function and by composition of the wall. There are five general types of blood vessels: arteries, arterioles, capillaries, venules, and veins (➔ Fig. 1.4). As these types of blood vessels have to withstand different degrees of blood pressure, the composition of the wall varies among these types (see ➔ Table 1.1).

Arteries

Arteries carry highly pressurized, oxygen-rich blood away from the heart to other organs of the body. Because arteries experience high blood pressure and pulsatile flow as blood is ejected from the heart, they must be strong, elastic, and

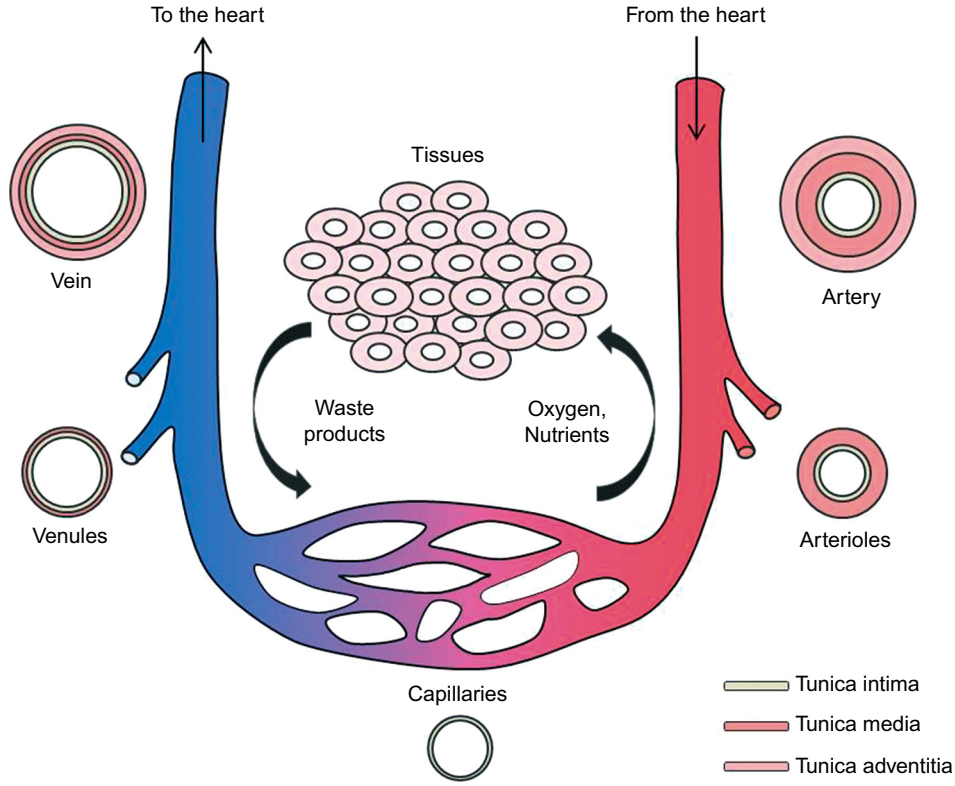


Fig. 1.4 Schematic drawing showing major structural characteristics of the different types of blood vessels.

flexible and, therefore, have a much thicker wall than veins. Arteries consist of three layers: tunica intima, tunica media, and tunica adventitia. The tunica media of an artery is very thick and contains more smooth muscle cells and elastic fibres than that of veins, allowing the arteries to be more contractile and elastic, respectively. The large arteries of the body contain a lot of elastic laminae that allow the artery to stretch and accommodate to high blood pressure. The arteries branch repeatedly into smaller and smaller vessels, eventually becoming arterioles. According to size and function, arteries can be divided into two groups:

1. *Elastic arteries* are found close to the heart and receive blood directly from the heart. These arteries are called elastic because the tunica media is dominated by elastic laminae that give the vessel wall great elasticity, helping the artery to stretch in response to high pulsatile blood pressures during each heartbeat. The aorta, pulmonary trunk, and the larger arteries that originate from them, are

Table 1.1 Summary of the characteristics of different blood vessels

Type of vessel	Actions	Structure vessel wall	Structure fits function
Artery	Carries blood away from the heart to the arterioles at high pressure	Three-layer thick wall (endothelial lining, middle smooth muscle, and elastic tissue layer, outer connective tissue layer; strong, elastic and flexible; narrow lumen)	Strong, elastic walls and narrow lumen help to maintain high blood pressure
Arteriole	Helps control blood flow from arteries to capillaries	Similar three layers as arteries but thinner; very narrow lumen	Vessel wall helps control blood flow by constricting or dilating
Capillary	Supply tissue with nutrients and gases and removal of waste products	Single, thin layer of endothelium	Thin wall brings blood into close contact with tissue; allowing diffusion
Venule	Connects capillaries to veins	Thinner wall than arterioles, less smooth muscle and elastic tissue; extremely porous wall	Porous wall makes it easy for fluids and blood cells to pass through
Vein	Carries relatively low-pressure blood from venules to the heart	Similar layers as arteries but thicker; thin middle layer but thicker outer layer; contains valves; wide lumen	Thin wall and wide lumen allow housing of a large volume of blood and offers less resistance to blood flow

types of elastic arteries. Their tunica intima is thicker than that of muscular arteries and is surrounded by an internal elastic lamina, which is less well-defined because of the abundance of elastic laminae. The tunica media of elastic arteries is much thicker compared to other arteries. It is primarily made up of multiple elastic laminae alternating with thin layers of smooth muscle cells and collagen fibres, which together form a lamellar unit. The external elastic lamina is difficult to distinguish from other elastic lamellar units in the tunica media. The tunica adventitia appears thinner than the tunica media and contains vasa vasorum, as the walls of these arteries are too thick to receive enough oxygen and nutrients from blood flow in the vessel lumen. The vasa vasorum supplies both the tunica media and the tunica adventitia with oxygen.

2. *Muscular arteries* are medium-sized arteries, which distribute the blood to various tissues and organs. These types of arteries include the femoral, brachial, and coronary arteries. The diameter of the muscular artery lumen is on average 0.1 to 10 μm. The tunica intima of muscular arteries is thinner than those of elastic arteries. The tunica media consists mostly of multiple layers of smooth muscle cells and less of elastic laminae. The elastic laminae are confined to two circumscribed rings: the internal elastic laminae and the external elastic laminae. The thickness and appearance of the tunica adventitia is variable. The greater amount of smooth muscle cells combined with less elastic laminae results in less elasticity but a better ability to constrict and dilate.

Arterioles

Arterioles are the smallest arteries of the body and have the same three layers as the larger arteries. The critical endothelial lining of the tunica intima is intact, and it still rests on the internal elastic laminae, which is not always well-defined in histological sections. The tunica media generally consists of less than six layers of smooth muscle cells and there is no external elastic lamina. The tunica adventitia is about the same size as the tunica media. The arteriole lumen is around 10–100 μm in diameter. As arterioles have a small diameter, they generate a great resistance to blood flow and are critically involved in slowing down blood flow. Smooth muscle cells of the tunica media form concentric rings that control distribution of blood flow by either contracting or dilating lumen size. Normally, smooth muscle cells are slightly contracted, causing the arterioles to maintain a consistent vascular tone.

Capillaries

Blood moves from the arterioles into the capillaries, which are tiny, narrow, thin-walled vessels that connect arteries with veins. Capillaries are the smallest of all blood vessels, about 5–8 μm in diameter, and blood pressure further drops as it encounters extra resistance flowing through the capillaries. Their walls consist of a single layer of endothelial cells and an underlying basement membrane, often accompanied by pericytes. The basement membrane keeps cells in place and is largely made up of proteins. Capillaries have no tunica media or tunica adventitia. The diameter of a capillary is just wide enough to allow single red blood cells (erythrocytes) to pass through. The thin wall of the capillaries facilitates its primary function: exchange of oxygen, nutrients, and other substances, between blood and the underlying tissue. Most capillaries are organized into a network called a capillary bed. Based on the morphology of their endothelial layer, capillaries can be classified into three different types.

1. *Continuous capillaries* consist of an uninterrupted, continuous lining of endothelial cells, which are joined by tight non-permeable junctions, and a complete basement membrane. The continuous capillaries have a low permeability to molecules; they only allow small molecules, like water and ions, to diffuse through the tight junctions, which have gaps of unjointed membrane called intercellular clefts. They are commonly found in skin, muscles, lung, and central nervous tissue.

2. *Fenestrated capillaries* have leakier intracellular junctions and perforations in the endothelial cell body, called fenestrae or pores. The fenestrae are present at both the luminal and basal surface of the cell, and the endothelial cells are surrounded by a continuous basement membrane. Fenestrated capillaries are much more permeable compared to continuous capillaries, and allow larger molecules and a limited amount of proteins to bypass the endothelial cells. The extent of fenestrae may depend on the physiological state of the surrounding tissue, as their numbers may depend on the need to absorb or secrete. They are found in tissues that participate in fluid exchange, including endocrine glands, intestinal villi, and kidney glomeruli.

3. *Discontinuous capillaries*, also called sinusoids, are the largest of all capillaries and have larger, open spaces in the endothelium, containing a lot more intracellular clefts. They are very permeable (leaky) and allow large molecules, including red and white blood cells and various serum proteins, to pass through the intracellular spaces of the endothelium. Discontinuous capillaries contain a basement membrane that is often incomplete. These discontinuous capillaries are found in areas where the exchange of substances is advantageous, i.e. in the liver, haematopoietic organs (spleen and bone marrow), and some endocrine organs.

Venules

Blood flows from the capillary beds into very small veins called venules (10–200 μm in diameter). Venules allow deoxygenated blood to return from the capillary beds to the larger blood vessels called veins. Venules consist of a tunica intima, a thin tunica media, and a tunica adventitia. The vessel wall of venules is thinner than arterioles and is extremely porous, making it easy for fluids and blood cells to pass through their walls. Venules can be further subclassified into muscular (50–200 μm) and post-capillary venules (10–50 μm). The post-capillary venule starts where two capillaries from the capillary bed come together. It is a non-muscular vessel, as the tunica media consists of an incomplete layer of pericytes and scattered smooth muscle cells. Instead, the post-capillary venule has a thin, very permeable endothelial layer, making it the preferred site of white blood cell (leucocyte) adhesion and transmigration. Hence, the response of the vasculature to inflammation is generally localized in post-capillary venules. During inflammatory responses, vasoactive substances act on the endothelium, which results in extravasation of fluid and the migration of leucocytes into the tissue. Inflammation in post-capillary venules will be covered in more detail in ➔ Chapter 4. Post-capillary venules join together, forming larger muscular venules. In the muscular venules, the pericytes are replaced by one or two layers of smooth muscle cells.

Veins

Multiple venules unite to form veins. Veins carry deoxygenated blood back from tissues and organs towards the heart. One difference between veins and arteries is the direction of blood flow. As in arteries, veins have three layers. However, veins have much thinner walls than arteries, as they are distant from the heart and subsequently experience less pressure from the blood flow. The tunica intima and tunica adventitia are similar in structure to arteries, though the tunica media is much thinner. The tunica intima consists of the endothelial lining with its basement membrane, and surrounding internal elastic laminae. The tunica media of veins is thin and only contains a few smooth muscle cells and elastic laminae, whereas the tunica adventitia is much thicker, containing collagen and occasionally some smooth muscle cells and elastic fibres. In general, veins are larger in diameter (varying from 1 to 15 μm) and contain a wide lumen, which, together with the thin walls, allow the accommodation of a large blood volume. Most of the blood volume of the body, around 60%, is contained within veins at any time. Because of these thin walls and a small medial layer, veins do not have the same elasticity and vasoconstriction capacities as arteries. Blood is displaced through veins by contraction of the surrounding muscles and pressure gradients that are created during inhalation and exhalation. Compared to arteries, veins are frequently irregular of shape and softer. Some veins also contain valves, particularly veins in the legs, which prevent backflow of blood as it travels back to the heart. Veins can be classified into four main types as shown in ➔ Box 1.2.

Box 1.2 Classification of veins

1. *Pulmonary veins* carry oxygenated blood from the lungs to the left atrium of the heart.
2. *Systemic veins* return deoxygenated blood from the rest of the body to the right atrium of the heart.
3. *Superficial veins* are located close to the surface of the skin and are not located near a corresponding artery.
4. *Deep veins* are located within muscle tissue and typically near a corresponding artery.

Lymphatic vessels: secondary drainage system

The general structure of lymphatic vessels is based on the three tunica described. Lymphatic vessels are lined by endothelial cells and have a thin layer of smooth muscle cells followed by the adventitia. They are part of the lymphatic system that transports fluid away from tissues and plays an important role in the body's defence system. The fluid that lymphatic vessels carry is not blood, but is clear fluid, called lymph, that comes from blood plasma that exits blood vessels at the site of the capillaries. Further details can be found in ➔ Chapter 9.

Ageing and the vascular wall

The prevalence of cardiovascular disease increases progressively with age. To understand why ageing is closely linked to cardiovascular disease, it is essential to know what happens to our vessels during normal ageing. Ageing is a natural biological process that begins as soon as adulthood is reached and it causes diverse detrimental changes in cells and tissues. A series of structural, architectural, and compositional modifications take place in the vasculature during ageing (➔ Fig. 1.5). As such, with increasing age, blood vessels lose their flexibility and structural integrity, which diminishes the ability of blood vessels to expand and contract efficiently. In addition, age-related vascular alterations lead to loss of adequate tissue perfusion (ischaemia), insufficient vascular

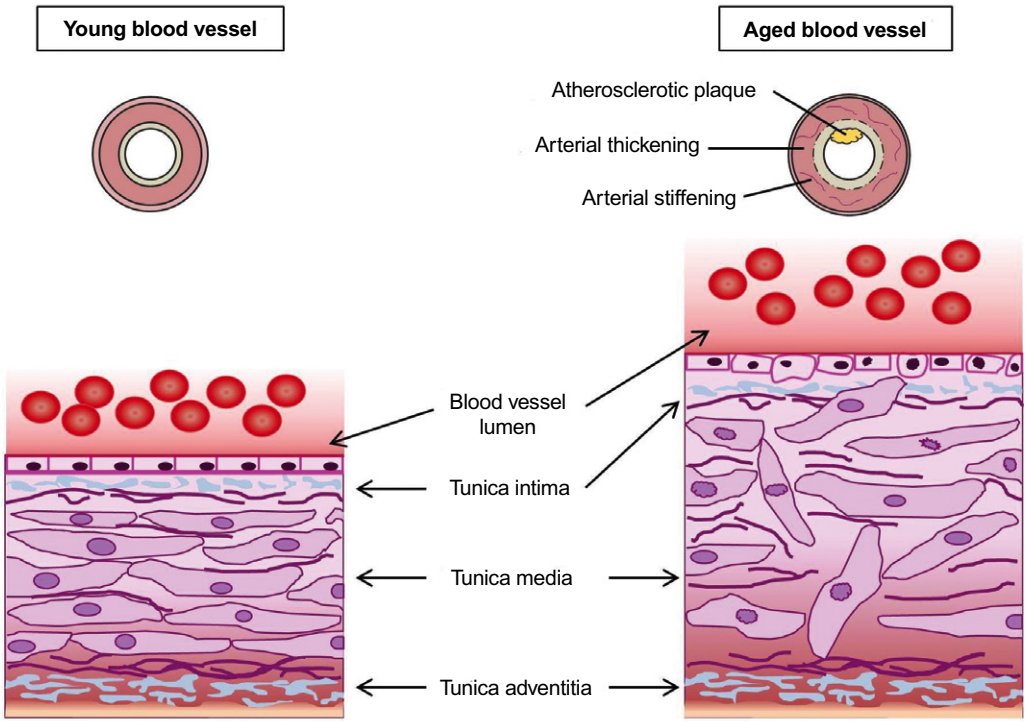


Fig. 1.5 Schematic view of the structural changes of the vessel wall during ageing.

growth, or excessive remodelling. Eventually these changes affect cardiovascular performance and set the stage for the onset of several cardiovascular diseases, including hypertension (high blood pressure), atherosclerosis, stroke, and aneurysm formation.

Age-related structural changes

At a microscopic level, principal age-related structural changes of the vasculature include an increase in vessel lumen size, and thickening and stiffening of the intimal and medial layers. Important structural changes causally related to vessel wall thickening and stiffening include vascular smooth muscle enlargement and relocation to the subendothelial space, increased extracellular matrix accumulation (particularly rich in glycosaminoglycans), and increased deposition of lipids and calcium salts. While the content of collagen increases, elastin fibres become disorganized, thinner, and fragmented.

As an individual grows older, the endothelial barrier of the tunica intima becomes damaged and some of its specialized functions are blunted. The endothelial barrier becomes porous (leaky), the self-renewal process weakens, and endothelial signalling is modified. For example, with increasing age endothelial cells produce substances that signal blood cells to adhere to the endothelial layer of the tunica media instead of smoothly flowing through the blood vessel lumen. Additionally, endothelial cells transmit signals to the underlying vascular smooth muscle cells in the tunica media that prompt these cells to change. These changes result in vascular smooth muscle cells translocating and moving towards the site of injury, where they reposition in the tunica intima just beneath the endothelial layer. At this site, the vascular smooth muscle cells multiply and produce matrix proteins, which eventually results in thickening of the tunica intima. Moreover, with age, some of the vascular smooth muscles of the tunica media die, increasing the workload of the remaining vascular smooth muscle cells and causing them to grow larger. Some changes cause vascular smooth muscle cells to switch from a contractile state to a state in which they produce excessive amounts of collagen proteins and other matrix substances, thereby creating an imbalance between the elastin and collagen content of the tunica media. The ratio of collagen to elastin increases in favour of collagen, which is 100 to 1,000 times stiffer than elastin, resulting in a stiffer wall and a less compliant blood vessel. Moreover, vessel wall stiffening has been associated with the formation of cross-links between glucose and collagen. Growing levels of cross-links reduce elasticity of the vessel wall, as these cross-links glue together important proteins of the extracellular matrix, thereby degrading its primary structure and preventing it from functioning correctly. Ageing also affects elastin, as it becomes overloaded with calcium, stretches out, and eventually becomes fragmentized and disorganized.

Related diseases

Due to structural changes in the vessel wall with age, many of the arteries are less able to withstand the forces of pulsating blood. High systolic blood pressure, which is commonly observed in the elderly, may exacerbate this problem. As a result, the vessel wall becomes weakened and more prone to develop several vascular diseases, including aneurysms. Moreover, ageing increases the incidence and severity of atherosclerosis, as the age-related changes of the vessel wall make it easier for fatty substances to accumulate inside of the blood vessel. Several studies suggest that exercise, good nutrition, and therapeutic interventions can slow down the ageing process occurring within blood vessels. For example, in the elderly who regularly exercise, arterial stiffening is less pronounced.

Summary

The vessel wall consists of three different layers termed tunica intima, tunica media, and tunica adventitia. The main components of these layers include endothelial cells, vascular smooth muscle cells, cytoskeleton proteins, and extracellular matrix proteins. Interaction between these components of the different layers determines the biological and physical properties of the blood vessel. Changes and damage to these components and cellular constituents contribute to the pathogenesis and progression of several vascular diseases, as well as to ageing of the vasculature. The subsequent chapters in this book will cover, in greater detail, related diseases of the vasculature.

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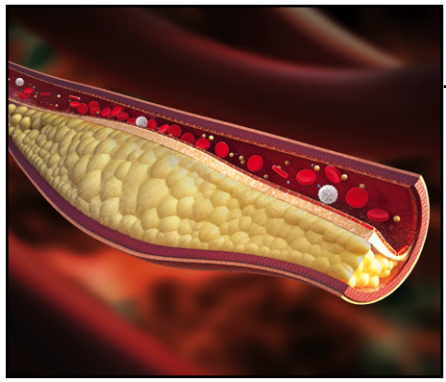

CHAPTER 2

Physiology of blood vessels

Victor W.M. van Hinsbergh

Content

Introduction

The complexity of a multicellular organ and organism can only be achieved and maintained if oxygen and nutrients are continuously available. To that end, a delicate network of blood vessels distributes and delivers these factors throughout the body. The blood that fills this delicate network bears nutrients for metabolism, oxygen carried by red blood cells, leukocytes, platelets, and many proteins with physiological, haemostatic, and immunological functions. The driving force for bringing the blood into the tissues is heart contraction. Circulating blood passes the heart twice via separated compartments. In this way the circulation consists of two loops: the systemic circulation and the pulmonary circulation. While the pulmonary circulation primarily is involved in oxygen uptake and carbon dioxide delivery, the systemic circulation distributes the blood to various tissues. The parallel orientation of individual vascular networks of the systemic circulation enables a regulated distribution to each individual tissue. Subsequently, delivery of oxygen, nutrients, and hormones, and removal of small waste products occur via the tiny capillaries. Altogether, the (cardio)vascular system has two main functions: distribution and exchange. This chapter focuses on general aspects of the physiology of blood vessels. After a short survey of the types of vessels present in the circulation, it will discuss how pressure and resistance determine the volume of blood that is locally delivered per time unit and, subsequently, how paracrine, neuronal, and humoral factors affect smooth muscle contraction that underlies blood pressure and volume distribution. Subsequently, it will discuss the exchange of solutes and macromolecules over the endothelium and its surface layer, the glycocalyx. As a constant volume is a prerequisite for adequate functioning of the circulatory system, mechanisms to keep that volume constant are briefly indicated.

Anatomy and physiology of blood vessels

The blood vessels conduct blood from the heart to the tissues and back, thus achieving continuous supply of oxygen and nutrients, removal of waste products, and—when needed—delivery of leukocytes to the organs. In one overall circulation cycle the heart is passed two times in order to pump the blood through the other tissues and lungs. Starting in the left ventricle of the heart, the blood flows into the systemic circulation through the aorta, and then into one of the

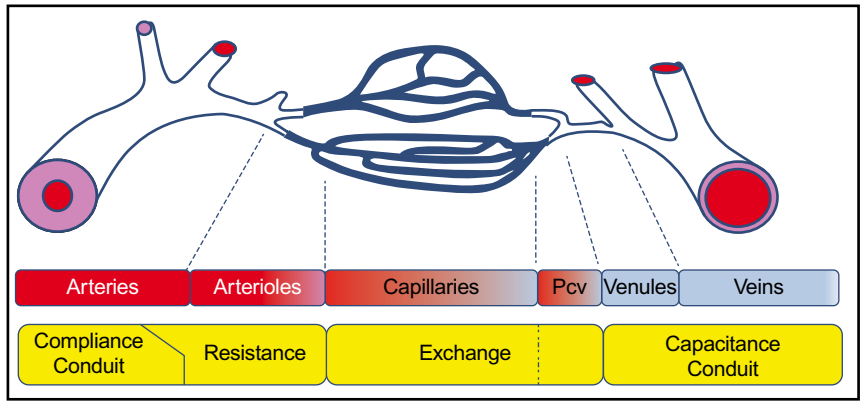

Fig. 2.1 Schematic representation of the vessel types along the systemic part of the circulation. General functions are indicated in the yellow bar. Pcv: post-capillary venules.

large conduit arteries that branch off from the aorta. These large conduit arteries subsequently subdivide into smaller conduit arteries, resistance arteries, and the microcirculation. In the microcirculation the blood vessels divide further into arterioles and finally into capillaries, the smallest blood vessels, which are pivotal for exchange of solutes. The capillaries then merge into venules, which further join into veins, flowing towards the right side of the heart (➔ Fig. 2.1). From the right side of the heart, blood flows into the pulmonary artery to enter the pulmonary circulation, which divides and merges likewise to the systemic circulation and brings back the blood via the pulmonary vein to the left side of the heart.

All large blood vessels are composed of three layers: tunica intima, tunica media, and tunica adventitia. The intima of healthy arteries is mainly a layer of continuous endothelial cells, which prevents intravascular clotting of the blood, regulates fluid and solute transport from the blood to the tissues and back, as well as local leukocyte recruitment and vascular tone. The media consists of multiple layers of smooth cells embedded in collagen and alternated by layers of elastin. The adventitia is a loose, fibrous tissue that contains vasa vasorum that nourish the outer cells of large arteries, as well as fibroblasts, and can harbour leukocytes, mast cells, and mesenchymal stem cells. At its outside it continues diffusely into a layer of perivascular fat tissue (PVAT) providing vasoregulatory adipokines to the arteries and arterioles. In atherosclerotic arteries the intima is thickened by accumulation of lipoproteins and lipid-laden cells underneath the endothelium (see ➔ Chapter 10).

The aorta and large arteries conduct the blood to the tissues and are characterized by a thick layer of smooth muscle cells in their media, which maintains a rather constant blood pressure. The compliance (ability to distend on increased pressure) of arteries not only helps to dampen the blood pulse generated by each heartbeat, but also—by recoil and thus reducing aortic volume—limits the decrease of the arterial hydrostatic pressure during diastole. Thus, it favours the continuation of flow into the distal tissue. Reflecting this property, aorta and large arteries are also indicated as compliance vessels.

Arteries branch into smaller arteries and arterioles, in which the thickness of the smooth muscle layers steadily decreases. These small arteries and arterioles are called resistance vessels, as they are the major site of resistance to blood flow. The inner diameters of the resistance vessels, in particular that of the precapillary arterioles, determine the perfusion of the connected capillary bed. The collective inner diameter of all resistance vessels together is a main determinant of blood pressure, together with cardiac output. Resistance vessels regulate tissue perfusion depending on the local demand, e.g. preferential perfusion through the muscle during exercise, while after a meal the splanchnic bed becomes strongly perfused. Neuronal factors, hormones, and tissue-derived paracrine factors modulate the perfusion of a tissue in order to meet its metabolic demand.

In capillaries, the exposure of blood to the endothelial surface increases by several orders of magnitude as compared to arteries and veins. Therefore, delivery of oxygen and nutrients, as well as removal of waste products, occur in the capillary bed. Capillaries are lined by endothelial cells only, but they harbour pericytes within their basement membrane. The post-capillary venules, which connect the capillaries with the larger venules, consist only of endothelium and have specific features. They are the first to respond to vasoactive agents and noxious stimuli by allowing temporarily protein leakage between the endothelial cells and facilitate the first recruitment of phagocytes after injury or infection. The endothelium of capillaries displays tissue heterogeneity. In addition to specific functions, the endothelial lining of capillaries can be continuous, fenestrated with diaphragms, or—as in liver and adrenals—discontinuous.

In this chapter we focus on capillaries with a continuous endothelium and refer for other endothelia to Aird (1).

The walls of veins are considerably thinner than those of arteries, with a distinctively thinner media. Limb veins contain valves, which facilitate blood delivery to the heart. Vein valves counteract the gravidity forces acting on the blood column in the low-pressurized veins by their ability to close the vessel when the blood tends to flow backward. In this way they suit the main vein function: namely, facilitating the return of the blood from tissues to the heart. Veins are easily distended and contain the larger portion of the blood of the circulatory system; therefore, they are also called capacitance vessels.

A final group of vessels is the lymphatic system (see ➔ Chapter 9). The lymphatics collect the excess of interstitial fluid from the tissues and drain it back into the circulation via the collecting duct that ends in the vena cava. The tiny endings of the lymphatic vessels in the interstitium are open, so that excess interstitial fluid can enter it. The small lymphatic microvessels join bigger ones, until they are all collected in the lymphatic duct. The propulsion of lymphatic fluid towards the vena cava is facilitated by valves in the lymphatics and by a lymphatic tone wave, which both propel lymph into the right direction.

Flow and blood volume distribution

Generation of systolic and diastolic blood pressures

The dual pumping action of the heart generates the force that propels the blood through the circulation. The compression force exerted by the heart muscle on the blood causes ejection of the blood into the arterial tree and an increase in the local blood pressure. Return of the blood during the subsequent relaxation of the heart is prevented by closure of the aortic valve. The blood pressure is highest in the aorta arch at maximal heart contraction (systolic pressure) and decreases in the aorta arch when the blood spreads out along the vascular tree reaching the diastolic pressure just before the next heartbeat. The difference between systolic and diastolic pressures is called the pulse pressure.

Because the vascular resistance of large arteries is small, the systolic and diastolic pressures decrease only slightly during passage of these vessels. This enables the physician to determine systolic and diastolic blood pressures in the upper arm on a routine basis using an inflatable cuff wrapped around the arm and attached to a manometer and a stethoscope placed at the brachial artery (the auscultatory method) or a modern variant. Continuous telemetric recording of systolic and diastolic blood pressures is also possible. In mice this can be done by placing a catheter in the jugular artery coupled to an implanted signalling device. A tail-cuff method is also used frequently, but this method is more sensitive to disturbances and the unwanted effects of anaesthetics.

Mean arterial pressure

In contrast to the only slight decrease of blood pressure along the large arteries, a substantial blood pressure drop is found during passage of the blood through small arteries and arterioles. This decrease is largely caused by energy-consuming friction that is generated when the blood drags along the vessel wall. It results in much lower blood pressure values in capillaries and veins. Simultaneously, the difference between systolic and diastolic pressures gradually disappears leading to a loss of pulse pressure in capillary and distal vessels. In line with that, not the systolic or diastolic arterial blood pressures, but the mean arterial pressure (MAP) is most indicative for physiological perfusion studies. As the diastolic period lasts longer than the systolic one, the mean arterial pressure is not the arithmetic mean of the systolic and diastolic pressures, but the geometric mean of both, which lies a bit closer to the diastolic value. The MAP can be estimated from the diastolic pressure + 1/3 pulse pressure—MAP equals closely to:

$$P_{dias} + 1/3(P_{sys} - P_{dias}).$$

The MAP can be used as one of the parameters to calculate the systemic vascular resistance:

$$(SVR) \text{ as } SVR = (MAP - CVP)/\text{Cardiac output},$$

in which CVP is the central venous pressure (vein pressure at the entrance of the right atrium), a relatively low value (for pragmatic reasons often considered negligible) as compared to MAP.

Pulse pressure

In addition to raising the blood pressure, the heart beat also generates a pulse wave that can be monitored as 'pulse' at various sites to determine the heart rate. The pulse is determined by the pulse pressure and moves over the arterial tree at its own speed, independent and much faster than that of the flow. Its shape and speed change along the arterial tree for several reasons. First, the pulse is not only determined by the heartbeat, but also by the compliance of the vessels, in particular the thoracic aorta. Furthermore, vibrations caused by closure of the aortic valve quickly dampen and are only detectable in the first part of the aorta, while reflections caused by arterial branching differentially affect the

final shape of the pulse pressure profile in various parts of the arterial tree.

Compliance

The compliance (C) of a blood vessel segment is the ability to host volume at a change of pressure and is defined as relationship between volume and pressure:

$$C = \Delta V/\Delta P.$$

The compliance of an artery is largely determined by the content and elastic properties of the elastin fibres deposited in the arterial media, while toned smooth muscle cells and collagen fibre networks, which are filled with water-accumulating proteoglycans, have stiffer and less compliant properties. The compliance of the arterial wall is highest in the highly elastic wall of the thoracic aorta and becomes less when the distance to the heart increases and the arteries gradually alter into a stiffer phenotype. The stiffer the vessels are, the higher the propagation speed of the pulse will be. As a consequence, the pulse wave travels two times faster in large arteries and even four times faster in small arteries than in the aorta. In stiffer vessels the start of the pulse becomes steeper because these less compliant vessels absorb less of the sudden increase in pressure. The compliance of the aorta is also of eminent importance in guaranteeing a steady flow through the vascular bed, in particular the tissue capillaries. Because of the elasticity of the compliant vessels, the aortic distension absorbs volume when the heart contracts, while the return of the aorta to its original diameter also delivers the absorbed volume to the circulation, so that sufficient pressure remains during diastole to guarantee continued flow. Furthermore, it dampens together with the vascular resistance the pulsatile character of the flow contributing to a continuous laminar flow through the capillaries and nourishment of the tissues. With advanced age, changes in the original composition of the vessel wall, including accumulation of calcium, can markedly reduce the compliance of large arteries. This can affect the functioning of the microcirculation. Vascular stiffness can be evaluated by determining the pulse velocity over the arterial tree. Its alteration is a clinically relevant parameter.

Pressure, flow, and vascular resistance

The flow of fluid runs from high to low hydrostatic pressure. In the vascular system, hydrostatic pressure is generated by pump action and gravitational forces. In addition, the shape of a vessel (diameter, surface) and fluid viscosity also contribute to the effective flow of blood. The hydrostatic pressure generated by the pumping heart falls along the vascular tree mainly by friction between blood and the vessels. The pressure drop along a vessel segment (ΔP), which represents the effective perfusion pressure for that segment, is given by the equation:

$$\Delta P = \text{Flow} \times \text{Resistance}.$$

Note that this equation is the hydrodynamic equivalent of Ohm's law for electrical circuits:

$$\Delta\text{Voltage} = \text{Current} \times \text{Resistance}.$$

The flow (volume/time) in a capillary determines the volume of blood that is available per time unit for exchange with the tissues. The resistance to flow depends on blood viscosity (η), length of the vessel (L) and, in particular, the radius (r) reflecting the diameter of the vessel. As the resistance is inversely related to the fourth power of the radius, while vessel length and blood viscosity are linearly related, the diameter of the vessel is a major contributor to vascular resistance to flow, e.g. every two-fold decrease in radius causes a 16-fold increase in resistance. This relationship is summarized as:

$$F = (\Delta P.\ r^4)/(\eta.L)$$

and is known as the Poiseuille–Hagen equation. Hence, the large conduit arteries provide little resistance to flow, by which only a small decrease in effective perfusion pressure (ΔP) occurs, while the smallest arteries and tiny arterioles (resistance vessels) contribute together to considerable (50–70%) reduction. Changes in diameter caused by contraction of their smooth muscle cells largely contribute to vascular resistance of individual tissues and overall blood pressure regulation.

Vascular resistance in parallel and serial positioned vessels

The hydrodynamic version of Ohm's law provides insight into the relative contribution of various parts of the vasculature to the overall vascular resistance. One aspect is that the parallel organization of the vascular beds of tissues helps to reduce the overall vascular resistance. Modulation of individual resistances by dilation or contraction of their resistance vessels is the prime mechanism to increase or decrease blood flow relative to other organs. In contrast, the serial organization of resistances of arteries, arterioles, and capillaries causes the resistances to accumulate. Given an overall vascular resistance, one can calculate the relative resistance that each type of vessel contributes. With decreasing diameter of the vessel, the resistance increases dramatically. As a result, the contribution of large arteries to vascular resistance is minimal, and even if up to 50% of

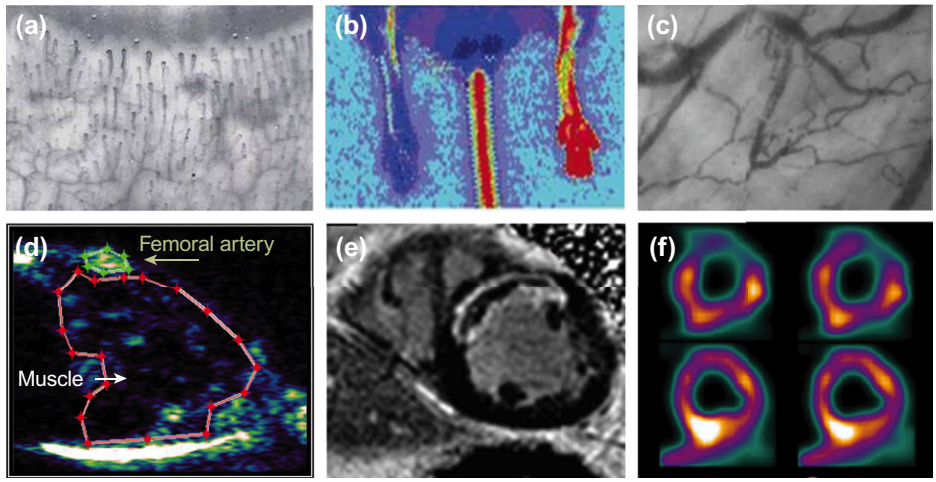

Fig. 2.2 Imaging of blood flow. Blood flow can be measured by various imaging techniques. They vary from nailfold video-capillary microscopy (a), which is used to estimate capillary blood flow in the nailfold of humans, to sophisticated techniques such as $^{15}O\text{-}H_20$-positron emission tomography (PET) (f), combined with CT-images, or Gadolinium-enhanced MRI measurements (e), which show the influx and accumulation of $^{15}O\text{-}H_20$ and Gadolinium, respectively, in tissues *in vivo*. Laser Doppler flowmetry (b) is most frequently used locally on a large conduit vessel to measure flow-mediated vasodilation. It is also used to evaluate the overall perfusion pattern of mouse hindleg to evaluate the perfusion recovery collateralization. The overall evaluation method, however, is sensitive to the penetration of the laser in the tissue and skin and muscle perfusion require different intensity settings. By contrast enhanced ultrasound (d), one can measure the influx and accumulation of microbubbles in the microvasculature after an acoustic destruction of existing microbubbles at the site of study, e.g. muscle. As the assay can be repeated, the effect of specific agents or hormones can be directly determined. Finally, in addition to intravital microscopy, sidestream-dark field flowmetry (SDF) (c), allows the visualization of the microcirculation and movement of red blood cells and leukocytes based on concentrically placed light-emitting diodes. It is relatively inexpensive and can also be used at the bedside. The applications of the latter technique are growing and vary from non-invasive studies on the tongue microcirculation to applications during surgery.
(Courtesy of Drs E. Serné, N. van Royen, C. Boer, E. Eringa, and P. Knaapen.)

the vessel is occluded, the relative contribution of an artery to vascular resistance usually remains percentual. On the other hand, one may expect that the vascular resistance in the capillaries is the highest. However, as each arteriole feeds a number of parallel capillaries, and possibly by involvement of precapillary shunts, the contribution of the arterioles to the overall vascular resistance appears to be more prominent. However, it should be noted that resistance measurements in the capillary bed have been limited, that no information is available of the contribution of the glycocalyx in arterioles and capillaries, and that contraction of pericytes will prevent the movement of red blood cells, by which the capillary becomes considered as non-perfused rather than fully resistant to flow.

Viscosity and laminar vs. turbulent flow

The Pouseuille–Hagen equation shows that viscosity is a determinant in blood flow. As blood displays a high viscosity in part by red cell interaction, increases in haematocrit may favour resistance and reduce blood flow. However, it appears that the contribution of haematocrit to viscosity markedly decreases in arterioles and other microvascular vessels, probably due to altered interaction between the red blood cells. Nevertheless, increased interactions of red blood cells can affect vascular resistance and perfusion in pathological conditions.

Usually the blood streams in a laminar flow pattern and the given laws are based on the presence of laminar flow (➔ Fig. 2.2). As soon as the blood becomes turbulent, the internal friction increases, resulting in a steeper drop in pressure over a given vessel segment (see also ➔ Chapter 12). In addition, the turbulent flow causes another activation pattern of endothelial cells other than laminar flow, often enhancing expression of inflammation-favouring genes. In contrast, arterial laminar flow generates anti-inflammatory effects on the endothelium and enhances the production of the important vasodilator nitric oxide (NO), both by gene induction and cell activation. NO also limits, together with prostacyclin, the activation of blood platelets.

Control of blood pressure and flow

The smooth muscle cells in the arteries and arterioles have a physiological tonus, which is largely controlled by α-adrenergic stimulation by norepinephrine released by sympathetic nerves, circulating vasoactive agents, and vasodilating factors released by the endothelium. This tone gives the artery a specific diameter. The vessel diameter can be reduced or enlarged by smooth muscle contraction or relaxation respectively, which, according to the Poiseuille–Hagen

equation, causes a significant decrease or increase of flow, in particular in the arterioles. Prolonged changes in blood pressure can induce remodelling of smooth muscle cells and structural adaptation of the vessel diameter.

Smooth muscle contraction and relaxation

Smooth muscle cell contraction is achieved through interaction of myosin and actin fibres aligned in parallel within the cell. This occurs after stimulation of the influx of calcium ions into the cytoplasm and subsequent activation of the enzyme myosin light chain (MLC) kinase (➔ Fig. 2.3). The phosphorylated MLC initiates movement of myosin along so-called F-actin fibres leading to cell contraction. Contraction continues until MLC is dephosphorylated by MLC phosphatase. Accordingly, inhibition of the MLC phosphatase by the RhoA-activated enzyme Rho kinase extends the contraction of smooth muscle cells.

Physiological relaxation of vascular smooth muscle is induced by increasing the cyclic GMP (cGMP) or cAMP concentration in smooth muscle cells. Cyclic GMP can be generated by NO-mediated activation of soluble guanylate cyclase, or by activation of the intracellular domain of membrane-bound ANP-receptors, which carries intrinsic guanylate cyclase activity. The generation of cGMP and subsequent activation of cGMP-dependent protein kinase results in a downregulation of the influx of Ca^{++} ions into the cell; therewith it overrules other contraction-inducing mechanisms. Inhibition of contraction is also achieved with vasoactive agents that elevate cAMP in smooth muscle cells, which leads to inactivation of MLC kinase and inhibition of contraction. Cyclic AMP generation can be induced by various agents, including epinephrine (via β-adrenoreceptor stimulation).

Many vasoactive agents such as norepinephrine (via α-receptors), angiotensin II, and endothelin-1 enhance cytoplasmic calcium levels in, and contraction of, smooth muscle cells when acting directly on these cells. Ca^{++}-channel blockers, ACE inhibitors preventing the formation of angiotensin II, angiotensin receptor inhibitors, and endothelin antagonist, are currently used to prevent smooth muscle contraction in order to lower blood pressure. Inhibition of Rho kinase may provide another route to blood pressure lowering, but is still under study. Furthermore, inhibition of lymphocyte release from lymph nodes also has an effect on blood pressure regulation, which may involve several effects, including a reduction of sympathetic stimulation.

Limitation of smooth muscle contraction can be achieved both by direct inhibition of the pathway that causes contraction or by raising intracellular concentrations of the endogenous molecules that cause relaxation. Vasorelaxing agents aim to deliver NO to smooth muscle cells (nitro-vasodilators) or to enhance intracellular cGMP (phophodiesterase-5 inhibition). In addition, many approaches to protect the vasculature include stimulation or restoration of the ability of endothelial cells to produce NO.

Paracrine regulation by the endothelium: NO and other factors

When vasodilator agents activate the endothelium, usually the endothelial nitric oxide synthase is stimulated, generating NO. The unstable NO rapidly diffuses into the underlying smooth muscle cells, where it activates guanylate

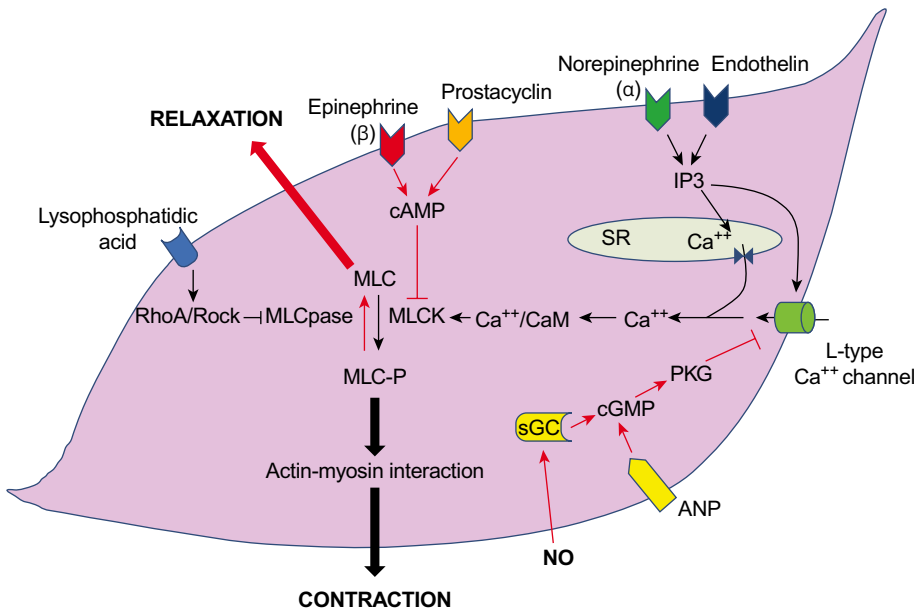

Fig. 2.3 Mechanisms involved in smooth muscle contraction (black arrows) and relaxation (red arrows). →: stimulation; ⊢: inhibition. Pink: smooth muscle cell. Abbreviations: SR, sarcoplasmic reticulum; IP3, inositol-trisphosphate; CaM, calmodulin; MLC, myosin light chain; MLC-P, phosphorylated MLC; MLCK, MLC kinase; cAMP, cyclic AMP; cGMP, cyclic GMP; ANP, atrial natriuretic peptide; PKG, cGMP-dependent protein kinase; NO, nitric oxide; sGC, soluble guanylate cyclase; Rock, Rho kinase.

cyclase. The formed cGMP subsequently overrules the mechanisms that maintain contraction in smooth muscle cells and hence induces relaxation and vasodilation. While NO is a dominant factor in large arteries, another group of factors, endothelium-derived hyperpolarization factors (EDHFs), can also contribute to the dilatation of resistance vessels. Several candidates have been proposed as EDHFs, including soluble factors such as hydrogen peroxide, epoxyeicosatrienoic acids, and factors generated by arachidonic P450 epoxygenase. Furthermore, endothelial cells can produce prostacyclin—and, in smaller vessels, prostaglandin E2—inducing cAMP elevation in smooth muscle cells, and subsequent vasodilation. The effects of NO and prostacyclin extend beyond smooth muscle contraction. These mediators also reduce the activation and aggregation of blood platelets. Furthermore, NO reduces inflammatory activation of the healthy arterial endothelium itself.

The high shear forces that are exerted on the arterial surface by laminar blood flow stimulates the long-term expression and acute activity of NO synthase and hence the production of NO in endothelial cells. In this way, the endothelium influences the tone of the arterial media, even in resting conditions. The ability of the endothelium to respond rapidly to a change in blood flow can be tested in the brachial artery by measuring flow-induced vasodilation after interruption of the arterial perfusion using an inflated cuff. Subsequent restoration of the flow is immediately followed by vasodilation.

In disease, the ability of endothelial cells to produce NO can be markedly decreased. This accompanies a state that is indicated as endothelial dysfunction, a state in which the endothelium is also more prone to inflammatory activation. However, one has to realize that the term 'endothelial dysfunction' is used for many different conditions varying from decreased laminar flow exposure to enhanced exposure to reactive oxygen species, from disturbed vasoregulation in diabetes to a reflection of atherosclerosis. A dysfunctional endothelium not only displays reduced NO production, but can also produce endothelin-1, a potent vasoconstrictor, at an increased rate. This can play a substantial role in vasoconstriction of resistance arteries.

Neuronal control: sympathetic regulation

An important contribution to vascular constriction and tone is made by sympathetic innervation. The majority of the vasculature is innervated by sympathetic nerves, except for capillaries and post-capillary venules. In arteries and arterioles, which are most densely covered by sympathetic nerve endings, released norepinephrine contributes to their vascular tone and resistance, and consequently to blood pressure regulation. This effect is predominantly mediated by α1-adrenoreceptors, which are present on all innervated vessels. In small arteries and arterioles also α2-adrenoreceptors are present, which give a similar but less profound effect. The importance of the sympathetic innervation for vascular tone is demonstrated by the vasodilation that occurs when sympathetic innervation is suddenly blocked by sympathectomy or pharmacological inhibition of α-adrenoreceptors. In veins, regulation of the vein diameter by norepinephrine from sympathetic nerves contributes to the volume regulation of these capacitance vessels. Vein contraction drives blood in the direction of the heart and helps to increase stroke volume and cardiac output. Adrenergic stimulation of resistance vessels and veins often takes place simultaneously, but can occur independently.

While parasympathetic innervation is important for the cardiovascular system by innervation of the cardiac tissue, most blood vessels are devoid of parasympathetic innervation. A few exceptions exist in specific tissues (e.g. the corpus cavernosum). Innervation of resistance vessels in striated muscle is complicated as nerve fibres of cholinergic nerves to myocytes run in parallel with sympathetic ones. Spill-over of the neurotransmitters norepinephrine or acetylcholine may occasionally occur. In such a way acetylcholine may reach small arteries in the heart. A small part of circulating catecholamines is spill-over of norepinephrine generated by nerves.

Feedback regulation by baroreceptors

The arterial hydrostatic pressure is continuously monitored by arterial baroreceptors, in particular, localized in the carotid sinus. These baroreceptors monitor the distension of the carotid body wall and signal to the brain medulla, where sympathetic and parasympathetic (vagal) stimulations are coordinated. They cover a large pressure range (about 60–180 mmHg) by a gradual adaptation of their overall firing intensity. Firing of the baroreceptors has—via nerve cell stimulation in the medulla—an inhibitory effect on sympathetic stimulation of blood vessels, while it enhances parasympathetic stimulation of the heart. When the arterial pressure falls, firing of these receptors drops. This results in sympathetic activation and release of norepinephrine in, and contraction of, the vessel wall. While the carotid body baroreceptors are most dominant, the aortic arch also contains baroreceptors.

Humoral control

The circulating blood contains a variety of factors that, depending on their concentration, can also affect the contraction status of smooth muscle cells and hence the

hydrostatic pressure within blood vessels. They comprise circulating catecholamines, products of the renin–angiotensin–aldosterone system, atrial natriuretic peptide (ANP), and many other vasoactive agents, including vasopressin and bradykinin. Many vasoactive substances have receptors both on the vascular endothelium and smooth muscle cells. Thus it often happens that, while a vasoactive agent is able to activate smooth muscle cells and to induce contraction, its binding to healthy endothelium activates a pathway that induces release of NO and overrules this contractile effect. The result of these two effects is vasodilation. If the endothelium is damaged or the endothelial ability to release NO is impaired (endothelial dysfunction), contraction will occur as yet.

Catecholamines

In addition to neuronal release of norepinephrine, circulating catecholamines also affect vasoregulation. The dominant circulating catecholamine is epinephrine (about 80%), which has much higher affinity for β-adrenergic receptors than norepinephrine that mainly acts through α-adrenergic receptors. At low concentrations epinephrine preferentially binds to β2-adrenoreceptors. β-Adrenergic stimulation by epinephrine binding causes cAMP generation and subsequent relaxation of the smooth muscle.

Vasoregulation by the renin–angiotensin–aldosterone system

Another humoral system that is involved in the regulation of blood pressure and volume is the renin–angiotensin–aldosterone system. Renin is mainly produced in the kidney (by the juxtaglomerular cells that are in communication with afferent and efferent arterioles) and converts angiotensinogen (synthesized by the liver) to angiotensin I. β-Adrenergic stimulation and hypotension in the kidney artery are among the stimulators of renin release. Angiotensin I is subsequently converted into angiotensin II by the enzyme angiotensin converting enzyme (ACE), which resides on the surface of the endothelium, particularly in the lung vascular bed. In humans, angiotensin II can also be generated by the enzyme chymase, which is secreted by mast cells, e.g. in atherosclerotic or inflamed arteries. Effects of angiotensin II are multiple and all result finally in an increase in the arterial pressure. This can occur directly by activation of AT1-receptors on smooth muscle cells or indirectly via activation of AT1-receptors of nerve cells, both in the brain medulla and by reduced re-uptake of norepinephrine at the sympathetic nerve endings in the arterial wall. Among the other effects of angiotensin II are stimulation of aldosterone production by the cortex of the adrenal gland, which causes fluid retention (which in turn increases blood volume), and stimulation of the release of vasopressin from the pituitary gland, which also leads to fluid retention. Long-term exposure to increased levels of angiotensin II causes hypertrophy of the resistance vessels and long-lasting hypertension. Almost all individual components of the renin–angiotensin system (except renin) have also been encountered in the arterial wall itself at low concentrations, including a homologous ACE2, but their contribution to physiology and pathological changes is still debated. ACE2 converts angiotensin I in angiotensin (1-9). Inhibition of ACE or AT1 receptors has been proven to be an efficient way of ameliorating high blood pressure in certain clinical conditions. However, a new light was shed on the action of ACE inhibitors by proteomic analysis of their effect. An important aspect of their action was not directly related to angiotensin II–AT1 receptor interaction but involved their inhibitory effect on the degradation of bradykinin that also occurs by ACE. This inhibition results in elevated bioavailability of bradykinin, contributing to vasodilatation.

Atrial natriuretic peptide (ANP)

A counter-regulatory system for the renin–angiotensin–aldosterone system is the release of ANP by atrial cardiomyocytes, when the atrial wall is distended (e.g. by hypervolaemia). ANP induces smooth muscle relaxation by activating ANP-receptors that stimulate generation of cGMP in smooth muscle cells. It also counteracts sympathetic constriction of resistance vessels, and reduces the blood volume and vascular resistance over prolonged time periods by regulation of the sodium and water balance by the kidney. In addition, ANP directly reduces central venous pressure and consequently stroke volume and cardiac output.

Adipokines and perivascular adipose tissue (PVAT)

Conduit and resistance arteries also respond to circulating factors that are released by adipose tissue. In the last decade it has become clear that arteries, arterioles, as well as veins are embedded in a thin layer of adipose tissue, so-called perivascular adipose tissue (PVAT), which directly delivers vasoactive factors to the adjacent vessel wall. These cytokines activate intracellular 5'AMP-activated protein kinase (AMPK) via adiponectin receptors on endothelial cells (stimulation of NO synthesis; inhibition of endothelin-1 release) and have a vasodilatory effect in healthy arteries by acting on smooth muscle cells (via voltage-dependent [K(v)] K^+ channels). The volume of PVAT increases dramatically in obesity and the vasoregulatory properties change from vasodilatory into vasoconstricting ones, as the spectrum of released cytokines alters completely.

Autoregulation

In addition to neuronal, humoral, and paracrine regulation, smooth muscle cells also display autoregulation. This means that after moderate vasoconstriction, vascular smooth muscle cells of a tissue adapt their tension to the original more constricted state. This occurs within a limited pressure range, the autoregulatory range, and proceeds independently of nerve stimulation or blood-borne factors. Although the precise mechanisms underlying autoregulation are still unclear, it is generally thought that autoregulation involves several components. A short-term component involves the adaptive response of smooth muscle cells to increases in radial strain evoked by increased hydrostatic pressure. Generation of vasodilator molecules by a drop in oxygenation and metabolic changes has been proposed to underlie long-term component of autoregulation.

Exchange in capillaries

The ultimate goal of the circulation is the delivery of oxygen and nutrients to, and removal of waste products from, the tissues. To that end oxygen is taken up in the lungs and transported by erythrocytes; nutrients are delivered to the blood in the gastrointestinal tissues; and waste products are extracted and handled mainly by the lung (CO_2), liver, and kidney. In addition, the blood transfers hormones from endocrinal glands to the target tissues. Because of the dimensions, the surface area of the vascular lining per ml of blood is dramatically higher in the capillaries than in the proximal and distal vessels of the circulation. Hence, exchange between blood and tissues occurs primarily via the capillaries. Before discussing details of capillary permeability, we first introduce the concept of vasomotion, the control of fluid entry into the capillaries regulated by precapillary arterioles, and the glycoclayx, the polysaccharide-rich surface layer that covers the luminal side of the endothelium.

Vasomotion

While a laminar flow reaches the capillaries, the constriction state of the precapillary arterioles determines whether a capillary is perfused or not. By the subsequent opening and closure of these precapillary arterioles (vasomotion) capillary perfusion is regulated. Therefore, the passage of blood cells through the capillaries usually proceeds intermittently. This process is called flowmotion. Vasomotion is regulated by metabolic factors in the microvasculature, in particular the oxygen tension. The higher the oxygen demand, the more capillaries will be simultaneously perfused (capillary recruitment). When the rhythmic changes in flowmotion, determined by Laser–Doppler flowmetry, were analysed by Fourier analysis, several different frequency patterns became visible. These patterns have been attributed to different sources (including endothelium, vascular smooth muscle, and neurogenic function). Nevertheless, they converge in vasomotion as the driving force.

The glycocalyx

The endothelium is covered at its luminal surface by the glycocalyx, a gel-like layer consisting of membrane-bound proteoglycans covalently linked to glycoaminoglycans, glycoproteins, and glycolipids. The polysaccharide glycoaminoglycans mainly consist of various forms of heparan-sulphates and hyaluronic acid. The glycocalyx has been estimated to be 0.5–1 μm thick, but can vary in different vascular beds. It harbours specific biomechanical properties, prevents direct contact of blood cells with the endothelial membrane, and contributes to mechanotransduction. Furthermore, it displays directly, or by binding various proteins, various anti-inflammatory and anti-coagulant properties. Furthermore, *in vivo* it has molecular sieving characteristics, it plays an important role in controlling endothelial permeability for macromolecules, while it is permeable for oxygen, water, and small molecular solutes.

Exchange over the endothelium

Any factor in the blood that will be delivered from the capillary into the tissue cells has to pass the endothelium. Such factors range from gases like oxygen to proteins like albumin, from water to lipids and hormones. ➔ Figure 2.4 summarizes several pathways that are involved. In the non-activated continuous endothelium they comprise diffusion (oxygen, lipids), hydrostatic exchange via interendothelial clefts (water, ions), and delivery via receptors and caveolar vesicles (proteins and other macromolecules).

The physicochemical properties of oxygen allow an easy diffusion through both the cell membranes and the cytoplasm. Small lipophilic factors are thought to use lateral diffusion along the cell membrane or after membrane passage via binding to specific carrier proteins, such as fatty acid binding protein.

Microvascular endothelial cells are connected to each other by junctional complexes that reside in intercellular clefts. In contrast to epithelial cells and blood–brain barrier endothelial cells, the tight junctions between most continuous endothelia are incomplete mosaic structures. Only the adherens junctions form an uninterrupted belt of cellular junctions that separates the luminal from the basolateral side of the cell. This allows the free passage of water and solutes between the cells. Extravasation of water and low

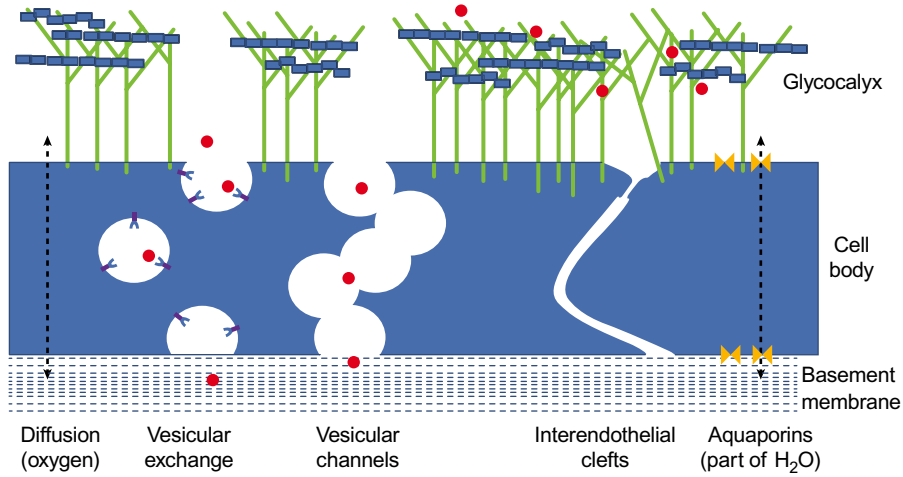

Fig. 2.4 Major mechanism of exchange in the non-stimulated healthy capillary. The blue box represents the endothelial cell, with, on its luminal side (top), the glycocalyx consisting of proteoglycans (green) and glycosaminoglycans (blue chains), and at its abluminal side (bottom) the basement membrane. The intercellular clefts can open in the post-capillary venules after exposure of the endothelium to vasoactive agents, such as histamine. Angiogenic factors, in particular VEGF-A, stimulate the generation of vesicular channels and open the clefts in post-capillary venules and capillaries.

molecular mass solutes is primarily driven by the hydrostatic pressure difference between the blood at the luminal side of the endothelium and interstitial compartment at its basolateral side. Spaces in the glycocalyx are large enough to allow the free diffusion of oxygen, water, and ions. From the water that passes the endothelium of continuous capillaries, 90% is estimated to proceed through the intercellular clefts, while exchange via small caveolar vesicles that ferry between the luminal and abluminal side of the cell is limited (5%). The endothelial membrane also harbours aquaporins, so-called water-only channels that facilitate water exchange. Although the exact contribution to water exchange is still being debated, it has been estimated that these aquaporins contribute ≤10% of the hydraulic conductance in most microvessels of healthy tissues. Exceptions are found in the capillaries forming the blood–brain barrier and microvessels in swollen muscle after exercise in which a higher contribution has been estimated.

Proteins like albumin pass poorly through the clefts of non-stimulated endothelial cells but are transferred over the endothelial layer via caveolar vesicles, usually after binding to a specific receptor and to a minor part via pinocytosis or channels formed by fused vesicles. For example, albumin binds to a specific receptor albondin/gp60 in non-brain continuous endothelial cells. Albondin facilitates accumulation of considerable amounts of albumin in the interstitial space. Comparable receptor-mediated exchanges were also observed for insulin (via the insulin receptor) and other hormones.

This picture changes dramatically when the adheren junctions open, as occurs in inflammatory conditions. In such conditions the glycocalyx barrier disappears and subsequent opening of junctions between adjacent endothelial cells allows massive efflux of fluid and macromolecules, such as nutrients, to fuel the inflammatory cells, and complement factors and fibrinogen, which can help to attack or immobilize infectious agents. A similar hyperpermeability response is observed after exposure to angiogenic stimulators, in particular vascular endothelial growth factor (VEGF-A). After extravasation of fibrinogen, fibrin or a fibrinous exudate is rapidly formed. Fibrin not only limits blood loss and spread of infection, but also provides a temporary repair matrix.

Transmicrovascular fluid exchange

The circulation with its sealed endothelium acts as a sealed water container to which physical laws of pressure and volume can be applied, as explained. How does this act when nutrients have to be delivered to the interstitium of tissues? It is generally accepted that in capillaries a gradual hydrostatic pressure fall occurs from the arteriolar side to its venular side. This has been verified in capillaries of the human nailfold, in which blood pressure steadily decreases along the capillary from 35–45 mmHg at the beginning to 12–15 mmHg at the end (measured at heart level). According to the classical concept of Ernest Starling (1896), hydrostatic pressure causes fluid leak through the endothelium of the first part of the capillary, which delivers nutrients and hormones to the tissue. The loss of fluid increases the concentration of macromolecules and hence the oncotic pressure. Indeed, the oncotic pressure in blood is mainly determined by its content of macromolecules, because intravascular small solutes like ions are in equilibrium with their counterparts in the interstitium. As along the capillary the hydrostatic pressure gradually decreases, while simultaneously the oncotic pressure increases, a point is reached where fluid is reabsorbed into the blood (➔ Fig. 2.5a). This was thought to occur at the distal half of the capillary—at the venular side—and

to balance the extravasated fluid at the arteriolar side. This principle of Starling is reflected in the equation:

$$Jv/A = Lp \{(Pc - Pi) - \sigma(\pi p - \pi i)\},$$

in which Jv/A represents the volume filtration per unit of endothelial surface area, Lp the hydraulic conductivity of the barrier, and (Pc – Pi) and (πc – πi) the differences in intravascular and interstitial hydrostatic and oncotic pressures, respectively; σ is the average colloid osmotic reflection coefficient; πp is the oncotic pressure of the plasma in the capillary.

The glycocalyx cleft model

While this concept has been accepted for more than 100 years and has provided much insight, in the last decade this concept had to be modified when more sophisticated techniques became available. Several observations did not fit with the original concept. First, when investigators became able to determine protein concentration locally in the interstitial fluid, they found that the protein concentration of the interstitial fluid, just beneath the endothelium, was not low, as assumed by Starling's concept. Second, albumin by itself has a profound effect on limiting macromolecular passage, a property that requires the positively charged arginine residues in the albumin molecule, suggesting binding to negative charges. Albumin accumulation by binding to the negatively charged glycocalyx contributes to the enhanced sieving properties of the glycocalyx and markedly limits penetration by macromolecules. As a consequence, protein extravasation—and the oncotic pressure gradient generated by it—remains minimal, and fluid extravasation spreads steadily over the endothelium of the entire capillary length

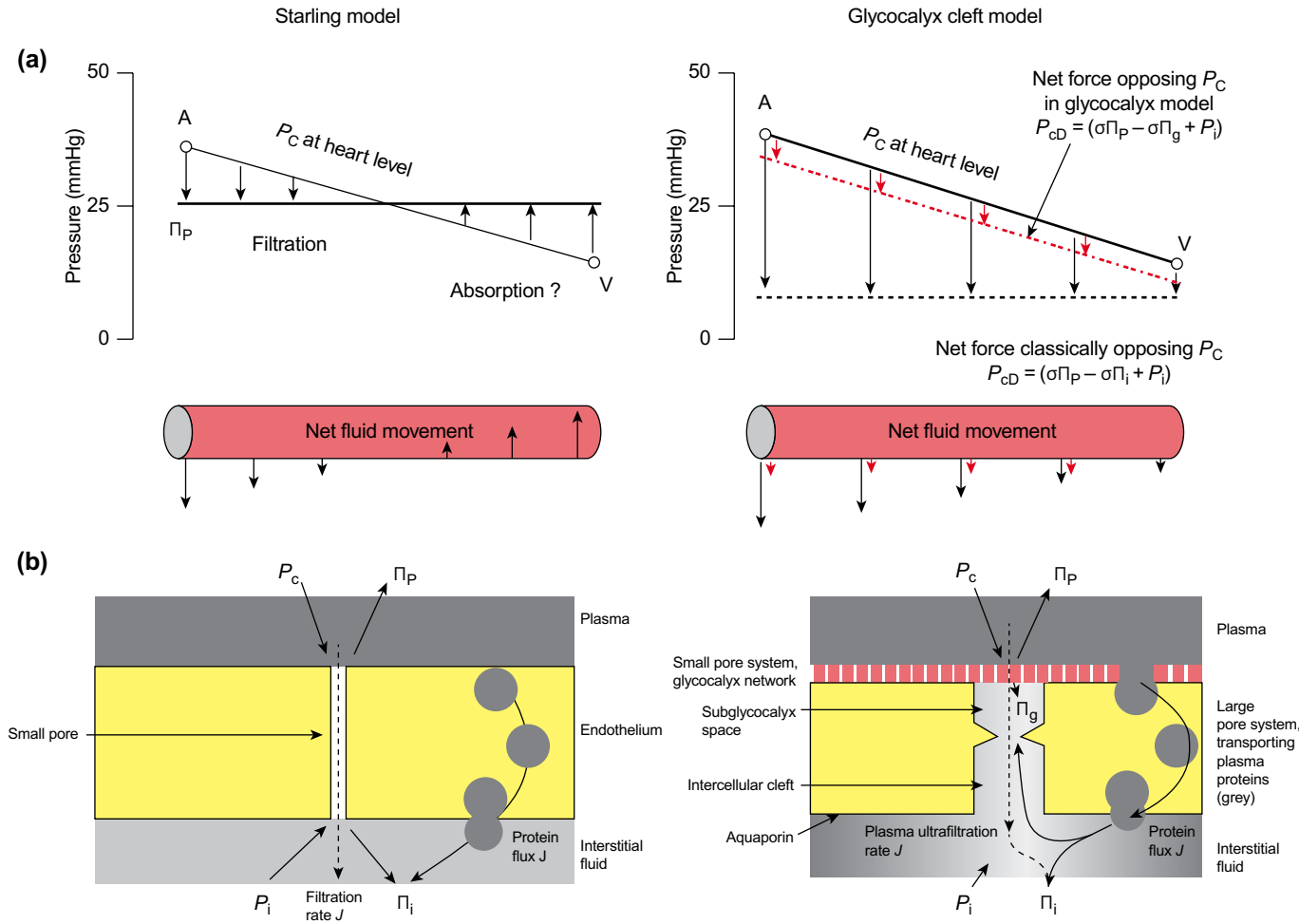

Fig. 2.5 Exchange of fluid in capillaries according to the model of Starling and the glyocalyx cleft model. (a) Net fluid exchange based on hydrostatic and oncotic pressures. (b) An endothelial cleft between two yellow endothelial cells. While the change in oncotic pressure forces the reflow of fluid in the distal part of the capillaries in the model of Starling, fluid extravasation proceeds steadily along the capillary in the glycocalyx cleft model, because macromolecules do not pass further than the pink glycocalyx layer (except for vesicular exchange). Top: blood compartment; bottom: interstitial compartment.
(Reproduced from R. Levick and C. Michel, Microvascular fluid exchange and the revised Starling principle, Cardiovascular Research, 87: 2 (2010) with permission from Oxford University Press.)

driven by the hydrostatic pressure (➔ Fig. 2.5a, right panel). Indeed, it was found that fluid is not reabsorbed at the venular side of capillaries in many tissues, including muscle, skin, and lung. Instead, interstitial fluid excess drains back into the circulation via the lymphatics, which deliver their content into the vena cava.

From these considerations Levick and Michel challenged the Starling principle and revised it by proposing that the semi-permeable barrier of a healthy endothelium is not formed by the entire endothelial cell body, but that only the surface-covering glycocalyx layer harboured the oncotic gradient in Starling's equation (the glycocalyx cleft model). Therefore, they modified the original Starling equation in such a way that πi (oncotic pressure in the interstitium) was replaced by πg (the oncotic pressure at the basis of the glycocalyx):

$$Jv/A = Lp\ \{(Pc - Pi) - \sigma(\pi p - \pi g)\}$$

The glycocalyx cleft model is schematically depicted in ➔ Fig. 2.5.

Although the glycocalyx model does not specifically mention a contribution of the endothelial junctions to the barrier for macromolecules of continuous capillaries, adherens junctions do play such a role. On the one hand, adherens junctions may act in a healthy endothelium to optimize maintaining a low protein concentration in the lower part of the interendothelial clefts. On the other hand, they provide an additional barrier (double barrier concept), which prevents protein leakage in conditions in which the glycocalyx becomes disturbed. After disintegration of the glycocalyx, opening of the adherens junctions is required for rapid extravasation of plasma proteins into the tissue interstitium. Opening of interendothelial junctions occurs in inflammation and angiogenesis-prone conditions, and in a transient way after exposure of postcapillary venules to vasoactive agents, such as histamine.

Consequences for diabetes and inflammation

Loss of glycocalyx size and function can occur in metabolic disease. In diabetes, a considerable reduction of the glycocalyx has been estimated using sidestream darkfield imaging. This has an effect on protein loss in the kidney (starting with microalbuminuria), but probably bears consequences for capillary exchange in general. One may anticipate that without functional glycocalyx fluid and protein exchange in tissue capillaries tend to the orginal model of Ernest Starling.

In inflammation the glycocalyx loses its integrity and the adherence junctions in the interendothelial clefts can open. This permits not only a massive extravasation of fluid and solutes, but also the extravasation of plasma proteins, including fibrinogen and complement factors. This supports the inflammatory process. Simultaneously, it will also change the nature of capillary exchange. Large openings at the junctions cause extravasation of plasma, i.e. fluid with dissolved proteins. As this has limited effect on the oncotic pressure of the remaining blood, fluid excess is not reabsorbed, but must drain back via the lymphatics. Hence, such increased protein extravasation bears impact on the use of fluids, colloids, and albumin to treat blood hypovolaemia that can occur in sepsis as compared to hypovolaemia due to trauma-induced blood loss.

Interstitial fluid pressure and oedema

The above depicted concepts all assume that the interstitial volume remains unaltered because it is regulated by capillary filtration and equilibration by lymph flow. While this has been accepted since Starling's concept, recent data have indicated that the interstitial volume can become altered in inflammation and trauma-related conditions. In such cases a change in the intercellular tension in the connective tissue can occur. This intercellular tension is generated by the combined interplay between the extracellular matrix (in particular collagens) and collagen binding β1-integrins on interstitial fibroblasts. When the integrin–matrix interaction ceases, the collagen microfibrils can give way, which is accompanied by swelling of the under-hydrated proteoglycans, which normally are caged by the collagen network. This not only causes a variant form of oedema, but also provides an additional force (reduced interstitial pressure) to facilitate fluid drain from the capillaries.

Exchange in post-capillary venules

Post-capillary venules represent a specific region of the vascular tree, in which the endothelial cells are not, or very poorly, covered by pericytes or smooth muscle cells. These areas are more sensitive to the action of vasoactive agents not because they can affect vasoregulation in these areas, but they can easily affect the endothelial barrier function. Initially they induce leakage in small spots along the post-capillary veins, which become leaky for a short time period (minutes) and alternate each other in the course of time. High exposure or continuous activation of the endothelium by mast cells in the interstitium causes leakage of proteins along the entire post-capillary venules. The post-capillary venules also act as the prime area for the initial extravasation of neutrophils and monocytes and are the main area to respond to inflammatory mediators like TNFα by synthesizing new leukocyte

adhesion molecules. The extravasation of lymphocytes requires different types of receptors, such as present on high venule endothelial cells of lymphoid organs.

Volume regulation and haemostasis

Volume regulation and preventing blood loss

The functioning of the cardiovascular system, seen as a pressurized blood container, critically depends on a constant fluid content. At a given vascular resistance blood pressure will change if the blood volume changes. The fluid balance of the intact circulation compartment, which is normally in equilibrium with the interstitial compartment, is largely controlled by the kidney. As indicated, the renin–angiotensin–aldosterone system and ANP are important regulators of the body's fluid balance. For further details the reader is referred to Peti-Peterdi et al. (2).

However, if the vascular system is severely damaged, major blood loss will interfere with the pump function of the heart and regulation of blood delivery to the tissues. To prevent serious blood loss the cardiovascular system harbours several defence mechanisms. First, upon cutting a muscular vessel, contraction of the damaged vessel segment is induced. This helps the haemostasis system forming a haemostatic plug by platelet activation and aggregation, which is subsequently enforced by fibrin threads thus forming a stabilized haemostatic plug (see Chapter 18). Platelets can also plug small leaks in the microcirculation. Furthermore, interstitial fibroblasts produce considerable amounts of tissue factor, a protein that initiates the extrinsic coagulation cascade, in particular around the arterioles.

Maintaining blood fluidity

The counterside of this protective system against vascular blood loss is intravascular thrombus formation that can obstruct the flowing blood and threaten the distal tissue that then becomes devoid of oxygen and nutrients. However, in healthy people this danger is efficiently counteracted by the vascular endothelium throughout the whole circulation, albeit with local adaptations. The endothelium of healthy vessels acts on three levels. First, it produces a number of proteins that prevent thrombus formation. It limits coagulation by binding antithrombin III, facilitating protein C activation by thrombomodulin, and releasing an inhibitor of the tissue factor pathway (TFPI). Furthermore, it reduces platelet aggregation by producing NO, prostacyclin, and ADP-degrading ectonucleotidase, and repels platelet binding by heparin sulphates. Finally, it releases tissue-type plasminogen activator, a fibrinolysis catalysing enzyme that becomes active as soon it encounters fibrin. By these anti-thrombotic activities the endothelium is pivotal for an undisturbed circulation. If the balance between pro- and anti-thrombotic factors is disturbed, thrombus formation or bleeding will occur, as is further discussed in Chapter 18.

Recommended reading

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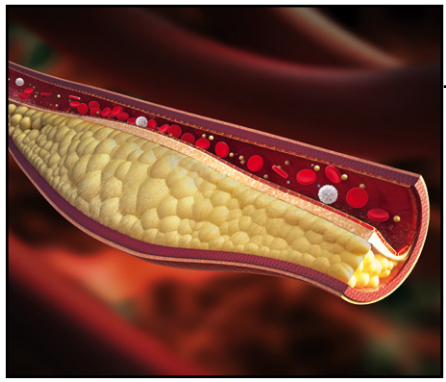

CHAPTER 3

Physical processes in the vessel

T. Christian Gasser

Content

Introduction

The evolution of multicellular animals was associated with a more and more organized circulatory system. Simple diffusion of extracellular liquid evolved into flow-driven by an archaic heart, into a highly organized system in mammals, and included blood flows related to the specific metabolic demands of organs. The latter was made possible by the heart's pumping ability and was regulated by peripheral resistances, which together generated the arterial blood pressure (1). This led to the complex cardiovascular system seen in humans, the analysis of which involves many physical disciplines.

Cardiovascular function critically depends on the proper interaction between blood and the vessel wall, and its malfunction may result in vascular pathologies such as aortic valve stenosis, aortic aneurysms, alterations in macromolecule transport and gene expression, calcification, inflammation, and neoangiogenesis. Consequently, haemodynamics-based biomechanical factors of the cardiovascular system are a common denominator of cardiovascular pathologies.

The present chapter aims at reviewing biomechanics-related physical processes in vessels with the focus on larger arteries. Central to this review are load-transition mechanisms in blood flow and the vessel wall, mechanical stress that develops in blood and vascular tissue, as well as solutions to the continuum mechanics' equilibrium equations that arise directly from Newton's second law of mechanics.

Despite the fact that analytical solutions of the equilibrium equations are clinically very important, the derivation of such solutions requires rather severe and idealized modelling assumptions. Investigating the consequences of Newton's principle for complex vascular domains requires approximate numerical methods like the Finite Element Method (FEM) or Computational Fluid Dynamics (CFD). The continuous advance of such methods makes it possible nowadays to explore biological systems and processes in much greater detail. This advances our understanding of the physiological and pathological mechanisms of the cardiovascular system, its interaction with medical devices, drug delivery pathways, the interplay between structure and cardiovascular function, mechanotransduction, and the like. A note: Throughout this chapter units are given in square brackets.

Fundamental biomechanics' relations and terminology

Stress

Stress is a measure of load that acts at the material particle (solid or fluid) and represents the force normalized by the area through which it is transmitted. Normal stress $\sigma = N / A$ is given if the force acts normal to the area (Fig. 3.1a). Shear stress $\tau = S/A$ is given if the force acts in parallel to the area (see Fig. 3.1b). In general, normal and shear stresses appear together, and in three dimensions the stress state is given by three normal and three shear stress components. The unit of the stress is Pascal [Pa}, which is the stress that arises if the force of 1 Newton is distributed over an area of 1 m^2. Stress rates are used to describe how stress changes over time and space (2) in order to analyse time-dependent problems such as deformation of fluid flow or viscoelastic solids.

Strain

Strain is a measure of particle deformation (solid or fluid) and, similar to stress, both normal strain and shear strain occur (see Fig. 3.2). Normal strain $\varepsilon = v / L$ reflects the change of length, while shear strain $\gamma = u / L$ reflects the change of angle. Strain is a dimensionless quantity and the strain state in three dimensions is given by three normal and three shear strain components, respectively.

In order to analyse fluid mechanical problems (or time-dependent solid mechanical problems), strain rates (2) are used to describe how strain changes over time and space.

Constitutive model

A constitutive model is a mathematical description of the material's constitution (solid or fluid). A sub-class of constitutive models for solids relates stress and strain, i.e. specifies how much stress develops at a certain state of strain, and vice versa.

Elastic (or hyperelastic) models for solids assume that the deformation energy, i.e. the mechanical energy required to deform the solid, is fully recovered after unloading. A linear elastic isotropic solid is an example of such a model whose properties are described by Young's modulus and Poisson's ratio. Young's modulus denotes a material's stiffness and Poisson's ratio denotes the interaction between normal strains. Vascular tissue is more complex and cannot be described by a linear elastic solid but shows a strong non-linearity (see Fig. 3.3).

Viscoelastic models (a class of dissipative models) assume that deformation energy is only partly transformed into tissue deformation, and that some part of the energy is transformed into heat, i.e. cannot be recovered by unloading. Yet another class of constitutive models relates to multiphasic materials. Specifically, a poroelastic model regards an elastic solid (skeleton) phase immersed in a fluid phase, while a mixture model assumes that the different phases co-exist in space, i.e. can simultaneously occupy the same position in space.

Constitutive models are also used to describe the mechanics of fluids, i.e. how shear stresses of a fluid particle develop with respect to its strain rate. A Newtonian fluid has a constant coefficient of viscosity, a modelling assumption that holds for most homogeneous liquids. However, blood shows several non-Newtonian effects and can only be approximated by a Newtonian model at very high strain rates.

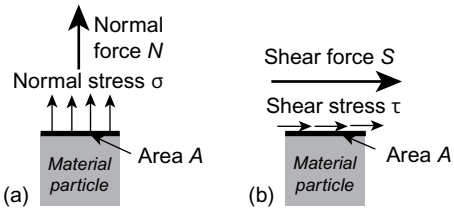

Fig. 3.1 Definition of normal (a) and shear (b) stress acting on the material particle.

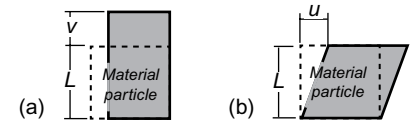

Fig. 3.2 Definition of normal (a) and shear (b) strain that deform the material. The dashed line denotes the undeformed material particle.

Strength

Strength denotes the stress level at which the tissue mechanically fails, i.e. ruptures. Different types of strengths are defined, depending on how stress is actually applied. For example, the ultimate tensile strength (UTS) is the stress level at which tissue fails under slowly increasing tensile stress.

In order to characterize strength under multi-axial stress conditions, equivalent stresses, like the von Mises' equivalent stress σ_M, are used, and the tissue is assumed to fail if

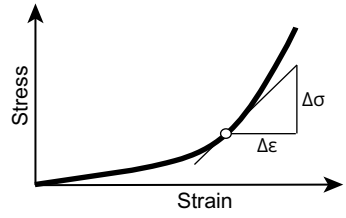

Fig. 3.3 Typical stress strain properties of vascular tissue. Vascular tissue is nonlinear, such that tissue stiffness $k = \Delta\sigma/\Delta\varepsilon$ is not constant but changes with strain.

σ_M reaches the tissue strength. Such equivalent stresses are related to the expected failure mechanism, i.e. if the failure appears brittle or ductile, different equivalent stresses are used.

Stiffness

Stiffness $k = \Delta\sigma / \Delta\varepsilon$ is a tissue-specific parameter that describes how stress increases with increasing strain, i.e. it is the tangent to the stress strain curve (see Fig. 3.3). Vascular tissue is nonlinear, such that its stiffness is not constant but depends on the strain. Consequently, the same vessel will have different stiffness at different inflation pressures.

Isotropy, anisotropy, and incompressibility

A material is isotropic if the mechanical properties are independent of the spatial orientation. This is not the case for vascular tissue, which clearly shows anisotropic properties, i.e. tensile tests on wall strips along the circumferential and the axial vessel directions reveal different results.

A material is incompressible if the volume of the material particle (solid or fluid) does not change under loading[.].

Equilibrium equation

At any time, the external forces acting on a solid or on fluid particles need to balance the internal forces, i.e. stresses. Specifically, each point in the tissue or blood of density ρ has to satisfy the equilibrium relation:

$$\operatorname{div} \boldsymbol{\sigma} = \rho \frac{\mathrm{D}\mathbf{v}}{\mathrm{D}t} \tag{3.1}$$

with $\boldsymbol{\sigma}$ and $\mathbf{v}$ denoting the Cauchy stress tensor and the velocity vector of the solid or fluid particle, respectively. This equation is a direct conclusion of Newton's second law of mechanics (3). Here, boldface letters denote tensor or vector quantities and div(•) denotes the divergence operator known from standard tensor algebra.

Equilibrium equation (3.1) can be evaluated within an Eulerian (typically used in fluid mechanics) or Lagrangian (typically used in solid mechanics) point of view, such that the material time derivative $\mathrm{D}\mathbf{v}/\mathrm{D}t$ is either $\partial\mathbf{v}/\partial t + \operatorname{grad}\mathbf{v}\cdot\mathbf{v}$ or simply $\partial\mathbf{v}/\partial t$, respectively. Here, grad [•] and [•]·[•] denote the gradient operator and the inner vector product, respectively.

Expressing the stress $\boldsymbol{\sigma}$ in equation (3.1) by a Newtonian fluid model leads to the Navier–Stokes equations, which are frequently referred to in fluid mechanics.

Finally, in order to complete the biomechanical description, boundary conditions and initial conditions specify how the analysed solid or fluid domain interacts with its surrounding, and denote its respective conditions at the beginning of the calculation. The whole set of equations can either be solved numerically (using FEM or CFD) or, if possible, analytically.

Blood flow

The cardiovascular system shows an oscillating flow in larger vessels (aortic arch: –20 to 60 [cm/s]; abdominal aorta: –10 to 60 [cm/s]; common iliac artery: –7.5 to 60 [cm/s]) that becomes a unidirectional flow in smaller vessels. Blood's main objective is to meet the metabolic needs of organs, and it can influence biological processes through biomechanical factors like wall shear stress (WSS) (4), vortical structure dynamics (5), and the like.

Blood rheology

Blood is a suspension of cells in plasma—erythrocytes (6–8 μm), leukocytes (10–15 μm), and thrombocytes (2–3 μm), and shows significant non-Newtonian effects (6). The shear rate at the vessel wall (boundary layer flow) is a function of plasma viscosity (7), whereas erythrocytes strongly influence whole blood viscosity (see Fig. 3.4). At regions of very low shear rates, erythrocytes join each other, leading to a Rouleaux formation, and these solid-like obstacles severely increase blood viscosity (see Fig. 3.4). With slight fluid motion these obstacles break up leading to a fast drop of blood viscosity at low shear rates. At higher shear rates the highly deformable erythrocytes deform and align with the bloodstream, which further reduces the blood's flow resistance (see Fig. 3.4). Finally, at shear rates larger than about 100 [s^{-1}], blood viscosity becomes insensitive to the shear rate (see Fig. 3.4) and the Newtonian assumption of constant viscosity is justified.

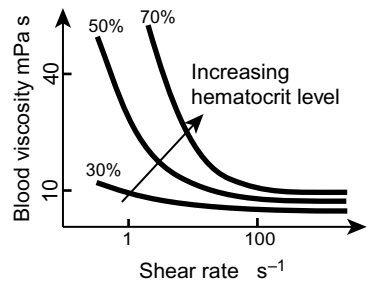

Fig. 3.4 Dependence of whole blood viscosity on the shear rate and the haematocrit levels.

Due to the high volume ratio of elastic cells in blood, it also shows solid-like viscoelastic and thixotropic properties. Besides the properties of the experimental model and method, vascular control mechanisms too, like metabolic autoregulation and/or modulation of endothelial function, can modify blood rheology. This could explain why blood is less viscous with *in vivo* conditions compared to *in vitro* conditions (8) (in ➔ Chapter 2).

Blood flow modelling

Despite earlier biomechanical work being based on the Newtonian behaviour of blood, many recent analytical and CFD investigations capture blood shear-thinning properties via the Carreau–Yasuda model (9). The Quemada model (10) goes one step further and considers the dependence of viscosity from the shear rate and the haematocrit. Finally, for specific biomechanical investigations, modelling blood viscoelasticity, anisotropy, shear-induced migration effects, and the like, might be required.

Blood flow phenomena

Blood flow is highly influenced by vessel geometry and shows, already at steady-state conditions, many complex flow features. The study of many of these features require either CFD simulations or direct experimental measurements using methods like particle image velocimetry (PIV).

Laminar and turbulent flow

A laminar flow occurs when a fluid flows in parallel layers, with no disruption between the layers, i.e. adjacent layers slide past one another like playing cards. In contrast, a turbulent flow shows chaotic/random motion, such that its velocity field varies significantly and irregularly in both space and time.

A laminar flow establishes if the fluid viscosity is large enough to dissipate the flow's kinetic energy, i.e. viscous forces dominate over inertia forces and the flow does not break up into smaller flow structures. If this is not the case, the flow becomes turbulent, causing the formation of eddies (vortices) at many different length scales, which in turn leads to flow regimes that show chaotic particle velocities. By an essentially inviscid mechanism, large-scale structures (at high turbulent kinetic energy) transmit kinetic energy to smaller and even smaller structures. This produces a cascade of eddies (vortices) along which energy is transmitted all the way down to the smallest possible length scale (Kolmogorov length scale), where viscosity dominates and the (remaining) kinetic energy is finally dissipated (13). Turbulent flows strongly promote the mixing of fluid particles and, in the ventricles, the Kolmogorov length scale is of the order of 100 μm (14).

In contrast to the deterministic description of laminar flows, the chaotic nature of turbulent flows is described by statistical methods, such that mean together with superimposed stochastic fluctuations determine flow velocity. Under physiological conditions, turbulent flow is seen in the ventricle and first aortic segments (14), In addition, jets that frequently develop in pathological vessel constrictions, quickly lead to a turbulent flow (15, 16).

Flow in circular tubes

Putting aside dissipation, i.e. regarding blood as an ideal (non-viscous) fluid, the Bernoulli equation relates kinetic energy to the potential (pressure) energies. The Bernoulli equation allows a first estimation of the average flow velocities that develop in a tube, or a network of tubes (6).

A more detailed analysis of blood flow is possible by solving the equilibrium equation (3.1). For example, a Poiseuille flow establishes in a straight circular tube of radius R (m) through which a Newtonian fluid of dynamic viscosity μ (Pa s) flows at steady-state. Specifically, at the radius r (m) within the tube (see ➔ Fig. 3.5a) the flow velocity reads as follows. Units associated with the introduced parameters are given in square brackets.

$$v_p = \frac{R^2}{4\mu}\left(\frac{\Delta p}{\Delta l}\right)\left[1-\left(\frac{r}{R}\right)^2\right]\text{[m/s]},$$

where $\frac{\Delta p}{\Delta l}$ [Pa/m] denotes the pressure gradient, i.e. how fast the pressure decreases along the flow direction. The Poiseuille flow defines a quadratic velocity profile with respect to the radius r (see ➔ Fig. 3.5a), and, at the flow rate $q = 2\pi \int v_p r \, dr$ [m³/s], it predicts a WSS of $\tau = \frac{4\mu q}{\pi R^3}$ [Pa]. Finally, it can easily be reformulated into the Hagen–Poiseuille law:

$$\Delta p = \frac{8\mu q \Delta l}{\pi R^4}$$

which nicely illustrates that a significant pressure drop Δp can only be achieved in the vascular bed, i.e. where the vessel radius R is small.

For a non-Newtonian fluid, the velocity profile (and also WSS) differs from the Poiseuille flow. Specifically, for a shear-thinning fluid like blood, the equilibrium equation (3.1) predicts a more plug-like profile (see ➔ Fig. 3.5a).

Blood flow in larger arteries is pulsatile, which naturally has a strong influence on the velocity profile. Considering

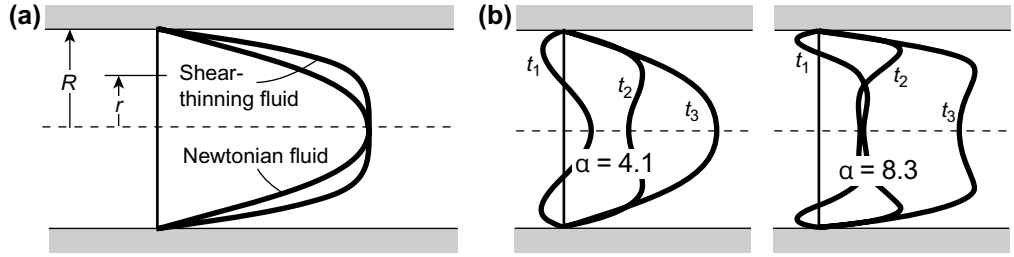

Fig. 3.5 Laminar flow profiles that establish within a circular tube. (a) Steady- state. Newtonian fluid defines a quadratic profile (Poiseuille flow), whereas a shear-thinning fluid leads to a more plug-like profile. (b) Periodic flow. Flow profiles for Wormersley numbers α = 4.1 and α = 8.3 at different times t_1, t_2, t_3 are shown.

blood of the density ρ [kg/m^3] and the kinematic viscosity $\nu = \mu / \rho$ [m^2/s], the equilibrium equation (3.1) leads for periodic (harmonic) flow conditions to the Womersley velocity profile (17):

$$v_w = \frac{P}{i\omega\rho} \exp[i\omega t]\left[1 - \frac{J_0\left(-\sqrt{\frac{i\omega}{\nu}}\, r\right)}{J_0\left(-\sqrt{\frac{i\omega}{\nu}}\, R\right)}\right] \text{[m/s]},$$

where P denotes the pressure amplitude that oscillates at the angular velocity ω. Here, $i = \sqrt{-1}$ is the imaginary unit, and t denotes the time at which the velocity is computed. In addition, exp[•] and J_0 [•] are the exponential function and the Bessel function of the first kind of order zero, respectively. The Womersley number $\alpha = \sqrt{\omega R / \nu}$ determines the shape of the velocity profile, and full details to derive the Womersley velocity profile is given elsewhere (8) (➔ Chapter 2). Profiles permutate significantly from the parabolic profile of unidirectional flow (see ➔ Fig. 3.5), and reversal flow starts in the laminae near the wall (see profiles labelled by t_1 in ➔ Fig. 3.5b). In addition a higher Womersley number increases the velocity gradient at the wall, such that it has a similar effect on the viscous drag than increasing the flow through the tube (18).

If a non-uniform flow is forced to turn, the equilibrium equation (3.1) predicts like a Poiseuille flow, the rotational or swirl components of velocity that develop (19); a phenomenon known at least since the last century (20, 21). In particular, finite curvature is found to reduce WSS from the inside to the outside of a bend. Practically, the curvature of vessels will always significantly influence blood flow in larger vessels (22) (see ➔ Fig. 3.6). CFD studies of anatomically realistic geometries observed, inter alia, that vortices joined each other and ended up in very complex flow conditions, which, however, were still laminar. For a detailed analysis of the effect of shape on steady-state and pulsatile flows in geometries like branches, anastomoses, stenosis, and the like, see Doorly and Sherwin (23).

Boundary layer flow

A boundary layer flow is the fluid flow in the immediate vicinity of a bounding surface. Due to the high shear rates in the boundary layer, effects of viscosity are significant when compared to the inertia effects of the fluid particle. If the flow in the layer that is closest to the bounding surface reverses its direction, the boundary layer can separate into a broader wake and trigger the formation of a vortex. At that point, WSS is zero and the boundary layer suddenly increases its thickness.

The natural tendency of deformable cells to move away from boundaries (24) creates a cell-free layer adjacent to the vascular wall, comparable in size to the size of a single cell. Due to the drift of the deformable cells away from the wall, the near-wall region is occupied by plasma.

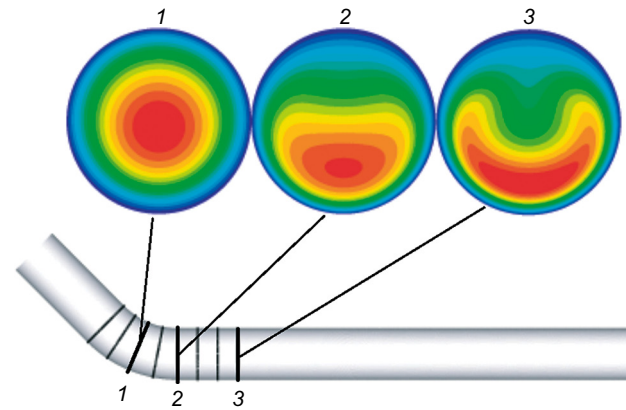

Fig. 3.6 Flow through a bent circular tube. Sectional axial flow profile at salient locations along the bend. Red and blue colours indicate high and low axial flow velocities, respectively.
(Reproduced from D. Doorly and S. Sherwin. Geometry and flow. In L. Formaggia, A. Quarteroni, and A. Veneziani, editors, Cardiovascular Mathematics, volume 1 of MS&A, pages 177–209. Springer, Milan, 2009 with permission from Springer)

Vortex flow

A vortex or a vortical structure is a region within which blood essentially has a swirling or rotating motion. Rapid turning of the flow direction (see Fig. 3.6) and flow separation are two mechanisms able to trigger vortex formation, and further mechanisms have been proposed (25). Vortices can move, develop, and interact with each other leading to a complex flow pattern (26). Vortices may appear, relatively weak compared to the mean flow, and hence are not always straightforward to detect, especially in complex cardiovascular flows. Most important, blood within the vortex is shielded from its surrounding and this may promote platelet activation, thrombin formation, and the like (5, 27).

Jet flow

A jet is a fast stream of fluid that can develop in stenotic vessels, pathological heart valves, or similar flow constrictions. A jet has high momentum and can travel long distances without dissipating, and the break-up of hairpin vortices may lead to a transition from a laminar to a turbulent flow some distance away from the constriction. The acceleration of the fluid through the stenosis results in WSS magnitudes that far exceed upstream levels, but low WSS levels accompany the flow separation zones immediately downstream of the constriction (15, 16).

Vascular wall

Despite the fact that mechanical properties of vascular tissue change along the vascular tree (for arteries, see Cox (28)), the general mechanical characteristics exhibited by vascular walls are the same. This section reviews some of these properties and relates them to load transition at the microstructural tissue scale.

Basic mechanical wall properties

Healthy arteries are highly deformable, composite structures and show a nonlinear stress-strain response, with typical mechanical stiffening at about physiological strain levels (29) (see Fig. 3.7). While the low strain properties are determined by elastin, mechanical stiffening is related to the gradual recruitment of the embedded collagen fibrils. This was concluded after comparing elastase-treated and collagenase-treated tissue samples to their untreated controls (30, 31).

Vascular wall stiffness and strength are directly related to collagen content in the wall, such that the collagen-rich abdominal aorta is stiffer than the collagen-poor thoracic aorta (32, 33, 34). Apart from the amount of collagen in the wall, its spatial orientation (35, 36) is a critical microstructural parameter that influences the anisotropic mechanical properties of vessel walls (37, 38).

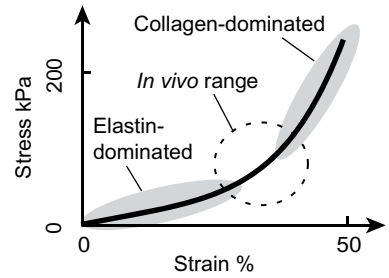

Fig. 3.7 Nonlinear stress strain properties of a vessel wall at simple tension. At about *in vivo* deformation, a transition from the soft elastin-dominated to the stiff collagen-dominated properties is observed.

Vascular tissue contains a large amount of particularly non-mobile fluid, and for many loading conditions, especially *in vivo*, the stress-induced flow of fluid in and out of the wall can be neglected. A volume change of only 0.06% has been experimentally observed for arteries (39), such that a nearly incompressible homogenized solid is suitable for many biomechanical applications.

The arterial wall continually adapts to its mechanical environment through growth, atrophy, remodelling, repair, ageing, disease, and the like. These adaptations are the reason that intact but unloaded arterial segments are not stress-free or strain-free and further deform when dissected into circumferentially aligned (32, 41) or longitudinally aligned (42) strips. This demonstrates that the load-free configuration of the vascular wall is residually stressed (or strained). Models for calculating the *in vivo* stress distribution across the vessel wall critically depend on such residual stress or strain (43). Another consequence of vessel adaption is the development of *in vivo* axial pre-strain, and vessels shorten on removal from the body (44). Axial pre-stretch reduces with age (45) and defines the 'crossover strain' (46, 47) of the vessel's length–force characteristics.

Time-dependent effects

Even in the absence of muscle tone, arteries exhibit hysteresis under cyclic loading, stress relaxation under constant extension, and creep under constant load (48). Viscous effects typically increase from proximal and collagen-poor to the distal and collagen-rich arteries. The structural origin of irreversible deformations in the vascular wall is not yet fully understood, but small proteoglycans (PG), such as decorin, could play a critical role (49, 50). Specifically, slow (viscous) deformation based on a sliding-filament mechanism of the two-fold helix of the glycan (49) could

explain the large portion of macroscopic viscoelasticity seen in arterial tissues. In addition to PG-related mechanisms, interstitial fluid flow and smooth muscle cell (SMC) related dissipative mechanisms probably contribute to the time-dependent response of the blood vessel.

In vitro testing of vascular tissue typically displays pronounced stress softening under the first few loading cycles. Vessels exhibit a nearly repeatable cyclic behaviour once stress softening is complete, i.e. the artery is said to be preconditioned (48). The microstructural mechanisms behind preconditioning are unclear, but macromolecule unfolding, as well as irreversible PG deformation, could play an important role.

Mechanical damage and failure effects

Mechanical force is transmitted from the macroscopic (tissue) length-scale down to the atomistic length-scale, and different microstructural constituents are loaded differently. Consequently, raising the macroscopic load leads to local stress concentrations in the tissue, and, if high enough, starts damaging it at specific spots. For example, micro-defects, such as breakage and pull-out of collagen fibrils, gradually develop, which in turn weakens the tissue. In healthy tissues at physiological stress levels, healing such defects is required in order to maintain the tissue's structural integrity. However, at supra-physiological stress levels or for diseased tissues, healing cannot fully repair such micro-defects and the tissue continues to accumulate weak links, which in turn irreversibly diminishes its strength. If the damage level, i.e. the number of defects per tissue volume, exceeds a certain threshold, micro-defects join each other and form macro-defects. Finally, a single macro-defect can propagate and rupture the tissue. Damage and failure-associated inelastic phenomena in the vascular wall are not yet well understood. *In vitro* supra-physiological inflation (51) indicates that softening and plastic phenomena may interfere with each other, and there might be some relation between preconditioning and damage-related phenomena (52, 53).

Active mechanical wall properties

The media consists of complex three-dimensional networks of SMC, elastin, and bundles of collagen fibrils. SMC cover between 20% and 60% of the tissue and provide the vessel with contractile properties to regulate blood flow through vasoconstriction and vasadilation, respectively. Besides being related to hormonal stimuli, neural stimuli, and drugs, the SMC tonus is strongly related to the vessel strain (54, 55) (see ➔ Fig. 3.8).

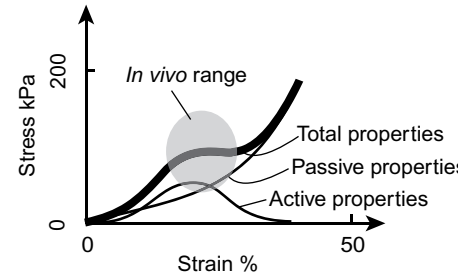

Fig. 3.8 Superposition of passive and active properties define the vessel's total stress strain properties. At *in vivo* deformation smooth muscle cells (SMC) are able to contribute most active mechanical stress.

Stress and strain state in the vessel wall

Wall stress and strain are the mechanical responses to external forces acting on the vessel and are distributed differently throughout the vessel layers. In contrast to strain, stress cannot be measured but has to be predicted (calculated) by solving the equilibrium equation (3.1) together with certain boundary and initial conditions. This equation can only be solved analytically (exactly) for a very few, rather simple problems. One such solution is the well-known Laplace equation that allows us to compute the circumferential wall stress $\sigma_\theta = \frac{pd}{2h}$ [Pa] in a thin-walled circular tube of diameter d [m] and wall thickness h [m] that is inflated at a pressure p [Pa]. However, complex constitutive properties, large deformability of vascular tissue together with complex vessel geometry, especially at regions vulnerable to diseases, makes the derivation of analytical solutions impossible, and stress (and strain) calculations require approximate numerical approaches like the FEM.

The media is formed by discrete medial laminar units (MLU) (56), a structure that is nicely visible in elastic arteries but that becomes more and more blurred in muscular arteries. MLU thickness is independent of the radial location in the wall and the number of units increases with increasing vessel diameter, such that the tension carried by a single MLU in the normal wall remains constant at about 2 [N/m] (56). However, this homeostatic target can clearly not be maintained in diseased vessels, and, in an aneurysm wall, for example, the tension can reach several hundred times this value.

The adventitia contains fibroblasts and fibrocytes embedded in extracellular matrix (ECM) of thick bundles of collagen fibrils. The adventitia is encompassed continuously by loose connective tissue that anchors the vessel to its surrounding. Specifically, the strong bundles of collagen in the adventitia allow very high stresses to be carried, such that the adventitia shields the biologically much more vital

medial and intimal layers from supra-physiological stress, i.e. mechanical overloads.

The ECM is critically involved in load transition from the organ length-scale to the cellular length-scale and provides an essential supporting scaffold for the structural and functional properties of vessel walls. The three-dimensional organization of elastin, collagen, and PGs is vital to accomplish proper physiological functions. The ECM, therefore, rather than being merely a system of scaffolding for the surrounding cells, is an active mechanical structure that controls the micro-mechanical environment, i.e. the amount of stress and strain that is transmitted to the individual cells of vascular tissue. At physiological loads, only 6% to 7% of collagen fibres are engaged (57, 41), such that vascular tissue can cope with much higher stresses than physiological ones prior to mechanical failure.

Vessel tissue modelling

In order to analyse physical processes in the vasculature, constitutive models for vascular tissue are required. The literature on such constitutive models is rich and the level of modelling is strongly linked to the objective of the biomechanical computation. Specifically, hyperelastic models (43, 58, 59, 36), pseudoelastic models (48), viscoelastic models (60), poroelastic models (61), viscoplastic models (53), and damage models (62) are all present in the literature.

Constitutive models of the arterial wall, or layers of it, are either based on a purely phenomenological approach (43, 58) or take structural information of the underlying histology into account (36, 59, 63, 64, 65). A structurally-motivated model facilitates our understanding of the tissue's function and provides an insight into the tissue's response to a given mechanical loading (66).

Vascular tissue is a dynamical structure whose mechanical properties adapt to changes in its environment. Consequently, a class of constitutive descriptions aims at addressing the turnover of key vascular constituents (64, 67, 68).

Blood/vessel interaction

Vascular structure interferes with blood flow and vice versa, either by active contraction of the heart muscle or simply by a passive mechanical reaction to the pulsating flow in larger arteries, for example. For such applications, wall mechanics influence blood flow and vice versa, such that rigid wall models are unrealistic and fluid structure interaction (FSI) modelling is required. Here, the equilibrium equation (3.1) is solved in both domains, i.e. the blood-filled lumen and the vessel wall, and compatibility conditions are enforced at the interface between them. Staggered and monolithic (also referred to as loosely coupled and strongly coupled) strategies are used to solve the FSI problem. Despite the fact that this describes the most general approach of blood/vessel wall interaction, sub-classes of problems with particular solutions are known for specific applications; some of them are discussed here.

Wave propagation

The elastic energy stored in vessels coupled to the mass of blood (and tissue) define the physical conditions for propagating pulse waves. Under the simplified conditions of a circular and thin-walled vessel of radius r [m] and thickness h [m] filled with blood of density ρ [kg/m^3], the pulse wave velocity is given using $v_{PW} = \sqrt{\frac{kh}{2r\rho}}$ [m/s] (known as the Moens–Korteweg equation (69, 70)). Here, k [Pa] denotes the incremental Young's modulus, i.e. the circumferential vessel stiffens at inflation. The Moens–Korteweg equation derives from the equilibrium equation (3.1) under the assumption of small deformations on top of the inflated vessel geometry. Due to the nonlinear stress-strain properties of the vessel wall (see Fig. 3.7) the incremental stiffness k is not constant either (see Fig. 3.3), such that the pulse wave velocity v_{PW} intrinsically changes with blood pressure (71).

Different techniques for the *in vivo* measuring of the pulse wave velocity are known (8), such that vessel stiffness can be non-invasively estimated with the help of the Moens–Korteweg equation.

Permeability

A pressure gradient exists between the arterial circulation and the interstitial pressure in the adventitia, generating a transmural fluid flow radially outwards through the arterial wall. This hydraulic conductance from the lumen to the adventitia conveys substances that can be retained, modified, and activated during their mass transport through the wall (1).

The radial flow flux is determined by the tissue's permeability, i.e. a measure of the ability of a material to allow fluids to pass through it. In the vasculature, permeability is closely related to the endothelial layer that facilitates a selective transport of small molecules (ions, water, and nutrients) and even whole cells (lymphocytes) in and out of the vessel (72). Consequently, the endothelium is a semi-selective barrier between the vessel lumen and surrounding tissue,

which controls the passage of materials and the transit of white blood cells into and out of the bloodstream. The blood–brain barrier (BBB) is probably the most prominent of such filters. Despite vascular permeability being a key physiological mechanism of the microcirculation, it is also vital to supply cells in tissues that are not penetrated by vasa vasorum, such as the media layer of large arteries, valve leaflets and the like.

Knowing the permeability k_p [s/N] (or [Darcy]) and the pressure gradient $\frac{\Delta p}{\Delta x}$ [Pa/m] along the x-direction, allows the computation of the fluid flow $q = -k_p \frac{\Delta p}{\Delta x}$ [m³/s] that establishes along the x-direction. This relation holds for isotropic material, i.e. a tissue whose permeability is independent of the flow direction. For an anisotropic tissue, three such equations apply that involve the three (non-equal) permeabilities along the tissue's three principal directions. Originally, this relation was experimentally derived by Darcy, but it also follows from the equilibrium equation (3.1) via homogenization, i.e. averaging properties over a certain domain. Image modalities, such as diffusion magnetic resonance imaging (MRI), essentially measure the permeability of tissue to water. The permeability of vascular tissue is not a constant but controlled by electrical resistance (73), chemical potentials, tissue stretch (74), and the like.

Poromechanics

Despite interstitial fluid not being particularly mobile in the vessel wall, it can influence the tissue's continuum mechanical properties. Consequently, tissue deformation interacts with interstitial flow and vice versa, phenomena that can be studied by the theories of mixtures and poroelasticity. The theory of mixtures is based on diffusion models (75–78) while poroelasticity models the interaction of deformation and fluid flow in a fluid-saturated porous, elastic medium (79). Despite the fact that mixture theory is more general than poroelasticity, by providing better conceptual mechanisms to integrate physical situations (80), poroelasticity was recently extended to model blood flows through the beating myocardium, for example (81).

Conclusion

Physics, and especially biomechanics, plays a prominent role in the study of the cardiovascular system. Cardiovascular function critically depends on the proper mechanical interaction between blood and the vessel wall, and haemodynamics-based biomechanical factors of the cardiovascular system are a common denominator of cardiovascular pathologies.

Newton's second law of mechanics allowed us to explore many physical phenomena of the cardiovascular system and led to several useful mathematical relations with direct clinical application. Although traditional applied mechanics' concepts are directly applicable, to a certain extent, to solve cardiovascular problems, the inherent complexity of this system remains a challenge. Simple, handy analytical solutions to Newton's law are always linked to strong model simplifications, such that they always have clear application limits.

Investigating clinically relevant problems, i.e. drawing conclusions from the equilibrium equation (3.1) for complex cardiovascular domains requires numerical tools like FEM and CFD. When providing input information, such as three-dimensional geometry or blood velocities at the boundaries of the computational domain, such tools synergetically combine with modern image modalities. However, close interaction between engineering and medical disciplines is critical to the successful exploration of the related physical phenomena.

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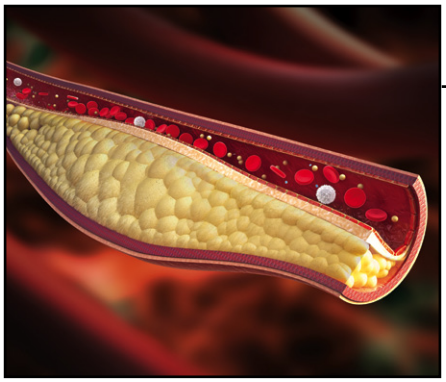

CHAPTER 4

Immunology of the vessel wall

Göran K. Hansson

Content

Introduction

The vasculature is in direct and continuous contact with cells and molecules of the immune system. Indeed, the immune system is dependent on the vasculature for its development, maturation, reactivity, and effector mechanisms. However, different vessels play entirely different roles in immunity. This chapter will describe the general structure of the blood vessel and the general features of its cells, with a perspective on their role in immunity. It will focus on the artery as a battleground for atherosclerosis and the arteritides.

The general structure of the vessel wall includes a flat, single-cell layered endothelium on a rather primitive basement membrane, a subendothelial tunica intima with few cells, a tunica media consisting of vascular smooth muscle cells interspersed with elastic lamellae, and an adventitial layer with connective tissue molecules, including collagen, fibroblasts, and immune cells, nerve endings, and small vessels called vasa vasorum that provide blood supply for the vessel itself (also see Chapter 1). In contrast to the intimal–medial and medial–adventitial borders that are demarcated by elastic lamellae, no distinctive anatomical structure marks the border between the adventitia and the surrounding connective tissues. Instead, the adventitia continues into a looser connective tissue rich in adipocytes.

Immune access through the microvasculature

The general organization of the vessel wall differs, for obvious reasons, between arteries, veins, and capillaries. The latter constitute the largest part of the vasculature and the site of virtually all exchange processes between blood and tissues. The capillaries consist of an endothelial layer, a basement membrane, and a layer of pericytes interspersed with connective tissue structures. This simple organization provides optimal opportunities for the exchange of gas and nutrients but reduces the defence capacity to that of the endothelium itself. Similarly, the post-capillary venules with their flexible endothelial junctions and minimal medial structures permit efflux not only of fluid and nutrients, but also emigration of leukocytes. Therefore, venules are instrumental in inflammatory reactions, by their capacity to increase fluid transport to cause oedema formation, as well as their expression

of leukocyte adhesion molecules that instigate leukocyte recruitment to infected sites.

The intact artery wall is non-permissive for immune reactions

The organization of the artery wall contrasts strikingly to the primitive, flexible structure of the microvasculature. The artery is highly structured yet contains very few specialized immune cells (➔ Fig. 4.1). Instead, it can be viewed as an 'iron curtain' aimed at preventing access by immune cells. Its first component is a tight wall, the endothelium, which is surrounded by demarcation zones and several fences. The first demarcation zone, the intima, contains a loose extracellular matrix with a few patrolling mononuclear phagocytes. Outside it, the internal elastic lamina represents a fence that permits access only through a limited number of holes.

The next zone, the media, is a no man's land for immune cells. Not only does the anatomical organization of the media prevent accumulation of immune cells, it lacks vasa vasorum in its inner part. To access the media, a monocyte or lymphocyte therefore has to penetrate the luminal endothelium, traverse the intima, and pass through openings in the internal elastic lamina. Once in the media, the immune cell will receive strong inhibitory signals to prevent it from becoming activated or producing bioactive immune mediators. This immune inhibition is conferred by the medial smooth muscle cells, which produce tryptophan-derived

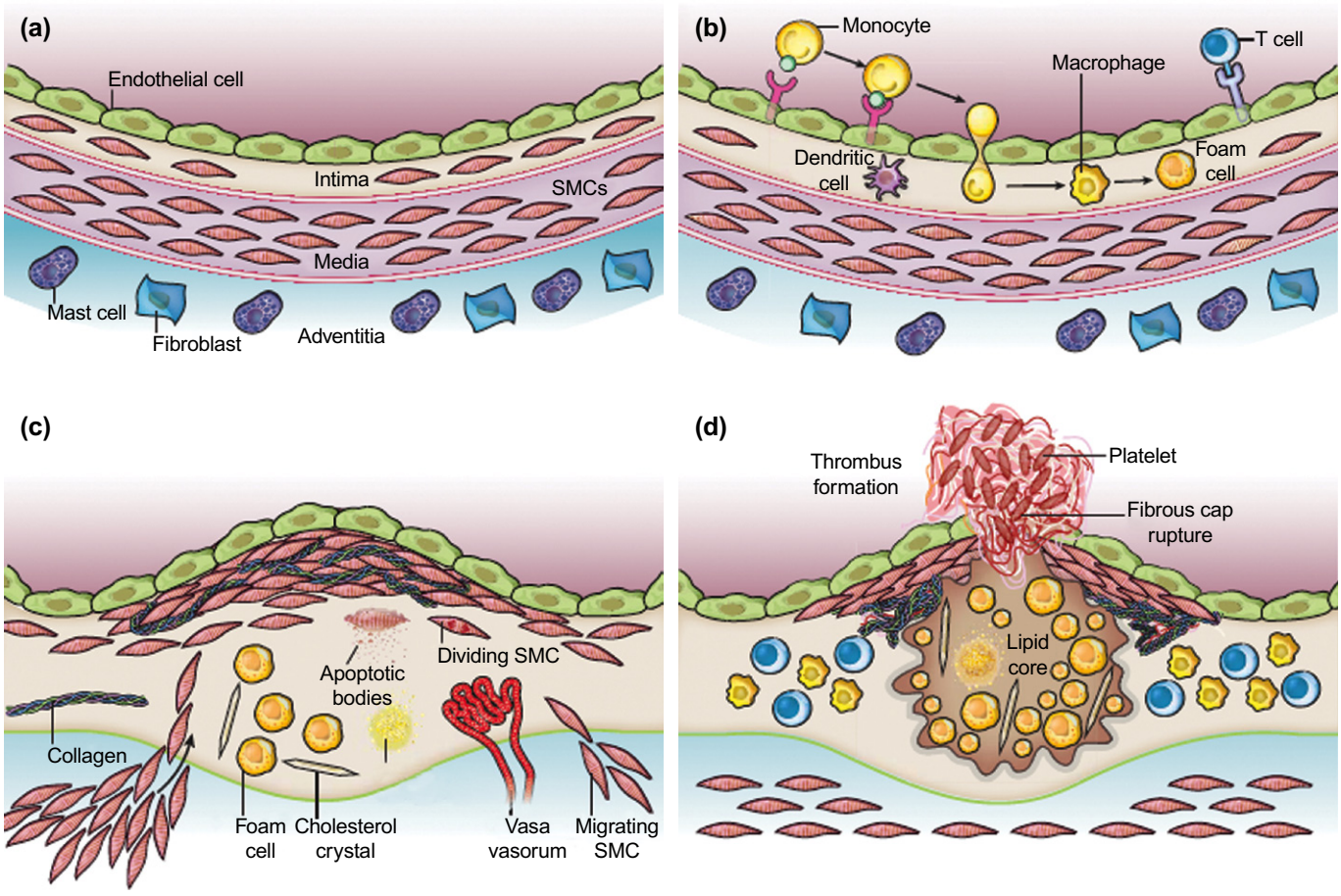

Fig. 4.1 Structure of the normal and atherosclerotic artery. (a) The normal artery is depicted, with its flat endothelium covering the tunica intima containing a few smooth muscle cells in a loose extracellular matrix. Underneath it, the tunica media is comprised of smooth muscle cells together with elastic lamellae. Outside of the media, the tunica adventitia contains several different cell types in a connective tissue matrix. (b) The atherosclerotic process is characterized by infiltration of immune cells, mainly monocytes and T cells, into an enlarged intima with lipoproteins, cell debris, and cholesterol. (c) A chronic inflammatory lesion develops, with repair processes including recruitment of smooth muscle cells and vasa vasorum, and formation of a fibrous cap covering the necrotic core portion of the lesion. (d) Clinical complications such as infarction or stroke occur if thrombi form on the lesions. Atherothrombosis is often triggered by fissuring of the plaque cap, leading to exposure of thrombogenic material (shown in the figure). Alternatively, it may arise due to endothelial surface desquamation (not shown).

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anti-inflammatory kynurenines generated through the indoleamine dioxygenase (IDO) pathway (1). In addition, these cells can express the immunosuppressive cytokine, transforming growth factor-β (TGF–β), which is deposited in the extracellular matrix of the artery (2). Therefore, the molecular conditions of the arterial media make it an immune-privileged area similar to the eye or testis.

The adventitia is entirely different from the inner parts of the artery wall, also with respect to its immunological properties. If the intima and media represent a dead man's zones around an iron curtain, the adventitia is the free world beyond them. Here, a multitude of different cell types intermingle in a loose connective tissue that permits dynamic interactions. A rich supply of blood and lymph offer an excellent transport capacity for cells and molecules, while autonomic nerve endings provide regulatory signals that integrate the perivascular tissues with those of the organism at large.

The endothelium—first line of defence

Endothelial cells are constantly exposed to the circulating blood and with it to all kinds of potential dangers, including bacteria, viruses, and microbial toxins. To handle such challenges, the endothelial cell is capable of mounting innate immune responses and also of recruiting professional immune cells to a site of attack.

Endothelial receptors of innate immunity

The normal endothelium expresses several pattern-recognition receptors, including Toll-like receptors (TLR) 2 and 4 (3). In addition, several other TLRs can be expressed upon inflammatory activation of the endothelial cell; for instance, by interleukin (IL)-1 or lipopolysaccharide (LPS) derived from Gram-negative endotoxins. Endothelial TLR expression is also enhanced at sites of disturbed blood flow in the vascular tree (4). Ligation of cell-surface bound TLRs by microbial toxins triggers a signal transduction cascade that instigates production of proinflammatory mediators, including cytokines, chemokines, eicosanoids, and reactive oxygen species (5).

Endothelial cells can also express intracellular pattern-recognition receptors, including endosomal TLR-3, -7, and -9, and also the cytosolic nuclear oligomerization domain (NOD)-like receptors (5, 6). Although expression of these receptors is minimal in normal arteries, endothelial cells of inflamed and atherosclerotic arteries express substantial amounts of intracellular pattern-recognition receptors.

Enzymatic signalling cascades downstream of signalling pattern-recognition receptors transduce receptor ligation into novel gene expression. Proinflammatory pattern-recognition receptors typically activate NF-κB, mitogen-activated protein kinase (MAPK), and interferon-response pathways in a complex, interactive pattern with crosstalk between cascades. Some aspects of these intracellular signals are identical to those elicited by proinflammatory cytokines, such as interleukin-1.

Finally, endothelial cells express several endocytic pattern-recognition receptors of the scavenger-receptor family. Expression of CD36 by the arterial endothelium may be important in hyperlipidaemia and atherosclerosis, whereas its expression in the microvasculature has been associated with malaria, particularly due to the capacity of CD36 to bind *Plasmodium*-coated erythrocytes (7, 8). Additional scavenger receptors that can be expressed by endothelial cells include LOX-1, SREC-1, and SR-B1 (9, 10).

Endothelial leukocyte adhesion molecules

The capacity to recruit blood cells is for endothelial cells. Platelet–endothelial interactions are key in haemostasis and emigration of granulocytes, monocytes, and lymphocytes play a central role in host defence. P-selectin is constitutively expressed by the endothelium, whereas E-selectin is inducible (11–13). Proinflammatory stimuli such as TNF, IL-1, and LPS induce surface expression by endothelial cells of E-selectin that interacts with carbohydrates of blood group antigen Lewis^x type on myeloid cells and T cells. In addition, these proinflammatory stimuli also induce endothelial expression of several other leukocyte adhesion molecules (LAM), including the Ig-like transmembrane receptors, ICAM-1 and VCAM-1, which ligate integrins CD11/18 and VLA-4, respectively. The combinatorial expression of endothelial LAM and leukocyte integrins and selectins provides a sophisticated anatomical and temporal control of the inflammatory process, by determining the type and place for recruitment of a certain type of myeloid or lymphoid cell to the vasculature.

Endothelial antigen presentation

The endothelium shows a remarkable capacity to contribute not only to innate, but also adaptive immune reactions. When exposed to allogeneic T cells, endothelial cells present their MHC molecules, thus triggering immune attack (14). Under more physiological conditions, endothelial cells express MHC class I molecules and can be induced to express MHC class II molecules when exposed to interferon-γ, a cytokine produced by T and NK cells (15).

MHC class II molecules (in humans, HLA-DR, DQ, and DP) bind peptides derived from endocytosed antigens to cognate T cells; the type of response elicited depends on the state of the responding T cell, as well as signals derived from the antigen-presenting cell. When cytokine-activated endothelial cells present antigen, they can activate effector T cells. This can lead to secretion of proinflammatory mediators, inflammation, and cytolytic attack, depending on the precise circumstances. However, endothelial cells cannot activate naïve T cells, this being a property of specialized dendritic cells of the immune system (16). When activating effector T cells, endothelial cells are significantly less effective than macrophages but more effective than fibroblasts or vascular smooth muscle cells. These differences are due to the capacity of the different cell types to express costimulatory factors and cytokines that enhance T cell activation. In addition, signalling molecules expressed by endothelial cells, such as CD31, may inhibit any immune responses (17, 18).

With respect to their role in adaptive immunity, endothelial cells have been termed 'semi-professional' antigen-presenting cells. They are probably very important as propagators of immune reactions; for instance, when antigens are distributed systemically during a pathogen challenge or when an organ is afflicted by an infection or an autoimmune reaction.

Medial smooth muscle—trying to stay out of trouble

Vascular smooth muscle cells (VSMC) of the media are specialized in tonic contraction and matrix production. They normally participate in inflammatory reactions by modulating vascular tone but have a limited capacity to contribute in immune reactions. The smooth muscle cell population that resides in the intima displays more immune-related activities, such as cytokine secretion. Depending on the strength or length of stimulation, 'synthetic' and 'contractile' smooth muscle cells can adapt certain features of immune cells, including MHC gene expression and cytokine secretion.

Autocrine production of immunomodulatory cytokines

Vascular smooth muscle cells (VSMC) produce transforming growth factor-β (TGF-β), a cytokine with powerful fibrogenic activities that contribute to the stability of the artery wall (19, 20). It is also an important anti-inflammatory and immunosuppressive cytokine (21). When produced by regulatory T cells, it can inhibit ongoing immune responses and bias immune reactions towards anti-inflammatory effector pathways. Smooth muscle production of TGF-β can have similar effects, although post-translational processing is critical for the action of this cytokine. Vascular TGF-β tends to be deposited in latent form on the extracellular matrix and is released upon conversion to active cytokine by enzymatic attack (19, 22). TGF-β contributes to the maintenance of a vascular immunoprivilege and plays an important role in stabilizing the vessel wall during scar formation after injury and fibrous cap formation on atheromata.

Indoleamine 3-dioxygenase (IDO) is expressed by vascular SMC and may also contribute to the medial immunoprivilege (1). This enzyme converts the amino acid tryptophan to kynurenine, which is further degraded to the bioactive compound, 3-hydroxyanthranilic acid (3-HAA) and eventually to quinolinic acid. 3-HAA is a powerful immunosuppressive and anti-inflammatory compound that inhibits immune activation, promotes formation of regulatory T cells, and dampens inflammatory reactions (23). 3-HAA is a powerful anti-atherosclerotic agent, both by reducing vascular inflammation and by lowering plasma lipids (24–26).

Proinflammatory signals arouse SMC

VSMC of the normal artery wall express few if any TLR proteins. However, TLR expression can be induced by proinflammatory stimuli or infection. For instance, LPS induces TLR4 expression by cultured VSMC (27). This suggests that VSMC display low levels of functional TLR4 receptors that, upon ligation, initiate a signal that is transduced into abundant TLR4 gene expression.

VSMC and endothelial cells can produce, as well as respond to, interleukin-1α and β (28), although this process is tightly regulated (29). IL-1 can, in turn, initiate secretion of large amounts of IL-6 (30). The latter cytokine exerts various metabolic effects, locally and systemically, and therefore serves as a bridge between inflammatory and metabolic responses.

As a consequence of stimulation with TLR ligands and proinflammatory cytokines, such as IL-1 and TNF, VSMC turn on a proinflammatory gene-expression programme. It includes the enzymatic apparatus required for prostaglandin E1 production (31), an array of pattern-recognition receptors (5), and the leukocyte adhesion molecule, VCAM-1 (32). Through the action of these mediators, the VSMC population modulates into a proinflammatory phenotype that is permissive for vascular inflammation.

VSMC in adaptive immunity

VSMC respond vividly to immune cytokines produced by Th1 and Th17 cells of the immune system. Interferon-γ,

produced by Th1 cells and NK cells, induce MHC class II gene expression in VSMC (33, 34). In contrast to endothelial cells, MHC-II + VSMC do acquire antigen-presenting capability but may enhance immune reactions as third-party stimulators of T-cell activation (35). Furthermore, interferon-γ inhibits actin gene expression, contractile capacity, and collagen production in VSMC (36, 37). This response helps the blood vessels to adapt to conditions during an immune response to pathogens but may be detrimental under conditions of pathological chronic inflammation.

Activated Th17 cells produce IL-17A, a cytokine with immune modulatory and profibrotic activities. VSMC respond to IL-17A by enhanced proliferation and collagen production (38). This response helps initiate repair processes and can contribute to formation of fibrous caps that stabilize atherosclerotic plaques.

The proinflammatory, NF-kB activating cytokines TNF and IL-1 synergize with interferon-γ that operates through the Jak/STAT1 pathway in inducing inflammatory gene expression. Among the set of genes expressed, nitric oxide synthase (NOS)-2 exerts important effects on vascular function (39, 40). It is a highly efficient catalyser for the conversion of arginine into citrulline and nitric oxide (NO). NO produced through this pathway can effect vasorelaxation by acting on VSMC and inhibit platelet aggregation (40). This mechanism contributes to vasodilation and vascular patency in inflammation and after tissue injury.

The vessel wall under attack

Attacks on the vessel wall, by infectious agents, toxic metabolites, or mechanical strain, are handled by mobilizing the immunological response capacity of the tissue. The response differs significantly depending on the site of attack. Whereas the intima has poor capacity for immune reactions and the media is immunoprivileged, the adventitia can mobilize strong immune reactions that involve organization of several sets of immune cells into tertiary lymphoid structures.

The enemy within—attack from the lumen

Pathogens and pathogenic stimuli that reach the artery from the lumen are primarily handled by activation of innate immune responses. They include expression of pattern-recognition receptors, production of proinflammatory cytokines, eicosanoids, and nitric oxide, secretion of cytokines and chemokines, and release of proteolytic enzymes. Chronic stimuli operating from the lumen tend to cause mononuclear cell infiltrates with monocytes, monocyte-derived macrophages, and T cells.

An intense intimal immune response is observed in chronic allograft rejection, when CD8 + T cells attack 'foreign' allogeneic MHC molecules on the graft endothelium and subendothelial cells. This leads to dense mononuclear infiltrates dominated by cytotoxic T cells that attack and destroy vascular cells and may cause intimal thickening, obstruction of blood flow, thrombosis, and ischaemia. Hence, the term chronic allograft vasculopathy has been coined for such chronic vascular rejection. It is alleviated by immunosuppressive drugs but remains a major problem in organ transplantation.

Atherosclerosis is a chronic intimal inflammation that is elicited by accumulation of cholesterol-rich lipoproteins in the intima (➔ Fig. 4.1) (26, 41, 42). Preferentially occurring at sites where transendothelial permeability has increased due to haemodynamic disturbance, cholesterol accumulation in intimal macrophages leads to formation of cholesterol microcrystals that activate an inflammasome (43, 44). This, in turn, leads to processing and release of IL-1β, a proinflammatory cytokine that initiates vascular inflammation. In addition, peptide sequences in apoB100, the protein component of low-density lipoprotein (LDL), can activate inflammatory responses with ensuing chemokine production in the artery wall (45). Finally, oxidatively modified LDL particles may directly ligate TLR4, thus initiating inflammatory reactions.

CD4 + T cells are recruited to the forming lesion by leukocyte adhesion molecules and chemokines produced as a consequence of innate immune activation (46). A proportion of these T cells recognize LDL as an (auto)antigen and are activated in the artery (26, 47, 48). A prevalent T-effector cell response is of the Th1 type that involves secretion of interferon-γ, TNF, and other proinflammatory cytokines (49). These mediators operate together with cell-surface molecules to promote macrophage activation, endothelial activation, VSMC modulation, and the development of a chronic intimal inflammation.

In addition to Th1 cells, effector T cells of the Treg and Th17 subtypes may operate in the lesion (38, 50). The former inhibits immune responses and inflammation, whereas the latter promotes fibrosis and cap formation. Therefore, these two T cell types should be considered as atheroprotective.

The adventitia under attack

Complex, adaptive immune responses develop in the adventitia and the periadventitial connective tissues. Antigens reach the adventitia via the vasa vasorum and also through conduits that are fibrous protein structures along which macromolecules can travel from the inner layers of the artery. Antigen-presenting cells in the adventitia

include occasional dendritic cells and significant numbers of macrophages.

T- and B-cell activation can be observed in the adventitia in several pathologies. A striking example is giant cell arteritis (51). In this condition, clonal CD4 + T cells, largely of the Th1 and Th17 types, aggregate around the external elastic lamina, where they promote formation of macrophage-derived giant cells. Several possible antigens have been identified serologically and proposed to play aetio logical roles. They include the nuclear envelope protein, lamin C, the iron-storing protein ferritin, and proteins of *Staphylococcus epidermidis*. However, no definitive data are yet available regarding the aetiology of this condition.

In advanced stages of atherosclerosis, large tertiary lymphoid structures may develop in the adventitia and periadventitial tissue (➔ Fig. 4.2) (52). They have been termed adventitial tertiary lymphoid organs and contain germinal centres with B cells going through differentiation to centrocytes and plasma cells. Surrounding them, dendritic cells, T cells, and macrophages form organized structures of interacting cells.

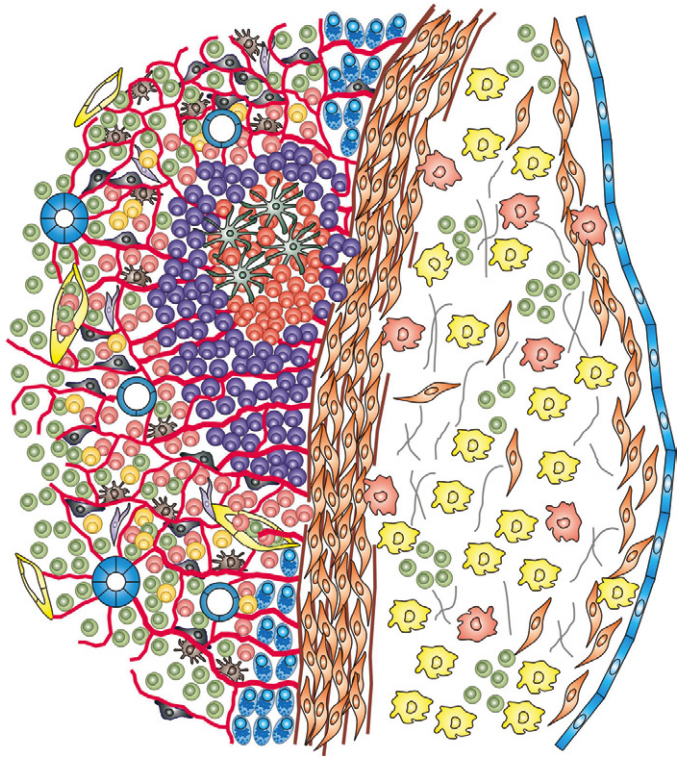

Fig. 4.2 Tertiary lymphoid structure in the adventitia of atherosclerotic artery. The intima (right) contains an atherosclerotic lesion with macrophages (yellow and pink), T cells (green), and smooth muscle cells (brown) under the endothelial layer (blue). The media is composed of smooth muscle cells (brown) and elastic lamellae (brown). The adventitia contains multiple cell types, including the round germinal centre with B cells in various differentiation stages (violet, orange) and plasma cells (blue), as well as T cells (green), dendritic cells (tree-like, brown), follicular dendritic cells (tree-like, green), tissue macrophages (violet, elongated), endothelial cells, some of which are forming high endothelial venules (blue), and lymph vessels (yellow) and acellular conduits (red).
(Reproduced from Gräbner R, Lotzer K, Dopping S, Hildner M, Radke D, Beer M, et al. Lymphotoxin beta receptor signaling promotes tertiary lymphoid organogenesis in the aorta adventitia of aged *ApoE*$^{-/-}$ mice. J Exp Med. 2009;206(1):233-48 with permission from The Rockefeller University Press.)

Adventitial tertiary lymphoid organs are sites of antibody production, including antibodies to plasma lipoproteins. Interestingly, deposits of ceroid-containing oxidized lipids are also found here, suggesting that they may serve as antigenic stimuli for antibody production (53).

Lymphatic vessels drain the adventitial side of the artery, their contents being delivered to periarterial and other regional lymph nodes (➔ Fig. 4.3). Primary immune reactions to artery-derived antigens take place here. It has been proposed that dendritic cells patrolling the artery pick up antigens such as accumulating plasma lipoproteins, structural proteins, and components of microbial pathogens *en route* to draining lymph nodes (54–57). Here, they activate naïve T cells and the microenvironment determines the differentiation pathway of the activated T cell. For instance, IL-6 produced in atherosclerotic lesions may synergize with local transforming growth factor-β to induce differentiation of lipoprotein-reactive T cells into Th17 effector cells that promote collagen synthesis and fibrous cap formation in the lesion (38).

Among immune effector cells of the adventitia, mast cells are of particular interest as they produce important vasoactive mediators, including histamine and leukotriene C4, anticoagulant heparin, platelet-aggregating-factor, and several enzymes (58). Mast cells are typically activated when their Fc receptors ligate IgE molecules but they can also be activated by pattern-recognition receptor ligation and by complement activation. By producing serotonin, mast cells provide a signal between the immune and nervous systems.

The adventitia contains autonomous nerve endings that impact on vascular permeability. Transmitters of the autonomic nervous system may also modulate immune activation (59) and the adventitia may be a site of such interactions.

In conclusion, the immune response capacity of the normal artery ranges from simple, innate immune activity of the intima, via an actively immunosuppressed media, to a complex immune activity in the adventitia. Disease conditions change this general pattern drastically, an important example being atherosclerosis. Thus, the atherosclerotic intimal lesion is a site of adaptive, as well as innate, immune reactions, and the adventitia surrounding lesioned arteries may even develop tertiary lymphoid organs characteristic of infectious and autoimmune diseases.

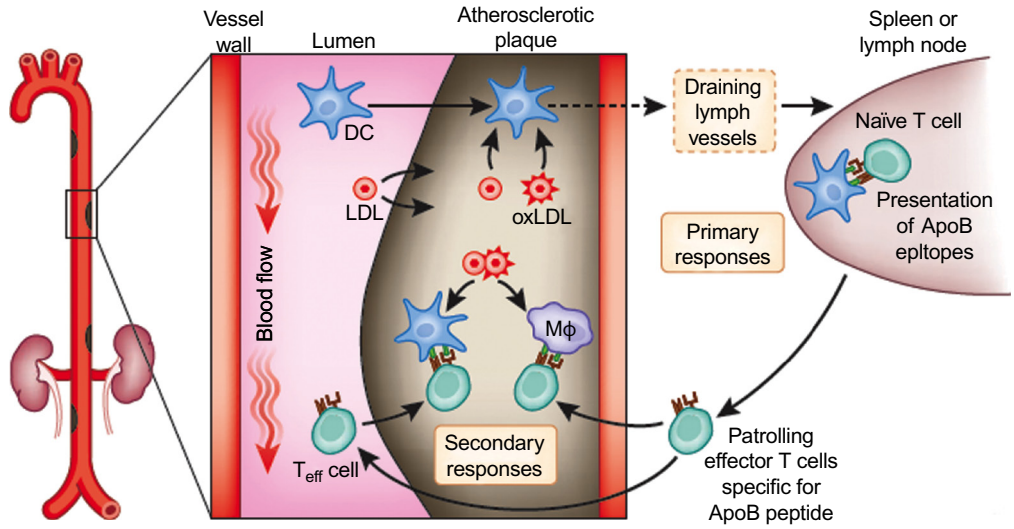

Fig. 4.3 T cell activation in the artery wall. Dendritic cells (DC) may pick up antigen, such as low-density lipoprotein (LDL), while travelling through the artery wall. When they reach the draining lymph nodes, DC present antigen to naïve T cells. In the case of LDL recognition, oligopeptides of the LDL protein, apoB act as epitopes for clonotypic T cells. Upon activation, T cells differentiate into effector T cells (T eff), the phenotype of which depends on signals present in the micromilieu during activation. T eff cells patrol tissues and may reach atherosclerotic lesions through the bloodstream. Here, T eff cells can be reactivated by antigen-presenting DC and macrophages (MΦ); this leads to secretion of cytokines that can accelerate or modulate disease development. (Reproduced from G K Hansson and A Hermansson, The immune system in atherosclerosis. Nat Immunol 2011; 12:204–212 with permission from Nature Publishing Group.)

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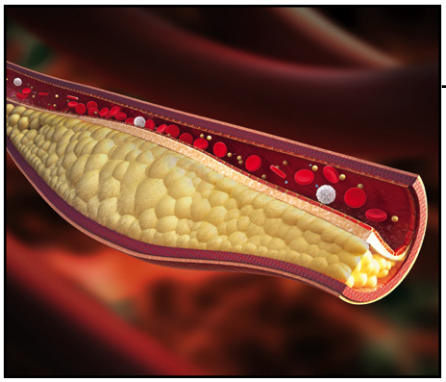

CHAPTER 5

Animal models to study pathophysiology of the vasculature

Wenduo Gu, Yao Xie, and Qingbo Xu

Content

Introduction

Vascular disease, especially arteriosclerosis, which is the principal cause of heart attack, stroke, and gangrene of the extremities, remains a major contributor to morbidity and mortality in the Western world (1). Various factors, including genetic polymorphism, hypercholesterolaemia, modified lipoproteins, hypertension, diabetes mellitus, autoimmune responses, infections, and smoking, were identified as being involved in the development of cardiovascular diseases. However, the aetiology and pathogenesis of many vascular diseases still have not been fully elucidated. Arteriosclerosis is characterized by smooth muscle cell hyperplasia or hypertrophy and matrix protein accumulation in the intima and/or media with or without lipid deposition, resulting in thickening and increased stiffness of the arterial wall (2). Arteriosclerosis includes (spontaneous) atherosclerosis, accelerated arteriosclerosis (namely, transplant arteriosclerosis), restenosis after percutaneous transluminal coronary angioplasty, and vein graft atherosclerosis (3). The atherosclerotic lesion is defined by arterial intimal cell proliferation, lipid accumulation, and connective tissue deposition. Depending on their size and composition, the lesions are usually divided into fatty streaks, predominantly consisting of lipid-rich macrophages and T lymphocytes within the innermost layer of the artery wall, and plaques, which are advanced stages of the lesions as suggested by their name 'atheroma' (4–6). The three major cellular components of human atherosclerotic plaques are the smooth muscle cells, which dominate the fibrous cap, the macrophages, which are the most abundant cell type around the necrotic core, and the lymphocytes, which are mainly ascribed to the fibrous cap (7). Although clinical investigation is an important approach for understanding the aetiology and therapy of these diseases, animal models prove to be effective tools for understanding the pathogenesis and intervention as well.

The first vascular research with animal models dates back to 100 years ago (7), which was a rabbit model published by Ignatowski in 1908. Knowledge of the pathogenesis and therapy of atherosclerotic disease and the use of animal models in arteriosclerosis research have evolved almost simultaneously. The use of animal models in the study of arteriosclerosis is essential in a set of aspects.

For instance, evaluation of a risk factor as a single independent variable, with almost complete exclusion of other factors, can best be performed in animals free of intercurrent diseases or abnormalities and with well-known genetic characteristics (8). On the other hand, the role of vascular injury due to angioplasty, alloimmune responses, or vein grafts can be investigated alone or in combination with other factors that either aggravate or have beneficial effects (9). Furthermore, experiments using animals are the only way to develop and test new diagnostic, preventive, and therapeutic procedures for both ethical and practical reasons. The investigator can choose the species, time, and method, and obtain samples of both tissue and serum as well, as these methods would be difficult, if not impossible, in studies with human subjects.

The rabbit is the first and one of the most frequently used animals at all points of arteriosclerosis research. Lesions analogous to those in humans have been induced in the rabbit by a variety of methods. Since rabbits are very sensitive to a cholesterol-enriched diet, hypercholesterolaemia and atherosclerotic lesions in the intima of large arteries can be obtained shortly after administration of the diet. The Watanabe heritable hyperlipidaemic (WHHL) rabbit, which is an excellent model of homozygous familial hypercholesterolaemia in humans, has also been used.

Rats are insensitive to a cholesterol diet, but are used as a model for transplant arteriosclerosis and restenosis after angioplasty. Actually they are the first animal models of endothelial injury mimicking angioplasty in humans (10). Recently, transgenic and knockout rats have been generated allowing greater insight into the genetic basis and pathogenesis of vascular diseases. These animal models display a great potential for studying diet-induced atherosclerosis and for new drug discovery.

Swine, as a close-to-clinical model, have become increasingly popular these days. Available evidence indicates that the pig is an optimal model for vascular disease because of the similarities with humans. The pig genome is highly similar to humans. Its chromosome structure is comparable to that of humans. This similarity with humans in cardiovascular anatomy, physiology, lipid profile, and lipoprotein metabolism has also been demonstrated. In addition, arterial structure and its response to hypercholesterolaemia and platelet aggregation resembles that of humans. Importantly, swine models can be used for vascular grafting, which is similar to human situations.

Due to well-defined genetic systems of transgenic and knockout mice, a number of investigators have used the mouse as an experimental system for arteriosclerosis research (8). Hundreds of inbred lines have been established, and the genetic map is relatively well defined, and both congenic strains and recombinant strains are available to better serve genetic experimentation. In just a few years, murine lipoproteins have been characterized, genetic variants of apolipoproteins identified, and genetic variation in susceptibility to atherosclerosis among inbred mouse strains demonstrated (11–13). In addition, reduced experimental animal numbers can be achieved due to inbred strains having low variability, which is also of economic benefit. The mouse is becoming a widely used model for studying all aspects of arteriosclerosis, and thus, this chapter will discuss all types of mouse models for vascular disease, highlight the usage of rat, rabbit, and pig models, and attempt to update progress in the use of animal models for studies on vascular diseases.

Mouse models of atherosclerosis

Atherosclerosis is now a leading cause of human death characterized by the development of atheromatous plaques within the vessels. Human atherosclerosis undergoes stages as follows (14). First, the adhesion of leukocytes to activated endothelial cells induces the migration of more leukocytes from blood and the maturation of monocytes into macrophages, which will uptake lipids to produce foam cells. Second, the migration of smooth muscle cells from the media to intima, followed by the proliferation of SMCs, and releasing extracellular matrix macromolecules. Smooth muscle cells and macrophages undergo apoptotic death during the process of plaque formation, leading to the release of lipids in the central zone of plaque. In a typical atherosclerosis, three distinguished areas can be identified via different proportions of SMC, macrophage, matrix, and inflammatory cells: fibrous cap, lipid zone, and basal zone (2). In more advanced plaques, cholesterol crystal cores and microvessels are the biomarkers. The final stage involves some serious complications of atherosclerosis, including thrombosis, plaque rupture, calcification, and intra-plaque haemorrhage (15).

In the 1960s, Wissler used a high-fat diet to induce atherosclerosis but the diet was toxic to mice (16). Then Paigen (4) modified the formula; however, it still has disadvantages. Even after modification, the diet-induced atherosclerosis in mice was not as obvious as that in larger animals. The mouse is naturally resistant to atherosclerosis and the lesions in mice are very small and limited to the early fatty-streak stage. In 1992, a great progress was made by genetic manipulation. With the help of genetic techniques, apoE-, LDL receptor-deficiency mice, as well as other genetic mouse models, were created to induce more human-like atherosclerotic lesions

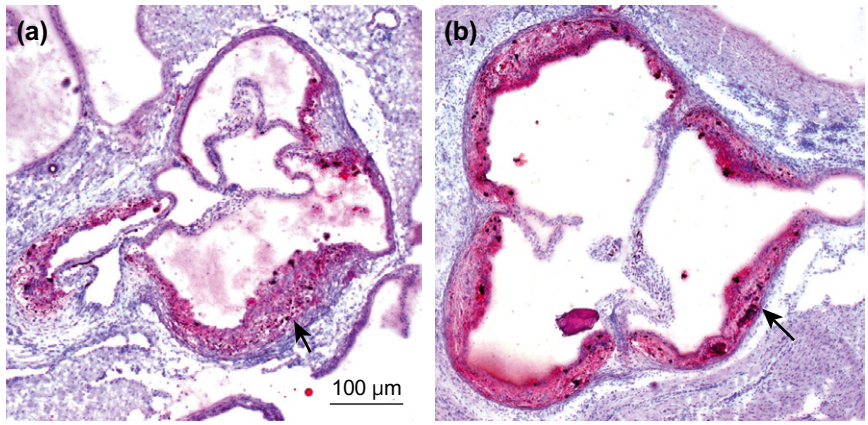

Fig. 5.1 Image representation of aortic root lesions of atherosclerosis from apoE (a) and LDL (b) deficient mice at age of 36 months with normal chow diet.

(➔ Fig. 5.1) (17). Numerous animal models based on transgenic mice were invented then to mimic various kinds of pathological states in humans.

At present, the mouse is the most widely used species in the cardiovascular field. In atherosclerosis studies, although there are some variances between mice and humans, such as different heart rates, atherosclerosis generation time, and site of atherosclerosis, they still share a lot of critical features (➔ Table 5.1). The second mouse model frequently used for vascular disease is the aneurysm model, which has provided multiple insights into the mechanisms of human aneurysms. Furthermore, both atherosclerosis and aneurysm-using mouse models share fundamental advantages, such as their low cost of maintenance, ease of breeding, ease of genetic modification, and variable ways to monitor the atherosclerotic process (7). However, mice and humans have different lipid profiles, limiting the use of mouse models in medical research. The main circulating cholesterol in mice is high-density lipoprotein, and it does not have cholesterol-ester transfer protein. In addition, the mouse hardly absorbs dietary cholesterol—this is the reason why wild-type mice are normally resistant to atherosclerosis. Therefore, scientists have used DNA technology to overcome this limitation. Now both apoE- and LDL receptor-knockout mice are widely used in the investigation of a variety of vascular diseases (7). These mice will not show obvious vascular lesions when fed a chow diet but may present lesions following an atherogenic diet. Meanwhile, other genetic-deficient mice, such as hepatic lipase-deficiency and human apoB100 expression, have also been generated to be used in the atherosclerotic field.

Table 5.1 Mouse models of vascular diseases

Advantages	Disadvantages
Small, easy to raise, handle and perform surgery.	No naturally occurring atherosclerosis.
Large groups.	Few biological samples (small).
Ease of genetic modification.	Technical difficulties (small).
Low cost of purchase and maintenance.	Unstable atherosclerosis.
Many well-established mouse models to induce atherosclerosis.	Different sites of atherosclerosis and aneurysms.
Minimal amounts of drugs or compounds are needed due to small size.	Different lipid profile compared to humans.
Short lifespan.	
Fully recognized genome.	

Diet-induced atherosclerosis

At present, experimental mice are fed on a high-fat diet and many other kinds of ingredients emerge according to the researchers' requirements. Atherosclerosis induced by this method is different depending on the ingredients within the diet. Wild-type mice have naturally a very low concentration of triglycerides and LDL in the serum, but have a very high HDL concentration, which would contribute to reducing atherosclerosis. Under certain circumstances, for instance, by feeding a Western-type diet that contains 35% fat, 50% carbohydrate, 15% protein cholesterol (0.5 to 1%), and cholic acid (0.1% to 0.5%), mice can be induced to raise their LDL and triglyceride levels and can display mild atherosclerosis. The cholic acid in Western-type diet can prevent cholesterol from converting into bile acid. In addition, the presence of cholic acid helps absorb the fat and cholesterol, which increases the level of cholesterol in the circulation (18). It is worth noticing that cholic acid has an effect on inflammation and lipoprotein metabolism, which are also the key factors of atherosclerosis. Atherosclerosis is induced with an atherogenic diet in apoE knockout mice and LDL-receptor knockout mice, which has dramatically accelerated the research in atherosclerosis (19).

Lesion quantification

For quantification of atherosclerotic lesions, there are two methods in mouse models with the most frequently used mode being the quantification of lesion size in the aortic root (5). This mode of analysis was initially described for mouse atherosclerosis in the much cited publication from Paigen and colleagues (5). Another common mode is the quantification of lesions on the intimal surface of the aorta, a process that is frequently referred to as '*en face*'. A similar form of analysis was commonly used in larger animal models, prior to its introduction into the mouse atherosclerosis field by Palinski and colleagues. There are also less commonly used forms of analysis such as quantification of the innominate artery. This form of analysis was described initially by Paigen et al. (20) to quantify lesion size formed in C57BL/6 mice fed a diet enriched in saturated fat, cholesterol, and cholate. A novel approach for quantification of lesions was needed, because small lesions form only in the aortic root of these mice, despite the relatively severe dietary manipulations. A detailed description of this analysis has been published previously (21). The analysis of this region can be performed on either paraffin-embedded tissues or frozen tissues with the majority of studies that quantify atherosclerotic lesion size in the aortic root performed on sections that have been cut using a cryostat. This method is preferred because of the greater ease with which the tissue can be orientated, which will be discussed later. Also, sections that will be stained for neutral lipids need to be frozen sections, since lipids are removed in the process of embedding tissues in paraffin.

Advanced lesions—plaque rupture

Recently, mouse models were also used to study plaque rupture (22). Mature atherosclerotic plaque in humans can be divided into two phenotypes: stable and 'unstable' or 'vulnerable', with the latter terms pathologically referring to plaques that are characterized by a large lipid core with extensive calcification (40–50% of the total plaque volume), thinner fibrous caps (sometimes less than 100 μm), and greater infiltration of macrophages in the plaque area than stable plaques (23). Mouse models that faithfully present morphological similarity with human plaque rupture or identical plaque vulnerability are rare. Spontaneous plaque rupture in apoE knockout mice was reported from two laboratories almost at the same time. Rosenfeld et al. (24) demonstrated intra-plaque haemorrhage and rupture of plaque in chow diet-fed apoE-deficient mice between 24 and 60 weeks of age. Similar observations were reported in apoE-deficient mice fed with a diet containing 21% pork lard and 0.15% for 14 months (25). Investigation on LDL receptor-deficient mice also showed signs of intra-plaque haemorrhage and rupture when fed on a cholesterol-enriched diet. Thrombus formation is rare in mice, unlike in humans, which may be attributed to differences between the vessel morphology and shear stress between them. Thus, it has to be kept in mind the difference of plaque rupture between humans and mice.

Mouse models of aneurysms

Aneurysm is a localized, blood-filled, and balloon-like bulge in the wall of a blood vessel. It is a result of a weakened blood vessel wall caused by either a congenital condition or acquired disease. Normally, aneurysms undergo three key steps: proteolysis, inflammation, and smooth muscle cell apoptosis (26). Aneurysm is a permanent dilation of the arterial wall, which can cause a sudden death due to vessel rupture. At present, more than 8% of men aged over 65 years suffer from abdominal aortic aneurysms without any previous symptoms (27). An ideal animal model should be the mirror of the pathology of human aneurysms. Mouse models for aneurysms are widely used to produce disease by chemical approaches or gene manipulation. The purpose of most of these models is the reduction of aneurysm severity by administration of matrix metalloproteinase inhibitors. At present there are several mouse models for aneurysm established.

Angiotensin-II-induced aneurysms

Angiotensin-II is proven to promote the severity of aneurysms when it is infused into apoE$^{-/-}$ and LDL receptor$^{-/-}$ mice with a subcutaneous osmotic minipump (28). Unlike the most common location of human abdominal aortic aneurysms, which is the infrarenal aorta, angiotensin-II-induced aneurysms usually occur in the suprarenal aorta (15). Infusion rates of angiotensin-II that generate aneurysm range from 500 to 2,500 ng/kg/min (29). Most studies have used the rate of 1,000 ng/kg/min that generates aneurysm in more than 80% of hyperlipidaemic male mice. The calculation of the dose of angiotensin-II to be given to the mouse is determined by the required infusion rate and the weight of the mouse, taking into consideration the weight of the mouse over the duration of the 28-day infusion. The infusion rate of angiotensin-II is based on the daily dose, weight of the mice, and the infusion rate of the specific batch of Alzet pumps (28). Most of the studies implant the pumps in the subcutaneous space. Because female mice groom more extensively than males, they have a greater propensity to open the incision site. The quantification of the aneurysm severity is challenging due to the heterogeneity

of aneurysms. There are three common approaches to the quantification of experimental aneurysms, which are: (1) percentage of incidence, (2) severity based on arbitrary comparison, and (3) measurement of the physical dimensions. Histologically, cross-sections of the aortic aneurysms (2.5mm in thickness) are made between the superior mesenteric and right renal arteries. The lumen and adventitial circumferences at the maximal expanded portion of the suprarenal aorta are quantified by imaging systems, which are then used to calculate the luminal and outer diameters of the vessel (28). The wall thickness is calculated from the difference between the luminal and outer diameters.

Angiotensin-II-induced aneurysms have been used in many studies to define mechanisms of the disease. The extrapolation of findings requires that the model resembles that of the human disease. In mouse models, the accumulation of macrophages will take place initially in the adventitia, followed by their infiltration into the media layers of aneurysmic areas. Within 4 to 10 days, a rapid lumen expansion can be detected because of the trans-medial breakage (29). Rupture of the aorta is reported in some experimental mice but most of them survive. Thrombi in ruptured aorta of survived mice leads to complex inflammatory process which involves the infiltration of macrophages, T and B lymphocytes. Within 28 days, marked re-endothelialization over the disrupted media and neovascularization throughout the aneurysmal tissue can be detected. (30). In addition, matrix metalloproteinase 9, chymase, and angiotensin-converting enzyme (31) are also reported to be upregulated during the late-stage or after-effect of angiotensin-II administration in hyperlipidaemic mice. Overall, the infusion of angiotensin-II into hyperlipidaemic mice is a highly reproducible model for the production of aneurysms. Although the model does not recapitulate all aspects of the human disease, many facets are present in both human and mouse aneurysmal tissue making it a model for defining mechanisms involved in the initiation and propagation of aneurysms.

$CaCl_2$-induced aneurysms

Extracellular administration of $CaCl_2$ around the target artery was first applied to a rabbit common carotid artery, leading to dilatation of vessels, loss of endothelial cells, disruption of the internal elastic lamina, atherosclerosis, and inflammation (32). Using a similar method also succeeded in murine models. Aneurysms can be induced with $CaCl_2$ in mice at 8–12 weeks. After anaesthesia, the abdominal aorta between the renal artery and the iliac bifurcation is exposed by blunt dissection. A piece of gauze soaked with 0.5 M $CaCl_2$ is applied uniformly along the abdominal aorta from the left renal artery to the bifurcation for 10 minutes. The aorta and the abdominal cavity are then rinsed with 0.85% sodium chloride three times before the incision is closed. The mice are allowed to recover under a warm blanket and are closely monitored until they regain consciousness. Mice are then placed into a clean cage and kept under standard housing conditions. Aneurysms develop in aorta in 3 weeks, which can be quantified as described.

Using this model, the mechanisms of aneurysm development have been studied, in which the destruction of extracellular collagen and elastin fibres is believed to be one of the most important reasons in aneurysm formation. By combining genetically altered mice and the $CaCl_2$ injury method, scientists found matrix metalloproteinase 2 and 9 deficient mice were not able to form aneurysms. Matrix metalloproteinase 12 (18) and TIMP-2 (33) deficient mice showed attenuated aneurysms compared with wild-type mice. Inflammation also plays a critical role in the $CaCl_2$ model. Proinflammatory cytokines, including interleukin-1 and -6, can be upregulated under this situation. Another article focused on inflammation using CCR2, CCR5, and CXCR3 knockout mice in the $CaCl_2$ mediated model and found only CCR2 reduced the inflammation against aneurysm formation (34). Phosphorylation of both c-Jun N-terminal kinase and its substrate c-Jun is involved in aneurysm formation induced by $CaCl_2$ in mice. By administration of SP600125, an inhibitor of c-Jun N-terminal kinase, matrix metalloproteinase 2 and 9 both significantly decreased with an increase in matrix protein biosynthesis. As a result, c-Jun N-terminal kinase inhibition partly reduced aneurysm formation. In addition, PPAR-alpha was reported to promote macrophage infiltration and cathepsin-S activation. Thus, this model proves to be a powerful tool for understanding the pathogenesis of the disease (➔ Fig. 5.2).

Elastase-induced aneurysms

Like $CaCl_2$ models, elastase-induced aneurysms also involve metalloproteinase. Under anaesthesia, after isolating the abdominal aorta, the proximal and distal portion of the aorta is temporary ligated. A catheter is then inserted into the aorta by an aortotomy after which type 1 porcine pancreatic elastase is injected into the aorta by a syringe pump for 5 minutes. Finally, aortotomy and the wound are closed (35). Aneurysms will develop in this model in around 3 weeks.

It has been reported that aneurysms can be inhibited in matrix metalloproteinase 9-deficient mice but not in matrix metalloproteinase 12 knockout mice (36). The role of TIMP was also examined in this model and deficiency of TIMP generated a larger diameter of target artery and aneurysms (37). Nitric oxide is another involved pathway in the elastase model. Nitric oxide synthase 2 expression is unregulated in wild-type mouse in the elastase model with reduction of nitric

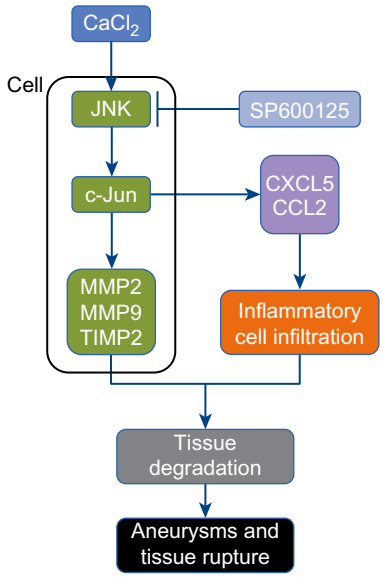

Fig. 5.2 Scheme of signalling pathways in $CaCl_2$ induced abdominal aneurysms. Activation of JNK/c-Jun pathway led to inflammatory infiltration by secreting chemokines and to tissue degradation by activating matrix metalloproteinase. The inhibitor of the JNK pathway, SP600125, was found to reduce the formation of abdominal aortic aneurysms

oxide synthase 1 and 3. However, male nitric oxide synthase 2-deficient mice were not affected in this aneurysm model but the size of aneurysms of female nitric oxide synthase 2-deficient mice was significantly increased, thus indicating a potential relationship between nitric oxide synthase 2 and female hormones (38). Aneurysms require extensive degradation of the artery wall matrix and cathepsin is one of the most potent elastases. In humans, cathepsin is highly expressed in aneurysm lesions. It is reported that cathepsin L deficient mice won't form aneurysms with the inflammatory cell number significantly lower than that of the wild type. A possible mechanism is that the absence of cathepsin L reduced monocyte chemotactic protein-1, macrophage, and T cells, thus reducing inflammatory cell accumulation, angiogenesis, and protease expression (39). In humans, there are multiple risk factors such as age, gender, and hypertension but no study has pinpointed the underlying mechanism yet. By using genetic mouse, however, scientists are able to focus on one particular gene and explore the role it plays in aneurysm formation. For example, mice without the matrix metalloproteinase 12 gene, which is the key element of aneurysms, won't form abdominal aortic aneurysms (40).

Mouse models of vascular injury

Recently, a number of interventions have been invented to accelerate the development of neointimal lesions in mice. By applying local interventions, researchers are able to not only reduce the experimental time, but also to select an optimal location and control the extent of injury. Balloon injury, guide wire injury, blood flow cessation, and stenosis collar were hence developed (9). Periarterial cuff placement was developed several years ago (41). This method allows the structural integrity of the endothelium to be maintained. Normally, under anaesthesia, the target artery is separated from surrounding tissue. A tiny polyethylene cuff will loosely sheath the artery and is tied with a suture. Since the cuff is larger than the vessel, it does not affect blood flow. Then the target artery is replaced and the wounds sutured. Electric injury to the carotid artery of mice can induce vessel injury, in which the artery should first be well isolated. A bipolar micro-coagulator can be applied, positioned perpendicularly to the longitudinal axis of the artery to do the injury. A very small current pulse can cause the injury and reducible neointima formation. The blood flow cessation model can be the easiest way to induce vascular injury. Remodelling is carried out with ligation of the target artery, i.e. common carotid artery near its bifurcation. Then the wound is sutured and the mouse is allowed to recover.

Guide-wire injury to the vessel can be achieved by three passages of a 0.25-mm diameter angioplasty guide wire. Under anaesthesia, the femoral artery can be separated clearly with the help of surgical microscope. A suture is inserted through the femoral artery in advance in preparation for further ligation. Then, an arteriotomy can be made and guide wire is inserted forward until the aortic bifurcation. The guide wire is then moved back and forth three times to ensure a complete endothelium denudation. After removing the guide wire, the arteriotomy site should be ligated immediately and the wound is sutured (13).

Stent implantation in the mouse model, along with administration of drugs and chemokines, is a powerful tool to help understand the mechanism and solutions of restenosis caused by a stent implantation in human beings. To implant a stent inside the common carotid artery, blood flow is firstly interrupted by binding a knot on both the internal carotid artery and the common carotid artery. Then, arteriotomy is performed on the external carotid artery and a silicon tube containing the stent is inserted through the external carotid artery to reach desired position in the common carotid artery. Afterwards, the silicon tube is removed through guide wire, allowing shape-memory expansion of the stent. In the end, a knot is bound tightly on the external carotid artery to close the incision site and the knots on the internal and common carotid artery are removed to recover blood flow. (42).

Compared to the mouse model of guide-wire injury, stent implantation mainly focuses on the mechanism of in-stent thrombosis after percutaneous transluminal (coronary)

angioplasty (PTCA) with stent implantation in human beings. The development of a miniaturized stent for mouse carotid artery, combined with different knockout mice, allows the study of particular molecular mechanisms for in-stent thrombosis and the tests of various stent-coating drugs. However, the mouse model of guide-wire injury may be more relevant to clinical intra-arterial manipulation but cannot represent the in-stent neointimal formation.

A major and common complication in vessel injury models is the development of neointimal lesions as early as the first week with the peak at 3–4 weeks. Using these models, smooth muscle cell migration and proliferation can be studied. Neointimal lesions usually stop growing in denuding injury models as soon as the luminal surface is re-endothelialized. Restricting the morphometric analysis to denuded segments assures that all measurements are performed on segments of vessels that have not been influenced by the variable of endothelial regeneration. Unfortunately, not many investigators rigorously control endothelial regeneration and clot-dependent effects on neointima formation. Like the rat balloon injury model, proliferation of SMC starts in the media within a few days after denudation and if no re-endothelialization occurs, proliferating SMC can be found in the neointima typically within a week. If re-endothelialization occurred before SMC can migrate into the intima, medial hypertrophy and hyperplasia will be observed since medial SMC proliferation precedes the arrival of SMC in the intima.

Mouse models of vein grafts

An alternative intervention used to mimic surgeries in humans is saphenous vein grafts, which is considered as a useful treatment for severe atherosclerosis. However, the later occlusion of the graft vessel is the main disadvantage of this surgical method. Vein grafting in mice is a very useful model for resembling aorta coronary vein graft bypass in humans. To perform the vein graft bypass experiments, the vena cava is collected from the donor and irradiated. Under anesthesia, the right common carotid artery of the recipient is separated from the bifurcation and then cut in the middle. The cuff, which is made from a nylon tube which has a 0.63mm outside diameter and a 0.5mm inside diameter, is placed at both ends of the artery. The donor's vein segment is then grafted between the two ends of the carotid artery by carefully sleeving the end of the vein over the cuff and ligating the end with an 8-0 suture. Different cell types as well as various chemokines could also be applied around the vein. One advantage of this model is that the thin vessel wall could easily be penetrated upon administration of virus or drugs on the adventitial side. (➔ Fig. 5.3) (43).

In mouse model of vein grafts, neointimal hyperplasia undergoes three processes. The first one is a marked early loss of smooth muscle cells, probably caused by biomechanical stress (44). The next involves massive infiltration of mononuclear cells (CD11b/18 +) (45). Increased adhesion and chemokines from smooth muscle cells and endothelial cells by biomechanical stress and dead cells from the early stage may drive continuous recruitment of inflammatory cells. At the final stage, cell differentiation, migration, and accumulation are involved. Vascular smooth muscle cells mainly contribute to neointimal hyperplasia (45). Others have tried to use various drugs or chemokines to reduce atherosclerosis caused by vein graft. For instance, in one article, CXCR4 is considered as the key element of atherosclerosis in vein grafts. They found neointimal hyperplasia is reduced if the recipient mice are CXCR4 $^{-/+}$ but no significant difference presents if the recipient mice are wild type (46). Another article revealed that a p53 deficient mouse displayed a two-fold neointimal hyperplasia compared to the wild type because of the reduction of cell death (47). Fibroblast growth factors (FGFs) also have an important role in the development of vascular disease. A dramatic decrease in neointimal formation in mouse models of vein graft was observed when the mice were treated with FGF receptor blocker (48).

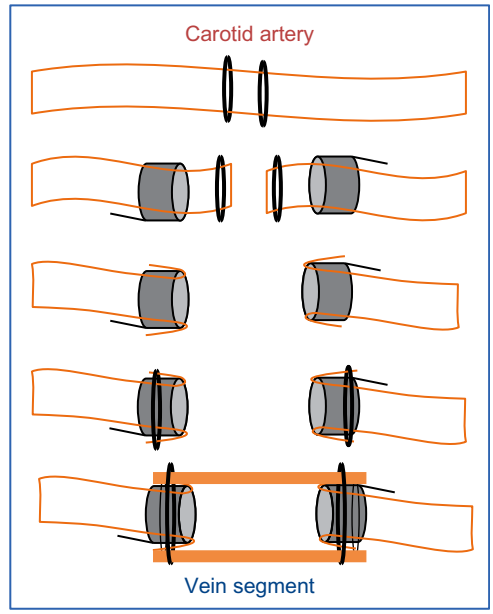

Fig. 5.3 Schematic representation of vein bypass graft. The right common carotid artery was ligated with an 8-0 silk suture, dissected between the middle ties, and passed through cuffs, respectively. The vessels, together with the cuff handle, were fixed with microhaemostat clamps, the suture at the end of the artery was removed, and a segment of the artery turned inside out to cover the cuff body. The vena cava vein segment was harvested and grafted between the two ends of the carotid artery by sleeving the ends of the vein over the artery-cuff and suturing them together with an 8-0 suture ligation.

Another advantage of this model is to clarify the origin of cells in neointimal hyperplasia. Traditionally, it was believed that smooth muscle cells in hyperplasia were from the media (49). Circulating stem cells actually participate in repairing damaged vessels, and about one-third of endothelial cells of vein graft cells are from the recipient circulation (50). In addition, in another research study, it was proved that about 22% smooth muscle cells are from the recipient and 69% are from the donor veins (51). What is more, adventitial stem cells may become the new suspect for cell proliferation in vein graft models. Sca-1^{+} progenitor cells applied to the adventitial side of grafted vein contribute about 30% of neointimal SMC in APO-E deficient mouse (52). Furthermore, this model could also been used for studying thrombosis formation and subsequent neointimal formation. A kinetic observation of vein grafts indicates how fibrin deposition and neointima formation develop in the mouse model (➔ Fig. 5.4), which could share some similarities with humans. Thus, this model could be useful for studying the pathophysiology of vessel grafts, as well as for potential drug screening.

Rat models

Despite the fact that the rat is resistant to atherosclerosis and its carotid artery does not have vasa vasorum (53), the rat carotid artery balloon injury model has been widely used to elucidate the vascular responses after injury and the mechanism of neo-intima formation and smooth muscle cell proliferation (54–56). However, the interspecies' differences should always be taken into consideration because some methods that are successful in preventing restenosis in rats were not effective clinically. For example, angiotensin-II was effective in the rat carotid artery balloon injury model in inhibiting restenosis (57, 58), but was ineffective in clinical trials (59, 60). Methodologically, the widely used model is to injure carotid artery using a Fogarty catheter. In brief, rats are anaesthetized by intramuscular injection of Zoletil (zolazepam hydrochloride and tiletamine hydrochloride; 20 mg/kg body weight) and Xylazine (10 mg/kg body weight). Carotid artery balloon injury can be performed in male or female rats. A 2F Fogarty catheter (Baxter Edwards) is introduced via an arteriotomy in the external carotid artery, and the catheter is then advanced to the proximal edge of the omohyoid muscle. To produce carotid artery injury, an inflated balloon with a calibrated device at 1.5 atmospheres is withdrawn three times. The external carotid artery is then permanently ligated with a 5-0 silk suture, and blood flow in the common carotid artery is restored.

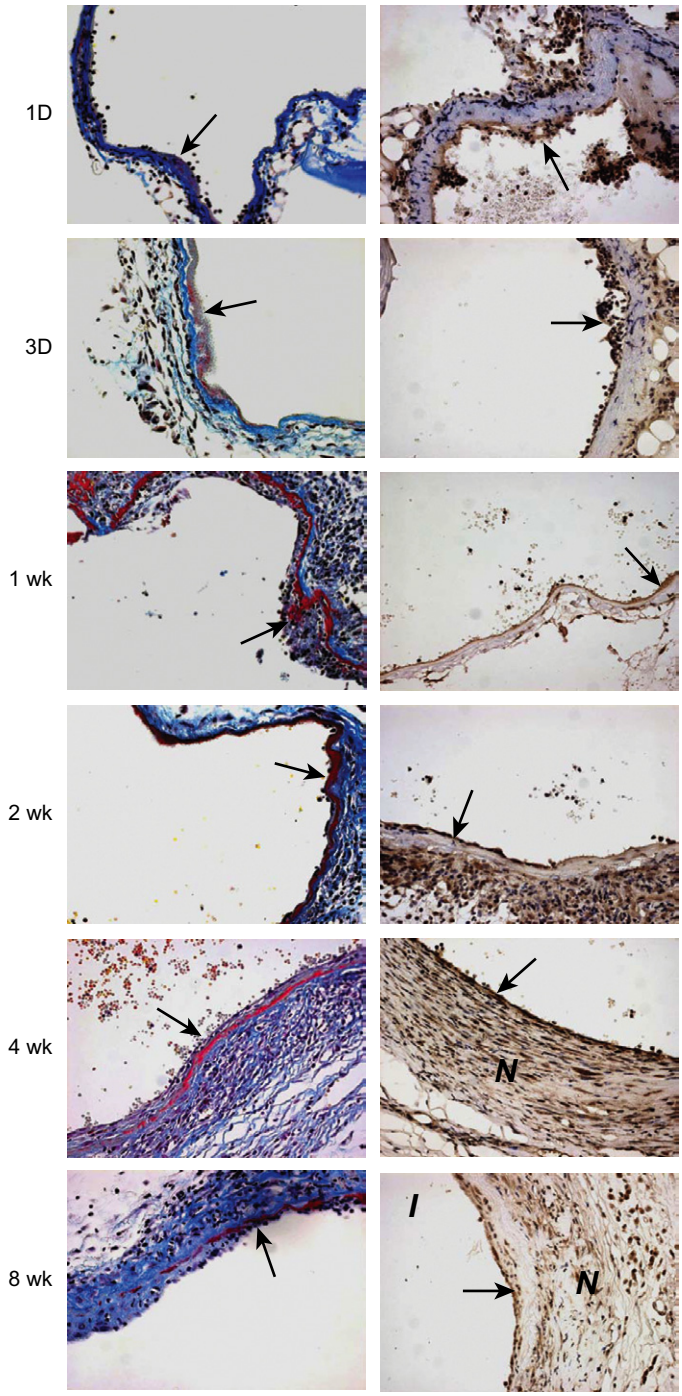

Fig. 5.4 Histochemical detection of fibrin formation in mouse vein grafts. Modified Martius Scarlet Blue (MSB) histochemical staining of grafts at 1 D (day), 3D, and 1, 2, 4, and 8 wk (weeks). Fibrin stains red, collagen is blue, nuclei are blue/black, and red blood cells are yellow. Arrows point to red fibrin staining; *N*, neointimal lesions. Original magnification ×40.

Apart from balloon injury in the carotid artery, intravascular stents have also been employed to study the pathophysiology of in-stent stenosis, which contributes to the high restenosis rate after intravascular stent intervention in human atherosclerotic diseases. Rat carotid artery, with its relatively small diameter, is mainly used to study

the mechanism of small artery restenosis after stent placement (61). As the size of the rat aortic artery is comparable with human coronary artery, several rat models deploying an intra-aorta stent have been described by trans-carotid artery stent placement, trans-iliac artery stent placement, or by trans-aorta stent placement (62–64). Trans-carotid stent placement enables researchers to put the stent in without too much interruption of the blood flow, but it requires delicate procedure performance, which is the main limitation of its wider usage (64). Trans-aorta stent placement proves to be relatively easier, but it is related with higher death risk caused by blood-flow blockage during the procedure (62). In this circumstance, Oyamada developed a novel trans-iliac aorta stenting procedure, which in part solves the problem (63), but its universal use needs to be further verified.

Rabbit models

Diet-induced atherosclerosis

The first animal model for atherosclerosis was published by Ignatowski in 1908, which was a rabbit model. Using the rabbit model, Ignatowski and colleagues tried to clarify the relationship between atherosclerosis and diet, demonstrating thickening of the intima of the aorta in the animals as a result of a high-cholesterol diet (meat, milk, and eggs). In addition, since people with suboptimal calorie intake are not affected by atherosclerosis, diet obviously is one major risk of atherosclerosis (65). Although rabbits are vegetarians and do not spontaneously develop atherosclerosis, atherosclerotic lesions can be induced if they are fed with a high-cholesterol diet (0.5–4%), which normally results in a blood cholesterol concentration several times that of human beings, thereby exhibiting the rabbits' sensitivity to cholesterol overload (66). However, the atherosclerotic lesions induced display a different lipid and macrophage composition from humans, which is mainly dominated by macrophage-derived foam cells (67).

To produce atherosclerotic lesions that show better human resemblance, several other models exist that induce more advanced atherosclerotic lesions by either intermittent feeding of a high-cholesterol diet (43) or by adding artery injury (e.g. balloon artery injury) to a hyper-cholesterol diet (42, 68). A simple rabbit atherosclerosis model was developed by Takashi and his colleagues who tried to standardize the protocol for getting a vulnerable plaque rupture model with a short term high-cholesterol diet. Atherosclerotic lesions in this model are histologically proven to be vulnerable plaques with high human resemblance. These models are largely impeded to elucidate the effectiveness of lipid-lowering drugs and the respective mechanism of neo-intima formation (42, 69). Another widely used rabbit model is the Watanabe hereditary hypercholesterolaemic (WHHL) rabbit, which endures hypercholesterolaemia due to a defect of LDL receptor (➔ Fig. 5.5). This offers an opportunity to study the natural process of the lesions developed in human familial hypercholesterolaemia (70).

Transgenic rabbit models

Apart from diet-induced atherosclerotic models, transgenic models of atherosclerosis in rabbits have been generated as well (71), including apoE2, apoE3, apoA-I, apoB-100, apo(a), lipoprotein lipase, and matrix metalloproteinase-12 transgenic rabbits, which were nicely reviewed by Watanabe in 2003 (72) (➔ Table 5.2) Although transgenic mouse models

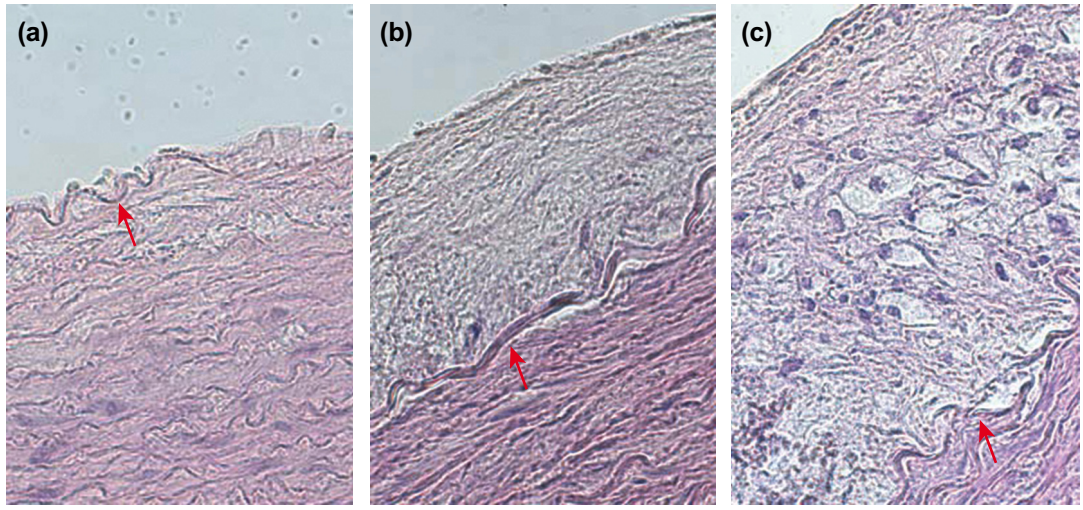

Fig. 5.5 Image representation of atherosclerotic lesions from New Zealand white rabbits with normal chow diet (a), with high-fat diet for 12 weeks (b), and Watanabe hereditary hypercholesterolaemic (WHHL) rabbit with high-fat diet for 12 weeks (c). Arrows indicate elastic intina.

Table 5.2 Transgenic rabbits for atherosclerosis

Transgenes expressed	Effects on atherosclerosis
Human hepatic lipase	Anti-atherogenic
Human lipoprotein lipase	Anti- or pro-atherogenic (Depedent on cholesterol levels
Human lecithin: cholesterol acyltransferase	Anti-antherogenic
Rabbit apoB mRNA editing protein	ND
Human apo(a)	Pro-atherogenic
Human apoA-I	Anti-atherogenic
Human apoB-100	ND
Human apoC-III	ND
Human apoE2	Atherogenic
Human apoE3	Atherogenic
15-lipoxygenase	Pro-atherogenic
Macrophage metalloelastase	ND

ND, not determined.
Source data from Fan JL, Watanabe T. Transgenic rabbits as therapeutic protein bioreactors and human disease models. Pharmacol Therapeut. 2003;99:261–282

are far more ubiquitously used, transgenic rabbit models have their unique importance in studying the atherogenic process, as rabbits are known to display similar lipoprotein metabolism (LDL-mammals) with humans, with mice being HDL-mammals (16), thus making the research conducted in transgenic rabbits more convincing than in transgenic mice when contradictory results appear, as is shown in the different results of the overexpression of apoE in transgenic mice and rabbits (7, 17). And these observations, which are different in transgenic mice and rabbits, even with overexpression of the same gene, are powerful evidence that interspecies' differences should not be ignored in any circumstance.

Plaque rupture models

Apart from stable atherosclerotic lesions, rabbits are also employed to study the mechanism of drug and device effectiveness on acute syndromes caused by atherosclerotic plaque rupture, such as myocardial infarction. In fact, apart from mouse plaque rupture models, the rabbit was the first animal model in which spontaneously ruptured plaque was created (27). In this model, two balloons are used, with the first balloon to de-endothelialize the aortic artery and the second indwelling balloon, which is vein scaffold covered to attach to the aortic wall (73). Scaffold is an acelluar, jugular vein-derived structure wrapped around the balloon, allowing the formation of an atherosclerotic lesion. Atherosclerotic lesions morphologically similar to those in human are formed in 4 weeks, after which the indwelling balloon is inflated until it induces the rupture of the plaque *in vivo*, allowing study of the consequence of plaque rupture. However, this model employs the balloon as the triggering factor of the plaque rupture, which in part is different from the human plaque rupture process, with plaque erosion sometimes happening without a crack in the fibrous cap (74). Other triggering factors of plaque rupture include the Russell's viper venom in New Zealand white rabbits with plaques induced by a high-cholesterol diet combined with balloon injury (40) and in Watanabe heritable hyperlipidaemic rabbits that are administered angiotensin II (74). Moreover, thrombosis formation in these models is confirmed by normal MRI imaging (15) and MRI with a fibrin-binding gadolinium-labelled peptide, EP-1873, as the imaging agent. There are still efforts invested in developing rabbit models with spontaneously ruptured plaques, but until now, we still need the combination of induced vascular injury and hyperlipidaemic diet to generate vulnerable plaques (18).

Drug and device effectiveness studies conducted on these induced rabbit models are of limited translational importance, not only because the atherosclerotic lesion composition is in some degree different from that of human beings, but also because the triggering events no matter pharmacologically or mechanically do not much mimic that happen in human beings. The spontaneous plaque rupture animal model, at least in part, serves as a solution of this problem, with selectively bred Watanabe heritable hyperlipidaemic rabbits with a 97% myocardial infarction incidence at 11–35 months (44). Using rabbits as an animal model of atherosclerotic and thrombotic diseases has well-documented advantages (e.g. cheap and easily manageable) and the aortic artery of the rabbits are the same size as human coronary arteries, providing researchers with a good opportunity to study intravascular interventions on rabbits. The drawback, which is the different composition of the atherosclerotic lesion from humans, is still obvious and needs to be taken into account.

Swine models

Although small animals are cheap and easy to manage, they are not as frequently used as large animals, such as swine and nonhuman primates, in translational researches, mainly due to the interspecies' differences, which should never be ignored when interpreting the data accumulated. Swine are excellent for an animal model studying atherosclerotic diseases, because of their similarity to human beings. First of all, swine can develop atherosclerotic diseases spontaneously and may undergo sudden cardiac death (66, 75, 76)

just like human beings. Second, the atherosclerotic lesion distribution of swine exhibits better human resemblance than small rodents in which lesions are mainly induced in larger arteries either by diet or by vascular injury. The size of the coronary artery is basically the same size as humans and instead of examining the drug and device effectiveness on large arteries in small animals, swine coronary artery emerges to be a better artery substrate. Moreover, the atherogenic and thrombogenic mechanisms and processes in swine are also similar to that of human beings; but the drawbacks are also well-documented: expensive price, large size, complex managing system, and intravascular intervention process, all hamper the generalized use of swine in translational research. There are now three kinds of literature-reported swine models of atherosclerotic disease, which are diet-induced atherosclerosis, the artery injury model (including balloon injury and intravascular stent), and the extra-corporeal arteriovenous shunt. They could be used separately or in combination with others to create ideal atherosclerotic models.

Diet-induced atherosclerosis

Pigs on a 50-day hypercholesterolaemic diet can develop atherosclerosis and the lesions are pretty much the same as in human beings, only not as severe in relatively younger animals (77). The relatively older pigs can develop lesions more advanced but their weight may easily exceed 100 kg and they are not easily managed, requiring more professional researchers (78). In this context, some studies employed miniature swine as the animal model to solve this problem, whose weight is a lot lower and its anatomical, physiological, and pathological similarity with human beings is not compromised (79), thus making miniature pigs more preferable than commercial pigs. However, miniature pigs are now more expensive, impeding generalized use.

There are now four promising pig models in translational research; these are the diabetic/hypercholesterolaemic pig model (80), LDLR−/− pig model of familial hypercholesterolaemia (81, 82), (PCSK9) gain-of-function mutant pig (83), and the metabolic disease pig model (Ossabaw Pig). It is nicely reviewed by Damir Hamamdzic and Robert L. Wilensky (84). The spontaneous plaque rupture model in pigs was reported in 39–54-month-old pigs that had inherited hyper-LDL cholesterolaemia (81). For the induced plaque rupture model, swine are a suitable model for intravascular intervention studies, since the size of their coronary artery is comparable to humans and the injury response, after balloon de-endothelialization and intravascular stent, is similar to humans, with the response more profound if the swine are on an atherogenic diet (85). In these animals, a lot of strategies, whether systemic drug-related or drug eluting stent-related, are proved to be useful in preventing restenosis after intravascular stent placement, including endothelin receptor antagonist (86) and localized stent eluted drugs such as methylprednisolone (87), prostacyclin analogue iloprost (88), paclitaxel, and rapamycin. Moreover, the consistency of the stent eluting rapamycin's safety and efficacy between swine models (89) and humans (90–93) serves as evidence of swine model sensitivity. Other than rapamycin, paclitaxel eluting stent also got positive results (94–97), in accordance with the data obtained in swine models (98–100). Thus, swine models provide valuable data for understanding the mechanisms, as well as therapeutic intervention, for vascular diseases.

Imaging in swine

Sudden death in patients with coronary atherosclerosis is the largest contributor to the mortality rate in developed countries these days, though the therapeutic strategies have been largely improved with new systemic drug therapies emerging and intravascular intervention methods modified (101, 102). For those who have undergone myocardial infarction, percutaneous intracoronary imaging such as coronary artery angiography and intravascular ultrasound, which is widely used clinically, enables doctors to obtain first-hand information about the plaque rupture location and the blood supply condition. However, they are invasive imaging methods and coronary angiography provides no further information, such as the plaque composition and risk of rupture, than the remaining lumen diameter of the artery. To evaluate the atherosclerotic plaque composition and its stability, more noninvasive imaging methods of predicative value are under development in order to prevent sudden cardiac death. In this context, magnetic resonance imaging, which can identify the character of atherosclerotic lesions (e.g. fibrous cap, lipid content, calcification, and haemorrhage), catches researchers' attention. As the size of the porcine coronary artery is pretty much the same as for human beings (44), Stephen G and colleagues utilized a clinical magnetic imaging system (a double-inversion-recovery fast-spin-echo sequence in a 1.5-T MR system) to evaluate the characterize the nature and quality of atherosclerotic lesions, matching the histology findings from a porcine model undergone balloon angioplasty (103). Contrast agents such as MS325 (104) and gadofluorine (105), were explored at the same time to identify atherosclerotic lesions and BMS-753951, an elastin-binding agent (106) was used to predict the extracellular matrix formation that is a major process in vascular remodelling after injury in porcine models. A further pilot study on diet-induced atherosclerotic mini-pig models shows that PET/MR (multi-sequence MR imaging

together with 10 minutes of PET emission data collection) is feasible in the assessment of atherosclerotic lesions preclinically, which takes the imaging method a step further to functional evaluation and requires more numerous animal studies (107). In conclusion, atherosclerotic porcine models, whether diet-induced or injury-induced, are suitable to explore the predicative value of novel imaging methods such as MRI in the detection of vulnerable plaques and the assessment of atherosclerotic lesions; further large-scale studies are needed.

Nonhuman primates

It is well known that nonhuman primates are the closest animal models to human beings, displaying the best similarity in the mechanism of atherogenesis, plaque rupture, restenosis, and therapeutic interventions in cardiovascular diseases (108). The first atherosclerosis model in nonhuman primates involved free-ranging howler monkeys (109), after which rhesus monkeys with a deficiency of LDL receptor then emerge as another atherosclerotic model (110, 111). Theoretically, nonhuman primates are the best animal model to study atherosclerotic diseases for reasons already described, however, their drawbacks are also obvious (e.g. expensive, hard to manage, require expertise to conduct experiments on, and have the risk for extinction). As a result, they are mainly reserved to evaluate therapeutic interventions just before clinical trials on human beings (112). For this reason, other large animals, such as swine, are more widely used; they are relatively more accessible and can create atherosclerotic lesions in a degree satisfying for getting results of predicative value.

Conclusion and perspectives

Recent studies aimed at testing and documenting either mechanistic or therapeutic interactions in animal models have significantly improved our understanding of pathophysiology in vasculature. For instance, smooth muscle cells in lesions of transplant arteriosclerosis in mice were derived from donor stem cells that were verified to be similar to the cell origin of lesions in humans (113). Therefore, because of results from animal studies, combined therapy, using dietary intervention and one or more drugs, e.g. statins, is employed in a variety of preventive and therapeutic approaches in humans. With the use of existing and newly developed animal models to study vascular disease, one can anticipate that there will be more opportunities to increase our comprehension of the lesion at the cellular and molecular levels.

Although mouse models are widely used by many laboratories, some problems often appear due to insufficient knowledge of the specific animal models, especially technical issues. Many mouse models involve techniques of microsurgery, which cannot be performed without proper training. Investigators, who are not experienced with the mouse model, would need to consider several issues before starting their experiments. On the other hand, larger animal models provided tools for investigating the pathophysiology of the disease with similarities to humans. Especially, translational research would require testing in swine models. In this regard, current progress is encouraging, although some technical issues need to be addressed. The present chapter has attempted to give a brief background on each model, to provide some methods and materials used for establishing the animal model, and to provide some examples for applications of the model. We believe that this chapter could be useful for researchers working with animal models in cardiovascular research. This knowledge should help us to acquire the scientific means to control the untoward disease process of vascular diseases in humans.

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SECTION II

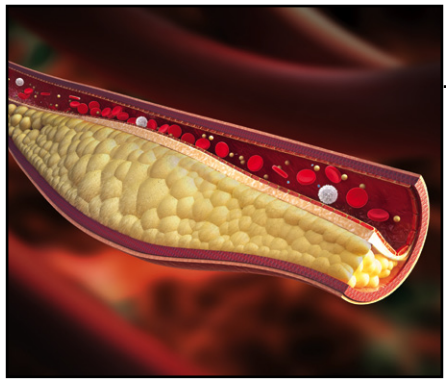
Biology of the vasculature

Section introduction

Marie-Luce Bochaton-Piallat

The vascular network is a complex system consisting of large and small vessels designed to supply oxygen and vital nutrients to distant tissues. Blood vessels share a common structure, including a monolayer of endothelial cells at the luminal side and a medial layer containing smooth muscle cells. Vascular pathologies are dominated by aberrant endothelial cell and/or smooth muscle cell behaviour; it is thus essential to have knowledge of the normal characteristics/functions of these cells to understand the pathophysiology of atherosclerosis and other vascular diseases, and to ultimately be able to revert the pathological cells back to their normal phenotype. Angiogenesis, arteriogenesis, and lymphangiogenesis, are processes involved in the remodelling of small vessels and play crucial roles in pathological conditions.

Chapter 6 provides a general overview on the structural heterogeneity of the endothelium. The most important physiological functions of the endothelium in blood vessels, such as transport of plasma molecules, regulation of vascular tone, synthesis and secretion of a large variety of factors, and maintenance of homeostasis, are described in detail. In Chapter 7, smooth muscle cells, which determine the structure of arteries and are decisive in the regulation of blood flow, appear to be highly heterogeneous throughout the vascular tree, depending on embryonic origin and local conditions. The underlying signalling pathways that determine smooth muscle cell phenotypic diversity and function are explained. Chapter 8 describes the formation of new blood vessels after birth by angiogenesis, i.e. capillary sprouting induced by hypoxia, and arteriogenesis, i.e. enlargement of pre-existing collateral arteries driven by fluid shear stress. The common and distinct cellular and molecular mechanisms driving the two processes in physiological and pathological situations are highlighted. Signalling pathways orchestrating venous/arterial specification are also designated. Finally, Chapter 9 deals with the lymphatic vascular system that forms one-way drainage channels transporting tissue interstitial components back to the venous circulation and passing through lymph nodes. Lymphatic vessels contribute to the regulation of interstitial fluid homeostasis, trafficking of immune cells, and absorption of dietary fats from the gut. The development of the lymphatic vasculature, as well as the role of the lymphatic system in pathological situations such as inflammation and primary (genetic origin) and secondary (damaged lymphatic vessels) lymphoedemas, are discussed.



CHAPTER 6

The endothelial cell

Ingrid Fleming, Brenda R. Kwak, and Merlijn J. Meens

Content

Introduction

Endothelial cells make up the monolayer of cells that line blood vessels and act as a physical barrier between circulating blood and vascular smooth muscle cells (➔ Box 6.1). For a relatively long time, endothelial cells were considered as little more than a nucleated protective anti-thrombogenic/anti-adhesive layer. Our appreciation of the metabolic activity of endothelial cells and its central role to the regulation of vascular homeostasis began in 1980 following on from the groundbreaking observations by Furchgott and Zawadzki (1), who initially identified the role of the endothelium in mediating vascular responses to acetylcholine. It is now generally appreciated that their strategic position determines, to a certain extent, their function, as endothelial cells tightly monitor the transport of plasma molecules, are involved in the regulation of vascular tone, the synthesis and secretion of a large variety of factors, and are implicated in the regulation of cell cholesterol, lipid homeostasis, signal transduction, immunity, inflammation, and haemostasis. Also, there are clear differences in the circulatory needs of different organs that are reflected in the tightness (junctional properties) of the endothelial cells, as well as their interaction with additional mural cells.

The endothelial organ

Endothelial cells can be regarded as a small organ in their own right, albeit with a very large surface area; the estimated total area of the blood/endothelial interface in humans is approximately 7,000 m^2. This has been calculated to correspond to a total endothelial mass in the range of about 1 kg. The term 'endothelial cell' represents a relatively heterogeneous group of cells that display differences in the characteristics of their intracellular junctions and can be classified as 'continuous', 'fenestrated', and 'discontinuous'. In addition, endothelial cells may differ in terms of morphology, mediator release, antigen presentation, or stress responses.

In intact vessels constantly exposed to flowing blood, endothelial cells take on an elongated elliptic form, orientated in the direction of flow. They are contact-inhibited cells with a very low turnover (estimates of half-life vary between 1 and 3 years). At vascular sites without a prevailing direction of flow, where turbulence and eddy circulation occur, endothelial cells display a polygon shape

Box 6.1 Endothelial cells

Endothelial cells are paracrine mediators of the vascular system and represent a functionally dynamic monolayer of cells that play many roles:

- They provide a non-thrombogenic surface by releasing platelet inhibitory substances such as prostacyclin (PGI_2) and nitric oxide (NO), and they generate binding molecules for inhibitors of the coagulation cascade.
- They interact with circulating cells of the immune system and are involved in their activation.
- They synthesize and release vasoactive signals that possess dilator or constrictor actions such as NO, prostaglandins (and other vasoactive derivatives of arachidonic acid), endothelial hyperpolarising factor (EDHF), and endothelin.

Endothelial cells are also actively involved in transporting macromolecules from the vascular lumen into the arterial wall or the extracapillary interstitium.

- They synthesize growth factors such as fibroblast growth factor (FGF), transforming growth factor beta (TGF–β), platelet-derived growth factor (PDGF), colony stimulating factor (CSF), and vascular endothelial growth factor (VEGF).
- They form connective tissue macromolecules such as basement membrane proteins, collagen, and proteoglycans.
- They are involved in the metabolism of circulating lipoprotein particles.
- They produce reactive oxygen species, such as superoxide anions, and can thus oxidatively modify low-density lipoproteins during their transit through the endothelium.

without detectable flow orientation. At these sites, endothelial cell turnover is higher. The endothelium is not uniform throughout the entire vasculature with regard to form or function. In some organs, endothelial cells are fenestrated and thus do not really function as a permeability barrier. In other organs such as the brain the endothelium has characteristic tight junctions and demonstrates only minimal permeability.

Endothelial phenotypes not only differ between species in different organs, but also between consecutive vascular sections. For example, in the kidney the endothelium is fenestrated in peritubular capillaries, discontinuous in glomerular capillaries, and continuous in other regions (2). While they were thought to be genetic pre-determinants of endothelial phenotypes (vascular patterning), it is now clear that endothelial cells are highly plastic and respond to environmental cues by alterations in differentiation.

The endothelial cell surface layer

The endothelium is frequently drawn as a thin (cell thickness 0.1–1 μm) monocellular lining of blood vessels; however, there is a remarkable heterogeneity in structure. Although most endothelial cells are very flat (a fact that minimizes diffusional distance), the endothelial cells of venules are tall, plump, and cuboidal (3). A large number of specific molecules with large extracellular domains reside in the endothelial membrane, and an additional polysaccharide layer on top of the glycocalyx, bound only by charge–charge interactions, was described as 'cell coat' (4). The endothelial glycocalyx (➔ Fig. 6.1) is a negatively charged gel-like surface structure of proteoglycans with its covalently bound polysaccharide chains called glycosaminoglycans, glycoproteins, and glycolipids that governs transcapillary fluid exchange. The glycocalyx has also been proposed to act as a biomechanical sensor for haemodynamic stimuli such as fluid shear stress (5, 6). Most electron microscopy studies indicate the presence of a glycocalyx with a thickness in the range of 20 nm. However, this now seems to be an underestimation as fixation methods for electron microscopy are likely to lead to a collapse of gel-like surface structures with high water content (7). More modern estimations describe the glycocalyx as being subdivided into bundles with an average thickness of 50 to 300 nm (electron microscopy) or even 2.5 to 4.5 μm (confocal microscopy), that are densely packed and can completely fill and cover fenestrae, at least in kidney glomerular endothelial cells (8, 9). The structure of the endothelial glycocalyx also seems to differ between fenestrated and continuous endothelium and its thickness and composition varies from organ to organ. Reduced glycocalyx thickness has been described at sites with low and/or oscillatory shear stress, such as the carotid artery bifurcation, and decreased thickness of the glycocalyx is associated with increased transcapillary transport of lipoproteins (6). Molecular components of the glycocalyx are cell-adhesion molecules involved in immune reactions and inflammatory processes, e.g. selectins and integrins and components of the coagulant and fibrinolysis pathways. Experimental studies suggest that shedding of the glycocalyx occurs in inflammation and the loss of the endothelial glycocalyx is associated with increased E-selectin mediated adhesion of tumour cells to the microvascular endothelial processes that may promote tumour metastasis (10).

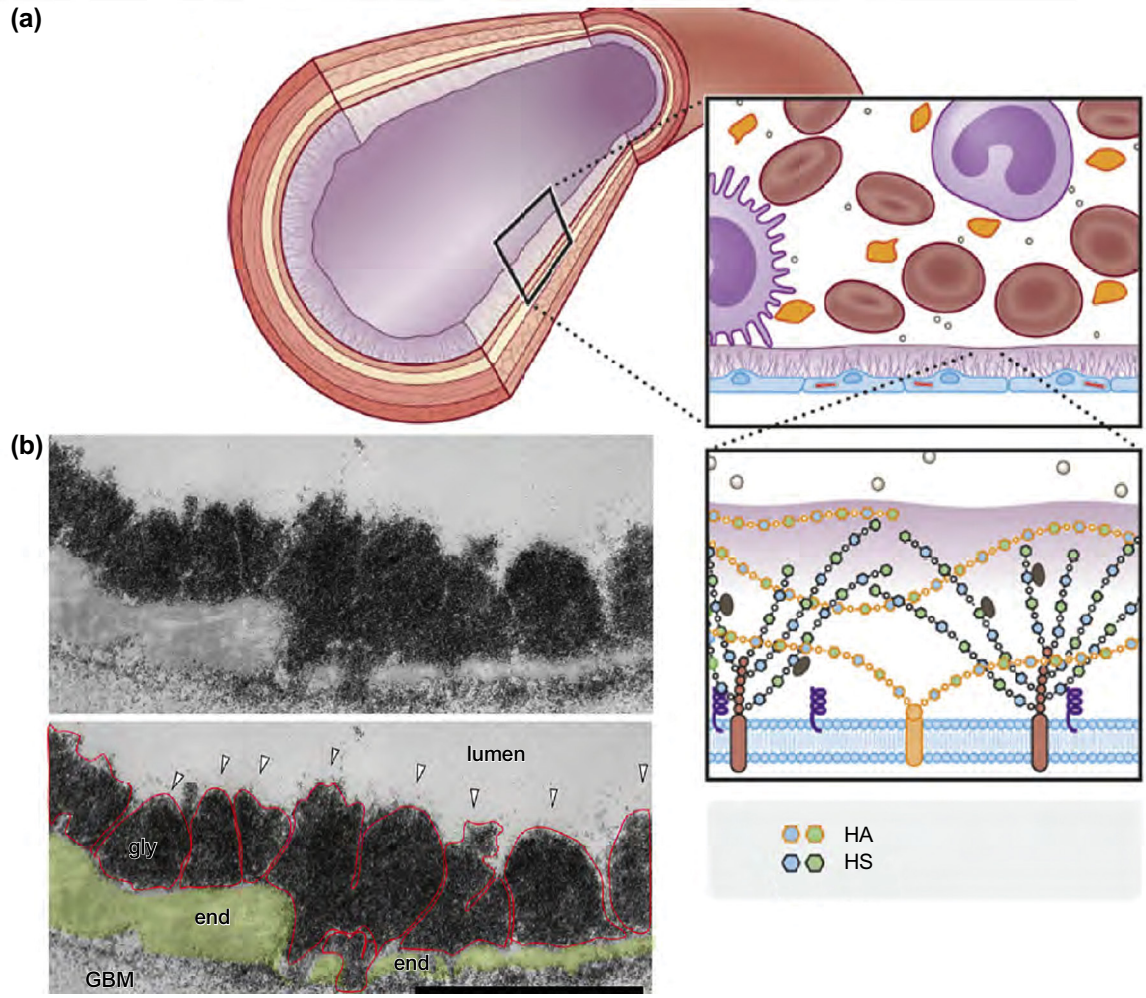


Fig. 6. 1 The endothelial gylcocalyx. (a) Schematic overview of the endothelial glycocalyx. Heparan sulphates, bound to a heparan sulphate (HS) core protein, and hyaluronan (HA), bound to, e.g. CD44, are the main constituents of the endothelial glycocalyx. The order and modification of disaccharide repeats within HS determine the binding site for specific proteins. (b) Region of the luminal face of the filtration barrier showing massive cThO2 staining. The glycocalyx layer is up to 300 nm thick. It is composed of packages resembling densely grown bundles, separated by narrow bright gaps. The distance between the packages varies from ~100 to 200 nm. Some staining by cThO2 can be observed also at the endothelial face of the GBM. Top: pure electron micrograph, Bottom: clarification of endothelium (end, green) and subdivision (red lines) of the endothelial glycocalyx (gly) into packages (arrowheads). Scale bar 500 nm. (Reproduced from Hegermann J, Lunsdorf H, Ochs M, Haller H. Visualization of the glomerular endothelial glycocalyx by electron microscopy using cationic colloidal thorium dioxide. Histochem Cell Biol. 2015 with permission from Springer.)

Given the relationship between the endothelium and the flowing blood, one important characteristic of the endothelial cell surface layer is its anti-thrombogenic properties. As the cell surface carries the adhesion molecules required for platelet–endothelial cell interaction, it is particularly important that several layers of control exist. The specific disruption of the glycocalyx, for example, results in thrombin generation and platelet adhesion. In the 'healthy' endothelium, the expression of many adhesion molecules is low or even absent—a situation partially attributable to release of NO and PGI_2. Indeed, it is the generation of these autacoids in response to fluid shear stress that largely prevents the aggregation of platelets. Under physiological conditions, the generation of thrombin is tightly regulated by the inhibitory actions of thrombomodulin, endothelial protein C receptor, and tissue factor pathway inhibitor, all of which are bound to the luminal endothelium. Activation or dysfunction of the endothelium results in the downregulation of these natural anti-coagulant mechanisms (11). Endothelial cells also synthesize and secrete both plasminogen activators (anti-coagulant) and plasminogen activator inhibitors (procoagulant), and the surface layer is the point of assembly of components of the fibrinolytic system, resulting in local stimulation of fibrinolytic activity. The metabolism and interconversion of extracellular adenine nucleotides via ecto-ATPase/ADPase (CD39) and ecto-5'nucleotidase (CD73) activities also takes place at the endothelial cell surface. The latter enzymes collectively dephosphorylate ATP,

ADP, and AMP with the production of adenosine; all of which are involved in modulating processes linked to vascular thrombosis. Therefore, a balance of extracellular ATP metabolism on endothelium controls platelet function with a direct effect on the prevention of thrombus formation (12).

Heterogeneity of endothelial cells

Morphological heterogeneity between capillary endothelial cells

Developmentally, endothelial cells arise from the mesoderm by the differentiation of haemangioblasts and/or angioblasts. However, other cell lineages may differentiate into endothelial cells as well, and vice versa endothelial cells may differentiate into other lineages (3). This is probably best illustrated by the fact that genetic studies aimed at using specific promoter constructs to label endothelial cells only manage to affect specific endothelial cell subsets (13). Thus there is no single molecule that can define all endothelial cells. Endothelial phenotypes within a single organism can be differentiated on the basis of morphology and permeability as well as on location, i.e. arterial, capillary, venous, or lymphatic endothelial cells.

In continuous capillaries, the luminal and abluminal membranes fuse only at the tight junctions, which represent the predominant pathway for the exchange of water, glucose, urea, and other hydrophilic molecules. Accordingly, the structure of individual tight junctions is the major determinant of vascular permeability in this type of endothelium and accounts, for example, for the tight blood–brain barrier of the brain microcirculation. Fenestrated capillaries are characterized by pores of 50–60 nm in diameter that are sealed by a diaphragm. Consistent with their presence at sites of infiltration, secretion, and absorption, fenestrated capillaries are more permeable to low molecular weight hydrophilic molecules and water. Since fenestrated endothelial cells are located in close proximity to epithelial cells, interaction between the two cell types has been proposed to trigger differentiation and the formation of fenestrae (2). Non-fenestrated, continuous endothelium is typically found in arteries, veins, and capillaries of the brain, skin, heart, and lung. Moreover, in straight sections of arteries, endothelial cells are long, flat, and narrow, and their nuclei are aligned in the direction of blood flow. At branch points and in areas of disturbed (non-laminar or oscillatory) flow they take on a more disordered structure. This is presumably a reversible phenomenon as exposing endothelial cells to altered flow patterns can alter their morphology. One of the best examples of this phenomenon studied endothelial cell orientation in canine arteries *in vivo*, which were excised and re-implanted after a 90° rotation. Within 10 to 12 days after intervention the endothelial cells had realigned themselves in the direction of blood flow (14). A similar phenomenon can be observed *in vitro* as endothelial cells cultured under static conditions possess a cobblestone morphology but when exposed to shear stress for 24 to 72 hours they also slowly develop an elongated and directed morphology.

Fenestrated endothelium is found in organs with functions linked to filtration and/or high levels of transendothelial transport (e.g. glomeruli, exocrine glands, gastric, and intestinal mucosa). Fenestrae are transcellular pores (70–100 nm in diameter) that extend through the full thickness of the cell, are frequently organized in arrays or 'sieve plates', and they constitute 30–50% of the glomerular capillary wall surface area. Most capillary fenestrae possess a thin 5–6 nm non-membranous diaphragm across their opening that may contribute to selective permeability (15, 16). In a single endothelial cell, fenestrae formation can be determined by the local environment and in particular by the local concentration of VEGF (17) in the case of the glomerulus, derived from podocytes (18). Mature glomerular endothelial cell fenestrae, however, lack diaphragms and thus the glycocalyx, which can completely fill and cover fenestrae in kidney glomerular endothelial cells (8, 9), may contribute to perm selectivity.

Discontinuous endothelium is found in certain sinusoidal vascular beds, most notably the spleen and liver. In the latter organ, endothelial cells possess fenestrations of 100–200 nm in diameter that lack a diaphragm and contain gaps (or large circular pores) 0.1–1μm in diameter, that are commonly also referred to as fenestrae. The basal membrane is either absent or involved in the gaps, which are not fixed structures but can undergo dynamic changes (3). Interfering with actin polymerization can induce marked and rapid changes in gap numbers, indicating that the actin cytoskeleton plays a major role in the regulation of endothelial cell porosity. Moreover, these pores can alter their size depending on extracellular calcium concentrations in liver sinusoidal endothelial cells.

Heterogeneity between arterial, venous, and lymphatic endothelial cells

During embryonic development, endothelial cell progenitors differentiate from mesoderm and form an irregular network or primitive vascular plexus. These early vascular structures undergo intense remodelling to form arteries, veins, and capillaries, steps that involve the recruitment of pericytes and smooth muscle cells to stabilize the vessel wall and provide a mechanism for the regulation of blood flow. Lymphatics then originate from the cardinal vein, to

complete the vascular network. The endothelial cells in the arteries, capillaries, veins, and lymphatics are exposed to markedly different haemodynamic environments and must perform distant functions, e.g. arterial endothelial cells should prevent the infiltration of circulating cells into the vascular wall, while veins are the preferred site for leukocyte extravasation and lymphatic endothelial cells facilitate fluid absorption and immune cell patrolling (19). For a long time the blood flow was thought to be a determinant of endothelial cell heterogeneity. However, over the last 20 years it has become clear that a combination of the local environment (including pressure and blood flow), as well as early genetic components, determine cell fate. Elegant experiments in zebrafish and mouse embryos have provided the majority of the molecular data that drive our current understanding of endothelial cell heterogeneity.

Molecular markers of arterial versus venous endothelial cells (➔ Fig. 6.2) include members of the Notch family, e.g. the receptors Notch1 and Notch4, as well as the ligands Jagged1, Jagged2, and Dll4. Also, the ephrinB2 ligand and its receptor, EphB4, are differentially expressed in the arterial and venous endothelial cells of the murine primary vascular plexus, even before the initiation of blood flow (19, 20). Despite interspecies' differences it appears that in mice, as well as in zebrafish, at least some venous cells have an arterial origin, as a small population of endothelial cell progenitors from the dorsal aorta relocates to the cardinal vein (21).

The development of the mammalian lymphatic vasculature is a stepwise process that requires the specification of lymphatic endothelial cell progenitors in the embryonic veins, and the subsequent budding of those progenitors from the embryonic veins to give rise to the primitive lymph sacs from which the entire lymphatic vasculature eventually derives. Interestingly, there are distinct and tissue-specific differences in the mechanisms by which the lymphatic vasculature arises and develops, so that visceral, dermal, intestinal, brain, and cardiac lymphatic vasculature all have their own unique and diverse developmental programmes (22, 23). For further details on the lymphatic vasculature, we refer the reader to ➔ Chapter 9.

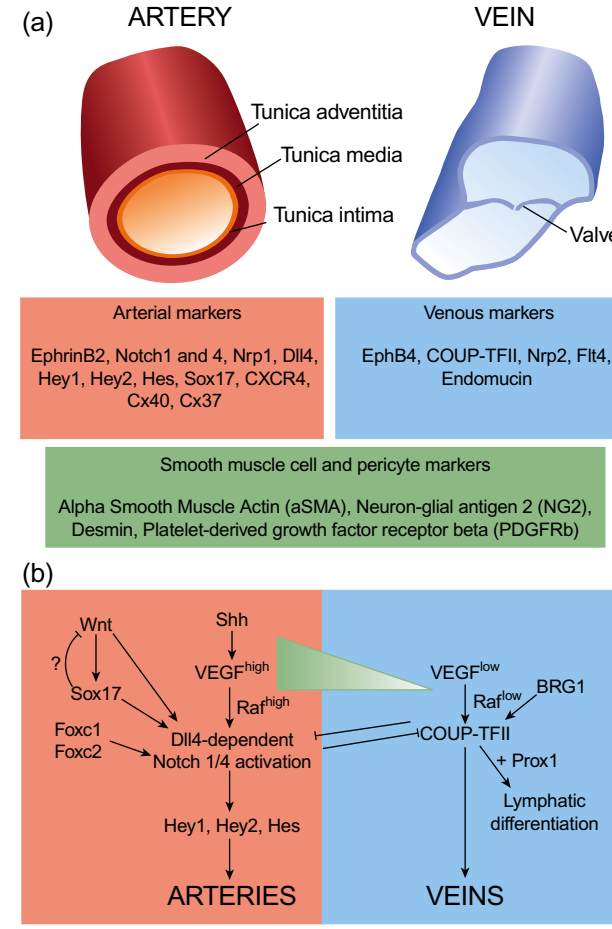


Fig. 6.2 Signalling pathways in the specification of arteries and veins. (a) Anatomically, arteries are characterized by a thick vascular wall while veins contain valves to keep blood flowing toward the heart. Nowadays, arteries and veins can be distinguished at the molecular level by the expression of several specific markers (see boxes). Smooth muscle cells and pericytes are more abundant around small and large arteries. Therefore, markers of these mural cells (green box) can be considered indirect markers of arteries. (b) Schematic representation of the signalling pathways involved in arteriovenous specification. Shh lies at the top of arterial specification triggering the expression of endothelial growth factor (VEGF) that, at high concentrations, promotes the activation of Notch signalling. Other factors, such as Wnt and the transcription factors Foxc1, Foxc2, and Sox17, cooperate to activate Notch signalling and the arterial fate. Vein progenitors, instead, are exposed to low VEGF concentrations and express chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) transcription factor that promotes venous specification by inhibiting Notch signalling.
(Reproduced from Corada M, Morini MF, Dejana E. Signaling pathways in the specification of arteries and veins. Arterioscler Thromb Vasc Biol. 2014;34(11):2372–7 with permission from Wolters Kluwer.)

Functions of the endothelial organ

The large heterogeneity of endothelial cells is intimately linked to the numerous physiological functions of the endothelium, some of which are typical for specific parts of the vascular tree and others are exerted throughout the body. The next paragraphs will systematically address some of the most important physiological functions of the vascular endothelium, i.e:

- *Modulation of vascular tone.* Endothelial cells secrete vasoactive agents that act in a paracrine fashion on adjacent vascular smooth muscle cells of resistance arteries to adjust local blood flow—for instance, flow-induced arterial dilatation in exercising muscle.
- *Regulation of blood–tissue exchange.* Endothelial cells in capillaries form a semi-permeable membrane to allow for

nutrient transfer into peripheral tissues while retaining blood cells and plasma within the circulation.

- *Endothelial cells sense the shear stress exerted by flowing blood.* This process is of particular importance in arteries and underlying important pathological processes, such as atherosclerosis.
- *The endothelium forms an anti-thrombogenic surface.* Nitric oxide (NO) and prostacyclin (PGI_2) are not only powerful vasodilators but also inhibitors of platelet aggregation. This prevents the circulating blood from clotting (thrombosis). The thrombotic process is described in detail in ➔ Chapter 18.
- *Endothelial surface enzymes modify vasoactive hormones in the bloodstream*. Endothelial cells express angiotensin-converting enzyme (ACE) at their luminal surface, which chemically converts secreted hormones to their active form. The large endothelial surface area ensures efficient conversion of the vasoactive precursors.
- *The endothelium participates in inflammatory responses.* In response to, for example, pathogen invasion, the venous endothelium close to the inflamed tissue starts to express adhesion molecules at its luminal surface. These surface molecules can capture circulating leukocytes and promote their transendothelial migration into the tissue. Details on the inflammatory process are described in ➔ Chapter 10.

Endothelium-dependent modulation of vascular tone

Local vascular tone is determined by a variety of factors such as neurotransmitters released from autonomic nerves, circulating vasoactive compounds, tissue metabolites, and endothelium-derived autacoids. A fundamental function of the endothelium is the regulation of contraction of the underlying vascular smooth muscle; an important basal regulatory function of the endothelium is brought about by several mechanisms:

- Degradation, conversion, or uptake of vasoactive signal molecules, such as oxidative deamination of catecholamines and serotonin, degradation of platelet-derived ATP and ADP, and hydrolytic cleavage of angiotensin I and bradykinin.
- Formation and release of the constrictor peptide endothelin.
- Formation and release of the vasoactive autacoids NO, PGI_2 and EDHF.

Endothelial cells and the adjacent smooth muscle occasionally form gap junction-like contacts via cellular protrusions penetrating the basement membrane and the electronic propagation of hyperpolarization (and, therefore, relaxation of the smooth muscle) by such contacts has been demonstrated. The formation and release of NO is the most important dilator produced by the endothelium and is generated in almost all organs. There is, however, a certain amount of redundancy as far as endothelium-derived vasodilator autacoids is concerned, as the release of PGI_2 and hyperpolarization-dependent mechanisms exist in varying dominance in different vascular beds—most notably in resistance arteries.

Endothelium-derived vasoconstrictors

Endothelin-1 is a 21 amino acid peptide that has powerful vasoactive properties and is produced by several different cell types, including cardiomyocytes, mesangial cells, different segments of the nephron, and endothelial cells. Endothelin-1 targets two G protein-coupled receptor subtypes, ET_A and ET_B receptors, the activation of which lead to distinct, and frequently opposing, effects. Despite early hopes that endothelin would turn out to be a major physiological regulator of vascular tone, the evidence supporting a major role was not convincing. Certainly NO appears to be a major determinant of endothelin-1 expression, as well as being a functional antagonist of its actions. Thus, the importance of endothelin may only really be clearly demonstrated in situations (hypertension, ageing, etc.) where NO bioavailability is decreased (24). There is, however, recent evidence to indicate that the ET receptors do play an important role in cardiovascular homeostasis, as arterial blood pressure is lower in mice lacking ET_A receptors specifically in smooth muscle cells (25, 26), as well as in mice lacking endothelin-1 specifically in endothelial cells (25, 27). Reviewing the recent literature it is certainly tempting to speculate that endothelin-1 is likely to exert many of its actions via the renal circulation (28).

Prostanoids can attenuate relaxations mediated by NO, PGI_2, and EDHFs via their actions on thromboxane/prostaglandin (TP) receptors and K^+ channels on vascular smooth muscle cells. Perhaps most is known about the synthesis and actions of thromboxane A_2 and prostaglandin H_2 (PGH_2). The inhibition of thromboxane A_2 synthesis partially inhibits contraction induced by ADP and endothelin but not the contractile response to acetylcholine, indicating that thromboxane A_2 is only one contracting factor released from endothelial cells. PGH_2 is the second most potent agonist at TP receptors and is more effective in activating TP receptors in vascular smooth muscle from hypertensive

than normotensive rats. Interestingly, PGI_2 can also elicit TP-receptor-dependent contractions (29). These factors are likely to be more important in pathophysiological situations.

Nitric oxide

The formation of NO from the amino acid L-arginine is catalysed by the endothelial NO synthase (eNOS) that is constitutively expressed in the endothelium. The activity of eNOS is largely stimulated by an increase in cytosolic Ca^{2+} and the binding of calmodulin (CaM). In addition to shear stress, a number of other physical stimuli, neurotransmitters, hormones, autacoids, and coagulation factors modulate eNOS expression and/or activity.

Robert Furchgott elegantly demonstrated that endothelial cells play a pivotal role in relaxations evoked by acetylcholine in isolated arteries (1). Thereafter followed the demonstration of NO production by the endothelium and the physiological effectiveness of L-arginine analogues (30), and eventually the isolation of the first NO-generating enzyme or NO synthase (NOS) (31). It is now general knowledge that NO is synthesized from the amino acid L-arginine by the NOS family of enzymes. The 'neuronal' (nNOS, NOS I, or bNOS) and 'endothelial' (eNOS or NOS III) NOS isoforms, which were named after the tissues in which they were first identified, are expressed constitutively and are generally regulated by Ca^{2+}/CaM, as well as by phosphorylation. The inducible NOS isoform (iNOS or NOS II), on the other hand, is not usually expressed in unstimulated cells; although exceptions to this rule of course exist. iNOS binds CaM so tightly that it is essentially Ca^{2+}-independent, and it generates NO in large quantities during inflammatory or immunological defence reactions. There are only a few intracellular mechanisms that regulate iNOS activity, which is usually determined by its expression level.

NO synthases are multi-domain enzymes consisting of an N-terminal oxygenase domain that contains binding sites for haem, L-arginine, and tetrahydrobiopterin (BH_4), and a reductase domain with binding sites for NADPH, FMN, FAD, and CaM. The active enzyme is part of a multi-protein complex consisting of the NOS dimer, each of which binds one CaM, as well as a spectrum of adaptor and regulatory proteins. NOS dimers contain one zinc ion, which is tetrahedrally coordinated to pairs of cysteine residues at the dimer interface. During the synthesis of NO, NADPH-derived electrons pass to flavins in the reductase domain and must then be transferred to the haem located in the oxygenase domain so that the haem iron can bind O_2 and catalyse the stepwise synthesis of NO from L-arginine. The association of CaM with its binding site is generally accepted to activate NO synthesis by enabling the reductase domain to transfer electrons to the oxygenase domain (32, 33). In addition to CaM, eNOS forms a large signalling complex with other proteins that regulate its function and intracellular localization, including caveolin-1, PECAM-1, Hsp90, and dynamin (➔ Fig. 6.3a).

The most recently characterized eNOS regulatory protein that is important for the regulation of NO bioavailability in the microcirculation is haemoglobin α (34). This protein seems to directly interact with the oxygenase domain of eNOS to inhibit NO production, as well as to scavenge NO via the haem group. Interestingly, haemoglobin α seems to be exclusively localized to myo-endothelial gap junctions in resistance arteries and has been proposed to act as a 'sink' for NO, diminishing its impact on the vasodilation of resistance vessels, at the same time as increasing that of prostacyclin (PGI_2) and hyperpolarization-dependent mechanisms.

eNOS is constitutively expressed but numerous physical and chemical stimuli affect eNOS levels *in vitro* and *in vivo*. For example, the fluid shear stress generated by the viscous drag of blood flowing over the endothelial cell surface is an important signal regulating eNOS mRNA and protein expression in cultured endothelial cells as well as in intact arteries. The signalling pathways involved in the regulation of eNOS expression are relatively complex but important roles have been attributed to the transcription factors NFκB and KLF-2, and eventually also to FoxO1 and specific microRNAs. Functionally, eNOS was initially classified as a Ca^{2+}/CaM-dependent enzyme with a low but measurable activity at resting levels of $[Ca^{2+}]_i$. It is now evident that eNOS can be activated by certain stimuli without a sustained increase in $[Ca^{2+}]_i$ being necessary. Shear stress can elicit Ca^{2+} transients; however, in both cultured endothelial cells and in isolated arteries there is a discrepancy in the time course of the Ca^{2+} response and the time course of the shear stress-induced production of NO. On the basis of such observations it was concluded that a sustained increase in $[Ca^{2+}]_i$ is not essential for the shear stress-induced activation of eNOS and led to the shear stress-induced activation of eNOS being referred to as 'Ca^{2+}-independent'. However, this is strictly speaking not the case since the chelation of intracellular Ca^{2+} also abolishes the response to shear stress (32). Rather the shear stress-induced increase in NO production is associated with eNOS phosphorylation and an increase in the sensitivity of the enzyme to Ca^{2+} so that the enzyme can be activated at resting Ca^{2+} levels (➔ Fig. 6.3b).

The relaxation induced by NO is largely due to the activation of soluble guanylyl cyclase in the smooth-muscle cells resulting in enhanced formation of cyclic guanosine monophosphate (cyclic GMP), a second messenger that initiates the lowering of cytosolic Ca^{2+} levels and the

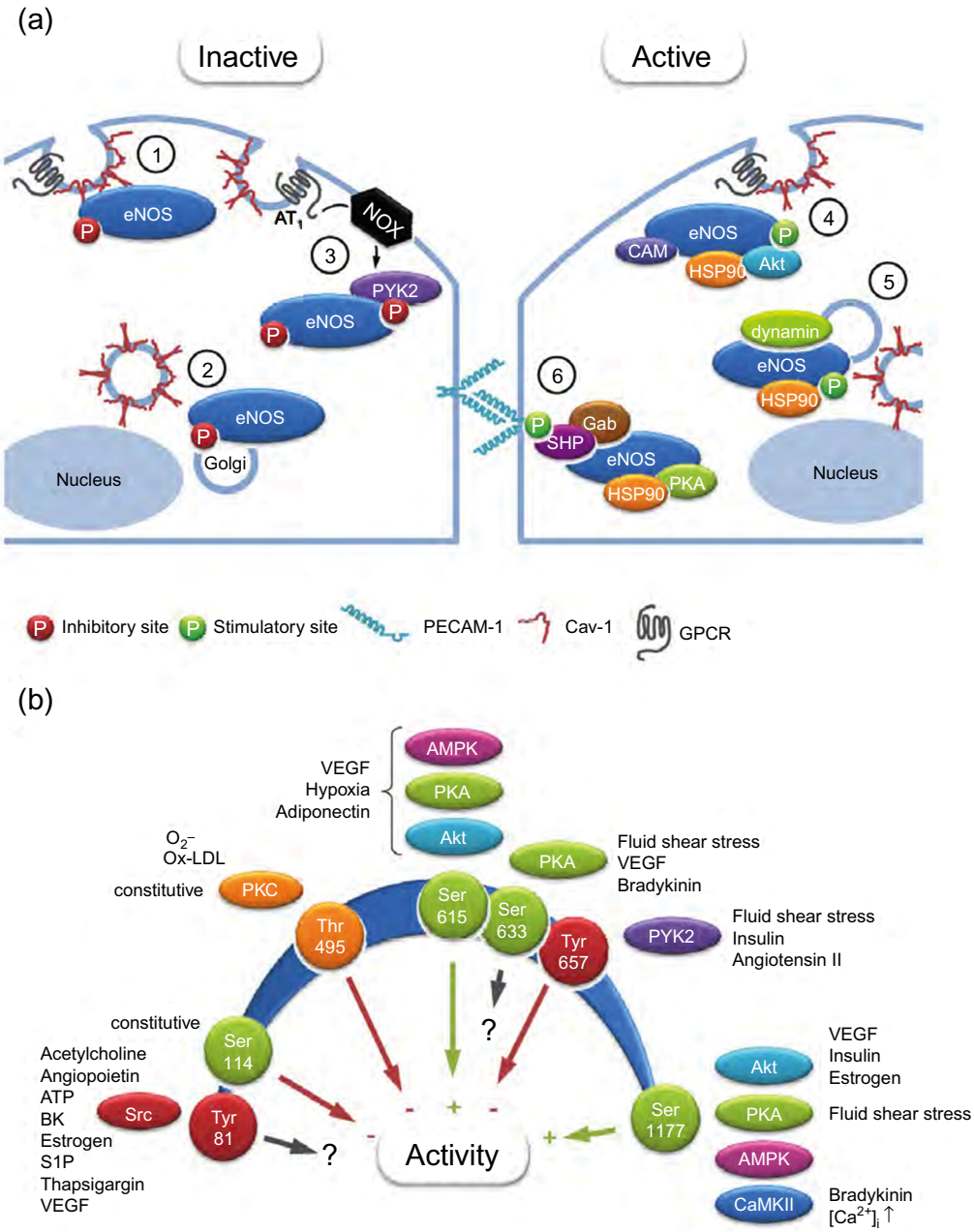


Fig. 6.3 Regulation of eNOS. (a) The functional eNOS protein is a dimer that is localized to the Golgi apparatus and plasma membrane caveolae. (1) In the inactive or basal state the protein in caveolae is coupled to Cav-1, which decreases its activity. (2) Moreover, eNOS is constitutively phosphorylated by PKC on Thr495, which prevents its association with CaM. (3) The enzyme can also be inhibited in conditions of oxidative stress as a consequence of the PYK2-induced tyrosine phosphorylation of eNOS. In response to cell stimulation (4 & 5) eNOS and Cav-1 disassociate (probably assisted by dynamin), Thr495 is dephosphorylated allowing CaM to bind to and activate the enzyme, and activating serine sites (e.g. Ser1177) are phosphorylated. (6) In endothelial cells exposed to shear stress eNOS is localized to cell–cell junctions where it can interact with PECAM-1 and the adapter protein, Gab-1, which in turn acts as a scaffold for kinases such as PKA. (b) eNOS can be phosphorylated on serine, threonine, and tyrosine residues, findings that highlight the potential role of phosphorylation in regulating eNOS activity. There are numerous putative phosphorylation sites but most is known about the functional consequences of phosphorylation of a serine residue (Ser1177) in the reductase domain, a threonine residue (Thr495) within the CaM-binding domain, and a tyrosine residue in the FMN-binding domain (Tyr657). The numbers refer to the human sequence (green arrows = activation, red arrows = inhibition, black arrow = no direct effect on enzyme activity). (Reproduced from Fleming I. Molecular mechanisms underlying the activation of eNOS. Pflugers Arch. 2010;459(6):793–806 with permission from Springer.)

dephosphorylation of the myosin light chain. A second well-established mechanism by which NO exerts its effect on cell function is the post-translational chemical modification of cysteine thiols in target proteins by a process known as *S*-nitrosation. The list of proteins that can be *S*-nitrosated has grown rapidly as the methods to detect the modification have grown more reliable. In endothelial cells, eNOS-derived NO is thought to regulate guanylyl cyclase

activity via *S*-nitrosation and modulate permeability at least partly via the *S*-nitrosation of junctional proteins including β-catenin and p120 catenin (35).

Prostacyclin

Endothelial cells produce and release PGI_2 in response to shear stress, hypoxia, and several other stimuli that increase endothelial cell Ca^{2+} levels and usually also increase NO production. The rate-limiting step of PGI_2 synthesis is the release of arachidonic acid from membrane-bound phospholipids by phospholipase A_2, which is activated by Ca^{2+}. Cyclo-oxygenases then convert arachidonic acid to prostaglandin H_2, which is further converted into vasoactive prostanoids, including PGI_2 and thromboxane A_2 (TxA_2), depending on whether the activity of the PGI_2 synthase or the TXA_2 expression is dominant. PGI_2 exerts its actions by binding to IP receptors on the plasma membrane of smooth muscle cells, which induces the activation of the adenylyl cyclase/cyclic AMP/PKA signal transduction pathway. Although PGI_2 is frequently referred to as the major prostanoid produced in endothelial cells, the balance between PGI_2 and TxA_2 production appears to be important for the regulation of vascular tone since TxA_2 is a potent vasoconstrictor.

Endothelium-dependent hyperpolarizing factors/mechanisms

The best characterized vasodilator autacoids are NO and PGI_2 but a substantial component of the vasodilator response observed in response to receptor-dependent agonists or increases in flow is insensitive to inhibitors of NO synthases or cyclo-oxygenases (COXs). The existence of a NO/PGI_2-independent component of endothelium-dependent relaxation is prominent in resistance arteries in some vascular beds, in particular coronary, mesenteric, and renal arteries. Since the NO/PGI_2-independent vasodilatation originally described was co-incident with vascular smooth muscle hyperpolarization, and was abolished by depolarizing concentrations of potassium, it was proposed to be mediated by an endothelium-derived hyperpolarizing factor or 'EDHF' (36).

Nowadays, the term EDHF has been recognized as an over-simplification, as there seem to be three principal components linked to hyperpolarization-dependent relaxations: (1) an increase in endothelial cell Ca^{2+} following cell stimulation that triggers K_{Ca} activation and the synthesis of a metabolite essential for the subsequent hyperpolarization; (2) K^+, released from endothelial cells via K_{Ca} channels, induces smooth muscle hyperpolarization by activating inwardly rectifying K^+ channels and/or the Na^+/K^+-ATPase on vascular smooth muscle cells; and (3) endothelial cell hyperpolarization is transmitted to the vascular smooth muscle via gap junctions (➔ Fig. 6.4). The strengths and weaknesses of the arguments for each of these specific types of EDHF have been discussed at length (37) and each of them appears to be valid in certain vascular beds. Interestingly, all of these mechanisms can be modulated by epoxyeicosatrienoic acids (EETs).

K^+ channel activation

Vascular smooth muscle cells *in vivo* are partially depolarized, largely as a result of a combination of physical factors, including longitudinal and circumferential stretch. This means that vascular smooth muscle cells are particularly sensitive to K^+ channel opening, which results in K^+ efflux and hyperpolarization. The latter decreases the opening of voltage-gated Ca^{2+} channels to reduce Ca^{2+} influx and results in smooth muscle cell relaxation. Indeed, endothelium-dependent agonists are reported to activate endothelial cell K^+ channels, elicit a small increase of K^+ ions in the intercellular space that results in the activation of either inwardly rectifying K^+ channels or the Na^+-K^+-ATPase to induce hyperpolarization and thereafter relaxation (38).

Gap junctional communication

Vascular cells *in situ* express relatively high levels of connexin proteins, which make up the junctions that electrically connect vascular cells with each other (39). Inter-endothelial gap junctional communication is important for the phenomenon of ascending vasodilatation that coordinates changes in blood flow at the level of an organ. Structurally somewhat more complicated myo-endothelial gap junctions connect endothelial cells and smooth muscle cells, passing through the basal lamina and contributing to the establishment of an endothelium–smooth muscle cell functional syncytium. NO/PGI_2-independent vasodilatation in a number of vascular beds was found to be sensitive to inhibitors of gap junctional communication or the genetic deletion of specific connexin proteins. Clearly this type of hyperpolarization response would not require a factor but be reliant on hyperpolarization mechanisms.

Epoxyeicosatrienoic acids

Originally, a role for cytochrome P450 (CYP)-derived metabolites of arachidonic acid in EDHF-mediated responses was implied on the basis of the fact that CYP inhibitors markedly attenuated NO/PGI_2-independent hyperpolarization and relaxation in various preparations. The metabolites in question are epoxides of arachidonic acid, so-called EETs. These substances elicit the hyperpolarization of endothelial and vascular smooth muscle cells by activating calcium-dependent K^+ (K_{Ca}) channels (40, 41), as well as the Na-K-ATPase (42). Exactly how these lipid mediators initiate responses is unclear but they are able to affect endothelial cell Ca^{2+}

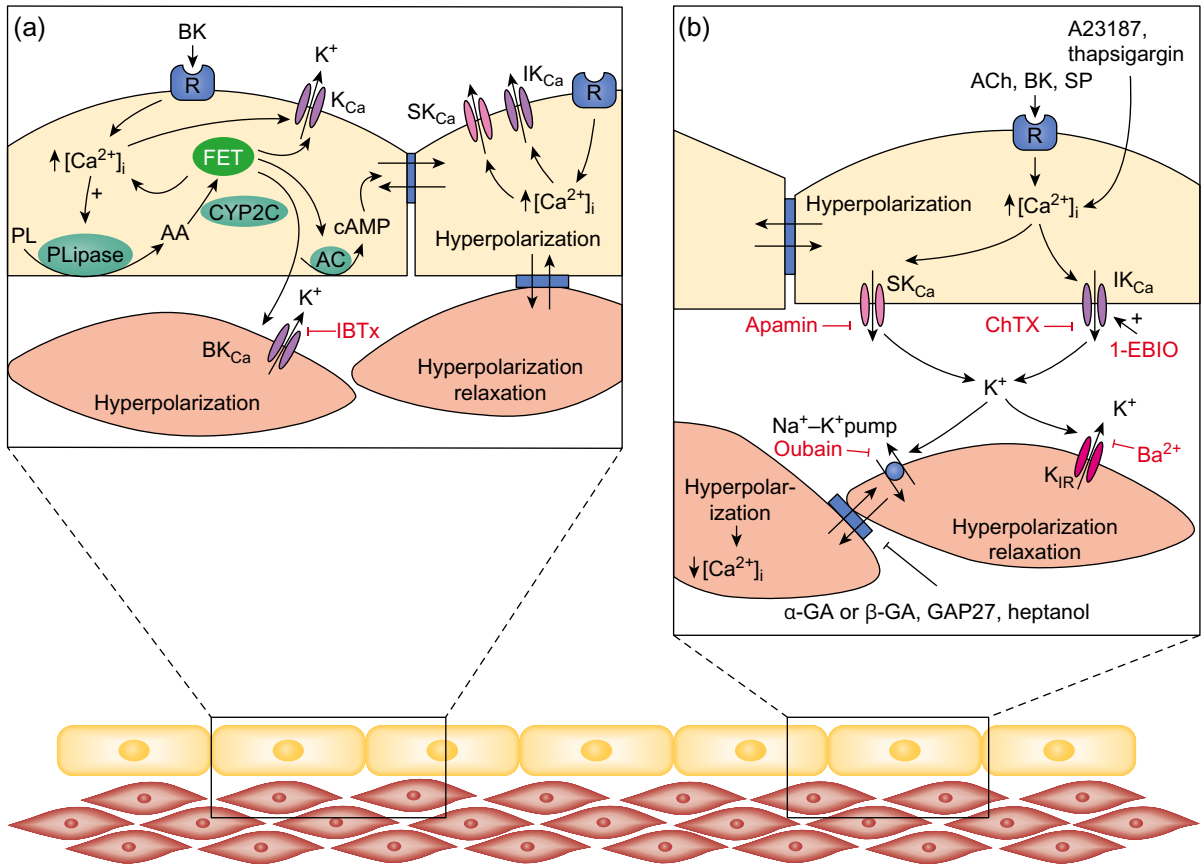


Fig. 6.4 Bringing the hyperpolarization concepts together. (a) Epoxyeicosatrienoic acids (EETs) might act as both intracellular and extracellular messengers. Following endothelial cell stimulation with bradykinin (BK), the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) increases and leads to the activation of a phospholipase (PLipase), which liberates arachidonic acid (AA) from membrane phospholipids (PLs). The subsequent activation of a cytochrome P450 epoxygenase (CYP2C) results in the generation of EETs, which in turn affect Ca^{2+} signalling, the Ca^{2+} sensitivity of Ca^{2+}-dependent K^+ (KCa) channels and the generation of cAMP by adenylyl cyclase (AC), as well as gap junctional coupling in the endothelial cells. EETs and/or their metabolites can also diffuse to smooth muscle cells and activate large-conductance KCa channels (BK_{Ca} channels), which are sensitive to iberiotoxin (IbTX). (b) The role of K^+ ions. Endothelial cell stimulation by receptor-dependent (e.g. acetylcholine (ACh), BK, and substance P (SP)) and receptor-independent Ca^{2+}-elevating agonists (e.g. the Ca^{2+} ionophore A23187 and the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin) initiates endothelial cell hyperpolarization by activating small- and intermediate-conductance K_{Ca} channels (SK_{Ca} and IK_{Ca} channels), sensitive to apamin and charybdotoxin (ChTX), respectively. Endothelial cell hyperpolarization leads to the accumulation of K^+ ions in the subendothelial space in concentrations sufficient to activate inwardly rectifying K^+ (K_{IR}) channels, which are blocked by Ba^{2+} and/or the Na^+–K^+-ATPase (blocked by low concentrations of ouabain). 1-Ethyl-2-benzimidazolinone (1-EBIO) is an activator of IKCa channels whereas α- and β-glycyrrhetinic acid (α- and β-GA), GAP27, and heptanol block gap junctional communication. (a and b) The role of gap junctions. The hyperpolarization of the endothelial cells, following K_{Ca} channel activation, can be transmitted along the monolayer of endothelial cells or towards the smooth muscle cells through gap junctions.

(Reproduced from Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. Trends Pharmacol Sci. 2002;23(8):374–80 with permission from Elsevier.)

signalling and thus K_{Ca} channel activity, as well as gap junctional communication. Therefore, the EETs may actually act by potentiating the hyperpolarizing mechanisms described.

Endothelial regulation of blood–tissue exchange

Capillaries are exchange vessels

Capillaries are extremely thin (diameter ranging from 5 to 10 μm), tube-like structures, which connect the terminal arterioles to post-capillary venules, hence linking the arterial and venous circulations. Essentially, a capillary consists of a single layer of thin endothelial cells covered by a basement membrane and, in fact, the smallest capillaries can even consist of a single endothelial cell wrapped around to meet itself at its two extremities. Importantly, the range of their diameters is large enough to permit passage of (deformed) red blood cells, and thin enough to allow for gas diffusion (diffusion distance ≈0.5 μm). Interestingly, the total surface area of the capillaries is around 2,500 cm^2. Moreover, their total volume has been estimated at ≈5 l, the same as the total volume of blood. Thus, the capillaries cannot be filled all at once

and, actually, in subjects at rest the capillaries and arterioles only contain about 7% of the total blood volume. Therefore, a continuous organ-to-organ redirection of blood, mediated by precapillary arteriolar contraction and/or dilatation, occurs to keep up with the metabolic demands of the body; e.g. during exercise, capillaries in skeletal muscle open, at the expense of those in the gut wall, and the reverse process occurs after a meal.

Capillary anatomy

As mentioned in ➔ Morphological heterogeneity between capillary endothelial cells, three anatomically distinct subsets (continuous, fenestrated, or discontinuous) of capillaries can be found in the body and each of these subsets has a distinct physiological function:

Continuous capillaries (➔ Fig. 6.5) can be found in the brain, lungs, skin, thymus, and muscles. As their name suggests, the endothelium in these capillaries is continuous (i.e. it does not display any fenestrae). Moreover, the basement membrane in this type of capillaries is continuous as well. Depending on the location in the body, endothelial cells in continuous capillaries can be connected via tight junctions (e.g. in the blood–brain barrier), occluding junctions, and/or gap junctions. Interestingly, two subtypes of continuous capillaries can be found: those with abundant transport vesicles (e.g. in muscles) and those with only few vesicles (e.g. in parts of the blood–brain barrier). Importantly, the permeability of this type of capillaries is rather limited due to tight junctions and tight intercellular clefts between the endothelial cells.

Fenestrated capillaries can be found in tissues that transport large amounts of lipids (e.g. intestinal villi, glomeruli, and choroid plexus). Endothelial cells in this type of capillaries are characterized by the presence of fenestrae, which can range from 10 to 100 nm in diameter. Moreover, depending on the exact capillary bed, e.g. in glomeruli, the fenestrae can be covered with a thin diaphragm. Importantly, even though the basal lamina in fenestrated capillaries is continuous, the perforations in the endothelial cells result in a relatively high permeability.

Discontinuous capillaries are present in bone marrow, spleen, and liver. They are characterized by large clefts between their fenestrated endothelial cells and gaps in their basal membrane. As one would expect, these anatomical features result in an extremely high permeability.

Capillary fluid exchange: general principles

To understand the mechanisms of capillary fluid exchange it is vital to first understand the concept of tissue

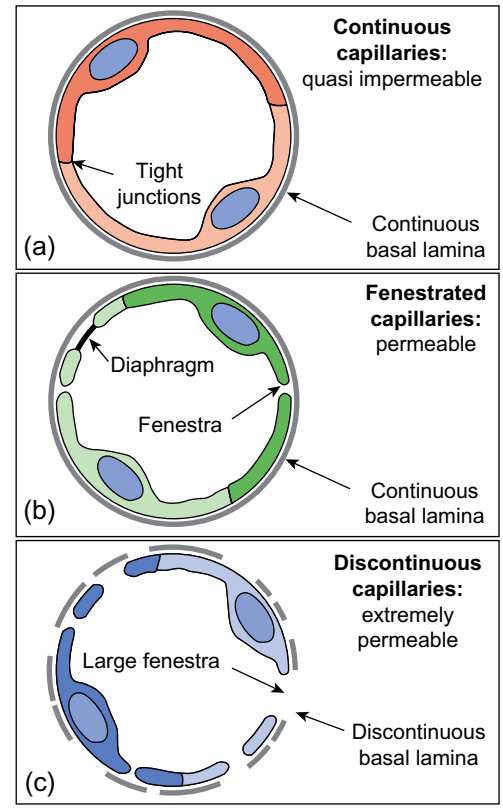


Fig. 6.5 Types of capillary endothelial cells. (a) Continuous capillaries are composed of continuous endothelial cells and a continuous basal membrane. Moreover, the endothelial cells can be coupled to one another via tight junctions and the intercellular clefts are tight as well. Therefore, continuous capillaries display relatively low permeability. This type of capillary can be found in the central nervous system, lung, skin, thymus, and muscles. (b) Fenestrated capillaries are characterized by a continuous basal membrane but, as the name suggests, their endothelium displays large numbers of fenestrae. These fenestrae can be covered by a diaphragm (e.g. in glomeruli), but this is by no means always the case. Importantly, because of the fenestrae in the endothelium, the permeability of this type of capillary is relatively high. Fenestrated capillaries can be found in intestinal villi, glomeruli, choroid plexus, and other endocrine organs. (c) Discontinuous capillaries are generally larger as compared to continuous or fenestrated capillaries. They consist of endothelial cells with large fenestrae and a fragmented basal membrane. Their permeability is extremely high and they can be found in bone marrow, spleen, and liver.

compartments. Basically, there are two primary fluid compartments in the body: the extravascular compartment and the intravascular compartment. The extravascular compartment, which can be subdivided into, for example, cellular, extracellular, and lymphatic compartments, is simply everything outside of the intravascular compartment. Fluids and ions move readily between both compartments depending on physical factors.

As mentioned, the movement of substances between the different tissue compartments is governed by physical constrains. These ensure that large molecules can normally not pass from the intravascular compartment towards the extravascular compartment. However, water and small

soluble molecules can pass between the different compartments, depending on hydrostatic pressure and osmotic forces. Importantly, oedema, i.e. swelling of tissues due to increased extravascular fluids, may occur due to an imbalance in fluid exchange between the tissue compartments. Therefore, it is essential to maintain fluid balance that occurs when:

Filtration = Reabsorption + Lymphatic flow.

Capillary fluid exchange: the four forces of Starling

Physical forces are the main driving factors behind fluid exchange in the capillaries (➔ Fig. 6.6). Basically, the most important are hydrostatic pressure and osmotic pressure (and the anatomical nature of the capillaries, i.e. fluid exchange happens much more readily in discontinuous and fenestrated capillaries, as compared to continuous capillaries). It all comes down to the following formula, which was proposed already in 1896 (43) and was later slightly revised (44):

$$NDF = (P_c - P_i) - \sigma(\pi_c - \pi_i),$$

in which, NDF = net driving force, P_c = capillary hydrostatic pressure, P_i = interstitial hydrostatic pressure, σ = reflection coefficient, π_c = capillary osmotic pressure, and π_i = interstitial osmotic pressure.

Essentially, the net driving force is determined by three factors:

1. The reflection coefficient. This reflects the capillary protein permeability to the proteins generating the osmotic pressure. As a matter of fact, when the capillary of interest is impermeable to protein, $\sigma = 1$.
2. The net hydrostatic pressure ($P_c - P_i$). Normally, P_c is much higher compared to P_i. Therefore, the net hydrostatic pressure in general drives fluid out of the capillaries.
3. The net osmotic pressure ($\pi_c - \pi_i$). In general, π_c is greater as compared to π_i (and π_c initially slightly increases when fluids enter the capillary bed, as initially fluid leaves the capillaries thereby concentrating the fluid and increasing this π_c (see ➔ Fig. 6.6). Therefore, fluid reabsorption can occur towards the end of the capillaries when no longer opposed by hydrostatic forces.

A theoretical experiment easily explains what happens to the physical forces driving capillary fluid exchange while blood runs through a capillary:

- Initially, when the blood arrives from the arterioles it generates a high P_c and it is characterized by a relatively low π_c (although still higher as compared to π_i). Thus, at the beginning of the capillary: $P_c > P_i$ and $\pi_c > \pi_i$. Hence, there is a positive net driving force, which will force fluid to leave the capillary and enter the interstitial space.
- At about two-thirds of the capillary's length, P_c has decreased considerably and π_c has slightly increased. Hence, net hydrostatic pressure and net osmotic pressure essentially cancel out each other. Thus, depending on the reflection coefficient, a state of zero fluid exchange may occur.
- Finally, towards the end of the capillary, P_c decreases even further. Therefore, $(P_c - P_i) < (\pi_c - \pi_i)$ and fluid reabsorption

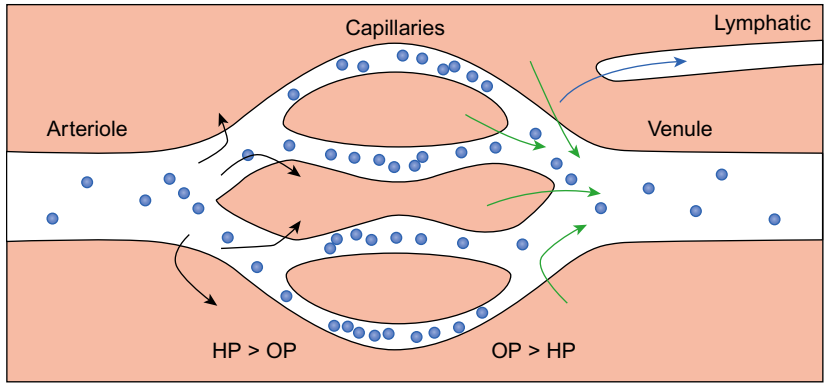


Fig. 6.6 Capillary fluid exchange. Capillaries link precapillary arterioles to post-capillary venules. Importantly, capillaries represent the main site of fluid exchange between the extravascular and intravascular tissue compartments. When fluid enters the capillaries from the arterioles it is characterized by a relatively high net hydrostatic pressure (HP) and a relatively low net osmotic pressure (OP). Hence, at the start of the capillaries, fluid will move from the capillary towards the interstitial space (black arrows). Towards the middle of the capillary the HP has decreased and the OP has increased because fluid drainage has concentrated the blood. Here, a balance between HP and OP may occur and when this occurs there is zero fluid flux. However, soluble molecules can still be exchanged between the blood and the interstitial space as these will move down their concentration gradient by diffusion. Towards the end of the capillaries HP drops even further and OP is still relatively high. Therefore, fluid reabsorption occurs (green arrows). In fact, the process of fluid reabsorption is so efficient that 90% of the fluid that has been drained from the blood in the beginning of the capillaries has been reabsorped at the end of the capillary bed. Normally, when the tissue displays proper fluid homeostasis, the remaining 10% of fluid moves back to the venous circulation via the lymphatics (blue arrow).

occurs. Importantly, in a healthy human, 90% of the fluid that moved from the capillary towards the interstitial space will be reabsorbed into the capillary, whereas the remaining 10% will move back via the lymphatic vessels towards the venous circulation.

One may wonder what the mentioned formula actually means for the total amount of fluid that is filtered by the capillaries. This depends on the capillary surface area (A) and the permeability of the capillary wall (K_f). Both reflect the anatomical properties of the capillaries, i.e. the permeability (K_f) of discontinuous capillaries is much greater as compared to the permeability of fenestrated capillaries. Moreover, the permeability of fenestrated capillaries is greater compared to the permeability of continuous capillaries. Finally, A depends on the length, diameter, and number of capillaries involved in the fluid exchange. Thus, to calculate the fluid flux (J_v) over the capillary wall one should use the following formula:

$$J_v = K_f A(P_c - P_i) - \sigma(\pi_c - \pi_i).$$

Capillary exchange of gases and soluble molecules

In addition to the exchange of water that occurs in the capillaries, capillaries also allow movement of gases and soluble molecules between the extravascular space and the intravascular space. The amount of movement of these molecules depends on diffusion (net movement down a concentration gradient) and is thus described by the First Law of Fick (45):

$$\frac{dn}{dt} = -DA\frac{\Delta C}{\Delta X}$$

In this formula dn/dt equals the flux in moles per second. Furthermore, *D* is the diffusion constant and *A* represents the surface area. Finally, ΔC and ΔX equal the concentration difference and the diffusion distance, respectively. With this formula in mind, it is easy to understand that there is more movement of soluble molecules and gases at about two-thirds of the capillary's length (since ΔC has increased because of the fact that fluids have moved towards the interstitial space due to physical forces).

Capillary exchange: bulk flow, vesicular transport, and active processes

Bulk flow, the movement of water together with its solutes, is especially important in glomeruli but can occur in nearly all tissues. It follows Poiseuille's equation of hydrodynamic flow and thus depends on both physical (i.e. hydrostatic and osmotic forces) and anatomical constrains (e.g. pore size and wall thickness). Finally, vesicular transport and other active transport processes can also be involved in movement of molecules from intravascular towards extravascular spaces, but these processes are generally considered to mainly reflect exchange of substances between individual cells and their immediate surroundings rather than reflecting exchange between intravascular and extravascular fluids.

Shear stress sensing

Blood vessels alter their morphology and function in response to changes in blood flow, and these responses are based on blood flow detection by the vascular endothelium (46). Arteries are exposed to a complex mechanical environment that they sense via numerous mechanoreceptors. On their luminal side, endothelial cells are constantly exposed to fluid shear stress, the frictional force generated by flowing blood (➔ Fig. 6.7). Arteries are also exposed to circumferential tensile stress, a perpendicular force generated by intraluminal pressure (➔ Fig. 6.7). These forces vary in time, magnitude, and direction according to vascular pulsatility and anatomy.

When blood is considered as a viscous fluid that moves along a solid boundary of a cylindrical-shaped conduit (the vessel wall), this results under laminar flow conditions in a parabolic blood velocity profile and the wall shear stress (τ) may be expressed by the following equation:

$$\tau = 4\,\eta\,Q/\pi\,r^3,$$

in which η represents viscosity of the blood, Q denotes the flow rate and r is the radius of the artery. The average wall shear stress in the healthy human aorta varies from 1 to 2 Pa and circumferential tensile stress varies from 1 to 2×10^5 Pa according to the anatomical site where it is measured. The situation is even more complex after arterial bifurcations and side-branches; these regions experience disturbed blood flow with repetitive phases of flow reversal resulting in steep spatial and temporal gradients in wall shear stress (47). Molecules integrated in the cell membrane of the arterial endothelium sense local changes in shear stress and transmit these signals into the interior of the cell, thereby evoking the following cellular responses:

- The endothelial response to acute variations in wall shear stress is closely linked to the physiological regulation of vascular tone and blood coagulation via the production of vasoactive compounds such as NO and PGI_2.
- Raised levels of shear stress represent an important stimulus for arterial remodelling processes, including arteriogenesis (collateral artery growth) (48) and angiogenesis (growth of new capillaries from pre-existing ones) (49), processes that involve cell proliferation, migration, and degradation of extracellular matrix.

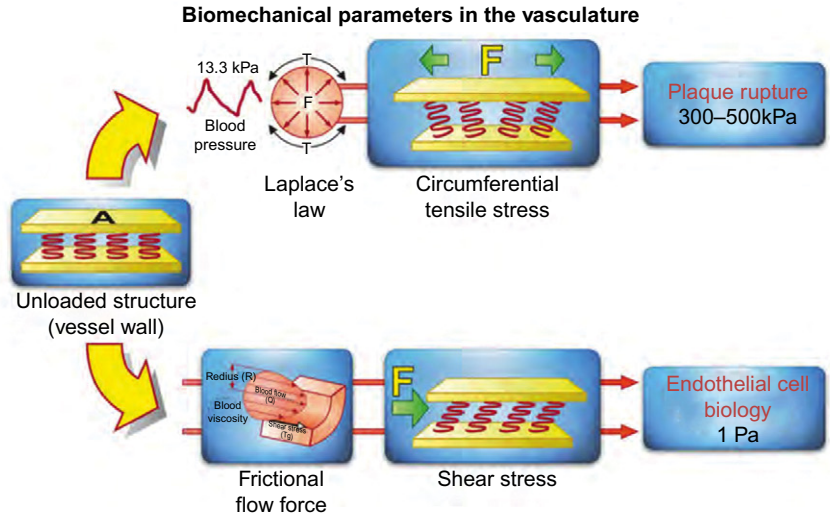


Fig. 6. 7 Biomechanical forces acting on the arterial wall. Blood pressure and blood flow induce forces in the vascular system that deform the vessel wall. When forces are to be compared they need to be normalized to surface area. Force per area is called stress and is expressed in N/m^2 or Pascal (Pa). Blood pressure produces a force directed perpendicular to the vessel wall. As a consequence, the cylindrical structure will be stretched circumferentially, resulting in a circumferential stress. Stress in the range of 300–500 kPa is associated with rupture of atherosclerotic plaques, a life-threatening condition. In contrast, the force induced by a difference in movement of blood and the non-moving vessel wall leads to a stress and strain parallel to the surface of endothelial cells. Due to its shearing deformation this is called a shear stress. This shear stress is of small amplitude (1 Pa) and exerts its main effects on endothelial cell biology through the activation of mechanosensitive receptors and signalling pathways.
(Reproduced from Kwak BR, Back M, Bochaton-Piallat ML, Caligiuri G, Daemen MJ, Davies PF, et al. Biomechanical factors in atherosclerosis: mechanisms and clinical implications. Eur Heart J. 2014;35(43):3013–20, 20a–20d with permission from Oxford University Press.)

- High levels of uniform shear stress in straight parts of arteries play an important role in maintenance of arterial homeostasis by regulating expression of anti-inflammatory genes in the endothelium through activation of key transcription factors such as KLF2, KLF4, and Nrf2 (50, 51).
- In contrast, the endothelium in regions of chronically disturbed (low, oscillatory) arterial flow displays increased expression and/or activation of the proinflammatory nuclear factor kappa B (NFkB), oxidant, and unfolded protein response signalling pathways, thereby contributing to an increased susceptibility to atherosclerosis at these locations (52, 53).

A large variety of membrane-associated molecules and microdomains have been proposed as potential shear stress sensors converting a mechanical signal into a chemical response, i.e. activation of intracellular signalling pathways. Shear stress-sensing mechanisms may roughly be divided in to three major classes: (1) specific molecules in the cell membrane, such as ion channels, receptors, adhesion molecules, and the glycocalyx; (2) specific membrane microdomains, like primary cilia and caveol; and (3) general cell-supporting structures such as the cytoskeleton and the lipid bilayer plasma membrane (54) (➔ Fig. 6.8).

Indeed, wall shear stress is known to activate a variety of endothelial ion channels, including transient receptor potential (TRP) channels and P2X4 receptors that are both signalling through a rise in intracellular Ca^{2+} concentration. Shear stress can also activate receptor-tyrosine kinases, such as the vascular endothelial growth factor receptor (VEGFR) and angiopoietin receptor, without the need for their natural ligands. Integrins may be involved in this ligand-independent activation of receptor-tyrosine kinases by shear stress. Integrins are transmembrane molecules with an extracellular domain that binds to extracellular matrix proteins and an intracellular domain that interacts with the cytoskeleton via focal adhesions and are thus ideally positioned for mechanotransduction. Changes in blood flow will also affect the conformation of the glycocalyx and the mechanical signal may be transduced to the cytoskeleton through the intracellular domain of glycoaminoglycans. Finally, molecules at cell–cell contacts in adherence junctions or gap junctions may serve as a mechanosensory complex to transmit the signal induced by mechanical forces from cell to cell. Although various mechanoreceptors seem to have a linkage to the cytoskeleton as a common denominator, wall shear stress may, however, also display direct effects on the actin cytoskeleton leading to alterations in gene expression of, for example, endothelin-1. As the physical properties of the plasma membrane (e.g. fluidity, pH, and temperature) affect the conformation and function of the molecules embedded in this lipid bilayer, the plasma membrane may be regarded

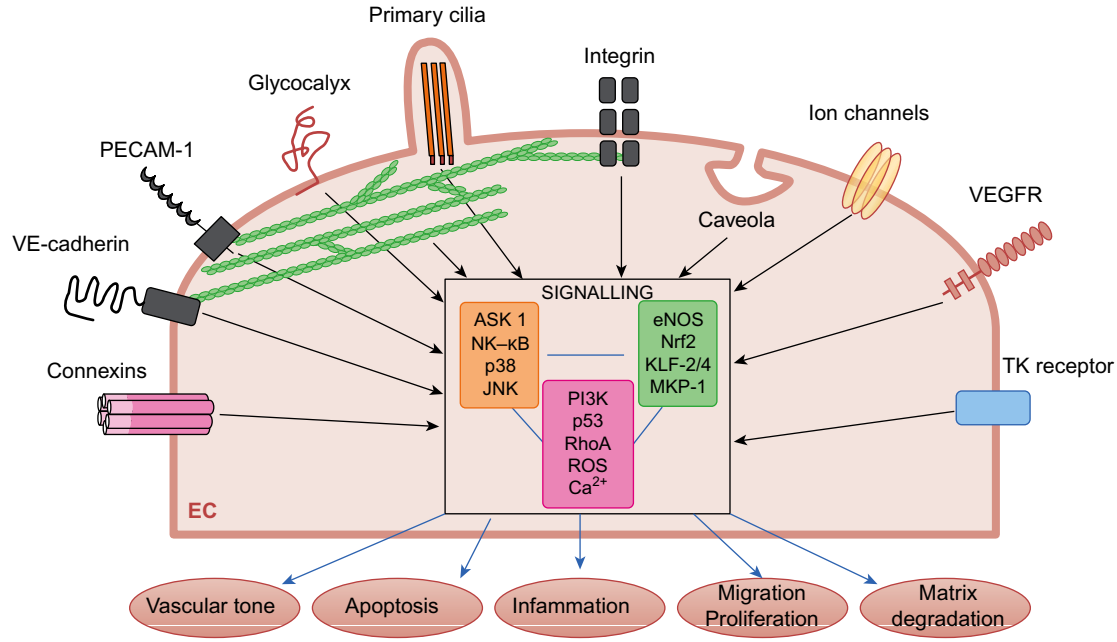


Fig. 6. 8 Mechanoreceptors and intracellular signaling in arterial endothelium. Schematic representation of a large variety of membrane-associated molecules and microdomains that have been proposed as potential shear stress sensors converting a mechanical signal into a chemical response. Shear stress activates receptor-tyrosine kinase, such as the vascular endothelial growth factor receptor (VEGFR) and PECAM-1, which regulate leukocyte adhesion and EC-EC coupling as well as mechanoresponsiveness. In addition to these mechanoreceptors, shear stress can also activate ion channels, actin filaments, caveolae, the glycocalyx, primary cilia, and adherence or gap junction proteins. Shear stress influences activation of ECs through multiple mechanisms that target the mitogen-activated protein kinases (MAPKs), nuclear factor kappa B (NFκB), and regulators of these pathways, including MAPK phosphatase-1 (MKP-1), Kruppel-like factors-2 and 4 (KLF-2/4), nuclear factor erythroid 2-related factor (Nrf2), and eNOS.
(Reproduced from Kwak BR, Back M, Bochaton-Piallat ML, Caligiuri G, Daemen MJ, Davies PF, et al. Biomechanical factors in atherosclerosis: mechanisms and clinical implications. Eur Heart J. 2014;35(43):3013–20, 20a–20d with permission from Oxford University Press.)

as an alternative common denominator for various mechanoreceptors. Of note, no information is yet available for discriminating which of the proposed candidates may be used to sense the subtle changes in wall shear stress as compared to the detection of circumferential stress in response to pulsatile flow or even to chronic hypertensive loading.

Conclusion

It was earlier thought that the endothelium merely forms a physical barrier separating blood and interstitial tissues. However, nowadays there is an overwhelming amount of evidence showing that the endothelium performs multiple tasks crucial to maintain homeostasis. For example, endothelial cells (1) secrete anti-inflammatory substances at locations exposed to high shear stress in conduit arteries, (2) release various important vasodilators, such as NO and EDH, in resistance arteries, and (3) regulate transport of fluids and/or proteins from the blood towards the interstitial space in capillaries. Moreover, given the fact that endothelial cells are directly exposed to blood, they do, at least in theory, form a highly promising therapeutic target in diseases such as atherosclerosis, hypertension, and oedema. It is, therefore, surprising that there are only very few therapies specifically aiming at this cell type. Thus, clearly, the development of drugs aimed at modifying endothelial function during pathologies should be one of the main focuses for cardiovascular researchers in the decades to come.

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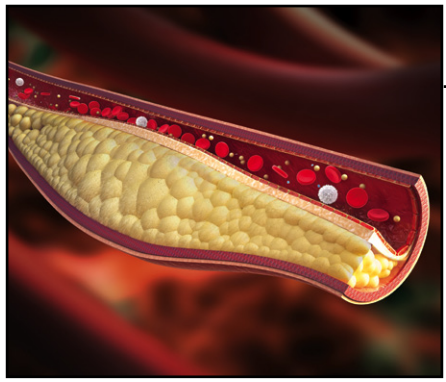

CHAPTER 7

Vascular smooth muscle cells

Marie-Luce Bochaton-Piallat, Carlie J.M. de Vries, and Guillaume J. van Eys

Content

Introduction

This chapter focuses on arterial smooth muscle cell (SMC) biology in the healthy vessel wall. The arterial wall contains multiple layers of SMCs, of which the number increases with vessel diameter providing structural support to these conduits. SMCs can contract and relax, thereby regulating the lumen of the vessels and thus blood supply to different parts of the body. A unique feature of SMCs is that these cells adapt their phenotype back and forth in between so-called contractile and synthetic SMCs in response to environmental cues. This knowledge is crucial to understand vascular pathologies such as atherosclerosis, stenosis, and hypertension. For a deeper understanding of SMC function, knowledge of the embryonic origin of these cells at different locations in the vascular tree and understanding the exact function of SMCs at those sites are indispensable. A wealth of information is available on the underlying signalling pathways and SMC-specific transcription factors that determine SMC function, which will be summarized.

Smooth muscle cell embryonic origin

SMCs of the vascular wall have distinct and well-defined embryonic histories depending on their localization in the body (for reviews see (➔ 1, 2)). Most SMCs are derived from local mesoderm cells, associating with the tube-like structures that are formed by endothelial cells in early embryogenesis. Endothelial cells play a key role in this process by attracting the mesodermal cells and promoting their final differentiation. In the descending aorta, however, SMCs initially arise from the lateral plate mesoderm but are shortly after replaced by SMCs derived from the paraxial mesoderm (somites) (3, 4). In addition, these somite-derived SMCs build up the renal and intercostal arteries (3). Cardiac neural crest cells, which are neuroectodermal derivatives, contribute to septation of the aorta and the pulmonary trunk. These cells also form the tunica media—vessel wall without endothelial cells and adventitia—of the ascending aorta, the aortic arch, and the common carotid arteries (5–7). Cardiac neural crest-derived SMCs are responsible for the elastic properties of the large vessels by promoting elastogenesis (8).

SMCs at the base of the aorta and the pulmonary trunk develop from the second heart field containing multipotent progenitor cells, which also contribute to the development of the myocardium (9).

Development of the coronary vessels is distinct from the systemic circulation, as both endothelial cells and SMCs arise from the proepicardium, a transient mesothelial cell cluster located at the venous pole of the heart. In addition to forming the epicardium, these proepicardial cells undergo epithelial-to-mesenchymal transformation and invade the myocardium to give rise to the coronary vessels (10–13). Finally, SMCs of the gut vasculature arise from the serosa mesothelium (14, 15).

The fate of mesodermal cells to SMCs is driven by several growth factors (for a review see (➔ 2)). In early embryos, cells of the so-called primitive streak develop into mesodermal cells upon stimulation by factors such as fibroblast growth factor 2 (FGF-2) and activin. The mesodermal cells subsequently transform, directed by factors like FGF-2 and bone morphogenetic protein 4 (BMP4), into paraxial and lateral plate mesoderm. A last developmental step, induced by transforming growth factor β1 (TGF-β1) and platelet-derived growth factor-BB (PDGF-BB), leads to the differentiation towards vascular SMCs. In addition, cells originating from the primitive streak may, driven by factors such as sonic hedgehog (shh) and BMP4, become neural crest cells, which again, under the influence of PDGF-BB and TGF-β, become SMCs of the ascending aorta. Final differentiation of proepicardial cells into coronary SMCs depends on PDGF and Notch pathways (for reviews see (➔ 16, 17)).

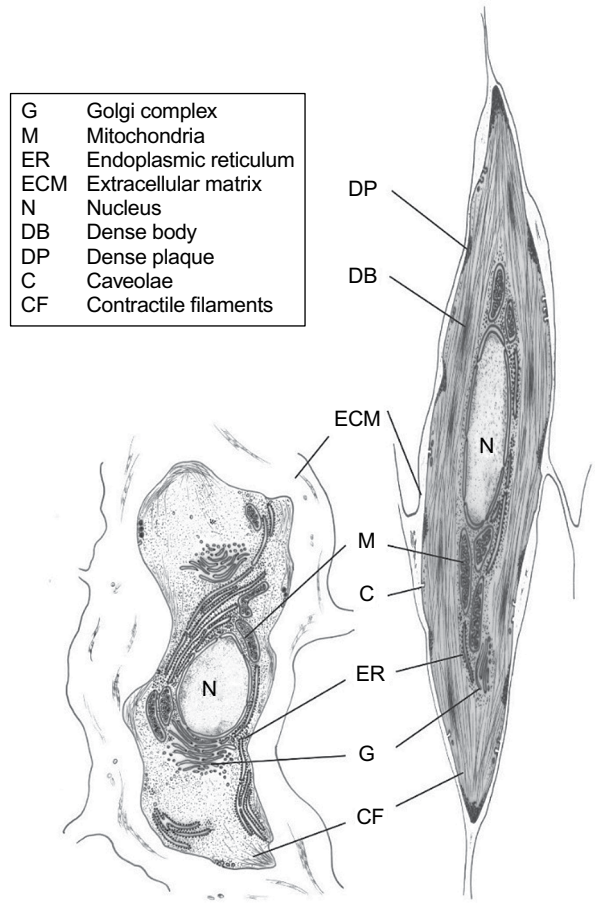

Fig. 7.1 Ultrastructural characteristics of contractile and synthetic SMCs. (Reproduced from Rensen SS, Doevendans PA, van Eys GJ. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. Neth Heart J. 2007;15(3):100-8 with permission from Springer.)

Ultrastructure of smooth muscle cells

During embryogenesis, early SMCs have been described as fibroblast-like cells that lack a basement membrane, but exhibit well-developed protein synthetic organelles, with a predominance of rough endoplasmic reticulum and Golgi apparatus. Gradually a build-up of myofilaments, at the cost of the synthetic machinery, occurs giving the SMCs a more muscle cell appearance, i.e. a contractile phenotype (➔ Fig. 7.1) (18–20). Contractile SMCs *in vivo* are elongated with a centrally located nucleus. The sarcoplasmic reticulum is poorly developed compared to the one found in cardiomyocytes. SMCs are surrounded by a basal lamina and the cells are connected to each other by gap junctions, important for the propagation of depolarizing currents. The myofilaments, mainly composed of actin and myosin, are not organized into distinct repeating sarcomeres, like in cardiomyocytes or skeletal myocytes, but are anchored to the dense plaques at the inner surface of the sarcolemma and intracellularly to the dense bodies (➔ Fig. 7.2). The dense plaques correspond to the transmembrane machinery (i.e. focal adhesions containing integrins and associated proteins, as well as desmosomes) linking the extracellular matrix (ECM) and the intracellular cytoskeleton. The dense bodies are similar to the Z-bands of the striated muscles and contain α-actinin, a cross-linked actin-binding protein, and two intermediate filament proteins: desmin, found in all muscle cells, and vimentin, expressed in all mesenchymal cells. In addition to their contractile apparatus, SMCs exhibit an extensive network of non-contractile cytoskeleton composed of intermediate filament proteins (desmin and vimentin) that bind to the dense bodies and desmosomes at the cell membrane but are obliquely oriented to the myofilaments (for a review see (➔ 21)).

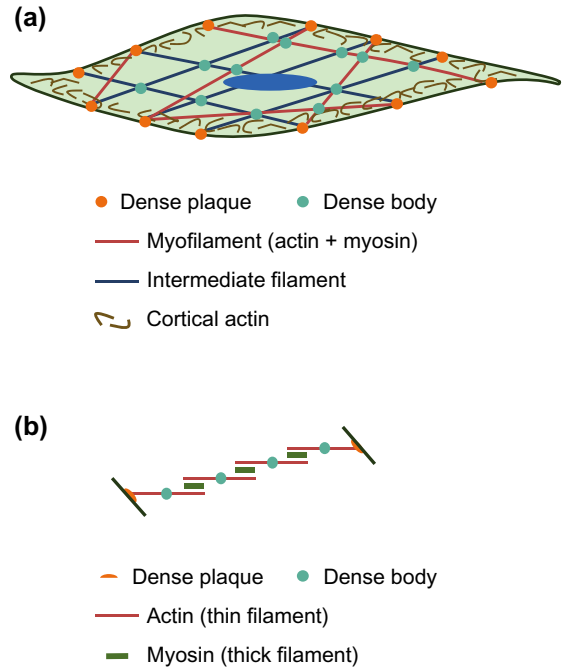

Fig. 7.2 Schematic representation of cytoskeletal apparatus in SMCs. (a) SMCs contains myofilaments (responsible for cell contraction) and bundles of intermediate filament proteins inserted into cytoplasmic dense bodies and dense plaques at the cell membrane. (b) Higher magnification view of the contractile apparatus of SMCs.

SMCs contain two smooth muscle actin (SMA) isoforms: α-SMA, which is the predominant actin isoform in vascular SMCs, and γ-SMA, which is highly expressed in enteric SMCs. Remarkably, γ-SMA is more expressed in the highly compliant vein than in the more stiff arteries (22). In addition, SMCs contain the two ubiquitous β- and γ-cytoplasmic actin isoforms. Thanks to the advent of specific antibodies, their cytoplasmic compartmentalization has been recently better clarified (for a review see (➔ 23)). Whereas α-SMA is restricted to the contractile actin filaments and is associated with myosin, the β-cytoplasmic actin is located around the dense bodies and plaques (24, 25). The γ-cytoplasmic actin is limited to the cortical actin compartment (25) (➔ Fig. 7.2).

The force of contraction developed by smooth muscle is similar to that of striated muscle, even if the proportion of myosin compared to actin is lower in smooth muscle than in striated muscle. This is due to the length of the mini-sarcomeres, which is higher in smooth muscle than in striated muscle. In addition, intermediate filament and microtubule networks participate to the contractile activity of SMCs. Moreover, binding of the contractile SMC machinery to the well-developed and complex ECM network contributes to the capacity of SMCs to generate high forces (for a review see (➔ 23)).

Smooth muscle cell phenotypic plasticity

Definition of smooth muscle cell phenotype

A key characteristic of adult SMCs is their lack of final differentiation. Skeletal and heart muscle cells undergo already during embryogenesis terminal differentiation and become contractile cells. As a consequence, changes in adult heart and skeletal muscle cells are limited to an increase or decrease in size. On the contrary, SMCs retain plasticity as they can (repeatedly) shift from a contractile to a so-called synthetic phenotype and vice versa, a process called phenotypic modulation (for reviews see (➔ 19, 26)). This phenotypic shift has been mostly described in a pathological context, since it contributes to processes such as atherosclerosis, stenosis, and hypertension (18, 27, 28). However, smooth muscle tissue, located around hollow organs that are subject to profound changes and mechanical forces, demands a flexible design. Structure and biochemistry of the contractile SMCs are geared for contraction, whereas the contractile capacity of SMCs with a synthetic phenotype is diminished and these cells synthesize ECM components and initiate cell division (➔ Fig. 7.1) (18, 19). Shifting between these two phenotypes allows smooth muscle tissue to cope with changing conditions by increasing or decreasing contractility, restructuring the ECM, and adapting the density of SMCs in tissues. Larger elastic arteries, such as the upper aorta or pulmonary trunk, contain many elastic fibres and thus the majority of the SMCs exhibit a synthetic phenotype with only a few contractile SMCs. In contrast, muscular arteries, such as coronary, mesenteric, and femoral arteries, contain mostly contractile SMCs and less elastic fibres. The SMC phenotype composition of blood vessels can change when conditions change or if blood vessels are transplanted into a new environment. For instance, the use of the saphenous vein in coronary artery bypass surgery causes extensive shifts in SMC phenotypes as well as in ECM composition (29). If the internal thoracic artery, a muscular artery, is used for bypass surgery changes in SMC phenotype and ECM are less drastic (30). Hence, SMCs of the vascular walls combine various embryonic backgrounds with environmental cues to regulate their phenotypic modulation, achieving optimum oxygen and food supply for the downstream tissues.

The concept of SMC phenotypic switch has been reinforced by the characterization *in vitro* of morphologically distinct SMC populations. Two different populations of SMCs were initially isolated from the rat carotid artery (31) and then from the rat aorta (32–34): (1) spindle-shaped SMCs, with a 'hill and valley' growth pattern obtained

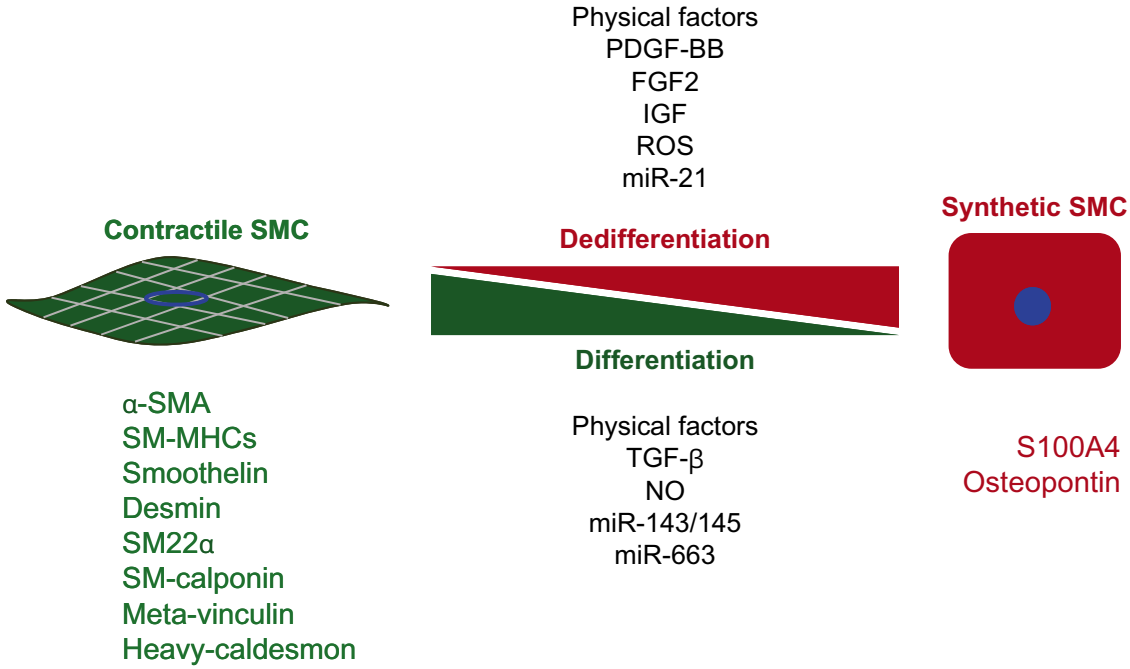

Fig. 7.3 Differentiation/dedifferentiation markers and factors involved in SMC phenotypic transition.

from the normal media and (2) epithelioid SMCs growing as a regular monolayer and exhibiting a cobblestone morphology isolated from the intimal thickening 15 days after endothelial injury (an experimental model of atherosclerosis). Similar SMC populations were further isolated from several species such as mouse, cow, pig, and humans (in the larger animals 'rhomboid' SMCs are similar to epithelioid SMCs), and from a variety of tissues and vascular locations (35–37). In particular, epithelioid and rhomboid SMCs can be isolated from the media of healthy arteries.

These phenotypic sub-populations differ not only in their location, but also in morphology, proliferative potential, migratory activity, and biochemical aspects. Epithelioid/rhomboid SMCs in intimal thickening are characterized by a poor level of differentiation, which is associated with enhanced proliferative, migratory, and proteolytic activities (for reviews see (➔ 19, 20)). The study of these SMC populations has extended our knowledge on SMC phenotype transition.

Smooth muscle cell differentiation markers

Switching from one SMC phenotype to the other is not an instant event. While adapting their phenotype, SMCs go through stages having characteristics of both synthetic and contractile cells. This gradual process and the differences in embryonic background already indicate that more than one or two marker proteins are required to determine the phenotypic status of a SMC. Coming from the morphological classification, spindle versus epithelioid/rhomboid, investigators have improved this subjective classification into a more distinct one by identifying protein markers specific for either the contractile or the synthetic phenotype (➔ Fig. 7.3).

α-SMA, the actin isoform typical of SMCs, is found in contractile as well as synthetic SMCs. α-SMA is by far the most used; it is expressed at early stages of development and represents the most general marker for the SMC lineage. Being an essential component of the contractile machinery, removal of α-SMA resulted in impaired vascular contractility and blood pressure control in adult mice (38). Expected lethality after knocking out the α-SMA gene was circumvented by surrogate activation of the skeletal actin gene.

With respect to intermediate filament proteins, vimentin, the marker of mesenchymal cells, is expressed in all SMCs. Desmin, which is a marker of muscle cells, is found in differentiated/contractile SMCs. Its content is high in muscular arteries and low in elastic arteries, where SMCs exhibit a more synthetic phenotype (39).

There are marker proteins that cover the two extremes of the entire phenotype gradient. Smooth muscle myosin heavy chains (SM-MHCs) and smoothelin have been found to be restricted to the contractile phenotype, whereas S100A4, a calcium-binding protein known as a mediator of cancer metastasis, and osteopontin, an extracellular matrix protein involved in bone mineralization, have been useful in identifying the synthetic phenotype (40–43). Mice deficient for SM-MHC, a central player in the contractile apparatus, died 3 days after birth (44), whereas smoothelin deficiency

in mice results in shortcomings comparable to that of α-SMA deficiency (45). S100A4 is upregulated in arteries from children with pulmonary hypertension (46), in human atherosclerotic and restenotic lesions (43, 47), and in human thoracic aortic aneurysms (48). Increased proliferation of pulmonary arterial SMCs has been observed in mice overexpressing S100A4 (49). Osteopontin has been described rather as an inducer of SMC proliferation than as a reporter of the synthetic phenotype.

Finally, proteins such as SM-calponin, SM22α, heavy-caldesmon, and meta-vinculin are also found in not fully differentiated cells or remain longer in cells on the way to the synthetic phenotype, and therefore may be considered as 'intermediate' markers. This is convincingly demonstrated in contractile SMCs that have been brought into culture. During the consecutive cell passages, markers of the contractile phenotype disappear, smoothelin and SM-MHC first. Since *in vivo* most SMCs will be of the quiescent contractile phenotype and the contractile apparatus is the dominant structure, it will come as no surprise that the majority of SMC markers are components of the contractile apparatus. Disappearance of the contractile markers has long been taken as a sign of modulation towards the synthetic phenotype, because of the paucity of true markers for the synthetic phenotype (for reviews see (➔ 19, 20)).

Smooth muscle cell variation by development, adaptation, and phenotypic modulation

Intrinsic variation in vascular SMCs starts by their different embryonic origin, as indicated. In addition, local conditions in terms of pressure, pulsations, flow, and turbulence vary substantially throughout the vascular system. SMCs respond to these dissimilarities by adapting their phenotype to the local situation. To make the aspect of phenotype transition even more complex, *in vitro* experiments revealed that established blood vessels contain sub-populations of SMCs. This indicates that, although there may be subtle differences between SMCs from different vessels, local conditions dictate the ratio between contractile and synthetic SMCs.

The phenotypic variation of SMCs derived from different tissues is the result of specific gene-expression profiles. Local environmental cues activate intracellular signalling pathways finally resulting in activation and silencing of sets of genes. To maintain the balance between the phenotypic subsets in the vessel wall, SMCs communicate with each other and with the endothelial cells that provide crucial environmental information. *In vitro* studies are suitable to identify factors that modulate SMC phenotype, such as growth factors and hormones. Also, proteins involved in cell–cell interaction and migration have been studied extensively in cultured SMCs (50, 51). However, research into the exact effect of pressure and flow requires *in vivo* studies; for instance, pressure and flow change as the blood runs down the aorta. The thoracic aorta has to withstand great changes in pressure caused by the pumping of the heart. The characteristics of SMCs in this segment are mostly synthetic as their primary task is to provide components for a strong and elastic ECM. SMCs in the abdominal aorta need to maintain pressure and thus most SMCs have a contractile phenotype. Since all SMCs of the aorta, with the exception of those in the ascending aorta, have the same embryonic origin, pulsation and pressure appear to determine the SMC phenotype (52–54).

An elegant experiment performed in transgenic mice showed that phenotypic destiny is at best temporary, i.e. the same cells can be contractile at one time point and synthetic at another (55). Mice were generated in which SMCs of the thoracic aorta express β-galactosidase at the moment they were contractile and thus coloured blue upon substrate addition. As expected, in the vessels of those mice some SMCs stained blue and some did not. Another mouse was engineered such that SMCs would colour blue once they had been contractile, even for a short while; all SMCs coloured blue. This indicates that the modulation of SMC phenotype between contractile and synthetic is not a rare event but rather an ongoing process.

Regulation of SMC differentiation marker expression

Gene regulation

A multitude of environmental and internal cues induce or repress the expression of specific gene sets, eventually leading to a more contractile or synthetic SMC phenotype. Marker proteins that are linked to defined phenotypic stages will be subject to gene regulation that is exemplary for other proteins of which expression is limited to the same phenotypic stages. Therefore, investigation of the gene regulation of marker proteins has been leading in SMC-specific gene-expression research. Both *in vitro* cell culture and transgenic mouse models have been applied to investigate the regulation of marker gene expression in SMCs. Classical straightforward reporter gene assays, assessing the effect of deletions and mutations of putative cis-acting elements of promoters of genes, such as α-SMA, SM-MHCs, smoothelin, SM22α, and SM-calponin, have been performed. Complementary, identification of factors that regulate SMC transcriptional activity have been investigated by established techniques such as footprinting, gel-shift assays, and chromatin immunoprecipitation (ChIP). Unfortunately, few if any studies are available in which the transcriptome of SMCs at different

phenotypic stages was studied. Here, we will describe the most important *in vivo* findings, occasionally supported by *in vitro* studies.

Because smaller promoter fragments, successfully used in *in vitro* studies, had no activity *in vivo*, investigators have come to prefer large fragments such as bacterial or yeast artificial chromosomes in transgenic mice. These large DNA fragments (>200 kilobases) are more likely to contain not only all the regulatory enhancer/repressor elements, but also the information for the correct higher order chromatin structure, which is known to influence transcription profoundly. The transcription pattern of reporter genes in transgenic mice carrying such large constructs mimics the expression profile of endogenous SM22α and SM-calponin (56–58). Alternatively, limitations of a small promoter fragment can be overcome by knock-in gene targeting, involving insertion of a reporter gene in the target gene. This way, all the regulatory sequences of a gene remain in place and will act as in the endogenous gene. This approach has been successful for, among others, α-SMA and SM22α. Introduction of mutations in enhancer regions allows analysis of a particular cis-element in transcription regulation (59). A third method to elucidate the effect of specific transcription factors on SMC phenotype modulation is the generation of mice deficient for these factors. Applying cre-recombinase technology can circumvent embryonic lethality, which often occurs in such mice (60, 61).

The most investigated and best-described SMC regulatory element is the so-called CArG box, which plays a central role in the regulation of the SMC differentiation programme. The CArG box consists of a $CC(A/T_6)GG$ motif. This specific sequence has been found (so far) in the promoters of all genes of which expression is restricted to SMCs. Often these promoters contain two or more CArG boxes that act in concert. Their position, as well as their distance, to the transcription start site is relevant for promoter activity. Serum response factor (SRF) is the transcription factor that binds to the CArG box, and SMC differentiation is SRF dependent (62). On the contrary, in non-SMCs, even overexpression of SRF will not activate SMC-specific promoters. Also, the expression level of SRF does not change during SMC modulation. These findings lead to the conclusion that SRF does not directly regulate SMC phenotypic modulation. Furthermore, SRF is subject to transcriptional and post-translational modifications that may contribute to selective promoter or cellular preferences. In line with these observations, it has been shown that the cofactors of SRF largely determine the outcome of SRF-mediated gene regulation in SMCs. A considerable number of cofactors have been identified, such as myocardin, Nkx3.1, Nkx3.2, MRTF-A, and GATA-4 (63). Of these factors, myocardin appears to be the most important. Myocardin stands apart from the other SRF cofactors because it is unable to bind DNA by itself and for full transactivation of SMC promoters it has to form dimers (64). In that constellation myocardin is a strong activator of transcription with up to a thousand-fold induction of SMC genes. Whereas SRF levels do not correlate with the SMC phenotype, myocardin levels do. Low myocardin levels are associated with the synthetic SMC phenotype and high levels with the contractile phenotype. With the data at hand, the generally accepted theory is that SRF serves as a docking platform, interacting with different co-activators and co-repressors. The composition of the SRF-complex is variable and determines the outcome of SMC-specific gene expression and thus contributes substantially to SMC heterogeneity (for a review see (➔ 65, 66)).

Epigenetics

In addition to gene expression regulation, programmed chromatin remodelling may dictate different subsets of SMCs with distinct function and phenotype. However, the data in this particular field are limited (for a review see ➔ (67)). In the early stages of life most SMCs will have accessible DNA in both contractile-specific and synthetic-specific genes, providing the potential to modulate to either phenotype (68). In a pathological setting, a chromatin remodelling mechanism is supposed to move SMCs towards a synthetic phenotype with relatively inaccessible 'contractile' genes. Data on SM-MHC CArG boxes support an accessible state of the chromatin structure of SMC-specific genes in SMCs but not in other cell types (69). Also, the histones of CArG-containing regulatory elements of SM-MHC, SM22α, and α-SMA have been shown to be acetylated (a characteristic of chromatin accessibility) in contractile SMCs. It is obvious that the chromatin status is another level that contributes to the regulation of phenotypic heterogeneity of SMCs.

MicroRNAs (miRNAs)

A third level of SMC phenotypic gene expression regulation is the post-transcriptional processing of mRNA of the SMC-specific genes. This includes processes such as alternative splicing, nuclear transport, and post-transcriptional modifications. In recent years miRNAs have been discovered to perform yet another level of gene-expression regulation by degradation or translational repression of target mRNA. miRNAs are a class of approximately 22 nucleotide-non-coding RNAs that act by pairing with target mRNAs. In mammalian cells miRNAs apply a mechanism of imperfect pairing, which means that on the one hand a given miRNA may have hundreds of different mRNA targets, and on the

other hand that a given target may be regulated by multiple miRNAs. A first indication of the effect could be deduced from Dicer knockout mice. Dicer is an enzyme crucial in the formation of miRNA. The blood vessels in SMC-specific homozygous Dicer-deficient mice, which die during embryogenesis, are dilated and thin-walled because of reduced SMC proliferation. Furthermore, the arteries of these mice exhibit reduced expression of SMC contractile genes, which results in impaired active and passive contractility (70).

In SMCs a number of active miRNAs have been identified. Here we will focus on the well-investigated miR-143/145 cluster that has been shown to be involved in the acquisition and maintenance of the SMC contractile phenotype (for a review see (➔ 71)). Expression of this cluster is largely regulated by the factors that also regulate contractile SMC marker proteins, such as SM22 and calponin. SMCs of mice, deficient for this cluster, have been demonstrated to be locked in a synthetic phenotype (72). This finding has been confirmed by several groups, using slightly different knockout mouse models (73–75). Expression of marker proteins for the contractile phenotype, such as calponin and smoothelin, was reduced by 50%, coinciding with a dramatic reduction in vessel contractility in response to angiotensin II (AngII) and phenylephrine. Remarkably, although expression of marker proteins runs parallel with that of the miRNAs 143/145 during embryogenesis, knocking out the miRNA cluster had no effect on the development of the mice (72, 73). A decrease in vessel wall thickness has been observed in knockout mice as a result of a reduced actin stress fibre formation (74). Other investigations have linked the miR-143/145 cluster to shear stress, as they are synthesized in endothelial cells and sent to SMC in exosome vesicles (76). Although the various studies show differences, all studies position the miR-143/145 cluster as a promoter of the contractile SMC phenotype, a modulator of actin dynamics and cytoskeletal assembly. More recently, also miR-663 has been identified as an miRNA that promotes the contractile phenotype and reduces SMC proliferation (77). Other miRNAs, such as miR-21 (78), miR-221, and miR-222 (79), generate opposite effects, driving SMCs towards proliferation. In summary, data on miRNAs so far have shown that they are an integrated part of the biology of SMC phenotype modulation (for a review see (➔ 80)). More recently, long noncoding RNAs have been added to the list of compounds that play a role in the control of SMC phenotype (for a review see (➔ 81)).

Extracellular factors influencing smooth muscle phenotype

Extracellular factors regulating SMCs can either be physical factors, including mechanical stimuli and cell–cell interaction, or biochemical factors, such as different hormones and growth factors.

Physical factors

The physical environment is important in shaping the SMC phenotype. Physical factors such as flow, pressure, and cell–cell interaction have a profound effect on the SMC phenotype. We mentioned the effect of the changing physical environment along the aorta, which forces SMCs to apply their phenotypic potential from synthetic in the thoracic aorta to contractile in the abdominal segment. The composition of the ECM produced by the synthetic SMCs displays unique elastic characteristics to the vessel wall, which allow the thoracic aorta to cope with the repeated increase and decrease in diameter caused by strong pulsatile waves of the pumping heart. The carotid arteries that branch off close to the heart are also rather elastic but contain more contractile cells than the upper segment of the aorta. Differently, the abdominal aorta and major vessels, such as the femoral and mesenteric arteries, have to comply with high pressure. SMCs in those arteries are muscular and support the flow generated by the heart.

Mechanical forces enhance the expression of both ECM and contractile proteins in SMCs. This is already the case in the developing embryo, where ECM synthesis, proliferation, and subsequent contractile SMC differentiation coincide with the onset of pressure in the embryonic circulation. In adult SMCs mechanical strain can lead to either increased synthesis of ECM components or in an increase in contractile proteins. In addition to the mechanical force, type/composition of the ECM and cell–cell interactions play a role in the phenotypic determination of SMCs.

Pressure and arterial stretch can be sensed directly by SMCs. Mechanical stretch is transmitted to the SMCs through focal adhesion that links the ECM and the intracellular cytoskeleton. Focal adhesions are macromolecular protein complexes consisting of transmembrane integrins that are associated with intracellular adaptors (paxillin, vinculin, and talin) and signalling proteins (focal adhesion kinases). Other structures such as caveolae, G-protein receptors, ion channels, and Rho family GTPases are also able to sense changes in physical forces (for reviews see (➔ 82–84)). Physical forces trigger perturbations of these mechanosensors, which are transduced into biochemical signals and initiate signalling cascades that lead to downstream changes in gene-expression patterns affecting the SMC phenotype. Additionally, sensing of changes in flow has to be mediated by endothelial cells. Shear stress can modulate endothelial cell function by sequentially activating mechanosensors, intracellular signalling pathways, and the expression of genes coding for specific (transcription) factors or miRNAs

involved in phenotypic modulation of the neighbouring SMCs (for reviews see (➔ 84, 85)).

In vitro studies in this field have fallen short in providing a complete picture. In addition to technical difficulties, SMCs placed in culture have a tendency to become synthetic. Depending on the cell cycle state of SMCs and/or the ECM environment (e.g. coating), contrasting effects of physiological strain on SMC phenotypes have been observed (for a review see (➔ 86)). For example, physiological cyclic strain can inhibit SMC cycle progression (87–89) and increases expression of differentiation SMC markers, such as h-caldesmon (90) and SM-MHCs (91). In contrast, SMC proliferation and increased ECM synthesis have been observed as well (92–94). Experiments in which SMCs and endothelial cells are co-cultured also have resulted in a modulation towards the synthetic SMC phenotype. Altogether, although physical factors have been identified as modulators of SMC phenotype, an understanding of the effects of these factors on phenotypic modulation of SMCs is far from complete.

Biochemical factors

The list of biochemical factors that influence SMC phenotype is long. Among these factors are growth factors, cytokines, and ECM proteins. This overview will be restricted to factors that are backed by a substantial amount of experimental data. These factors include PDGF-A/B, TGF-β, nitric oxide (NO), and reactive oxygen species (ROS) (➔ Fig. 7.3). In addition, compounds like IGF-I and IGF-II, endothelin-1, AngII, PPAR-γ agonists, and FGF and ECM components, such as heparin and collagen, have been shown to affect SMC phenotype but will not be discussed here.

PDGF-A and -B have been identified as homo- or heterodimers that influence SMC differentiation. In early embryogenesis PDGF is critical for endothelial cells to recruit SMC precursors. Mice deficient for PDGF-B and PDGF-receptor-α or -β have reduced numbers of SMCs. In adults, PDGF is known to regulate proliferation and migration after arterial injury. From these examples it is clear that PDGF drives SMC differentiation towards a synthetic phenotype (for reviews see (➔ 66)), as has been confirmed by *in vitro* experiments in contractile pig coronary artery SMCs (37, 43).

TGF-β is essential to induce SMC differentiation *in vivo*, as it plays a role in the migration of mesenchymal precursor cells towards endothelial cells and stimulates subsequent interaction between these cells during the formation of blood vessels. This is supported by the observation that *in vitro* TGF-β promotes multipotent (stem) cells to differentiate towards a contractile phenotype. In adult tissues TGF-β also stimulates a shift towards a contractile phenotype, as can be deduced from the increased expression of proteins such as α-SMA, SM-MHC, and SM-calponin, and the reduction of ECM degrading proteases. In addition, TGF-β suppresses cell division by inducing expression of the cell-cycle inhibitors p21 and p15. Although TGF-β has also been reported to stimulate the production of proteins like collagen and elastin, the net effect of TGF-β is a differentiation of SMCs towards the contractile phenotype (for reviews see (➔ 66)).

There is increasing evidence that small molecules like NO and ROS are regulators of SMC phenotype. NO can be generated by several enzymes, but the predominant enzyme in the vasculature is endothelial NO synthase (eNOS). Physiological levels of NO, generated by eNOS, promote the contractile SMC phenotype. In particular, NO interferes with SMC proliferation, possibly by downregulation of NFκB and/or AP-1. ROS are major modulators of NO availability. The interaction of NO and O_2^- radicals will result in the formation of peroxynitrite ($ONOO^-$). Several studies indicate that increased breakdown of NO by an enhanced production of O_2^- is a key mechanism leading to the decline of endothelium-dependent vasorelaxation. In addition, ROS affect intracellular and intercellular second messengers that modulate many downstream signalling molecules, like protein tyrosine kinases and tyrosine phosphatases, transcription factors, and ion channels (for a review see (➔ 95)). Induction of these signalling cascades leads to SMC proliferation and migration, change of endothelial function, modification of extracellular matrix, and expression of pro-inflammatory mediators. Besides, Ang II has been suggested to induce its pleiotropic vascular effects through NADPH-driven generation of ROS. Experimental results on ROS are not unequivocal. This may be due to the way ROS is generated, which largely determines the location of ROS in the cells. For instance, Nox1 and Nox4 can produce ROS; whereas Nox1 has been reported to decrease the expression of SMCs marker proteins, Nox4 has been implicated in the maintenance of the contractile SMC phenotype (for a review see (➔ 96)). Nevertheless, ROS are considered to promote the synthetic SMC phenotype and to be involved in pathologies such as hypertension and atherosclerosis.

Effect of ageing on SMCs

Ageing has a profound effect on the number, phenotype, and performance of SMCs in the medial vascular wall, and this coincides with a thickened intima, increased ECM deposition (in particular collagens I and III), and progressive calcification. This may be the consequence of confounding

factors such as failing endothelial cells, inhibition of stem cell differentiation, and damage by circulating cells. However, also a number of internal, sequential changes have been observed in SMCs during the ageing process. Ageing coincides in SMCs with a progressive shift from the contractile to the synthetic phenotype, a decrease in size, and finally senescence and/or apoptosis. Several underlying mechanisms involved in SMC ageing have been proposed (for a review see (➔ 97)). Telomere reduction, caused by repeated cellular division, stress, or impaired DNA repair, has been suggested to be at the basis of SMC ageing. Telomeres consist of DNA repeats at the end of chromosomes. Each cell division telomeres loose a number of these repeats and the loss of a major part of these repeats interferes with the cell cycle and drives the cell in a final G0 phase, hence senescence (98). The decreasing length of telomeres is counteracted by telomerase, an enzyme that restores telomere length. Telomeres in telomerase-deficient mice display a decrease of telomere length over consecutive generations resulting in impaired reproduction in the sixth generation. In SMCs, telomere shortening is associated with senescence and apoptosis (99–101). In addition, non-age-related factors, such as stress, have been shown to affect telomere length and drive cells towards senescence.

Best documented in SMC ageing are the effects of ROS, concentrations of which have been reported to increase with age in the arterial wall (102). High concentrations of ROS damage DNA in general, telomeres in particular, and proteins. In healthy SMCs ROS are kept under control by various superoxide dismutases. ROS-induced damage of DNA and/or proteins can lead to senescence or apoptosis (103). Also, AngII-induced senescence has been shown to be mediated by ROS via telomere attrition, as well as via a telomere-independent mechanism (99, 104, 105). Interestingly, ROS synthesis in the naked mole rat (the longest-living rodent) is extremely low, resulting in a superior vascular condition. All these data point towards ROS as a central inducer of a plethora of processes, which accelerate ageing. ROS are major modulators of NO availability and, therefore, increased ROS concentration with subsequent lowering of NO concentration will result in a lower blood vessel distensibility. This coincides with a phenotypic change in SMCs as can be deduced from gene-expression patterns, SMC proliferation, and increase of ECM deposition, in particular collagen, leading to increasing stiffness of the vessel wall. The combination of increased stiffness and reduced distensibility results in an impaired vasorelaxation.

Findings on ageing in SMCs under normal conditions are supported by observations in progeria patients. Progeria is a rare disease of accelerated ageing resulting in a lifespan of less than 15 years. Despite the fact that most of these patients die of cardiovascular dysfunction, investigation of the arterial pathology has been limited, mainly due to the low incidence of this disease. Progeria patients have a severe loss of vascular SMCs in the aorta and carotid arteries, which may cause aortic dilatation and rupture of atherosclerotic lesions. Two independent studies in transgenic mouse models of progeria confirmed the loss of SMCs in large vessels. In the large arteries of a transgenic mouse model carrying a mutated human lamin-A gene (a nuclear intermediate filament protein), a dramatic increase of ECM and calcification occurred at the cost of SMC numbers (106). The mouse histology is very similar to what has been seen in progeria patients. These results were actually a confirmation of an earlier study in a mouse deficient for an enzyme involved in lamin-A processing (107, 108). Heart muscle cells and SMCs have been suggested to be particularly vulnerable for mutated lamin-A because the constant movement of these cells will break-up the nuclear lamina, affecting DNA integrity and gene expression processes eventually leading to a cellular collapse.

Thus, findings in human specimens, results of *in vitro* studies, and models of accelerated ageing, all point at a phenotypic shift in vascular SMC leading to senescence/apoptosis of the cells and an ECM-enriched, calcified, and vulnerable wall in the ageing vasculature.

Concluding remarks

This review shows that SMCs in arteries are dynamic cell populations. The core of these populations is constituted by a broad spectrum of phenotypes that reaches from contractile to synthetic SMCs. Signalling pathways that regulate gene-expression patterns typical for contractile or synthetic SMCs have been unravelled by *in vitro* and *in vivo* (genetically modified mice) systems. Recently, the impact of specific miRNAs on SMC differentiation and phenotype switching has been described, which most likely will not be the last novel insight in SMC biology. However, the data available now have revealed only partially the complexity of vascular biology. Given that certain vascular pathologies are dominated by aberrant SMC behaviour, such as (in-stent) restenosis, vein-graft disease, and transplant arteriosclerosis, it is essential to have knowledge of the normal characteristics of these cells to be able to revert the pathological SMC back to its normal phenotype. With the information presently available on arterial SMC, future research linking SMC phenotypic diversity present at a particular location and pathological manifestations can be addressed.

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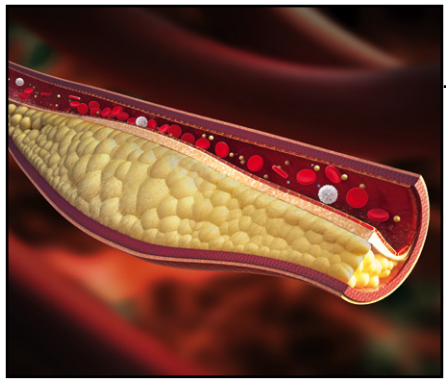

CHAPTER 8

Arteriogenesis versus angiogenesis

Peter Carmeliet, Guy Eelen, and Joanna Kalucka

Content

Introduction

Angiogenesis and arteriogenesis are different processes but are equally important. During angiogenesis, new capillaries sprout from pre-existing vessels to expand an initial vascular plexus (1). A primary cue for this is a lack of oxygen (hypoxia) and nutrients in the surrounding tissue, which cause non-vascular cells to start producing pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), and other pro-angiogenic factors. Collectively, these will 'activate' endothelial cells (ECs; the cells lining the blood vessel lumen) by inciting them to abandon their normal, quiescent state and to start proliferating and migrating (2). The resulting increase in capillary density of the plexus enhances perfusion of the tissue and secures adequate oxygen and nutrient supply. Angiogenesis occurs only rarely after birth in healthy conditions, but is crucial for wound healing and tissue regeneration, and also permits tumours to acquire the large amounts of nutrients required for excessive growth (3).

Although formerly considered a variant of angiogenesis, arteriogenesis covers two highly specific processes. In a developmental setting, arteriogenesis entails the formation of the arterial vessel network (versus the venous and capillary network) from the primitive vascular plexus (4). In adulthood, arteriogenesis more commonly describes the radial growth of arteries to increase bulk blood flow (5). It is then mainly considered to be a pathophysiological process in which, upon occlusion of a main feeding artery, pre-existing interconnecting collateral arterioles are remodelled into mature, large conductance arteries. The primary function of these collaterals is to serve as a natural bypass and to redirect and secure blood flow to peripheral tissues in need of nutrient supply. The drastic radial enlargement of the arterioles is not caused by passive vasodilation but by active growth involving EC activation and vascular wall remodelling. Remodelling of pre-existing vessels is a feature that distinguishes arteriogenesis from angiogenesis. Furthermore, unlike for angiogenesis, shear stress and not hypoxia is the main trigger for arteriogenesis (6, 7) (see ➔ Modulation of vascular growth).

Angiogenesis and arteriogenesis in the developing vascular plexus

The first organ to be formed during embryonic development is the cardiovascular system. The formation of the initial, primitive vascular plexus occurs through vasculogenesis. This process takes place during early embryonic development and consists of the differentiation of EC progenitors (mesodermal angioblasts) into primitive endothelial tubes devoid of mural cells either at the site of initial angioblast appearance or after extensive migration of the angioblasts (sometimes referred to as type I or type II vasculogenesis, respectively) (for a review see (➔ 8)). Alternatively, ECs have been postulated to originate from a precursor they have in common with haematopoetic cells, namely the haemangioblast (for a review see (➔ 9)).

Once the initial vascular plexus is formed, extensive expansion and remodelling start taking place. Several different types of vessel formation can expand the initial plexus, including vessel sprouting (commonly called angiogenesis) and vessel splitting (intussusception) (➔ Fig. 8.1a and b). Sprouting angiogenesis can be described as a series of continuous processes that are initiated by pro-angiogenic stimuli. When the balance of growth factors leans towards higher levels of pro-angiogenic stimuli (such as VEGF and FGF), endothelial cell–cell contacts loosen, the basement membrane is degraded, and pericytes (PCs) detach from the basal endothelial wall. Endothelial 'tip cells' are leading cells that migrate towards the source of the pro-angiogenic signal (10). Trailing behind the tip cell, endothelial 'stalk cells' proliferate to elongate the newly formed sprout. At the same time, 'phalanx cells' (so-called because of their typical cobblestone appearance (11)) form a lumen and anchor the sprout to the

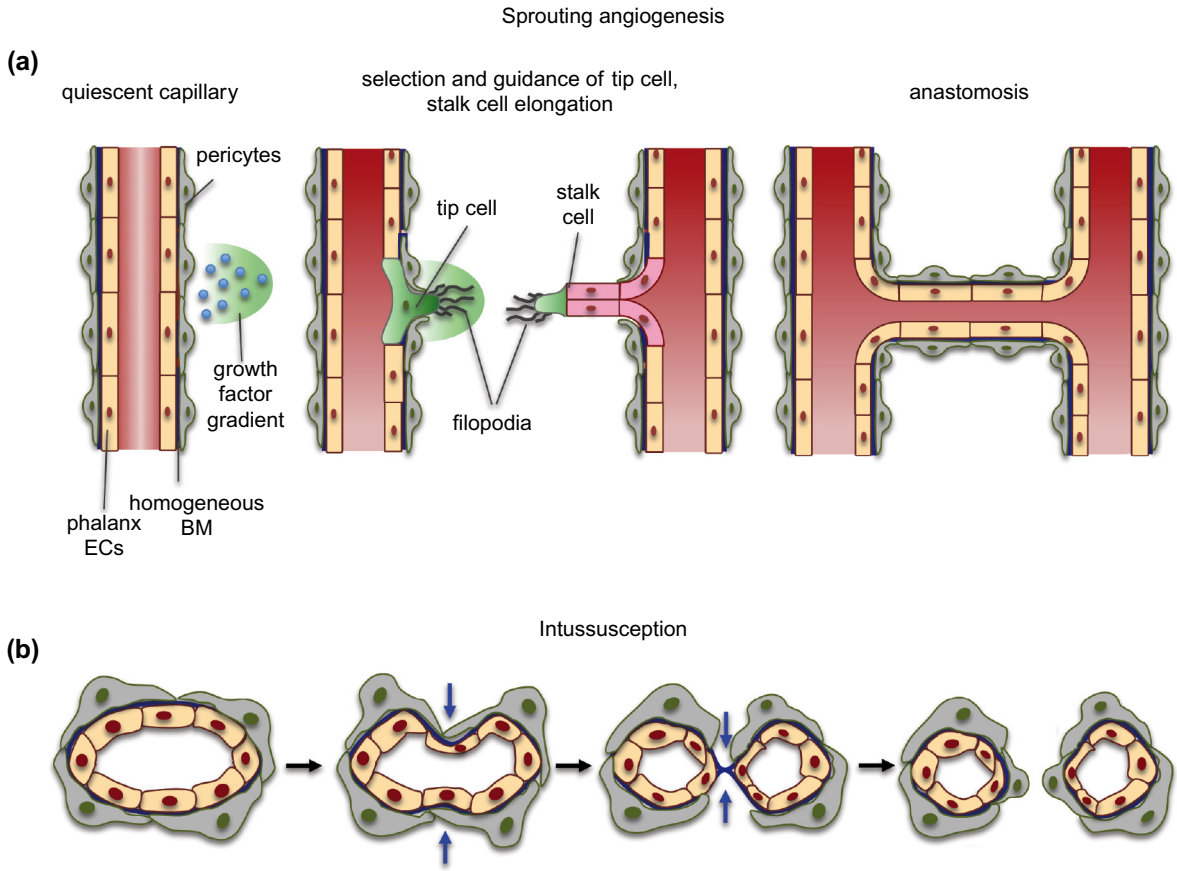

Fig. 8.1 Basic concepts in angiogenesis. (a) In sprouting angiogenesis a tip cell is selected in response to changes in growth factor gradients. The tip cell extends numerous filopodia in order to sense and respond to pro-angiogenic growth factors and guidance signals (such as VEGF). Stalk cells behind the tip cell proliferate and form a lumen. New sprouts anastomose to establish a perfused neovessel. (b) In intussusceptive angiogenesis a pre-existing vessel is split to give rise to two parallel daughter vessels. EC, endothelial cell; BM, basement membrane.
(Adapted from Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nature reviews Drug discovery. 2011;10(6):417–27 with permission from Nature Publishing Group.)

quiescent ECs of the existing vasculature (2) (➔ Fig. 8.1a). Newly formed sprouts eventually meet and connect through a process called anastomosis, creating a closed tubular system. Macrophages can facilitate the process of anastomosis, since they act as 'bridging cells' between anastomosing tip cells by secreting pro-angiogenic factors (12). Although tip and stalk cells arise from pre-existing blood capillaries, they bear distinct morphology and functional properties. While tip cells display a highly motile phenotype with multiple filopodia and cell protrusions, stalk cells have few filopodia and adopt various cell shapes (13). Perivascular cell recruitment is the last step in creating a fully functional blood vessel. A combination of genetic and computational models has revealed that competition for tip cell identity is a dynamic process in which cells shuffle between tip and stalk cell identities throughout the growth of the vessel to ensure that the most competitive EC is leading the tip of the sprout (14). Intussusceptive growth permits remodelling of the plexus and can increase the density of a capillary bed by creating two adjacent vessels from one single capillary. In brief, transcapillary tissue pillars are being formed when the opposing walls of a capillary protrude into the lumen to the point where their ECs make contact. The resulting endothelial bilayer is then perforated upon reorganization of EC intracellular junctions and the resulting hollow core/pillar is reinforced by pericytes and myofibroblasts, and the formation of an extracellular matrix. The circumference of the tissue pillar subsequently increases and the existing lumen is split in two to create two parallel vessels (15, 16) (➔ Fig. 8.1b). Of the two types, sprouting angiogenesis is better studied and understood. In disease (cancer), other modes of vessel formation exist (17) (see ➔ Therapeutic opportunities).

Upon further development, a primitive plexus no longer suffices to efficiently perfuse the organs and remodelling of the plexus into an ordered network of capillaries, arteries, and veins is required (2). Undifferentiated mural cells are recruited and 'muscularize' the nascent plexus. Arteries typically face high-pressure haemodynamic loads to transport blood to capillaries and are consequently covered by vascular smooth muscle cell (SMC) layers and a specialized matrix. Veins encounter lower pressure gradients, are thinner, and have fewer surrounding SMCs.

Most arteries feed into smaller size arterioles, which further branch into even smaller diameter capillaries (resembling a tree-branching pattern), with blood flowing from the proximal larger artery towards the distal extreme capillary. Formation of collateral arteries (collaterogenesis) occurs when an arteriole connects directly with another arteriole (mostly) from a neighbouring arterial tree (18) (➔ Fig. 8.2a). In these collaterals, blood does not flow along a proximal–distal axis but from the two opposite extremes towards the smaller branches on the collateral. The role of collateral arteries, which are the subject of pathological arteriogenesis in adult life, in embryonic development is less well understood (19).

Postnatal angiogenesis and arteriogenesis

During adult life, vessels are quiescent but retain the remarkable ability to sprout. Vessel growth during tissue regeneration, as occurs in wound healing or in the cyclical changes in female reproductive organs, are examples of non-developmental angiogenesis. However, active postnatal angiogenesis is mostly linked to uncontrollable vessel growth and deviant microvascular networks in pathological settings, such as diabetic retinopathy, inflammation, and tumourigenesis.

To acquire its own vasculature and secure nutrient and oxygen supplies, the growing tumour bombards ECs with pro-angiogenic factors. The ECs start branching chaotically and excessively, and build a highly abnormal and disorganized tumour vasculature (20) in which they fail to adopt their normal cobblestone-like morphology (➔ Fig. 8.3a). The ECs display increased loosening of cell–cell junctions, rendering the vessel highly permeable and accessible for cancer cells on their way to metastasis. Composed of morphologically aberrant, tortuous, and leaky vessels, which often lack normal venous versus arterial specification and normal blood flow dynamics, the excessive but inadequately perfused tumour microvasculature fails its primary function, namely the supply of oxygen and nutrients to the growing tumour. The hypoperfusion combined with the continuously increasing metabolic demand of the growing tumour mass not only fuel a nonstop cascade of aberrant non-productive angiogenesis, but also render the tumour microenvironment increasingly harsh. Interstitial pressure and acidity increase in the tumour microenvironment and allow only resistant cancer cells to survive, but promotes others to escape the hostile microenvironment, thereby favouring metastasis (17, 20). Importantly, the structurally and functionally abnormal vasculature and the increased tumour interstitial pressure also impede efficient delivery of chemotherapeutics to the tumour (21) (see ➔ Therapeutic opportunities).

Arteriogenesis in the adult setting denotes the formation of arteries upon obstruction of an adjacent arterial trunk in vascular occlusive diseases (e.g. myocardial infarction, stroke, and peripheral artery disease) (4, 5, 22). It remains debated if *de novo* formation by arterialization of capillaries

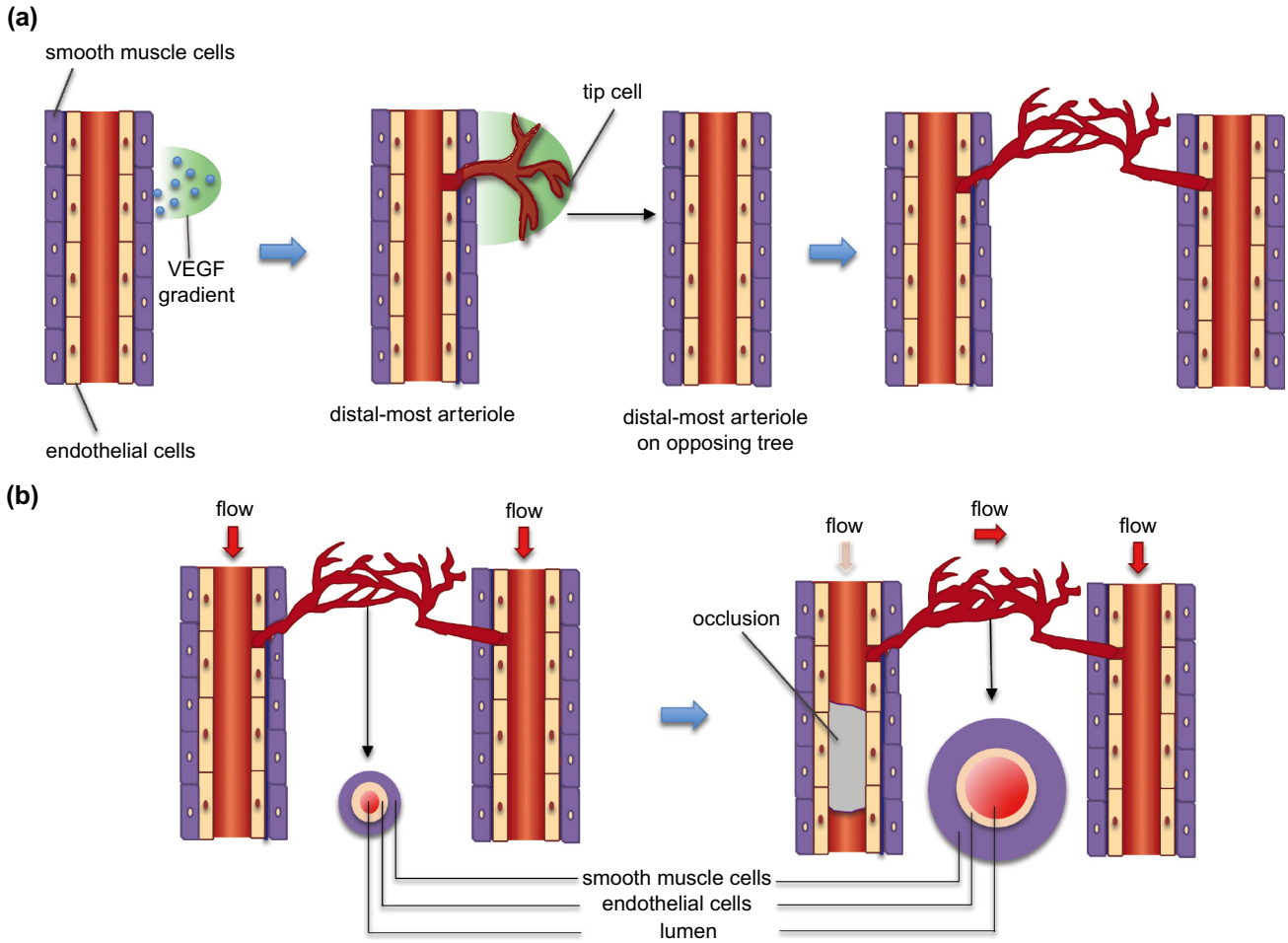

Fig. 8.2 Basic concepts in arteriogenesis/collaterogenesis, (a) Developmental formation of collateral arteries through a sprouting angiogenesis-like process. (b) Arteriogenesis is induced upon occlusion or stenosis of a major artery (indicated by grey plug). Blood flow is redirected to pre-existing collateral anastomoses where altered fluid shear stress initiates the remodelling processes leading to increased lumen diameter and increase in number of smooth muscle layers. VEGF, vascular endothelial growth factor.

contributes to this phenomenon or if it is exclusively a matter of enlargement of pre-existing collaterals (22) (➔ Fig. 8.2b). The massive (up to 50-fold) increase in diameter and concomitant thickening of the arterial wall upon acute arterial occlusion is unique for the collateral circulation and occurs during four distinct phases (for a review see (➔ 19)). During a first phase, ECs and vascular SMCs are activated to proliferate and the permeability of the collaterals is increased to allow their remodelling. Additionally, immune cells are recruited; the activated ECs have heightened expression levels of vascular cell and intercellular adhesion molecule 1 (VCAM1 and ICAM1, respectively), monocyte chemotactic protein 1 (MCP-1), ephrin B2, and VEGF, which is crucial for immune cell recruitment (see ➔ Inflammatory cells/macrophages as angiogenic and arteriogenic accessory cells) (➔ Fig. 8.3b). From the second phase on, SMCs take up a more prominent role in collateral remodelling; the dramatic increase in collateral wall thickness is mainly brought about by increased proliferation and migration of SMCs leading to a 20-fold or higher increase in their tissue mass (23). Reduced expression of contractility proteins (e.g. α-actin, calponin, and desmin) allows the SMCs to switch from a highly contractile to a synthetic phenotype (24). Lowered levels of the focal adhesion protein vinculin, which anchors SMCs in the ECM, possibly favour SMC migration in the vessel wall (25), while the ECM is progressively being broken down by matrix metalloproteinases (MMP) 2 and 9. In a third phase, the SMCs establish cell–cell contacts and reorganize into circular layers. Concomitantly, the ECM is remodelled further. In a final fourth phase, the new arterial circulation is 'refined' and the number of small, non-used collaterals is decreased to promote the larger ones. This 'pruning' action occurs by intimal proliferation leading to complete occlusion of the vessel (for a review see (➔ 19)).

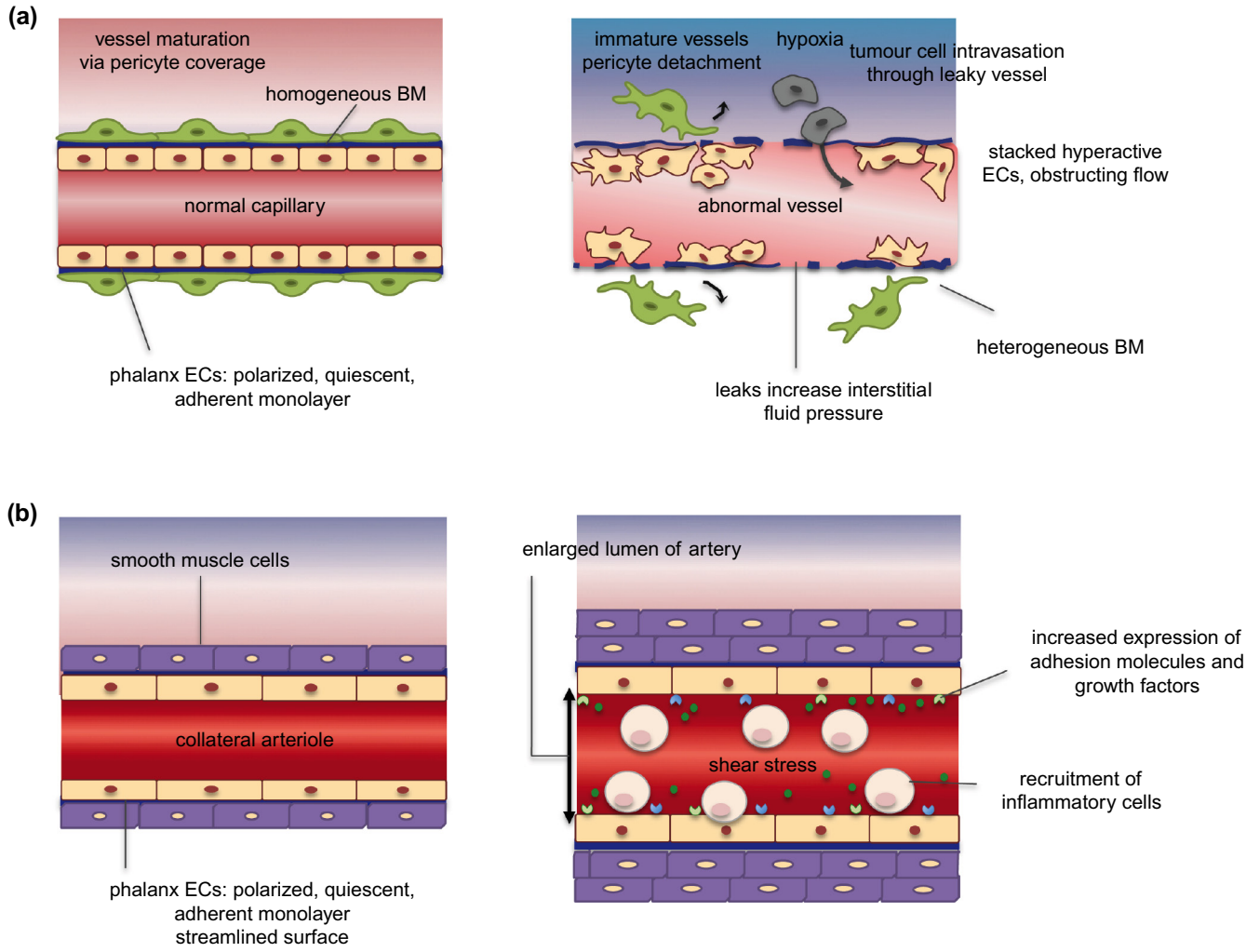

Fig. 8.3 Hallmarks of pathophysiological angiogenesis and arteriogenesis. (a) Compared with normal quiescent vasculature (left), tumour vasculature (right) displays several abnormal features like immature EC phenotypes, a heterogenous basal membrane, and lack of pericytes' coverage (reduced vessel maturation). Due to these abnormalities, tumour vessels fail to deliver oxygen and increase hypoxia in tumour tissue. (b) Upon arterial occlusion pre-existing collateral arterials (left) are being remodelled into larger diameter arteries (right). Mechanical (fluid shear stress) stimuli initiate arteriogenic signalling in resting ECs, which increase the expression of adhesion molecules to recruit inflammatory cells. Monocytes produce fibronectin, proteoglycans, and proteases to remodel the extracellular matrix, which allows remodelling processes to increase radial growth of the vessel wall. Abbreviations as in Fig. 8.1.
(Adapted from Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011;473(7347):298–307 with permission from Nature Publishing Group.)

Modulation of vascular growth

Hypoxia and angiogenesis

All eukaryotic organisms rely on oxygen (O_2) to support biological processes; therefore, a constant O_2 supply, maintained by the vascular system in mammals, is critical for proper tissue development, homeostasis, and function. Vascular dysfunction due to vessel occlusion or rupture can lead to decreased O_2 delivery and is a pathogenic driver in peripheral artery disease and ischaemic heart disease. Hypoxia emerges when changes in the O_2 supply or demand occur and is a common feature in both normal mammalian development and human disease. In regions of tissue ischaemia, angiogenesis and arteriolar growth have been observed. Tissue hypoxia drives the development of new blood vessels from existing blood vessels (angiogenesis), and the remodelling of existing collateral vessels (arteriogenesis), in order to secure sufficient tissue perfusion and oxygenation. Angiogenesis or capillary sprouting towards the ischaemic tissue remedies the local hypoxia, whereas arteriogenesis restores the bulk blood supply and bypasses the vascular occlusion.

Oxygen-sensing mechanisms are sensitive to fluctuations in intracellular oxygen concentrations and respond to low oxygen by rapidly accumulating hypoxia-inducible factors (HIFs), which are heterodimeric transcription factors that promote short- and long-term adaptation to hypoxia.

HIFα expression is tightly regulated by a family of enzymes known as the HIF prolyl hydroxylases (PHDs 1–3) (26). In the presence of oxygen, PHDs hydroxylate specific proline residues within the oxygen-sensitive α subunit of HIF, which is then tagged for proteasomal degradation and prevents the activation of HIF-signalling pathway (27). Under hypoxic conditions, HIFα is stabilized and activates transcription of its target genes in the nucleus. HIF rapidly increases O_2 supply through upregulation of the vasodilatory enzyme-inducible nitric oxide synthase (iNOS) (28). Nitric oxide (NO), the enzymatic product of iNOS, relaxes vascular SMCs, providing a short-term increase in blood flow. An increase in HIF-signalling can also activate the transcription of other pro-angiogenic genes like VEGF, angiopoietin-1 (ANG1), angiopoietin-2 (ANG2), Tie2, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and MCP-1. Induction of expression of these genes also facilitates adaptation to hypoxia, due to improved oxygen delivery as a result of more active angiogenesis.

Shear stress as a modelling force in arteriogenesis

Similar to angiogenesis, where new capillaries sprout from pre-existing ones, arteriogenesis also relies on the presence of pre-existing arterioles. In the adult, arteriogenesis occurs as a consequence of vessel occlusion, which leads to the remodelling and growth of collateral arterioles from pre-existing anastomoses. Angiogenesis is ischaemia-driven but for the initiation of arteriogenesis, hypoxia does not seem to be the driving mechanism (29).

The available evidence sustains the hypothesis that mechanical forces caused by the altered blood flow dynamics upon arterial occlusion lead to the induction of collateral remodelling. Elevated fluid shear stress (FSS) mediates the enlargement and remodelling of existing arteries and arterioles (arteriogenesis) in the adult. The increased FSS is a consequence of the pressure difference created by the occlusion, which heightens blood flow in nearby smaller arteries. This process is self-limiting because FSS will fall with increasing collateral vessel diameter. Increased FSS activates the endothelium and induces changes at morphological (e.g. changes in cytoskeleton (30)) and molecular levels.

An important consequence of the increased shear stress is the production of nitric oxide (NO) by ECs, which can diffuse through the basement membrane that separates ECs and SMCs. NO-mediated increases in vascular conductance allow for rapid greater collateral dependent blood flow to the tissue distal to occlusion, and is the first 'functional' (i.e. 'non-structural') response of the vessel towards the sudden increased need of nutrient supply (31). Targeted deletion of the endothelial NOS (eNOS) gene leads to a loss of vasodilatation but not arteriogenesis, whereas targeted deletion of inducible NOS (iNOS) leads to partial but not complete impairment of arteriogenesis. Only deletion of both eNOS and iNOS (primarily produced by recruited inflammatory cells) results in a complete loss of collateral vessel remodelling during arterial occlusion (32).

In a second stage, 'structural' responses ensure that the enlarged collateral vessel size is maintained. Several other important molecules that regulate vascular function and contribute to EC survival, such as PDGF-A or PDGF-B, are induced by shear stress (33, 34). FSS has also been shown to facilitate the expression of endothelial VEGF receptor-2 (VEGFR-2) leading to enhanced responsiveness to VEGF (for more details on VEGFR-2 actions see Molecular mechanisms of angiogenesis versus arteriogenesis) (35, 36). Gene expression analyses showed that FSS induced the shear stress sensitive gene transient receptor potential cation channel subfamily V member (Trpv4) (37, 38). Pharmacological activation of Trpv4 increased cerebral arteriogenesis and collateral flow (39); conversely, a Trpv channel-blocker tended to reduce collateral flow (37). In addition, FSS induces the activation of the endothelial NFκB pathway leading to induction of expression of various adhesion molecules, such as ICAM-1, ICAM-2, and VCAM-1 (40–42). The adhesion molecules on the surface of the endothelium recruit various immune cell subsets, which can act as regulators of arteriogenesis (➔ Fig. 8.3b). The role of monocytes/macrophages in these processes is discussed in the following section.

Inflammatory cells/macrophages as angiogenic and arteriogenic accessory cells

ECs change their gene expression in response to the alteration in shear stress, resulting in the upregulation of chemoatractants, chemokines (e.g. MCP-1) (42, 43), and adhesion molecules (ICAM-1 and integrins), and favouring the recruitment of inflammatory cells (44). In particular, monocyte migration and infiltration to the perivascular area of growing collaterals is associated with improved arteriogenesis. Pharmacological depletion of monocytes results in impaired arteriogenesis in rabbit and mouse models of hindlimb ischaemia (43, 45, 46). Furthermore, animals genetically deficient in MCP-1 or chemokine receptor-2 (CCR2) display reduced monocyte recruitment and impaired arteriogenesis (47).

Plasticity is a hallmark of macrophages and is reflected by their different activation states according to the stimuli present in the local microenvironment. The expression and composition of vessel- and tissue-derived cytokines is tightly regulated at the ischaemic site, and affects the angiogenic and arteriogenic function of macrophages. Recent classification proposes that macrophages have several phenotypic subsets ranging from classically activated (M1), induced by, for example, IFNγ (which enhances their cytotoxic activity), to alternatively activated (M2) promoted by IL-4, IL-10, or IL-13 (which results in the upregulation of VEGF and supports angiogenesis). At the site of inflammation, for example, macrophages are exposed to vessel- and tissue-derived cytokines, such as VEGF, ANG1, or ANG2, which reprogramme them to become highly angiogenic and arteriogenic accessory cells (48, 49). ANG1-mediated macrophage reprogramming results in the repression of PHD2, which further promotes an M2-like, pro-arteriogenic phenotype with increased expression of PDGF-B, neuropilin 1 (Nrp1; see Molecular mechanisms of angiogenesis versus arteriogenesis), chemokine (C-X-C motif) receptor 4 (CXCR4), and its ligand stromal cell derived factor 1 (Sdf1) (50). In addition, ANG1-mediated PHD2-repression enhances the expression of the ANG-receptor Tie2, which amplifies Tie2 signalling in a positive feedback loop and hence promotes vessel maturation (49). Macrophages that infiltrate the areas of collateral vessel development orchestrate the remodelling process that leads to the formation of collateral arteries, for example, by upregulating MMPs, which induces the required remodelling of extracellular matrix. Also they stimulate the proliferation of ECs and SMCs by secreting bFGF (51), PDGF (52), and VEGF in the infiltrated area.

Moreover, in different animal models of angiogenesis, M2-like activated macrophages support the proliferation and migration of ECs and promote vessel sprouting (12, 53, 54) (➔ Fig. 8.4). M2 macrophages possess high angiogenic capability and promote the release of VEGF from the tissue matrix, thereby enhancing vessel sprouting (55). Additionally, hypoxia was found to enhance ANG2 expression in murine and human macrophages, which may subsequently increase their pro-angiogenic function (28). Therefore, macrophages are a potential target for novel therapeutic avenues to treat ischaemic diseases (➔ Fig. 8.4). Of note, in addition to monocytes/macrophages, other immune cell types like T-lymphocytes play crucial roles in angiogenesis and arteriogenesis; these are not discussed here but more details can be found in the following references (56, 57).

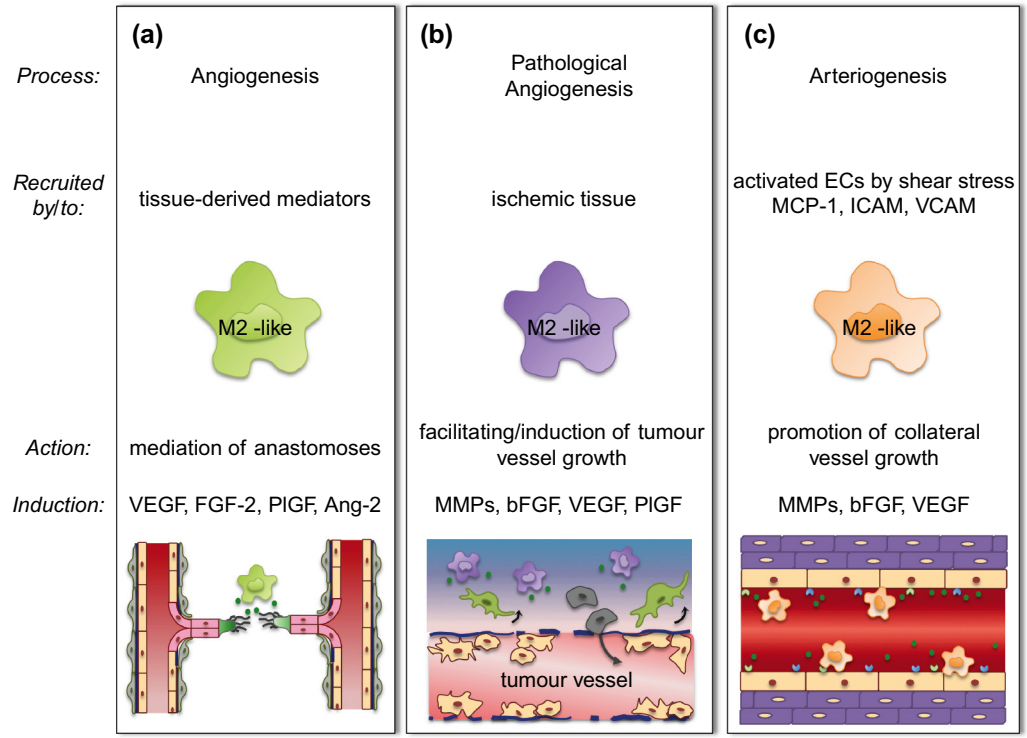

Fig. 8.4 Macrophages as mediators of angiogenesis and arteriogenesis. Macrophages are recruited to ischaemic sites where they (in response to tissue-derived factors) switch their phenotype to potent angiogenic and arteriogenic (M2- like) cells. MCP1, monocyte chemotactic protein 1; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; PHD2, prolyl hydroxylase 2; PDGF, platelet-derived growth factor; Nrp1, neuropilin 1; CXCR4, chemokine (C-X-C motif) receptor 4; Sdf1, stromal cell derived factor 1; MMP, matrix metalloproteinases; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; Ang2, angiopoietin-2.

Molecular mechanisms of angiogenesis versus arteriogenesis

Signalling pathways driving angiogenesis and/or arteriogenesis are numerous and complex; summarizing each of them is not within the scope of this chapter. Instead, we highlight the molecular mechanisms that dictate if ECs will form arteries or veins (leading to developmental arteriogenesis) and the molecular mechanisms underlying vessel sprouting and sprouting-like phenomena (leading to developmental/pathological angiogenesis and developmental collaterogenesis, respectively). Even though SMCs and other perivascular cells are crucial players in both angiogenesis and arteriogenesis, we will focus mainly on ECs here. Due to space limitations, mechanisms in mammalian systems will be presented. For the most recent insights in molecular mechanisms of angiogenesis/arteriogenesis in other model systems (e.g. zebrafish), the reader is referred to other reviews (58, 59).

Venous and arterial endothelial cell fate

Establishment of arterial and venous EC fate is among the earliest cell differentiation steps that take place in the developing vascular system. The patterning of artery and vein identity is essential for normal embryonic development and the subsequent function of the circulatory system. In recent decades, a variety of signalling molecules, such as VEGF-VEGFRs, the Delta–Notch system, and ephrin-Eph receptors have been identified to have a crucial function in vascular development. From the earliest stages of cardiovascular development, arterial ECs display the expression of transcription factors, signalling pathway, gap-junction proteins, and ECM molecules that are largely absent from venous ECs (4) (➔ Fig. 8.5).

Arterial differentiation is thought to occur in angioblasts exposed to higher VEGF concentrations, whereas cells exposed to lower concentrations acquire venous fate. The VEGF family of angiogenic regulators consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). The key role of VEGF-A has been demonstrated *in vivo* using genetic studies. The loss of a single allele of *Vegfa* is sufficient to induce early embryonic (embryonic day (E) 8.5-9.5) lethality due to a failure of primitive vascular formation (60, 61). Similarly, overexpression of VEGF-A results in embryonic lethality between E12.5 and E14 due to several vascular defects (62). VEGF-A exerts its angiogenic

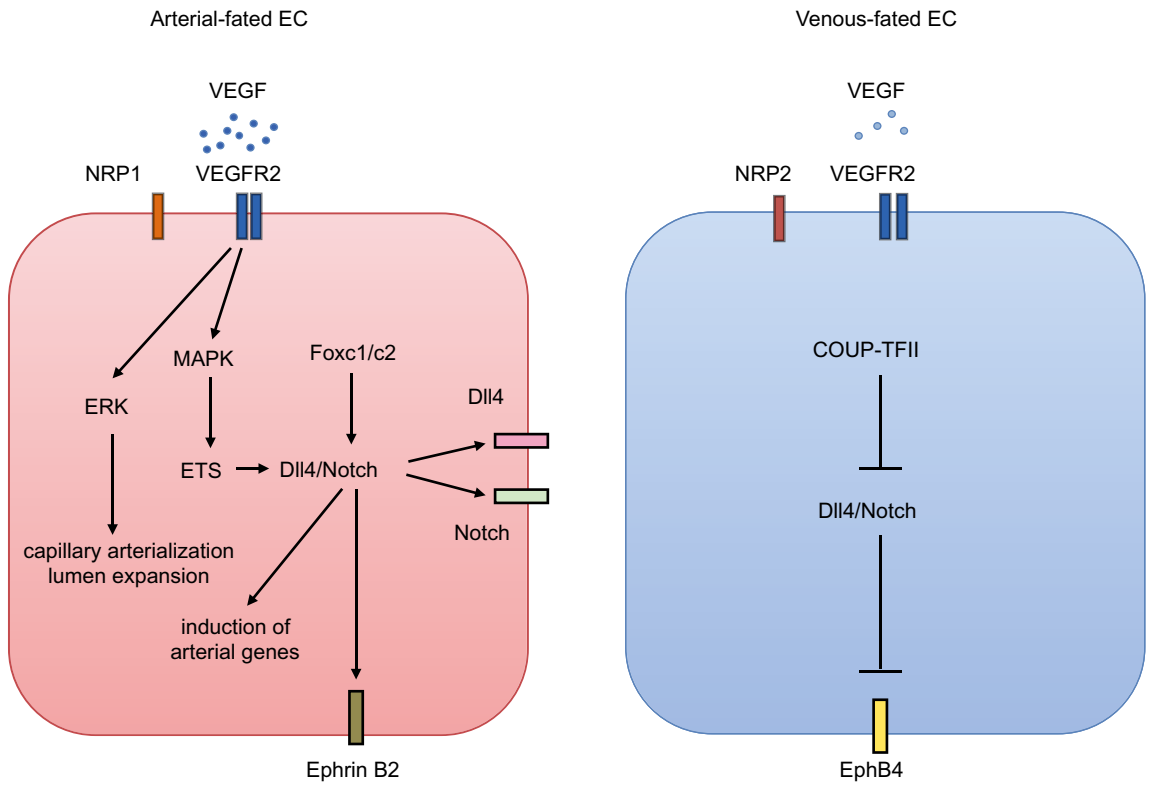

Fig. 8.5 Signalling pathways orchestrating venous/arterial specification. Simplified scheme of signalling cascades involved in arterial versus venous specifications. For details, see main text. Abbreviations as in Fig. 8.1 and 8.4; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; Fox, forkhead transcription factor; ETS, E26 transformation-specific sequence; VEGFR, VEGF receptor; EphB4, Eph receptor B4; COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; Dll4, Delta-like 4.

signalling by binding to vascular endothelial growth factor receptors (VEGFR-1 and -2), which are members of the receptor tyrosine kinase family. Binding of VEGF-A to VEGFR-2 induces direct effects on ECs by promoting their proliferation, survival, and vessel sprouting. Therefore, it is not surprising that homozygous disruption of *Vegfr2* induces a similar phenotype as loss of the VEGF-A ligand (63). Different splice variants of the *Vegfa* gene (Vegf120, Vegf164, and Vegf188) have distinct effects on vascular development. Genetically engineered mice that express only the VEGF-A_{120} isoform exhibit defects in retinal vascular outgrowth, whereas mice expressing only the VEGF-A_{188} isoform exhibit impaired retinal arterial development, but normal venous development (64). Only mice that express solely $VEGF_{164}$ display normal retinal vascular development.

Recent work has established that genetic pre-patterning mediated by Notch signalling also plays a primary role in regulating arteriovenous differentiation. During vascular development, Notch receptors and ligands are preferentially expressed in arterial ECs (65, 66). For instance, expression of the Notch ligand Dll4 is initially restricted to large arteries in the embryo, whereas in adult mice and tumour models, Dll4 is specifically expressed in smaller arteries and microvessels. Similarly to heterozygous VEGF-A deficient mouse embryos, heterozygous Dll4 deficient embryos exhibit embryonic lethality due to vascular defects, lack of expression of arterial markers, and defective arterial branching from the aorta, and even arterial regression (67–69). The Fox transcription factors, Foxc1 and Foxc2, can act as regulators of arterial cell specification by directly activating the Dll4 promoter. The Foxc-mediated induction of Dll4 expression was found to be enhanced by VEGF (70, 71). Recently, an intronic enhancer was identified in the *Dll4* locus that drives arterial-specific expression and can be bound by E26 transformation-specific sequence (ETS) factors. In arterial precursor cells, VEGF signalling activates the ETS factors in a mitogen-activated protein kinase (MAPK)-dependent manner to drive Dll4 expression (72).

Much like those lacking Dll4, mice deficient in the Notch1 and -4 receptors also display severe vascular remodelling defects (73). A number of studies elucidated the role of SoxF factors as interacting with Notch signalling in the context of arteriovenous specification (74, 75). In the absence of Sox17, for example, ECs lose arterial markers (74). Also, combinatorial deletion of SoxF and Notch activity causes loss of arterial identity (75). Jagged1, another Notch ligand, is expressed in SMCs surrounding the arteries and plays an important role in SMC maturation. EC-specific Jagged1 knockout does not affect arterial-venous differentiation per se but impairs VSMC differentiation (76). This observation indicates that Jagged1 expression in the arterial endothelium activates Notch in neighbouring cells, and that this function is critical for SMC differentiation.

In addition to VEGF-mediated activation of Notch-signalling, VEGF-A/VEGFR-2 signalling activates the endothelial extracellular-signal-regulated kinases (ERK) cascade, which is required for arterial gene expression (77, 78). VEGF-dependent activation of ERK is particularly important for arteriogenesis and for regulation of arterial branching and lumen size. Mice with mutations leading to reduced VEGF-dependent ERK activation show a defect in arterial but not in venous vessel formation (for a review see (➔ 4)).

Other proteins and/or signalling pathways fulfil determining roles in venous versus arterial fate specification. Neuropilins, for example, which are multifunctional non-tyrosine kinase receptors that bind to class 3 semaphorins and VEGF, also display distinct arteriovenous-specific expression patterns. NRP1 is expressed in arteries, whereas expression of NRP2 is restricted to veins (64, 79). Noteworthy, both neuropilins can bind to different VEGF isoforms possibly resulting in distinct signalling responses in arteries versus veins (80). Eph receptors and ephrin ligands mediate the mechanistic basis of border formation between arterial and venous ECs and prevent cellular intermingling. Ephrin/Eph signalling mediates crucial intercellular behaviour such as repulsion, adhesion, and motility (81). Ephrin B2 and Eph B4 have been identified as the first marker molecules of arteriovenous differentiation, with ephrin B2 being expressed by arterial and EphB4 by venous ECs (82). Thus, venous-fated EphB4-positive ECs migrate away from the arterial-fated ephrin B2-positive ECs in the precursor vessel towards the location of future (cardinal) vein. Moreover, ephrin B2 also controls VEGFR internalization and tip cell behaviour (82, 83). Deficiency of any of these molecules leads to prenatal death due to severe defects in arteriovenous remodelling.

Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) (also known as nr2f2), is a member of the orphan nuclear receptor superfamily and is expressed in venous but not in arterial ECs. It has been proposed that COUP-TFII acts as a positive mediator of venous fate by repressing Notch, which in turn upregulates ephrin B2 and downregulates EphB4 (84). COUP-TFII-deficient embryos die at E10.5 due to severe haemorrhage and oedema formation resulting from enlarged blood vessels and malformed veins (85). Targeted disruption of COUP-TFII results in venous acquisition of arterial markers (ephrin B2, Notch1) (84) and ectopic expression of COUP-TFII in ECs leads to the fusion of arteries and vein (for a review see (➔ 86)).

Other growth factors such as angiopoietins (ANG1 and 2) are essential for vascular homeostasis by promoting

vascular maturation and integrity. ANG2 is almost exclusively expressed by ECs, whereas ANG1 is expressed by numerous cell types, including mural cells (SMCs and perivascular cells), fibroblasts, and monocytes. ANG1 was shown to venous-specifically increase the blood vessel diameter by regulating EC proliferation (87). ANG1 conventional deficient mice show embryonic lethality due to defects in vascular maturation and heart trabeculation (88). Analogously, overexpression of ANG1 induces vascular remodelling that leads to the formation of vessels with a wider diameter (89). Vascular SMCs secrete ANG1 to stabilize ECs through Tie2, a specific receptor for ANG1. In the presence of angiogenic factors, sprouting ECs release ANG2, which antagonizes ANG1/Tie-2 signalling, causing mural cell detachment, vascular permeability, and promoting vessel sprouting (90).

Tip and stalk cell specification and interaction

During angiogenesis, new capillaries sprout from existing blood vessels. The process of tip and stalk cell differentiation is under the tight control of VEGF, as well as multiple other signalling pathways (2). In the current model of endothelial angiogenesis and the interplay between tip and stalk cell, Notch signalling is central to establish these cell identities. A new angiogenic sprout begins when extracellular VEGF-A binds VEGFR-2 on an EC, causing upregulation of its downstream target signalling pathway—Notch ligand/Delta-like-4 (Dll4). Dll4 expression is induced in response to VEGF-A, and activation of Notch by Dll4 on adjacent ECs results in the downregulation of VEGFR-2 in those cells. As an outcome of this interaction, one cell expresses high VEGFR-2 levels and its neighbours are rendered less sensitive to VEGF-A signalling (14). The cell expressing VEGFR-2 and Dll4 becomes a non-proliferative, migratory 'tip' cell. The Notch-expressing neighbours of the tip cell become 'stalk' cells that proliferate and contribute to the elongation of the nascent sprout. In addition, Notch receptor signalling also drives expression of VEGFR-1, which is a high-affinity/low-activity receptor that serves as a competitive inhibitor of VEGF signalling (91) (➔ Fig. 8.6). Precise regulation of Dll4 expression is achieved through the TEL/CtBP repressor complex at the *Dll4* promoter, which is transiently disassembled upon VEGF stimulation, allowing a temporally restricted pulse of Dll4 transcription (92). In line with a central function of Dll4 for vessel patterning dynamics, several other pathways, such as the Wnt/β–catenin pathway, converge on the transcriptional control of Dll4 (93). Importantly, inhibition of Dll4/Notch signalling increases filopodia and sprouting as a consequence of excessive tip cell formation (94, 95).

Dll4 is not the only Notch ligand involved in tip/stalk cell specification. Jagged1, which has high expression levels in stalk cells, is another crucial player. Genetic deletion or endothelial overexpression of Jagged1 *in vivo* resulted in reduced or increased sprouting, respectively. Endothelial Notch signalling has been shown more active in the absence of Jagged1, which suggests that Jagged1 is a negative regulator of Notch activity. *In vitro* studies confirmed that Jagged1 antagonizes Dll4-Notch signalling and leads to the opposite effect in that it promotes EC proliferation and sprouting. The antagonistic effects of the two ligands are controlled by Fringe glycosyltransferase-dependent modulation of Notch signalling (96). Most recently, phosphate and tensin homologue deleted on chromosome ten (PTEN) was identified as an orchestrator of Notch signalling responses preventing stalk cell hyper-proliferation. The induction of PTEN expression balances stalk cell numbers and coordinates their pattering. Upon endothelial deletion of PTEN, Notch signalling fails to arrest stalk cells resulting in defective sprout length and pattering (97).

As mentioned, tip cells extend numerous filopodia and are highly responsive to VEGF because of high VEGFR-2 expression levels. Mice deficient in NRP1 display blind-ended sprouts and glomeruloid tuft formation in the developing hindbrain caused by failure of tip cell filopodia to turn laterally and to fuse with filopodia of the neighbouring sprout (98). More recently, it has been shown that ECs that maintain expression of NRP1 preferentially take the tip cell position when competing with NRP1-deficient ECs during sprouting angiogenesis (99). The long-standing view on the importance of VEGF-binding to NRP1 during angiogenesis has recently been challenged by findings pointing to a VEGF-independent role for NRP1 in angiogenesis. Mice with a mutation in the *Nrp1* gene that prevents binding to VEGF (but also reduced NRP1 expression levels), are viable but display an increased rate of postnatal mortality and impaired angiogenesis in the retina and hindbrain (100). Likewise, NRP1/VEGF-A binding-deficient transgenic animals that express normal levels of NRP1 display normal viability and only mildly impaired retinal angiogenesis (characterized by delayed vessel outgrowth) (101). In contrast to EC-specific NRP1 knockout mice, which have severely impaired angiogenesis in the hindbrain accompanied by reduced VEGFR-2 expression and phosphorylation levels, expression of VEGF-A binding-deficient NRP1 results in normal VEGFR2 expression and only mildly reduced VEGFR2 phosphorylation levels (101). These results suggest that during angiogenesis, NRP1 function as a VEGFR-2 co-receptor that enhances VEGFR-2 activation.

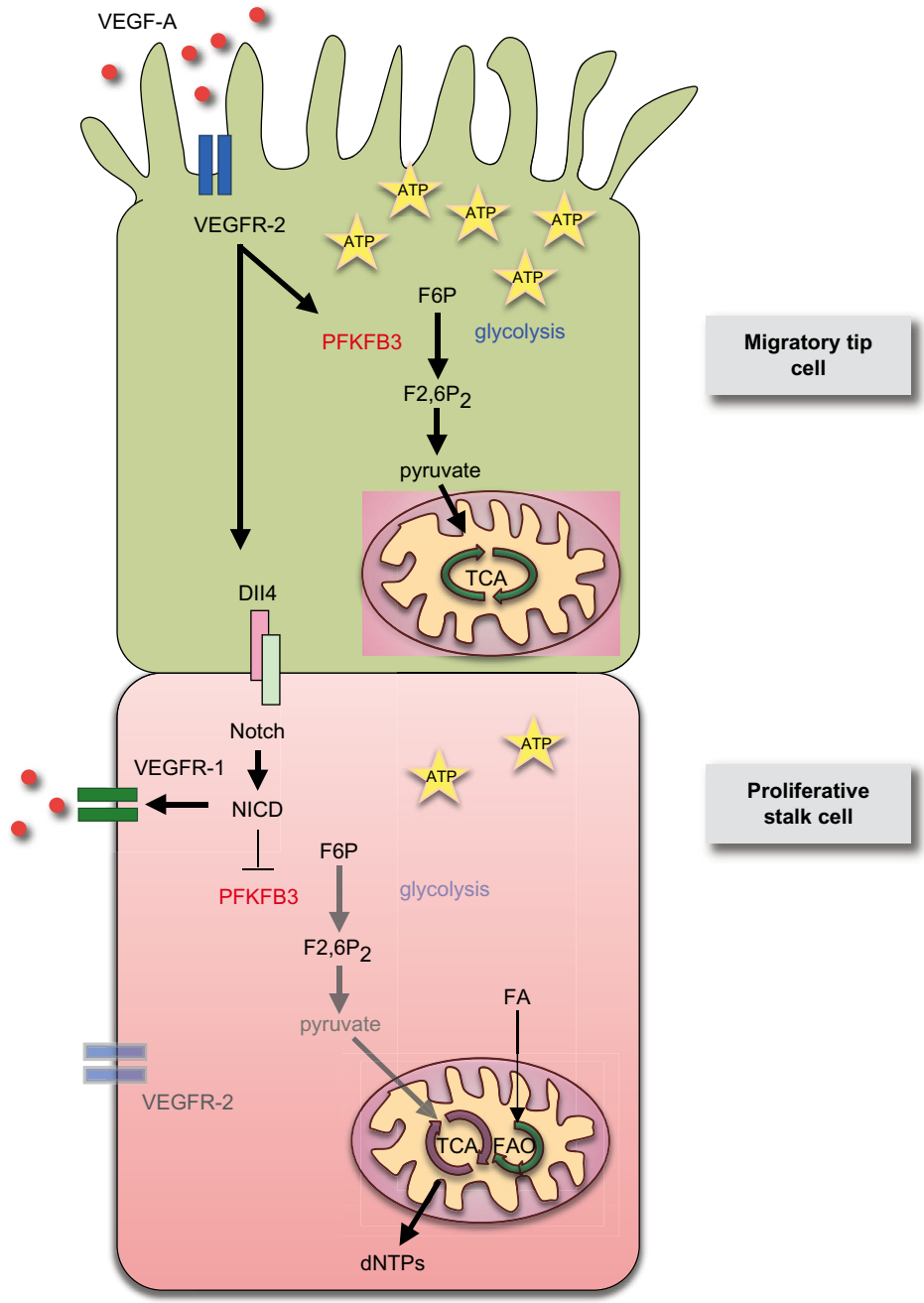

Fig. 8.6 Genetic and metabolic control of tip/stalk specification. Schematic of a tip (green) and a stalk (red) cell in a vascular sprout. In response to VEGF-A, VEGFR-2 increases the expression of Dll4, which activates Notch signalling in the stalk cell by downregulating VEGFR-2 and upregulation of VEGFR-1 expression. The activation of VEGFR-2 upregulates PFKFB3 levels and glycolysis for ATP production, whereas NICD in the stalk cell lowers PFKFB3 expression and glycolytic flux. In addition, proliferating stalk cells need fatty acid oxidation (FAO) and fatty acid (FA)-derived carbons for (among other things) *de novo* dNTP synthesis required for DNA replication. The role of FA-derived carbons and FAO in tip cells is unknown. VEGFR, VEGF receptor; PFKFB3, phosphofruktokinase-2/fructose-2,6-bisphosphatase 3; F6P, fructose-6-phosphate; $F2,6P_2$, fructose-2,6-bisphosphate; TCA, tricarboxylic acid; Dll4, Delta-like 4; NICD, Notch intracellular domain.

Stalk cells, as well as quiescent phalanx ECs, express Tie2, the receptor for both ANG1 and ANG2. Pericytes cover and stabilize these ECs by producing ANG1; ANG1 promotes a homomeric Tie-2 complex to form across endothelial cell–cell junctions and thereby promotes cell–cell adhesion, anti-permeability, and attenuation of angiogenesis. ANG2 is expressed at low levels in these stabilized ECs and it is stored in Weibel–Palade bodies, from which it can be readily secreted to control EC responses during vessel sprouting (102). Upon VEGF-induced vessel sprouting, ANG2 is induced in migrating and invading EC tip cells (103, 104). In contrast to ANG1, ANG2 sequesters Tie2 to cell–matrix

contacts and destabilizes vessels to facilitate sprouting. Independent of Tie2, ANG2 directly binds and activates integrins, which are induced in angiogenic ECs. This results in focal adhesion kinase phosphorylation and enhanced tip cell migration (105–107).

Interestingly, microRNAs (miRNAs) are emerging as new players in the regulation of vessel sprouting. Silencing of Dicer (a miRNA processing enzyme required for miRNA maturation) results in reduced sprouting of ECs (108). miR-126 has been identified as a pro-angiogenic miRNA able to enhance the response of ECs to VEGF-A signalling by repressing expression of *Sprouty-related protein 1* (SPRED1), a negative regulator of VEGF-A signalling (109). Another microRNA, miR-27b, controls venous specification and tip cell fate via its targets Dll4 and *Sprouty homologue 2* (Spry2) (110).

In contrast to postnatal collateral remodelling upon vascular occlusion, which has been broadly studied, it is not fully understood yet how collateral arteries differentiate from the circulation during development. A similar sprouting angiogenesis-like mechanism, as described, has been suggested for developmental collaterogenesis (111). More specifically, on the embryonic brain, VEGF-A/VEGFR-2 signalling and subsequent Notch/Dll4 signalling drive collateral formation. A tip cell phenotype is promoted in an arterial-fated EC of a distal-most arteriole of the cerebral artery tree. This tip cell then migrates over the pial capillary plexus followed by lumen-forming stalk cells and finally forms a nascent collateral by fusing with a distal-most arteriole of an opposing artery tree (111). The exact signalling mechanism causing this tip cell to fuse with an arteriole of an opposite arterial tree, rather than with nearby capillaries, is not fully understood. High versus low paracrine VEGF-A levels result in increased or decreased embryonic collateral formation, respectively. Furthermore, mice with an EC-specific VEGFR-2 deletion show fewer and smaller collaterals (111). Furthermore, pial collateral formation relates inversely to *Dll4* gene dosage; newborn *Dll4*$^{+/-}$ mice show increased numbers of pial collaterals caused by increased arterial branching morphogenesis during embryogenesis (112). Endothelial disintegrin-and-metalloprotease-domain 10 (Adam 10) promotes collateral formation through Notch and subsequent induction of a stalk cell phenotype, whereas Adam 17 reduces collaterogenesis by cleaving the VEGFR-2 intracellular domain and by altering cytoskeletal dynamics (111). Strikingly, the observed change in numbers of collaterals persisted into adulthood, which is indicative of embryonic collaterogenesis as a determining factor in the response to vascular occlusion in adulthood. Of note, even though an individual's collateral circulation might be embryonically/genetically defined, (pertained) physical exercise has been suggested to enhance collateral circulation in patients with coronary artery disease (113).

A role for cellular metabolism in angiogenesis and arteriogenesis

Until recently, only genetic signals were known to specify the tip versus stalk cell identity. However, emerging evidence reveals that cellular metabolism is also a key determinant of the EC subtype specification. Recent studies show that ECs rely on glycolysis for ATP production. Moreover, phosphofruktokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3), a key glycolytic regulator, controls tip versus stalk cell behaviour (114). In an *in vitro* angiogenesis model, silencing of PFKFB3 reduces glycolysis and vessel sprouting, whereas overexpression of PFKFB3 induces opposite effects (114). Moreover, EC-specific deficiency of PFKFB3 inhibits vessel growth and causes vascular defects in several *in vivo* models of angiogenesis. This is due to impaired stalk cell proliferation and tip cell migration (➔ Fig. 8.6). In fact, PFKFB3 knockdown not only negatively influences tip cell selection, but also abrogates increased tip cell competence upon Notch silencing *in vitro* (114). Strikingly, glycolysis even overrules the genetic (Notch) signals that regulate EC specification during vessel branching. Overexpression of PFKFB3 converted genetically instructed (by Notch overexpression) stalk cells into tip cells, while silencing of PFKFB3 caused opposite effects (114).

In addition to glycolysis, other metabolic pathways also contribute to EC behaviour; one of them is fatty acid (FA) metabolism. It was recently reported that FAs serve as a carbon source for endothelial DNA synthesis, putting FA metabolism into the spotlight as a potential basis for anti-angiogenic strategies (115) (see under Therapeutic opportunities). However, additional metabolic pathways, including the pentose phosphate pathway, amino acid metabolism, and others, have been poorly studied in ECs in relation to (pathological) angiogenesis (116, 117). Together, these findings identify EC metabolism as an important regulator of angiogenesis tightly intertwined with genetic angiogenic signals.

Given the recent nature of these seminal findings, which were made in models of sprouting angiogenesis, it remains to be determined if metabolism is a driver for arteriogenesis too. This is highly likely, especially in the adult setting of arteriogenesis, which is characterized by activation of ECs and SMCs, and massive proliferation and migration of SMCs; these phenomena are directly linked to changes in

cellular metabolism. Whether differences in cellular metabolism drive arterial versus venous specification, similar to the mechanism whereby it can drive tip versus stalk specification, remains to be studied. Irrespective of such a possible causal role, differences in cellular metabolism between different EC types have been reported. For example, pulmonary arterial ECs have a lower glycolytic flux but higher oxygen consumption rates than pulmonary microvascular ECs (118).

Therapeutic opportunities

Anti-angiogenic strategies to inhibit the excessive angiogenesis in the tumour microvasculature are mainly growth factor-centric (119). Even though these therapies have proven their value, their benefits have recently been questioned by the observation that overall survival did not necessarily improve (120); a proportion of tumours is inherently refractory or acquires resistance by developing escape mechanisms (120, 121). Anti-VEGF treatments (such as aflibercept or bevacizumab, to name only two) can incite tumours to start excreting alternative pro-angiogenic molecules such as FGF and placental growth factor (PlGF). VEGF inhibition can, on the other hand, increase hypoxia and interstitial pressure levels, and as such can create a milieu that promotes metastatic behaviour or selectively favours hypoxia-resistant cancer cells to thrive (120, 122). However, to date, clear evidence that anti-VEGF therapy would promote invasive and metastatic behaviour of cancer cells has only been convincingly shown in preclinical studies (123–126).

The tumour's ability to acquire vasculature by mechanisms other than sprouting angiogenesis can underlie resistance to VEGF inhibition. Co-option of the existing vasculature allows certain cancer cell types to obtain the necessary oxygen and nutrient levels without the requirement for angiogenesis; lung carcinoma cells from micrometastases, for example, switch to vessel co-option upon VEGF-A inhibition (127). Vascular mimicry, whereby cancer cells start behaving like ECs but with reduced VEGF sensitivity, represents another resistance mechanism (82, 128). Finally, tumours can recruit bone marrow-derived/endothelial progenitors to acquire vasculature through vasculogenesis (for a review see (➔ 129)).

These observations call for new and alternative anti-angiogenic strategies. Dll4, for example, has been considered an anti-angiogenic target based on its strong upregulation in tumour endothelium. Preclinical studies showed that inhibition of Dll4-Notch signalling leads to supernumerary but non-functional tumour vessels, resulting in decreased tumour growth (130–132). Given that the underlying mechanism is not entirely known, and in light of possible adverse effects, further research on the applicability of anti-Dll4 treatment is warranted (133, 134). ANG/Tie signalling represents another potential target. Antibodies targeting ANG2 were found to reduce tumour angiogenesis and to enhance the effect of anti-VEGF treatment on tumour vascularity and perfusion (135). Along the same line, the double anti-angiogenic protein (DAAP), a chimeric decoy receptor that simultaneously targets angiopoietins and VEGF, reduces tumour angiogenesis and growth (136). The complexity and involvement of ANG/Tie signalling, not only in sprouting angiogenesis but also in lymphatic and macrophage biology, necessitates further research on ANG/Tie as an anti-angiogenic therapy. Of note, in a recent phase III clinical trial with an ANG1-and ANG2-binding peptibody in ovarian cancer, the overall survival end-point was not reached, in spite of earlier reported promising data on progression-free survival (137).

A possible future strategy is to bypass growth factor signalling complexities and to hit the EC itself in such a way that tumour escape and resistance mechanisms have minimal to no impact on the treatment. The recent seminal findings on the crucial role of EC metabolism in vessel sprouting (114, 115) might open new roads towards such a kind of therapeutic intervention. Indeed, follow-up studies have already generated proof-of-principle for the use of PFKFB3 inhibition in treating pathological angiogenesis (138, 139). Pharmacological blocking of PFKFB3 with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) reduced EC glycolysis only transiently and partially (by approximately 40%); matching the increase in glycolysis levels in proliferating ECs (those actively participating in angiogenesis) versus quiescent ECs) but efficiently reduced pathological angiogenesis without harming the quiescent, healthy vasculature. This overrules the earlier belief that only complete and permanent glycolysis inhibition would have therapeutic benefits, even if it imposes risks for adverse effects. Blocking endothelial mitochondrial long-chain FAO with etomoxir also reduced pathological angiogenesis in a mouse model of retinopathy of prematurity (ROP). Intriguingly, ECs seem to rely on FA-derived carbons for dNTP synthesis more than various other cell types tested, including several cancer cell lines (115); this hints at a yet to discover therapeutic potential of targeting endothelial FAO.

Whether EC metabolism—and SMC metabolism by extension—could be the next focus in therapeutic arteriogenesis in tissue ischaemia too is an open question at present. In contrast to the anti-angiogenic strategies discussed, it would require gearing up EC/SMC metabolism in collaterals to enhance their rapid enlargement. (For overviews of currently tested/used pro-arteriogenic therapies and their outcomes see (➔ 140, 141).) Here, we will briefly highlight some major pitfalls

associated with these therapeutic approaches. The success of pro-arteriogenic strategies may be restricted by the fact that the same mechanisms (growth factor signalling and immune cell recruitment) that increase collateral expansion can also induce atherosclerotic plaque formation, creating an extremely delicate trade-off between both effects (*aka* Janus phenomenon) (142). For instance, local infusion of MCP-1 in hyperlipidaemic apolipoprotein E-deficient mice increased monocyte recruitment and collateral circulation following femoral artery ligation but also increased aortic atherosclerotic plaque surface (143). Likewise, macrophage recruitment might be considered a strategy to stimulate arteriogenesis. Also here, care should be taken not to skew the delicate equilibrium in macrophage subtypes within the plaque towards the more pro-atherogenic macrophage phenotypes (144, 145). Of note, the very driving force of arteriogenesis itself, shear stress, would cause atherosclerosis when increased. Another drawback of pro-arteriogenic treatments is the possibility of inducing tumour angiogenesis (again based on shared mechanisms); this requires careful monitoring, especially when sub-clinical tumours can be expected, as in older patients. Further research is needed to identify targets that are specific for arteriogenesis, such as endothelial extracellular receptor kinase (ERK; for a review see (➔ 4)), and to determine if and how these can be manipulated for pro-arteriogenic therapy.

Concluding remarks

The key concepts in angiogenesis and arteriogenesis, as well as underlying (molecular) mechanisms, overlap, and differences therein, have been presented. Both in development and in an adult setting, angiogenesis and arteriogenesis are of crucial importance given the vital role blood vessels play in nutrient and oxygen supply. Examples of postnatal, pathological angiogenesis, and arteriogenesis have been discussed: excess and abnormal angiogenesis in a tumour and arteriogenesis or acute remodelling of pre-existing collateral arteries upon arterial occlusion. These two settings require a virtually opposite therapeutic approach: *anti*-angiogenic treatment to block the excess angiogenesis versus a *pro*-arteriogenic approach to enhance collateral circulation and reduce severity of vascular occlusion disorders. The existing overlap in signalling cascades controlling these two processes makes it challenging to selectively target one of them without unwillingly favouring or repressing the opposite one. To improve selectivity in this 'balancing act', further understanding of the exact molecular mechanisms, as well as further identification of key players unique for one of the two processes, are required.

Recommended reading

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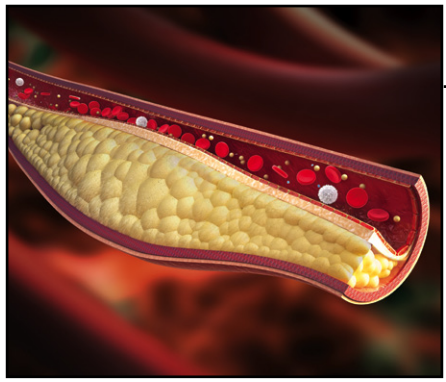

CHAPTER 9

The lymphatic system

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Content

Introduction

There are also in the body veins from the stomach, very small, and of all kinds, by whose means the food comes into the body.

Hippocrates

The blood vascular system transports oxygen, nutrients, and cells to the periphery, while removing the cellular waste products. During vascular network formation, blood vessels assemble to form a hierarchic pattern of arteries and veins, connected by a fine network of capillaries (1). In contrast to the blood vascular system, which is a closed circuit with a central pump—the heart—the lymphatic vascular system constitutes a one-way network of drainage channels that relies on smooth muscle cell (SMC) and skeletal muscle contractions, arterial pulsations, and intraluminal valves to transport the lymph in a unidirectional manner (1). These two vascular systems work cooperatively to ensure tissue homeostasis of the highly complex vertebrate body.

The first documented description of the lymphatic system may stem from the observations of Aristotle (384–322BC) and Hippocrates (460–377BC.) more than 2,000 years ago, when Aristotle described observing 'structures containing colourless fluid' (2). In 1627, Gaspare Aselli published the first formal description of the lymphatic vessels in his book '*De lactibus sive lacteis venis*' (2, 3). Further analysis succeeding Aselli's initial studies demonstrated that the 'white veins' he reported actually represented a vascular system separate from the blood vessels (4). In this chapter, we give an overview of the anatomy and function of the lymphatic system and discuss its role in pathological conditions.

Organization and physiological functions of the lymphatic system

The lymphatic system, present only in vertebrates, is composed of lymphatic vessels and lymphoid organs, such as the bone marrow, thymus, lymph nodes, spleen, Peyer's patches, tonsils, and the appendix. Lymphatic vessels are absorptive vessels that are found in almost every organ. As in the blood vascular system, lymphatic vessels are composed of morphologically, functionally, and

hierarchically distinct segments, which can be categorized into capillaries, pre-collectors, and collectors. An extensive network of lymphatic vessels drains virtually all organs. Lymph nodes are highly organized lymphoid organs that are located at the intersections of collecting lymphatic vessels (5). During the past two decades, tremendous progress has been made towards elucidating the anatomy and function of the lymphatic system. Recent experimental evidence, together with improved imaging technologies, have rekindled the debate about the extent of lymphatic vessel coverage of organ systems that were previously considered 'alymphatic', such as the eye and the central nervous system (6–10). In the following subsections, we will discuss the anatomy and the physiological functions, of the lymphatic vasculature.

Tissue fluid homeostasis

Fluid and solutes slowly extravasate from the blood capillaries into the interstitium and are returned to the circulation primarily via the lymphatic system (11), making the lymphatic system a major contributor to tissue fluid homeostasis. The filtrated protein-rich exudate in the interstitium (called lymph once it is within lymphatic vessels), is channelled to the lymphatic capillaries via low-resistance tissue conduits (12). The lymphatic capillaries are formed by a single layer of partially overlapping lymphatic endothelial cells (LECs), with discontinuous or no basement membrane and lack smooth muscle cell (SMC) coverage (Fig. 9.1). The one-way entry and transport of interstitial fluid and immune cells are facilitated by the special features of the oak-leaf shaped LECs,

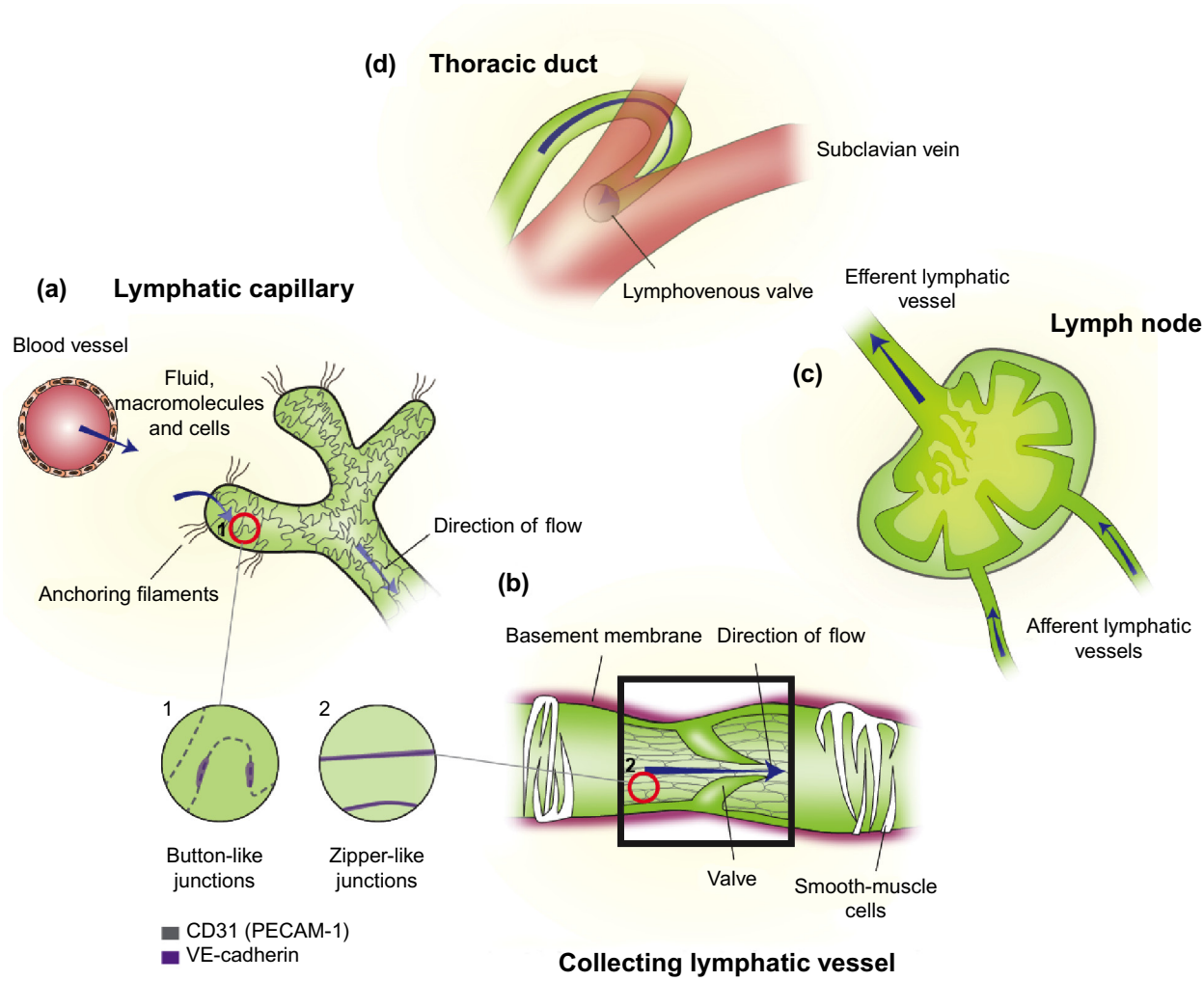

Fig. 9.1 Organization of the lymphatic vascular tree. (a) Lymphatic capillaries take up extracellular fluid, macromolecules, and cells from the peripheral tissues. Inset 1 shows button-like junctions that form the primary valves, which allow increased permeability of the lymphatic capillaries. (b) Lymph flows towards the collecting lymphatic vessels that contain intraluminal valves, which ensure unidirectional flow. Collecting lymphatic vessels are lined with LECs that are interconnected by zipper-like junctions (inset 2). (c) The antigen- and immune cell-loaded lymph is filtered through the lymph nodes. (d) Lymph finally reaches the largest collecting ducts and then returns to the venous circulation via the thoracic duct.

which are adjoined by interrupted 'button-like' junctions, rich in vascular endothelial cadherin (VE-cadherin) that generate primary valves (13, 14) (➔ Fig. 9.1a). Elastic fibres anchor the LECs to the surrounding extracellular matrix and function as mechanosensors that are able to detect increases in tissue pressure and open the primary lymphatic valves to permit interstitial fluid to enter the lymphatic capillaries during tissue swelling (15). These distinct properties of initial lymphatic vessels are quintessential for highly efficient absorption and transport of fluid, macromolecules, and immune cells. After entering the blind-ended lymphatic capillaries, lymph is transported towards the valve-containing collecting vessels by intrinsic contractions of the latter, external tissue compressions, a temporary increase in upstream pressure, or by a temporary decrease in the downstream pressure in the collecting lymphatic vessels (12).

In contrast to lymphatic capillaries, the collecting lymphatic vessels possess endothelia that are tightly interconnected with 'zipper-like' junctions and possess intraluminal valves, a basement membrane, and SMC coverage (➔ Fig. 9.1). A collecting lymphatic vessel segment flanked by two valves, called a lymphangion (16–18), is the contractile unit where lymph is propelled to the next compartment in a unidirectional manner (19).

The concept of 'lymphatic drainage' may seem straightforward; however, in order to drain tissue fluid, lymphatic vessels have to overcome a number of fundamental physiological forces. First, under normal conditions, the range of interstitial tissue fluid pressure is below atmospheric pressure in peripheral tissues (20), while the fluid pressure in lymphatic capillaries is above this level. Second, the intralymphatic pressure increases along the drainage route as lymph is propelled towards the larger collecting vessels and ultimately to the thoracic duct or right lymphatic trunk, which coalesce with the subclavian veins to return the lymph to the blood circulation (21). In contrast to the blood circulatory system, the lymphatic system is devoid of a central driving force; therefore, the lymphatic vessels need to contract rhythmically to transiently increase intralymphatic pressure in order to open lymphatic valves and to move the lymph forward (22). Once the fluid pressure within the preceding lymphangion returns to a lower level, the relatively higher pressure in the downstream lymphangion shuts off the intraluminal valves and prevents retrograde lymph flow (➔ Fig. 9.1). Importantly, lymphangions harbour an inherent pressure-sensing mechanism, which responds to changes only at relatively low-pressure ranges. For example, lymphangions are insensitive to changes in pressure at times of considerably high transmural pressures, partially explaining the lymphatic vessel malfunction in lymphoedema (23). However, while the potential hydrostatic pressure gradient from the foot lymphatics to the great veins of the neck is highly negative and opposes lymphatic flow, the gradient from the head to the great veins is positive, and thus favours the flow of lymph (19). Lymphatic drainage from the brain may thus represent an exception to these concepts.

Immune surveillance

Apart from their function in returning extravasated fluid back to the blood circulation, lymphatic vessels also play a major role in helping the immune system defend the body against disease. The immune surveillance hypothesis suggests that cells of the immune system constantly scan the organism to detect and destroy 'non-self' invaders, as well as transformed (cancerous) host cells. Most memory T cells patrol peripheral tissues to provide protection upon re-exposure to the same pathogen. To do so, they must continuously recirculate between blood vessels and lymph nodes (24). Dendritic cells (professional antigen-presenting cells) probe the interstitium to acquire free antigens to present to naïve T cells in the lymph nodes. Lymphatic vessels serve as 'express-highways' for the fast and efficient delivery of immune cells, as well as free antigens to the regional lymph nodes for the elicitation of appropriate downstream immune responses (25–27).

While free antigens diffuse through the extracellular fluid, antigen-bearing dendritic cells proactively migrate to the lymph nodes under the influence of chemotactic cues produced by the lymphatic vessels (28). LECs produce chemokines such as CCL21 and CCL19 that potently recruit mature dendritic cells that have upregulated the cognate CCR7 receptor expression, and guide their entry into the lymphatic vessels. By expression of CCL21, lymphatic vessels do not only attract activated dendritic cells, but also T cells, neutrophils, and M1 polarized (classically activated) macrophages (29). K14-VEGFR3-Ig transgenic mice expressing an inhibitor of lymphangiogenic growth factors ('VEGF-C/D trap') are devoid of dermal lymphatic vessels and hence of lymphatic drainage from the skin (30). This results in an apparent delay in T-cell responses to intradermal immunization with antigens (31), demonstrating the importance of antigen and activated dendritic cell transport to the lymph nodes in this process.

Lymphatic vessels also contribute to the maintenance of self-tolerance. High-level expression of the programmed death ligand-1 (PD-L1) in combination with low-level expression of co-stimulatory molecules on LECs was shown to effectively eliminate CD8 + T cells that recognize peripheral tissue antigens expressed and presented on major

histocompatibility complex I (MHC I) by the LECs (32, 33). Interestingly, lymph node LECs also express MHC II; however, rather than displaying peripheral antigens on MHC II, LECs supply dendritic cells with peripheral tissue antigens, which are later presented to CD4 + T cells to induce anergy. These data indicate that lymph node LECs act as a peripheral tissue antigen reservoir for CD4 T-cell tolerance (34).

The original immune surveillance concept developed by Burnet was based on the recognition and destruction of cancerous cells by the immune system (35). Lymphatic vessels transport antigens from the tumour microenvironment to the lymph nodes and, depending on the cues that are present in the lymph, tolerance vs. immune response to tumour antigens can be initiated (36). Lymph also carries self-antigens from its tissue of origin to the regional lymph nodes. These antigens can be taken up by the resident immature dendritic cells, which then have the ability to cross-present the lymph-borne antigens to alloreactive (reactive to non-self) T cells that induce self-tolerance by activating regulatory T cells (26, 37). In order for lymph-carried peptides to be tolerogenic, they have to be present in adequate concentrations for the antigen presentation. The self-antigens in lymph are present in high-nanomolar to micromolar amounts, comparable to the concentrations that are effective in peptide immunotherapy (38). Thus, the lymphatic system plays a central role not only in the mounting of adaptive immune responses, but also in boosting self-tolerance (27).

Uptake of dietary lipids

The small intestine is a highly vascularized organ whose major function is the absorption of digested nutrients from the diet. The intestinal villi are the functional units responsible for the uptake of nutrients from the gastrointestinal tract (39). They consist of enterocytes on the luminal surface, lamina propria, smooth muscle fibres, and blood capillaries that form a cage-like network surrounding a central lymphatic capillary called a lacteal vessel. Intestinal lymphatic vessels were discovered in 1622 by Gaspare Aselli, who coined the term lacteal, which stems from the word *lactis* (i.e. intestine) (2). Lacteals take up chylomicrons, very low-density lipoproteins (VLDLs), long-chain fatty acids and, importantly, fat-soluble vitamins secreted by the enterocytes. Lipid drainage by the lacteals is aided by the contractile activity of the surrounding smooth muscle cells (40). The whitish appearance of the lymphatic vessels after a meal is due to the composition of 'chyle', which is a lipid-rich emulsion of lymph. The majority of the absorbed nutrients, including short (2–6 carbon residues) to medium (6–14 carbon residues) chain fatty-acids, traverse through the blood capillaries of the intestinal villus and are transported initially to the liver via the portal vein (hepatic route) and then to the blood circulation. Passive diffusion across the endothelial cells of blood vessels is not feasible for particles of high molecular weight or colloidal nature, whereas the properties of LECs allow an increased permeability of the lymphatic vessels (41), facilitating the transport of larger particles into the lacteals. Chylomicrons (approximately 1.2 μm in diameter) and VLDLs, which are produced by enterocytes in addition to the long-chain lipids (longer than 14 carbon residues), are readily taken up and transported by lacteals.

The uptake, packaging, and secretion of lipids by the enterocytes have been studied intensively, but the process of lipid transport into lacteals is not fully understood yet. To date, two major mechanisms have been explored; (i) paraendothelial transport, where particles enter passively into lacteals from 'pores' at the apex of the lacteal or in between the LECs; and (ii) transendothelial transport across the LECs (42). Both means of transport take place as shown by studies using transmission electron microscopy. In a tissue-engineered model of intestinal lacteals (Caco-2 cells as the chylomicron packing enterocytes in co-culture with the LECs), both routes of lipid particle transport appear to function in parallel (43). In this model, lipid vesicles were observed both intracellularly and in association with cell–cell adhesions, with directional transport from the enterocytes towards the LECs.

Lymphatic dysfunction is associated with defects in the uptake and transport of lipids from the intestine. Mouse models with genetic defects in lymphatic vessels, such as Prox1 haploinsufficient mice (*Prox1*$^{+/-}$), show defective intestinal lipid uptake and transport and develop chylous ascites (44). Intestinal lacteals, unlike lymphatic vessels elsewhere, depend on VEGFC produced by the neighbouring cells for their maintenance. Conditional deletion of *Vegfc* or the Notch ligand *Dll4* results in lacteal regression and defects in lipid absorption in adult mice (45, 46). Mice deficient of the pleomorphic adenoma gene-like 2 (PlagL2) transcription factor, which is involved in the assembly of chylomicrons, develop a wasting syndrome and die shortly after birth due to lipid absorption defects (47). It has been shown that in these mice, the enterocytes are able to absorb nutrients and secrete lipid-loaded particles; however, due to their defective uptake to the lacteals, these particles accumulate in the intestine.

Considering their unique absorption and transport features, lacteals comprise an attractive route for the administration of therapeutic drugs. There are two major advantages in utilizing the intestinal lymphatic route for drug delivery. First, lacteals selectively take up certain types of

free fatty-acids and lipoprotein particles. This opens up new avenues for selective targeting via conjugation of active substances to such lipid species. Second, the drugs transported via intestinal lymphatics are delivered into the systemic circulation as they initially bypass the liver. Consequently, these drugs avoid the hepatic first-pass metabolism, enhancing their bioavailability after oral application, which could be critical for the delivery of liver-metabolized drugs (41).

Development of the lymphatic vasculature

In mouse embryos, lymphatic vessels begin to develop after the onset of blood circulation (48). LECs arise from a subpopulation of endothelial cells in the dorsal part of the common cardinal vein (➔ Fig. 9.2) (49, 50). The first LECs can be identified by their expression of PROX1, which is induced by the SOX18 transcription factor (51, 52). These lymphatic endothelial progenitor cells begin to proliferate and sprout away from the veins. These strings of migrating LECs coalesce to form the lymph sacs (49, 50). The cells connecting the lymph sacs and the cardinal vein form lymphovenous valves (53). Blood clotting by platelet aggregation induced by the interaction of platelet C-type lectin-like receptor 2 (CLEC2) with the lymphatic endothelial surface protein podoplanin (PDPN) is important in maintaining blood–lymph separation (54).

Subsequently, the majority of the lymphatic vasculature develops from the lymph sacs through the active migration and proliferation of LECs in a process termed lymphangiogenesis. Indeed, lineage-tracing experiments have indicated that the majority of the lymphatic vascular tree originates from blood vessels (55), as postulated in 1902 by the American anatomist Florence Sabin (56). However, researchers such as Huntington and McClure proposed that lymphatic vessels are formed by mesenchymal lymphangioblasts (for a review see (➔ 57)). A mesenchymal origin was indeed described in *Xenopus* tadpoles (58) and chick embryos (59). However, in mammals, only recent lineage-tracing studies have convincingly demonstrated that some lymphatic vessels do not originate from the

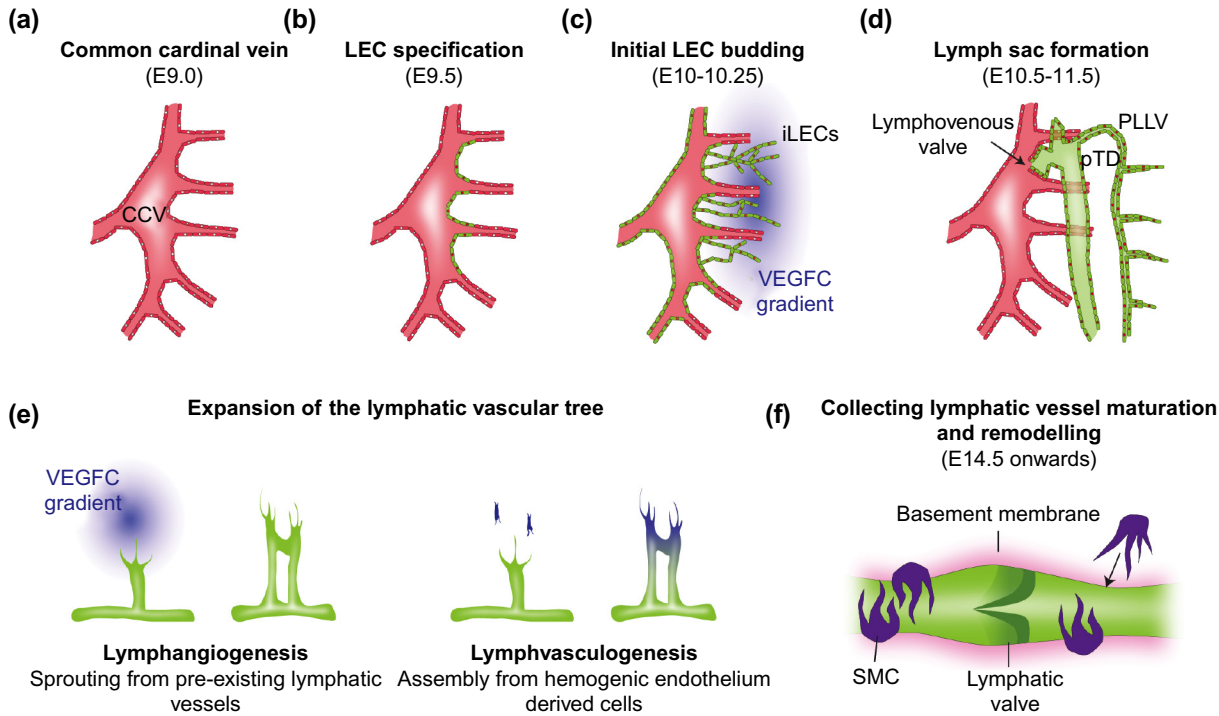

Fig. 9.2 Development of the lymphatic vascular system. (a) The common cardinal vein in embryos, lined by blood vascular endothelial cells (BECs) at E9.0. (b) Lymphatic endothelial cell (LEC, green) specification at E9.5, involving WNT5B, COUP-TFII, SOX18, and PROX1. (c) VEGFC-driven budding of the initial LECs (iLECs) at E10.25. (d) The lymph sacs, composed of the primordial thoracic duct (pTD) and the primordial longitudinal lymphatic vessel (PLLV) at E12.5 are bilateral structures from which the majority of the lymphatic vascular tree is derived. Platelet aggregation by PDPN/CLEC2 interaction maintains blood–lymphatic separation at the lympho-venous valve. (e) Expansion of the lymphatic vascular tree from the lymph sacs by two mechanisms: lymphangiogenesis and lymphvasculogenesis. (f) Maturation and remodelling of the lymphatic vessels into a hierarchical tree composed of capillaries and collecting vessels; the latter have valves and smooth muscle cell coverage.

(Adapted from Hagerling R, Pollmann C, Andreas M, Schmidt C, Nurmi H, Adams RH, Alitalo K, Andresen V, Schulte-Merker S, Kiefer F. A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. EMBO J. 2013 Feb 12;32(5):629–44.)

lymph sacs, but instead from haemogenic endothelial cells that trans-differentiate into LECs and incorporate into the growing vascular tree via a mechanism termed lymphvasculogenesis (60–62).

Subsequently, the lymphatic vessels undergo maturation by forming valves, recruiting smooth muscle cells, depositing basement membrane around the collecting vessels, and forming of button-type junctions in lymphatic capillaries (➔ Fig. 9.2) (13, 63). As the collecting lymphatic vessels mature, the expression of several LEC markers, such as PROX1, VEGFR3, and LYVE1, is decreased. However, PROX1 and FOXC2 expression remain high in lymphatic valves (64).

The past 20 years have seen substantial progress in the understanding of the molecular mechanisms involved in lymphangiogenesis (65). While vascular endothelial growth factor (VEGF) signalling via vascular endothelial growth factor receptor 2 (VEGFR2) has mainly been implicated in angiogenesis, VEGFC and VEGFD signalling via VEGFR3 is the main driver of lymphangiogenesis (65). VEGFR3 was one of the first lymphatic endothelial cell markers to be discovered (66). Early during embryogenesis, VEGFR3 is also expressed in blood vessels, and *Vegfr3* gene-targeted mice die at around E10.5 due to the defective development of the cardiovascular system (67). Also, during angiogenesis, blood vessels upregulate VEGFR3 expression in the tip cells of angiogenic sprouts, which in part drives angiogenesis (68, 69). However, later in development, VEGFR3 expression becomes largely restricted to lymphatic vessels and fenestrated blood vessels (68). VEGFR3 is activated by its two ligands, VEGFC and VEGFD. The secreted forms of VEGFC and VEGFD can bind only VEGFR3, but proteolytic processing enables binding also to VEGFR2 (70). VEGFR3 can form heterodimers with VEGFR2, which may lead to combinatorial signals by the intracellular tyrosine kinase domains of the two receptors (71).

In embryos, VEGFR3 is abundantly expressed in initial LECs committed to the lymphatic lineage. VEGFC is critical for driving LEC migration away from the cardinal veins, which fails in *Vegfc*-deficient mice (50, 72). Interestingly, *Vegfd* is largely dispensable for early lymphatic development (73), although its deficiency alters the calibre of initial lymphatics in the dermis, leading to reduced functional capacity (74). Also, the compound deletion of both *Vegfc* and *Vegfd* does not recapitulate the early embryonic lethality observed in *Vegfr3* null mice, suggesting that other factors may also activate VEGFR3 (75).

After the formation of the lymph sacs, VEGFC is predominantly expressed where lymphatic vessels develop, and it drives the expansion of the primitive lymphatic plexus (72). Mice heterozygous for *Vegfc* or for a dominant negative mutation in the tyrosine–kinase domain of *Vegfr3* fail to develop superficial lymphatic vessels and display lymphoedema (72, 76). Furthermore, the transgenic expression of soluble VEGFR3-Ig (VEGFC/D trap) after E14.5 inhibits lymphangiogenesis and induces partial regression of already formed lymphatic vessels, resulting in lymphoedema (30, 77). Interestingly, inhibiting lymphangiogenesis with VEGFC/D trap or VEGFR3-blocking antibody postnatally induces the regression of already formed lymphatic capillaries until 2 weeks after birth, but after 4 weeks of age, the lymphatic vessels begin to regrow despite sustained inhibition (78). In adults, prolonged VEGFC/D trap exposure does not seem to affect the established lymphatic vessels (79). However, conditional *Vegfr3* deletion for over 3 months induces a gradual hypoplasia of the intestinal lymphatic vessel network (80). Therefore, the requirement of VEGFR3 signalling gradually decreases as the lymphatic vessels mature, with the exception of lacteals that depend on VEGFC for their maintenance (45).

Collagen- and calcium-binding EGF domain 1 (CCBE1) protein has recently been identified in genetic screens in zebrafish as being indispensable for lymphangiogenesis (81) and was later linked to the Hennekam lymphangiectasia–lymphoedema syndrome (82). *Ccbe1*-deficient mice fail to form lymph sacs (50, 83). The mechanism by which CCBE1 affects lymphangiogenesis was found to be via the enhancement of VEGF-C/VEGFR3 signalling. CCBE1 is secreted by mesenchymal cells and binds the disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS3) metalloproteinase, which promotes the cleavage of VEGFC into its fully active form, thus enhancing its signalling via VEGFR3 (84, 85).

VEGFR3 signalling is also enhanced by neuropilin 2 (NRP2), an axon-guidance receptor that functions as a VEGFR3 co-receptor. Although NRP2 is expressed in both venous and lymphatic endothelium, *Nrp2*-deficient mice have defects in the lymphatic vasculature and not in the blood vasculature (86). *In vitro*, NRP2 binds VEGFC, VEGFD, and VEGF and interacts with both VEGFR2 and VEGFR3. However, genetic experiments have revealed that NRP2 mainly interacts with VEGFR3 *in vivo* (87). Lymphatic development is impaired in $Nrp2^{+/-}$; $Vegfr3^{+/-}$ mice, but not in $Nrp2^{+/-}$; $Vegfr2^{+/-}$ mice (87). NRP2, as such, is not likely to have a major signalling role although NRP2 is internalized upon receptor activation (88, 89). Therefore, NRP2 mediates tip-cell extension and guided-vessel sprouting in response to VEGF-C probably by enhancing VEGFR3 signalling in response to VEGFC/D gradients.

In addition to VEGFs and VEGFRs, secreted angiopoietin growth factors and their TIE receptors are also involved

in lymphatic and blood vessel development (90). While the overexpression of ANG1 via viral transduction resulted in TIE2-mediated lymphatic vessel sprouting (91), blocking ANG2 binding to TIE2-with antibodies impeded lymphatic vessel growth, filopodia formation, and LEC proliferation (92). *Ang2*-deficient neonatal mice presented tissue-specific lymphatic defects such as reduced lacteal growth and chylous ascites in the mesentery, and abnormal lymphatic plexus formation and reduced lymphatic drainage in the skin. This phenotype was rescued by the genetic substitution of *Ang2* with *Ang1* (93). ANG2 has also been implicated as an essential regulator of the poorly understood formation of button-like junctions in lymphatic capillaries (92).

One of the earliest markers of LECs is PROX1, which functions as a master-regulator that determines the lymphatic phenotype. Within the vascular system, PROX1 is expressed in lymphatic endothelial cells, venous valves (94), and on the concave side of cardiac valves (95). PROX1 expression is induced in LECs committed to the lymphatic lineage in the cardinal veins at E9.5 (96). *Prox1* null embryos fail to develop lymph sacs or lymphatic vessels (96). The initial LECs of the cardinal vein wall start to bud off, but in a non-polarized manner and this process is later arrested. Interestingly, these cells fail to express additional lymphatic endothelial cell markers, such as LYVE1, VEGFR3, and CCL21 (97). In contrast, the overexpression of PROX1 in blood vascular endothelial cells (BECs) upregulates the expression of LEC-specific markers (52, 98). Overall, these data suggest that PROX1 functions as a master switch for the specification of LEC fate (99). The SOX18 transcription factor has been shown to induce LEC specification upstream of PROX1 (51). The *Prox1* promoter contains SOX18-binding sites and SOX18 expression in initial LECs precedes the expression of PROX1. Ectopic SOX18 expression in BECs was sufficient to force the expression of PROX1 *in vitro*. Interestingly, however, *Sox18* knockout mice fail to induce *Prox1* expression only in the C57BL/6 background. In other mouse strains, the related SOX7 and SOX17 transcription factors were able to compensate for the loss of SOX18 (100). In contrast, NOTCH1 signalling, which regulates cell-fate decisions, has been shown to repress the emergence of LECs from the cardinal veins (101). Recently, WNT5B signalling was also implicated in the early specification of LECs in zebrafish (61).

Lymphatic vessel hyaluronan receptor 1 (LYVE1) is one of the most widely used markers for LECs. In adults, LYVE1 is highly expressed in lymphatic capillaries, but downregulated in collecting lymphatic vessels (102). Extralymphatic LYVE1 labelling occurs in a subpopulation of macrophages. (103). Developmentally, LYVE1 expression is the first indicator of lymphatic endothelial competence, even though LYVE1 is not required for normal lymphatic development or function (104).

Several signalling pathways are involved in the maturation of collecting lymphatic vessels. Importantly, the FOXC2/calcineurin/NFATC1 pathway is required for the maturation of collecting lymphatic vessels. Without *Foxc2*, the primitive lymphatic plexus maintains a high expression of PROX1, VEGFR3, and LYVE1, and fails to mature into functional collecting vessels and valves (64). Furthermore, the conditional deletion of *Foxc2* results in the regression of lymphatic valves (105). Also the conditional deletion of the calcineurin regulatory unit *Cnb1* leads to defects in the formation of lymphatic valves (106). Overall, FOXC2, PROX1, and also lymphatic flow, cooperate to activate calcineurin/NFATC1, which contributes to the proper formation and maintenance of lymphatic valves (106, 107). In addition, the connexin family of gap-junction proteins (CX26, CX37, and CX43) is involved in developmental lymphangiogenesis. *Cx37*-deficient mice lack lymphatic valve-forming cells and thus do not develop valves. *In vitro* flow analyses revealed that *Cx37* depletion reduces calcineurin/NFATC1 activation, and that PROX1, FOXC2, and oscillatory shear stress regulate the expression of *Cx37* (106).

Extracellular matrix proteins and their interaction partners are also involved in lymphatic vessel development and maturation. LECs of the collecting lymphatic vessels secrete the reelin glycoprotein into the ECM near the valves, which is associated with the migration and adhesion of SMCs to the vessel wall. Reelin-deficient mice have enlarged collecting lymphatic vessels that fail to mature, have less SMC coverage, and show impaired drainage (108). Integrin-α9 (ITGA9) is specifically expressed by the LECs of the lymphatic valves. Its interactions with the ECM protein, EMILIN1, are critical for lymphatic valve morphogenesis (109), and *Itga9*-deficient mice have abnormal lymphatic valves that are reduced in numbers (110).

Axon-guidance proteins, which have well-established roles in guiding angiogenic tip cells and axon growth cones, also play significant roles in lymphangiogenesis (111). The axon-guidance molecule semaphorin 3A (SEMA3A) and its receptors, NRP1 and plexin A1 (PLXNA1), are expressed in collecting lymphatic vessels and lymphatic valves. SEMA3A-NRP1 signalling is especially important for later stages of lymphatic valve morphogenesis and SMC coverage, but does not affect the early valve specification (112, 113).

Bidirectional signalling via the transmembrane ligand ephrin-B2 and its cognate transmembrane Eph4 receptor tyrosine kinases have been identified as important regulators of blood vascular development (114, 115). Both forward signalling via Eph4 and reverse signalling via ephrin-B2 are

required for lymphatic development (102, 116). While the tyrosine kinase domain of Eph4 is required for lymphatic valve development, the PDZ domain of ephrin-B2 is more important. Mice lacking the PDZ domain of ephrin-B2 displayed hyperplastic-collecting lymphatic vessels that lacked valves, while mice lacking the ephrin-B2 tyrosine residues displayed only minor defects (102, 116). In contrast, the inhibition of EphB4 with monoclonal antibodies or in EphB4 kinase mutation resulted in defective lymphatic valve development (116). Ephrin-B2 was shown to be required for the internalization of VEGFR3 and subsequent downstream signal transduction by the small GTPase Rac1, Akt, and the mitogen-activated protein kinase Erk (115).

Several other important factors in lymphatic vessel and valve formation have been identified. Activin receptor-like kinase 1 (ALK1) is a transforming growth factor (TGF)-β family type 1 receptor that is expressed by both BECs and LECs. Its ligand bone morphogenetic protein 9 (BMP9) was initially identified as a critical regulator of retinal angiogenesis. BMP9 also controls lymphatic vessel maturation and valve formation by inducing the expression of FOXC2, CX37, ephrin-B2, and NRP1 via ALK1 (117). In accordance, blocking ALK1 signalling resulted in defective postnatal lymphatic vascular patterning (118). Furthermore, the TGFβ receptors *Tgfbr1* and *Tgfbr2* in LECs are required for proper sprouting and patterning of lymphatic vessels *in vivo* (119).

An important function of the lymphatic vascular system lies in interstitial fluid drainage. Indeed, the absence of lymphatic vasculature in VEGFC-deficient mice results in severe oedema and subsequent embryonic death. Interestingly, it has been shown that the increasing interstitial fluid pressure during development promotes the expansion of the lymphatic vascular tree in order to meet the demand in tissue drainage. Mechanistically, LEC stretching induced VEGFR3 tyrosine phosphorylation and LEC proliferation via β1 integrins (120). In addition, haemodynamic forces within lymphatic vessels also drive the maturation of collecting lymphatic vessels (105, 121).

The elucidation of these mechanisms has significantly improved our understanding about the role of lymphatic vasculature in human disease, which will be highlighted in the next section.

The lymphatic system in pathology

Inflammation

Inflammation is the body's response to insults such as infection and injury and occurs during autoimmune conditions or cancer (122). Inflammation elicits changes in blood and lymphatic vessels, such as the activation of the endothelium, the increase in vessel permeability and, depending on the nature of the stimulus, profound angiogenesis and lymphangiogenesis in the inflamed tissue (1). Inflammation-associated lymphangiogenesis and lymphatic enlargement are observed in several inflammatory diseases including, but not limited to, psoriasis (123), atopic dermatitis (124), rheumatoid arthritis (125), chronic airway inflammation (126), and Crohn's disease (127). While newly formed blood vessels are pruned after the resolution of inflammation, in a *Mycoplasma pulmonis*-induced chronic airway inflammation model, the lymphatic vessels showed very little pruning after the resolution of inflammation (126), despite the administration of anti-inflammatory corticosteroid (128). However, the regression of lymphatic vessel after inflammation appears to be context-dependent, as they do regress in the lymph nodes in an experimental skin inflammation (129) or in the cornea in a suture-induced inflammation model (130).

The inflammatory response is required to protect the body from invasion by pathogens and for re-establishment of homeostasis; however, its prolongation would be at the expense of tissue integrity. As the number of inflammatory cells soars in the aftermath of inflammation, the cellular efflux and the removal of inflammatory cytokines and excess extracellular fluid from the affected tissue become of pivotal importance to resolve the inflammation. The concept of lymphatic vessel-mediated removal of these factors has been reinforced by mouse studies where lymphangiogenesis is induced or inhibited experimentally, both of which greatly affected the progression of inflammation. For instance, in two mouse models of skin inflammation, namely oxazolone-induced contact hypersensitivity (CHS) and ultraviolet B (UVB)-induced skin inflammation, the stimulation of lymphangiogenesis by the transgenic delivery of the lymphangiogenic growth factors VEGFC and VEGFD strongly impeded the development of acute inflammation (131). In a mouse model of myocardial infarction (MI), further induction of post-MI lymphangiogenesis by the administration of recombinant VEGFC(C156S) was reported to lead to a transient improvement of cardiac function (62). Moreover, in the chronic CHS model, transgenic delivery of VEGFC to the skin of mice completely inhibited the development of chronic skin inflammation (132). Conversely, the inhibition of VEGFR3 signalling by the systemic administration of receptor-blocking monoclonal antibodies prolonged the inflammation in the UVB-induced inflammation model (133), exacerbated the oedema formation in the CHS (132) and the *Mycoplasma pulmonis*-induced airway inflammation model (126), and exacerbated the inflammation in mouse models of chronic

inflammatory arthritis (134) and inflammatory bowel disease (135). Here, the essential component in limiting acute inflammation, as well as in resolving chronic inflammation, is not a mere expansion of the lymphatic vascular network, but also a functional activation of the lymphatic vessels. Interestingly, in the inflamed airways, existing lymphatics underwent button-to-zipper transformation, which could explain the reduced interstitial fluid uptake in the inflamed state; also, sprouting lymphatic capillaries have been reported to have zipper-like junctions (13, 63). Inflammation-associated lymphangiogenesis is also a critical component in transplant rejection (136, 137). In heart transplantation, chronic rejection induces marked VEGFC upregulation and lymphangiogenesis. Blocking VEGFC/D in this context prolongs allograft survival in part via inhibition of CCL21 production (137).

Lymphatic vessels also contribute to dampening of inflammation by regulating the levels of available inflammatory cytokines *in situ*. For example, in response to inflammation, lymphatic vessels upregulate the expression of D6, which is an atypical CC-chemokine receptor that scavenges pro-inflammatory chemokines and leads to their intracellular degradation upon binding. By these means, the LECs restrict the binding of inflammatory cells to the vessel surface (138). Indeed, mice deficient for D6 develop an aggravated skin inflammation in response to Freunds's adjuvant injection compared to wild-type mice (139). These data indicate that lymphatic vessels play an important role in the initiation (as discussed under Immune surveillance), as well as resolution of inflammation.

Lymphoedema

One of the main functions of lymphatic vessels is the clearance of excess interstitial fluid extravasated from blood vessels. The cardinal manifestation of lymphatic dysfunction is lymphoedema. Primary lymphoedemas are rare; they result from defects in genes involved in lymphatic vessel development, often involving the VEGFC/VEGFR3 signalling axis, while secondary lymphoedemas arise from damage to or physical obstruction of lymphatic vessels after cancer surgery, infection, or trauma (140).

Currently at least 19 genes have been involved in various primary lymphoedemas, which occur as largely isolated diseases or a part of a syndrome (140). We will briefly introduce two of these (for more information see (➔ 141)). ➔ Figure 9.3 illustrates some of the most common genetic defects behind the rare primary lymphoedemas.

The VEGFC/VEGFR3 signalling axis

Heterozygous mutations in the *FLT4* gene, which encodes for VEGFR3, comprise about one-half of hereditary lymphoedemas. Mice harbouring heterozygous mutations in the tyrosine kinase domain of *Vegfr3* and *Vegfc* heterozygous mice show a similar lymphoedema phenotype (72, 76). Thus, the missense point mutations in the tyrosine kinase domain of VEGFR3 result in defective lymphangiogenesis during development, and cause primary congenital lymphoedema, also known as Nonne–Milroy lymphoedema (142). Typically, this form of lymphoedema is apparent already at birth. Recently, heterozygous VEGFC mutations have been linked to a Milroy-like disease, which is indistinguishable from Nonne–Milory disease (143).

As introduced earlier, mutations of the *CCBE1* gene are associated with the Hennekam lymphangiectasia–lymphoedema syndrome. In addition to lymphoedema, these patients have a pathological dilation of lymphatic vessels (lymphangiectasia), systemically and viscerally, and mental retardation (82). CCBE1 has been shown to enhance VEGFR3 signalling by increasing VEGFC cleavage into its active, fully mature form by the ADAMTS3 metalloprotease, and possibly by other proteases (84, 144).

Mutations in the protein tyrosine phosphatase PTPN14 have been linked to lymphoedema–choanal atresia syndrome. PTPN14 may be involved in the dephosphorylation of VEGFR3, besides its other targets. *In vitro*, the loss of PTPN14 activity results in hyperactive VEGFR3 signalling (145). Thus, while further mechanistic studies are needed, it appears that an overactive VEGFR3 signalling pathway may also result in lymphoedema. Interestingly, downstream of VEGFR3, mutations in the phosphoinositide-3 kinase (PI3K) gene resulting in hyperactivation of PI3KCA (146) and AKT (147) have been associated with lymphatic malformations.

Transcription factors

The VEGFR3 downstream transcription factor FOXC2 has been associated with lymphoedema distichiasis syndrome, which is often characterized by distichiasis (two rows of eyelashes) ptosis, and yellow nail syndrome. As introduced earlier, mechanistic studies in mice have indicated that *Foxc2* is critical for the maturation of collecting lymphatic vessels. Lymphatic vessels in the Foxc2-deficient mice showed defective lymphatic valves and smooth muscle cell recruitment to lymphatic capillaries, which are probably responsible for the phenotype also in humans (148). Mutations in *SOX18*, which is involved in the induction of PROX1 during development, are associated with hypotrichosis–lymphoedema–telangiectasia syndrome (51, 100, 149). Mutations in the transcription factor *GATA2* have been associated with Emberger syndrome, a primary lymphoedema with myelodysplasia (150).

Gap-junction proteins are also required for lymphatic development. Mutations in two connexins have also been

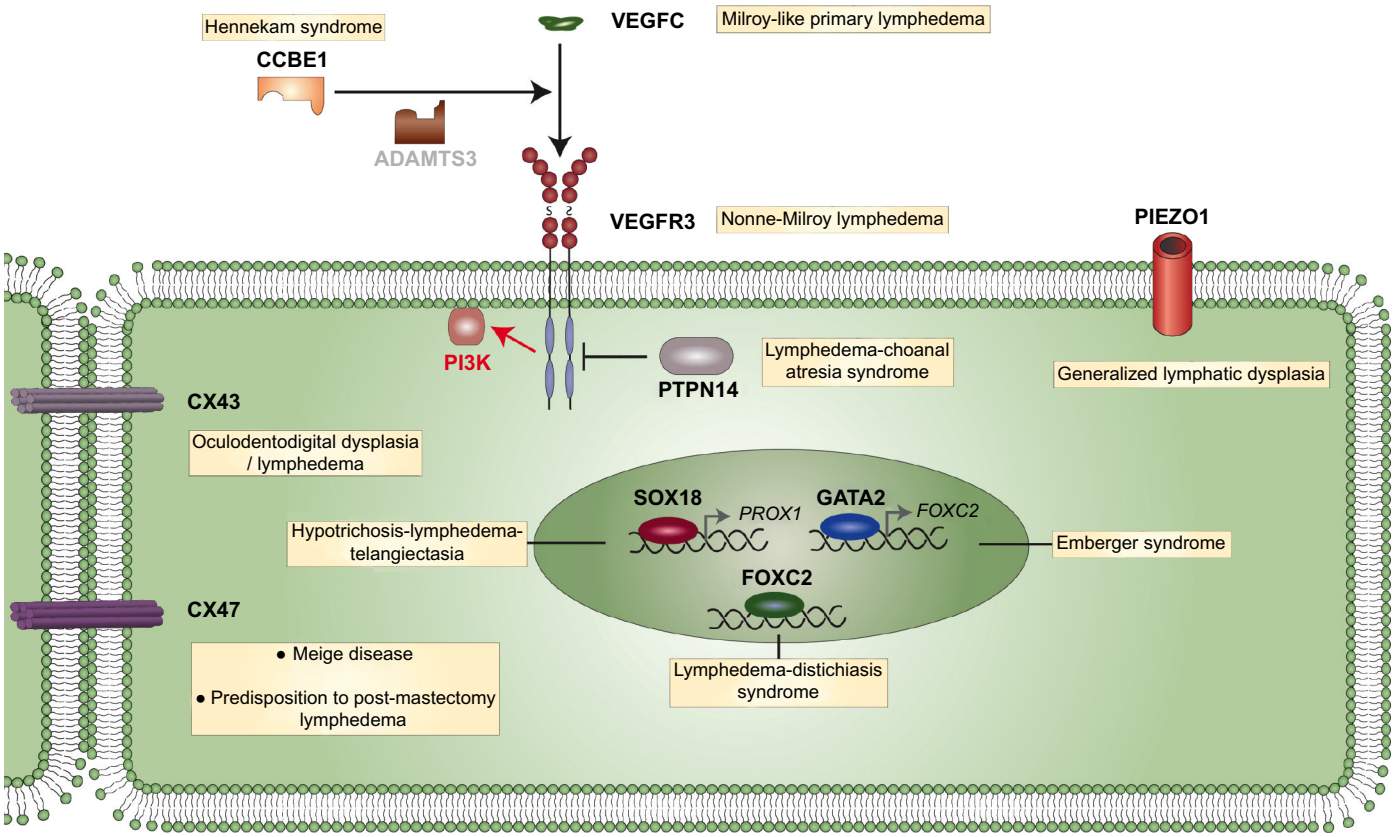

Fig. 9.3 Common genetic defects behind lymphatic malformations and rare primary lymphoedemas. Mutations in the VEGFC/VEGFR3 signalling pathway, which is required for lymphangiogenesis, account for over half of all primary lymphoedemas. Mutations in VEGFR3 and VEGFC are associated with Nonne–Milroy lymphoedema and Milroy-like primary lymphoedema, which are indistinguishable from each other. CCBE1, which enhances the cleavage of VEGFC into its fully active form by proteases such as ADAMTS3, is associated with the Hennekam lymphangiectasia–lymphoedema syndrome. Mutations in PTPN14, a protein tyrosine phosphatase that can dephosphorylate of VEGFR3, are associated with lymphoedema–choanal atresia syndrome. Activating mutations in the VEGFR3 downstream signalling protein PI3K (red) have been associated with lymphatic malformations. Mutations in SOX18, which induces the expression of *PROX1*, are associated with hypotrichosis-lymphoedema telangiectasia. Mutations in FOXC2, which is required for lymphatic valve development, are associated with lymphoedema–distichiasis syndrome. Mutations in GATA2, which is required for FOXC2 induction and lymphatic valve development, are associated with the Emberger syndrome, a primary lymphoedema with myelodysplasia. Mutations in the gap-junction proteins, CX43 and CX47, are associated with oculodentodigital dysplasia/lymphoedema and Meige disease, respectively. Mutations in CX47 are associated with a predisposition to develop post-mastectomy lymphoedema. PIEZO1 ion channel mutations are associated with generalized lymphatic dysplasia.

identified in lymphoedema. *GJC2* (*CX47*) is associated with hereditary lymphoedema II, also known as Meige disease (150, 151), and *GJA1* (*CX43*) with oculodentodigital dysplasia/lymphoedema (152). Interestingly, both CX47 and CX43 are expressed in LECs covering the upstream surface of lymphatic valve leaflets, and deficiency of *Cx37* or *Cx43* in mice results in defective valve formation, lymphoedema, and chylothorax (153). Recently, mutations in the mechanically activated ion channel PIEZO1 have been implicated in generalized lymphatic dysplasia with non-immune hydrops fetalis (154, 155).

In addition, several other genes have been associated with less well-characterized primary lymphoedemas. These include *KIF11*, and genes encoding the signal transducers *HGF*, *PTPN11*, *SOS1*, *KRAS*, *RAF1*, *RASA1*, *IKBKG*, and *HRAS* (140). To date, mutations in eight genes account for 40% of familial, 10% of sporadic, and 25% of primary lymphoedemas. It is unlikely that the remaining heritability is due to the 11 other known genes. Therefore, additional genes that are mutated in primary lymphoedema are likely to exist (140, 156).

While primary lymphoedemas are of genetic origin, secondary lymphoedemas are a consequence of damaged lymphatic vessels, most commonly due to surgery, radiotherapy, infections such as filariasis, and traumas (140). The most common cause of lymphoedema in developed countries is breast cancer surgery. Interestingly, mutations in *GJC2*, which encodes for connexin 47, predispose the mutation carriers to post-mastectomy lymphoedema (157). Globally, the most common cause of lymphoedema

is lymphatic filariasis, which is caused by mosquito-transmitted parasitic roundworms such as *Wuchereria bancrofti*, which may dwell in lymphatic vessels and lymph nodes for years, inducing inflammation and scarring (158).

Lymphoedema treatment represents a clinical challenge. Despite current treatment, the disease results in life-long debilitation. The application of lymphangiogenic growth factors constitutes a promising approach to repair the lymphatic vessel damage in secondary lymphoedema. In a model of post-mastectomy lymphoedema in mice, the transient overexpression of VEGFC via a replication-deficient adenoviral vector reconstituted the damaged vessel network. Network restoration was further enhanced by concomitant autologous LN transplantation (159). These types of approaches for lymphoedema treatment that have been validated in large animals are now being validated in clinical trials.

Concluding remarks

Lymphatic research has gained momentum during the past two decades through a more complete understanding of the role and function of the lymphatic system. Importantly, the control of lymphangiogenesis during pathological conditions is possible; and the inhibition of lymphangiogenesis in tumours or activation in inflammation may eventually lead to new therapeutic strategies. Besides their now well-established role in cancer and inflammation, lymphatic vessels may also contribute to the development of pathologies related to the central nervous system (8, 9). These new and exciting findings open up new avenues of research to underpin mechanisms of lymphatic involvement in such pathologies.

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SECTION III

Pathogenesis of atherosclerosis

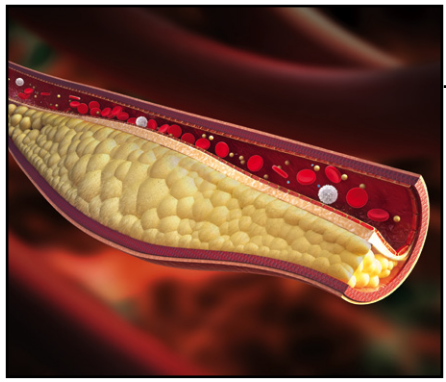
Section introduction

Imo Hoefer

Atherosclerosis is the primary underlying process of arterial narrowing and occlusion and, therefore, one of the main factors accountable for the commonest cause of death worldwide. The search for therapeutic strategies and the fight against cardiovascular disease heavily relies on a well-founded understanding of the mechanisms that lead to initiation and progression of atherosclerosis. In the past decades, our growing knowledge on its asymptomatic instigation by lipids, and sustainment and fuelling by inflammation towards symptomatic disease has enabled prevention programmes and specific treatments that have significantly reduced cardiovascular disease mortality and increased life-expectancy in Western countries.

Pathologists were the first to describe atherosclerotic lesions in the nineteenth century, but even 2,000-year-old Egyptian mummies show definite signs of atherosclerosis on computer tomography images. Based on pathological observations, two different schools of thinking initially evolved, the most popular being the lipid model, which described atherosclerosis as a disease of lipid accumulation in the vascular wall. This was modified to the response-to-injury model and later evolved into the currently valid inflammatory paradigm. Today, atherosclerosis is best summarized as a lipid-driven inflammatory disease, as outlined in Chapter 10. While the debate on the respective share of lipids and inflammation continues, there is broad consensus on the prime role of lipids in the onset of atherosclerosis. Blood transport of lipids occurs in the form of lipid-protein complexes of different composition, different characteristics, and varying functions. Chapter 11 deals with the various lipid fractions, their role as causal factors and biomarkers, and lipid metabolism aimed treatments.

Atherosclerosis is seldom confined to a single vessel or lesion. Nevertheless, it is not randomly distributed throughout the vasculature, but follows clear patterns. Some vessel segments are almost never affected, whereas other, biomechanically more exposed, parts are much more prone to atherosclerotic plaque formation. The prominent influence of biomechanical factors, such as flow and stress, in this context are detailed in Chapter 12. Biomechanical factors also form the basis for the increased inflammatory cell accumulation at these predilection sites. Macrophages were the first inflammatory cells to be observed in atherosclerotic plaques and were long considered the main cellular drivers. Their importance remains unquestioned, but there is much evidence for a complex interplay of resident vascular and circulating inflammatory cells that encompasses most blood cell fractions (e.g. monocytes/macrophages, T cells, neutrophils, etc.) on one side and vascular cells (e.g. endothelium, vascular smooth muscle cells, fibroblasts, etc.) on the other. The various cellular and molecular players in this multifaceted, multifactorial process are put in context in Chapters 13 and 14, respectively.



CHAPTER 10

Atherosclerosis—a short history

Claudia Monaco and Esther Lutgens

Content

A short history

The pathogenesis of human atherosclerotic lesions has long been debated and is still evolving nowadays. First conceptualized as a chronically evolving degenerative disease initiating in the mother's womb (1), then increasingly accepted as a dynamic process causing severe acute complications that jeopardize the blood flow to the heart (2).

Evolution of the hypothesis mirrored the progress of cellular and molecular biology, leading to progressive broadening of the understanding of cell types and molecules involved in atherogenesis. In this chapter, we first describe the current histopathological view on the pathogenesis of atherosclerosis. Subsequently, we touch on a historical perspective weaving in the fundamental discoveries that still influence our perception of this disease in humans.

Histopathology

Nowadays, atherosclerosis is considered as a lipid-driven inflammatory disease in which both lipids and immune cells play a major role (3). The disease is characterized by the presence of atherosclerotic plaques consisting of lipids, (immune) cells, and debris that form in the arterial intima. Plaques develop at predisposed regions characterized by disturbed blood flow dynamics, such as curvatures and branch points.

The first stage of atherosclerosis is characterized by activation, dysfunction, and structural alterations of endothelial cells leading to subendothelial retention of lipid components, such as low-density lipoproteins (LDL) (4). Once trapped, LDL particles are subject to modification by oxygen radicals (reactive oxygen species) and enzymes (myeloperoxidases and lipoxygenases), resulting in the generation of modified LDL and other lipid products that initiate the inflammatory process (5). As a response to injury, the endothelium becomes activated and immune cells are recruited. Once the immune cells have infiltrated into the subendothelial space, taken up lipids, and differentiated and/or polarized into activated immune cell subtypes, atherosclerotic plaques arise. Early plaques, called *fatty streaks* or *intimal xanthomas*, are typified by the presence of cholesterol-engorged macrophages and few T cells (6). These plaques are clinically silent and can either disappear or evolve into mature lesions over time.

When immune cell infiltration progresses, and lipid infiltration continues, plaques grow and progress towards *pathological intimal thickenings*, larger atherosclerotic lesions characterized by extracellular lipid pools, and some migrated smooth muscle cells and fibroblasts (6). Via intertwined immunological interactions between immune cell subsets, endothelial cells, platelets, and smooth muscle cells, but also via extracellular matrix production, remodelling, and degradation, an advanced, clinically relevant plaque stage develops: the *fibrous cap atheroma* (6). Fibrous cap atheromas are characterized by the presence of a necrotic core surrounded by a fibrous cap. Immune cell interactions in the fibrous cap atheroma typically occur at the shoulder regions, and can finally weaken the extracellular matrix, resulting in intraplaque haemorrhage. Within the fibrous cap atheroma types, *thin fibrous cap atheromas*, which have a fibrous cap thickness <65 μm, and most likely have a high chance of rupturing, as well as *fibrocalcific plaques*, plaques containing a high percentage of extracellular matrix and/or calcifications, can be discerned (6).

When atherosclerotic lesions progress, they are often characterized by thrombi. In the histopathological setting, thrombi-containing plaques are classified according to the thrombus-causing process: *rupture*, *erosion*, or, more rarely, *a calcified nodule* (6). These processes can occur in the setting of a fibrous cap atheroma or, in the case of erosion, a pathological intimal thickening. *Plaque rupture* is defined by an area of fibrous cap disruption whereby the overlying thrombus is in continuity with the necrotic core. *Plaque erosion* is identified when a thrombosed artery fails to show fibrous cap rupture. Often, the endothelium is damaged or absent at the site of thrombosis. A rare form of plaque thrombosis is the *calcified nodule*, a lesion with fibrous cap disruption and thrombi associated with eruptive, dense calcified nodules. Plaque thrombosis can lead to growth of the atherosclerotic plaque (upon healing) and/or occlusion of the artery, resulting in severe clinical complications such as myocardial infarction, stroke, peripheral arterial disease, or intestinal ischaemia (6).

Pathogenesis

The evolution of the current paradigm of atherosclerosis mirrors the progress of cellular and molecular biology, which has, over the past two centuries, led to progressive broadening of the understanding of cell types and molecules involved in atherogenesis. In the following sections, we will touch upon past and current theories that have contributed to our current view on the pathogenesis of atherosclerosis.

Evolving insights

The thrombosis model

Two centuries ago, in 1852 in Austria, von Rokitansky described the presence of mural thrombi in human lesions (7). He suggested that atherosclerotic obstructions were composed of organized thrombi (the so called '*encrustation*' hypothesis). Later, it was postulated that an encrustation of small mural thrombi existed at the sites of arterial injury. These thrombi would subsequently undergo organization by the ingrowth of SMC, and they would become incorporated into vascular lesions, causing luminal narrowing. Michael Davies substantiated this model as an important mechanism of coronary plaque progression in 1990 (8).

In 1856, Virchow, who was also based in Austria, observed lipid deposits in the atherosclerotic plaque (1). Based on these findings, Virchow proposed the '*infiltration*' theory based on the accumulation of plasma constituents in the intima of the artery. He dismissed Rokitansky's 'encrustation' hypothesis, as the localized intimal thickenings (atherosclerotic plaques) were subendothelial and 'hence could not be derived from surface deposits'.

The lipid model

Based on Virchow's observations, the Russian scientist Alexander Ignatowski induced atherosclerosis in rabbits by feeding them a diet of milk and egg yolk (9). Also in Russia, Anitschkov and Chalatov reproduced experimental atherosclerosis by adding pure cholesterol to rabbit food (10). This gave rise to the lipid theory of atherosclerosis that remained the dominant theory on the origin of atherosclerosis until the 1990s. The success of the theory was reinforced by the discovery of the LDL receptor by Goldstein and Brown. They found that the LDL receptor was able to regulate the systemic LDL blood levels via its rate of uptake through the hepatic LDL receptor pathway. This was evidenced by the massive accumulation of LDL in patients with homozygous familial hypercholesterolaemia due to the lack of functional LDL receptors (11, 12).

Animal models of hypercholesterolaemia have contributed invaluably to the understanding of the sequence of events in atherosclerotic lesions. Rabbits and nonhuman primates were first used, since they develop atherosclerosis after being fed a fat and cholesterol-containing diet. The data of Faggiotto et al. (13, 14) and Masuda and Ross (15, 16) in monkeys show that atherosclerotic plaque initiation occurs after 7 to 14 days of diet-induced hypercholesterolaemia, and consists of the attachment of large numbers of leukocytes, principally monocytes, to the surface of arterial

endothelium. The monocytes attach to the endothelial cells in clusters that appear to be randomly located throughout the arterial tree in all large- and medium-sized arteries. Within <1 month, large numbers of foam cells are found beneath the endothelial lining, leading to the formation of fatty streaks, i.e. intimal xanthomas. After approximately 5–6 months to 1 year, primates developed advanced stages of atherosclerosis, i.e. pathological intima thickening and fibrous cap atheromas. These advanced lesions initially formed at branches and bifurcations in the iliac arteries and subsequently at higher regions in the arterial tree. Changes towards fibrous cap atheroma formation occurred at the iliac bifurcation after approximately 7 months, in the abdominal aorta after 9 months, in the thoracic aorta after 11 months, and in the coronary arteries after a year.

Mice do not develop atherosclerosis spontaneously. The pioneering studies performed in the mouse were aimed at finding the most susceptible mouse strain. Paigen et al. (16) established that wild-type mice were relatively resistant to the disease and developed fatty streak lesions but not fibrous cap atheromas. The most susceptible strain was found to be the C57Bl/6 strain, which was thus used for the development of transgenic atherosclerosis strains (17). The most used atherosclerotic mouse is the ApoE knockout mouse, which is deficient in apolipoprotein E, an important ligand for lipoprotein clearance. As a consequence of this deficiency, mice develop severe hypercholesterolaemia and intimal xanthomas, pathological intimal thickenings, and fibrous cap atheromas, resembling those observed in humans (18, 19). These lesions are exacerbated when mice are fed a high-cholesterol, high-fat, Western-type diet. Another model that is frequently used is the LDL receptor deficient mouse (20), which mimics familial hypercholesterolaemia.

The response to injury model

On the basis of their studies in hypercholesterolaemic animals, Ross and Glomset (21, 22) proposed in 1976 that toxic injury to the endothelium, due to an increase of plasma lipoproteins, principally oxidized LDL, was the initiating event in atherogenesis. The resulting '*response to injury*' hypothesis of atherosclerosis proposed that endothelial denudation was the first step in atherosclerosis. Ten years later, Ross (23) updated this hypothesis because of the accumulation of information regarding the possible interaction between endothelial cells, macrophages, and SMC. This most recent version emphasized endothelial dysfunction and/or endothelial activation rather than denudation. This concept evolved again in 1992, on the basis of studies of experimental vascular injury by Fuster and colleagues. They proposed a broader theory (24, 25) that took into account the micro- and macro-thrombotic aspects, which are much more prevalent in human disease than in hypercholesterolaemic animal models. Fuster distinguished the severity of vascular injury into three types: Type I, functional alterations to the endothelial cells without substantial morphological changes; Type II, endothelial denudation and intimal damage with intact elastic lamina; and, Type III, damage extending to the media. They proposed that the extent of platelet deposition and the size of the thrombus are proportional to the severity of the vessel wall injury. When areas of the endothelium were gently denuded without deep intimal injury (Type I), they are rapidly covered by a monolayer of adhering, but not aggregating, platelets not associated with SMC migration and proliferation. SMC migration and proliferation occur only when regenerated endothelium is removed a second time and is followed by the formation of fibrin-platelet thrombus, or when the injury to the intima is so severe that the internal elastic lamina is disrupted (Type III). In both cases, the resulting mural platelet thrombus is covered rapidly with endothelial cells and invaded by proliferating SMC.

Several converging data showed that the initial response of the arterial wall is essentially similar for a wide variety of noxious stimuli (26) and can be (a) mechanical, such as balloon denudation of the endothelium, (b) chemical, such as exposure to oxidized LDL, fibrin, and homocysteine, or (c) immunological.

In all cases, the response of the local arterial wall is proliferative, characterized by (a) migration of SMC from the media into the intima, (b) rapid proliferation of SMC within the intima, (c) production of collagen and extracellular matrix, and (d) accumulation of lipid when blood cholesterol levels are elevated. Although the process begins as a physiological repair of the insult, it subsequently evolves towards a pathological alteration because of the severity of the insult and its persistence or recurrence (23).

Later, this theory was revised by Tabas et al. (27) with a new theory of lipoprotein retention that highlighted the role of matrix deposition and the binding of lipoproteins to the matrix component of the subendothelium leading to their retention. This phenomenon may be important in humans, as human lesions have precursors called intima cushions that are characterized by SMC proliferation and proteoglycan deposition.

The inflammatory model

The idea of atherosclerosis as an inflammatory disease was proposed already in the nineteenth century (1), but

firmer evidence for this view was presented through light microscopy studies in the 1960s, when lymphocytes were demonstrated in the adventitia surrounding the atheromatous arteries (28). In the 1980s, the introduction of immunohistochemical techniques to the study of the atherosclerotic plaque, provided definite evidence for the presence of macrophages (29), but also of T lymphocytes in an activated state in the atherosclerotic plaque (30) and quantitative analysis revealed that up to 20% of the cells in some regions (shoulder region) were T lymphocytes (31–33). Furthermore, even the earliest type of lesion, the fatty streak, was shown to contain T lymphocytes (34). Moreover, a wide range of pro-inflammatory cytokines, such as tumour necrosis factor (TNF) (35), and chemokines, such as monocyte chemotactic protein 1 (MCP-1) (36), were found at the plaque site. Contemporarily, clinical studies observed that elevated levels of C-reactive protein (CRP), an acute phase response protein (37), and its main inducer IL-6 (38) were associated with adverse prognosis in unstable angina patients. Elevated levels of CRP, IL-6, and TNF (39–41) were also found to be associated with adverse long-term prognosis in population studies. These findings provided the basis for an hypothesis of entirely new pathogenic mechanisms.

The current paradigm

Atherosclerosis was eventually defined by Ross in 1999 to be 'an inflammatory disease' (26): 'It is well established that lesions of atherosclerosis represent a series of highly specific cellular and molecular responses that can be best described, in aggregate, as an inflammatory disease'.

It would be a mistake to surmise that the different models on the pathogenesis of atherosclerosis that have been proposed are mutually exclusive. Modern biology, through the definition of the molecular mechanisms of disease, has reconciled the different interpretations of atherosclerosis. The molecular pathogenesis of an atherosclerotic plaque is the result of complex cell–cell interactions, intracellular signalling events, remodelling of the extracellular matrix, and the contribution of immune cells and inflammatory mediators. The most recent insights into the pathogenesis of atherosclerosis are described in the rest of Section III (this book).

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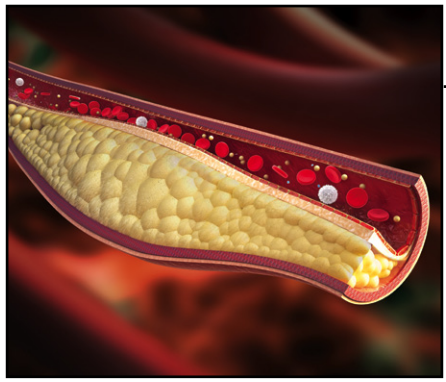

CHAPTER 11

Pathogenesis of atherosclerosis: lipid metabolism

Olov Wiklund and Jan Borén

Content

Defining plasma lipids and lipoproteins

The most abundant lipids in plasma are: triglycerides, cholesterol, cholesterol ester, and phospholipids. Since lipids are water-insoluble they have to be transported in microparticles—lipoproteins. All lipoproteins have a similar construction with a central core of lipophilic molecules (triglycerides and cholesterol esters) and a surface of partially water-soluble, amphipathic molecules (phospholipids and unesterified cholesterol). The lipoprotein particles also carry protein components with structural roles and key functions in the metabolism of lipoproteins (➔ Fig. 11.1).

Depending on function and composition, the lipoproteins are traditionally divided into classes primarily based on hydrated density (➔ Table 11.1). The largest and most buoyant lipoproteins are the triglyceride-rich chylomicrons and very low-density lipoprotein (VLDL). Denser, more cholesterol-rich particles are intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). High density lipoproteins (HDL) are richer in phospholipids and proteins. In addition to this classification system, subclasses have been introduced defining entities with different metabolic and pathogenic properties. Another classification system is based on the electrophoretic migration of the lipoproteins (➔ Table 11.1). The electrophoretic migration classification has some similarity with the classification according to density (➔ Table 11.1).

The apolipoproteins are a very diverse group of proteins and many apolipoproteins have been identified. The apolipoproteins are integrated into the structure of the lipoprotein particle and numerous of these have been shown to have essential functions for the metabolism and pathophysiology of lipoproteins in plasma. The distribution of the major apolipoproteins between different density classes are given in ➔ Table 11.1 and their major functions in ➔ Table 11.2.

Metabolism of lipoproteins

ApoB-containing lipoproteins

Triglyceride-rich lipoproteins are a mixture of lipoprotein particles synthesized either in the intestine (chylomicrons) or the liver (VLDL). Each lipoprotein

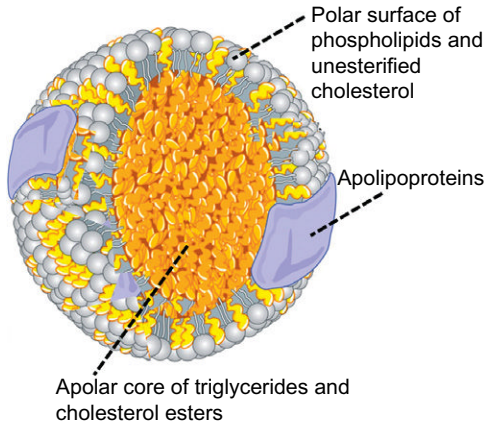

Fig. 11.1 Principal structure of plasma lipoproteins. The particle is built by a central core of hydrophobic lipids, triglycerides, and cholesterol esters, and a surface with amphipathic lipids, phospholipids, and unesterified cholesterol.

particle contains one molecule of the principal protein, apolipoprotein B (apoB). ApoB differs from other apolipoproteins in that it is nonexchangeable, i.e. it cannot transfer between different lipoproteins but remains bound to the particle on which it was secreted into plasma. The reason for this is the very strong lipid-binding characteristics of apoB (1).

ApoB is present in two different forms: apoB100 and apoB48. The shorter form, apoB48, is synthesized in the intestine and thus present on intestinal-derived chylomicrons and their remnants. The longer form, apoB100, is synthesized in the liver and is present on VLDL, IDL, and LDL. Interestingly, both apoB48 and apoB100 are coded by the same gene. ApoB48 (which corresponds to the amino-terminal 48% of apoB100, the full length form of apoB) is produced as a result of post-transcriptional cytidine-to-uridine (C-to-U) RNA editing of the apoB

Table 11.1 Classification and characteristics of plasma lipoproteins

Lipoprotein	Density	Electrophoretic mobility	Diameter (nm)	Triglycerides	Cholesterol	Phospholipids	Protein	Major apolipoproteins
Chylomicrons	>0.93	Origin	80–1200	85–95	2–5	3-8	1-2	B-48, A-I, A-II, A-V,
VLDL	0.93–1.006	Pre-β	30–80	50	22	19	8	B-100, A-I, C, E
IDL		β	23–35	20	38	23	19	B100, C, E
LDL		β	18–25	11	47	22	21	B100, C
HDL2		α	9–12	6	22	30	41	A-I, A-II, C, E
HDL3		α	5–9	6	15	23	55	A-I, A-II, C, E

Classification systems are based on density of the particles or the electrophoretic migration. The composition of the lipoproteins is characterized by the lipid content and different apolipoproteins.

Table 11.2 Major plasma apolipoproteins and their role in lipoprotein metabolism

Major Apolipoproteins	Molecular weight (Kd)	Major function in metabolism	Chromosome localization
A-I	29	Structural in HDL Reversed cholesterol transport	
A-II	9		1
A-IV	44	LCAT-activator	11
B-100	512	LDL receptor ligand	2
B-48	241	Chylomicron synthesis	2
C-I	7		19
C-II	9	Activator of lipoprotein lipase	19
C-III	9	Inactivation of lipase	11
D	19		3
E	32	Ligand for LDL receptor Activation of hepatic lipase	19
Apo(a)	280-800		6

transcript that generates a stop-codon and thus a truncated form of the full-length protein. The process is called 'RNA editing' and is mediated by the enzyme apobec-1. In humans, apobec-1 is expressed in the intestine only, but in certain animals, such as rodents and dogs, apobec-1 is expressed also in the liver (2).

ApoB48-containing lipoproteins carry dietary lipids that have been taken up in the intestine (3). Once synthesized, chylomicrons are secreted into the lymphatic vessels until they enter into the bloodstream at the left subclavian vein. Thus, they are delivered directly to peripheral tissues (heart, adipose tissue, and muscles) without first being metabolized by the liver. In the circulation, chylomicron-triglycerides are hydrolysed by lipoprotein lipase, which is present on the capillary surface, and the released free fatty acids (FFA) are absorbed by peripheral tissues and stored or used for energy production. The chylomicrons shrink in size when the triglycerides are removed forming chylomicron remnants. Importantly, even if the chylomicrons contain mainly triglycerides, they also contain some cholesterol esters. When triglycerides are removed from the lipoprotein particle, the cholesterol esters remain. This results in the formation of smaller remnants particles that are depleted of triglycerides and enriched in cholesteryl esters (➔ Fig. 11.2). The smaller chylomicron remnants are cleared from the circulation by the liver. Their clearance is mediated by two endocytic receptors: the syndecan heparan sulphate proteoglycan (HSPG) and the LDL receptor (4–7).

The liver secretes apoB100-containing triglyceride-rich VLDL particles. The liver secretes both larger $VLDL_1$ and smaller $VLDL_2$ particles. The larger $VLDL_1$ particles carry most of the triglycerides in plasma, and have been shown to be the major determinant for the variation of plasma triglycerides in both healthy subjects and individuals with type 2 diabetes (8–10). Several studies indicate that the fatty acids used for the biosynthesis of VLDL-triglycerides are derived from triglycerides stored in cytosolic lipid droplets (11–13). It is, therefore, not surprising that the secretion of large $VLDL_1$ particles is linked to the fat depots in the liver (14, 15). Indeed, subjects with non-alcoholic fatty liver disease (NAFLD) have increased $VLDL_1$ production (16).

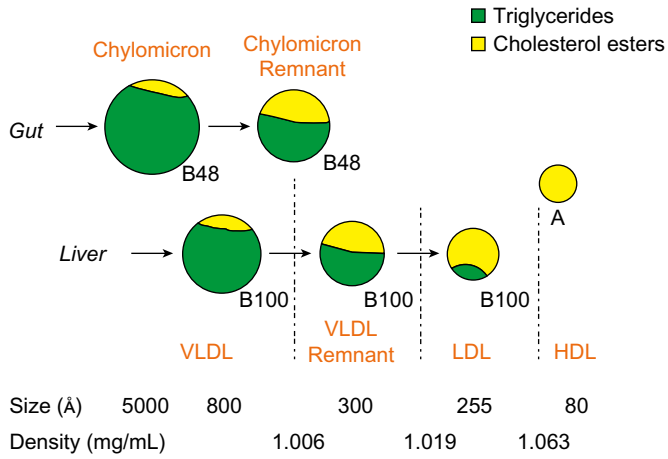

Fig. 11.2 Metabolism of apoB-containing lipoproteins.

Once in the circulation, VLDL-triglycerides are hydrolysed by lipoprotein lipase and subsequently stored in peripheral tissues or used for energy production. When VLDL-triglycerides are removed via lipoprotein lipase-mediated lipolysis, their density increases and they become IDL and subsequently LDL (➔ Fig. 11.2). The VLDL particles mainly contain triglycerides but also substantial amounts of cholesterol-esters. When triglycerides are hydrolysed by lipoprotein lipase, the cholesterol esters remain. Thus, the end-product LDL contains mainly cholesterol esters and is the major determinant of cholesterol in plasma. The LDL particle binds to the LDL-receptor and is thus cleared from the circulation by the liver. The plasma concentration of cholesterol is mainly dependent on the hepatic clearance of LDL particles. The liver produces around 70% of the total cholesterol in the body, but the amount of cholesterol synthesized is controlled by the amount of LDL-cholesterol taken up by the liver. Statins (or HMG-CoA reductase inhibitors) are a class of drugs used to lower cholesterol levels by inhibiting the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase. When the cholesterol biosynthesis is blocked, the cell responds with increased expression of LDL receptors on the liver surface to enhance the uptake of exogenous cholesterol to the liver. The protein PCSK9 also plays a major regulatory role in cholesterol homeostasis. PCSK9 binds to the epidermal growth factor-like repeat A domain of the LDL receptor, inducing LDLR degradation. Reduced LDLR levels result in decreased clearance of LDL from the circulation, which could lead to hypercholesterolaemia. Therefore, PCSK9 inhibitors have been developed as a novel strategy to enhance the expression of LDL receptors on the liver surface, and thus enhance clearance of LDL-cholesterol.

ApoB48- and apoB100-containing particles are partly cleared from the circulation by common pathways and, therefore, compete for clearance; particularly in the postprandial phase (10, 17). Increased secretion of VLDL from the liver is, therefore, an important predictor of postprandial accumulation of chylomicrons and chylomicron remnants (18). For many years accumulation of

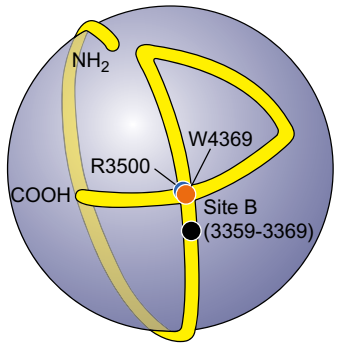

Fig. 11.3 The three-dimensional structure of LDL with its major apolipoprotein, apoB100.

chylomicron and chylomicron remnants were thought to be the major culprits in postprandial hyperlipidaemia (19, 20). However, chylomicron remnants are preferentially cleared by the liver compared to VLDL remnant particles. Therefore, the major increase in the postprandial lipoproteins after food intake occurs in the liver-derived VLDL remnant particles (21, 22).

The three-dimensional structure of apoB100 on the LDL particle is not known in detail (23) but experimental data indicate that apoB100 has an elongated structure encompassing the entire particle (➔ Fig. 11.3). The carboxyl-terminus of apoB100 folds back over the belt and crosses it around amino acid residue arginine-3500. The arginine-3500 binds to a tryptophan (residue 4396) stabilizing the carboxyl-terminus. The LDL receptor binding sequence in apoB100 (residues 3359–3369) resides in the domain, and its conformation seems to depend on this stabilization (24, 25), since a number of known mutations involving these amino acids break the arginine–tryptophan interaction and result in reduced binding of LDL to its receptor (24, 25). This autosomal dominant disorder characterized by reduced binding of apoB100 to the LDL receptor is called familial defective apoB100 (FDB). This causes impairments in LDL catabolism, resulting in increased levels of LDL-cholesterol in the blood.

Lp(a)

Lipoprotein(a) (Lp(a)) is a lipoprotein belonging to the LDL class (26). Structurally it is an LDL particle with another apolipoprotein, apo(a), attached to apoB via a disulphide bridge. Apo(a) is a protein with structural similarities to plasminogen. Apo(a) is built by cringle structures homologous to structures in plasminogen, but lacking enzymatic properties. Due to a variable number of cringle repeats, the molecular weight of apo(a) may vary within a wide range. The number of cringle IV type 2 repeats may vary between 10 and 50, and the molecular weight of apo(a) varies between 300 and 800 Kd (27). The function of Lp(a) is unknown. The concentration of Lp(a) in plasma is to a large extent determined by genetic factors. The number of cringle repeats is inversely related to plasma concentration. Furthermore, a number of SNPs regulating Lp(a) concentrations have been identified. In genetic studies, Lp(a) is found to be one of the main inherited causes of CVD.

HDL

HDLs are the most dense lipoproteins with the smallest diameter (28). HDL is often subdivided into HDL_2 and HDL_3 (➔ Table 11.1). HDL is a complex group of lipoproteins with variable composition and multiple functions, which as yet are incompletely understood. The major apolipoprotein of HDL is apoAI, which is about 70% of the protein content in HDL. The other main apolipoprotein is apoAII, which is about 15–20%. The rest is other apolipoproteins but also a number of other associated proteins, to a large extent of unknown function (29).

ApoAI is mainly synthesized in the liver, but to some extent also in the gut. The lipoprotein is secreted as apoAI in a lipid-poor, disc-shaped particle (pre-β HDL). The particle matures into HDL_3 and HDL_2. The maturation is mediated by the accumulation of cholesterol and phospholipids. This is the first step in the so-called reversed cholesterol transport. The uptake of lipids from peripheral tissue is mediated via interaction with ABC transporting proteins in macrophages and other cells (30). Unesterified cholesterol is esterified by the enzyme lecithin cholesterol acyltransferase (LCAT), and is building up the hydrophobic core of the particle. The transport of cholesterol from HDL to the liver is mediated by two pathways. The major pathway is mediated by cholesterol ester transfer protein (CETP). CETP transfers cholesterol ester between HDL and LDL in exchange for triglycerides. The cholesterol is integrated in the LDL particle and metabolized via the LDL receptor. The second pathway for 'reversed cholesterol transport' is a direct uptake of HDL via receptors (SRB1) in the hepatocytes (31).

In addition to the reversed cholesterol transport, a number of different functions have been ascribed to HDL. Suggested functions are anti-inflammatory, anti-oxidative, and improved endothelial and anti-thrombotic functions. Some of these functions may be mediated by the HDL-associated proteins, which include lipolytic enzymes, acute phase response proteins, immune response proteins, metal-binding proteins, and vitamin transport proteins. The clinical and biological relevance of these potential HDL functions are still debated (➔ Fig. 11.4) (32).

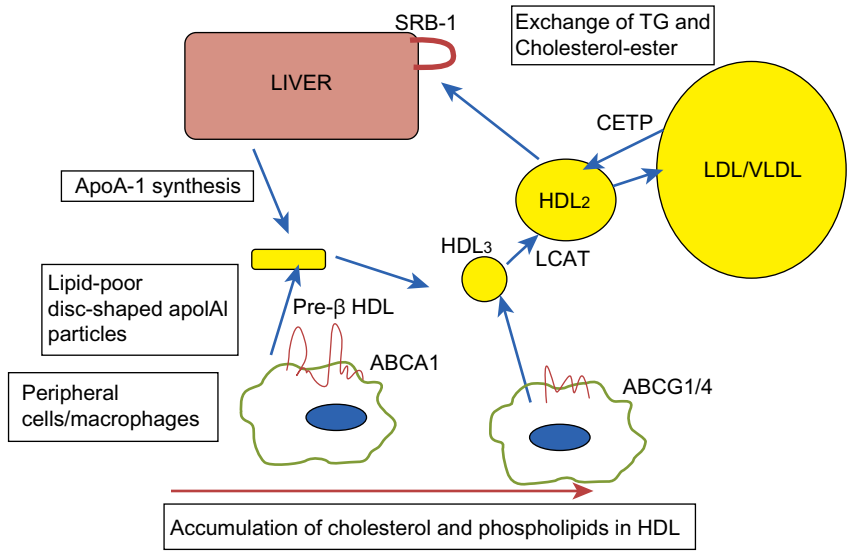

Fig. 11.4 A simplified scheme of the metabolism of HDL and the reveresed cholesterol transport. CETP: cholesterol ester transfer protein; SRB-1: scavenger receptor B1; LCAT: lecithin cholesterol acyl transferase.

Lipids and lipoproteins as risk factors for cardiovascular disease

Cholesterol and LDL

The association between cholesterol in plasma and cardiovascular risk is based on a large amount of evidence from different sources. Already in the late 1800s and early 1900s an association between cholesterol and atherosclerosis was suggested. Cholesterol crystals were shown in the atherosclerotic plaque and in the pioneering work by Alexander Anitschkoff it was shown that the cholesterol feeding of rabbits induces changes in the aortic wall similar to the atherosclerotic lesions seen in humans. Since then atherosclerosis has been induced in a large number of animal models with hypercholesterolaemia caused by diet or with genetic modification (33–37).

The epidemiological evidence on the association between plasma cholesterol, LDL-cholesterol, and cardiovascular disease is overwhelming. One of the first studies was the seven-country study, from 1955 (38) demonstrating the association between plasma cholesterol in different countries and CVD. This association has later been verified in a large number of studies and data from many studies have been summarized in a meta-analysis (39). In this analysis it has been demonstrated that plasma cholesterol is a risk-factor irrespective of age, sex, and other subgroups. The risk gradient may be less steep in the elderly than in the young. Hazard-ratio for ischaemic heart disease per 1 mmol of plasma cholesterol in the young is 0.45, while in the elderly it is 0.8. However, since the disease is much more common in the elderly, the absolute number of events explained by cholesterol is actually about ten-fold higher in the elderly (39).

Also, human genetics support the strong association and causality between plasma cholesterol (primarily LDL-cholesterol) and atherosclerosis. The most evident is familial hypercholesterolaemia (FH) (40). FH is caused by mutations in three different genes (LDL-receptor (LDLR), apoB, and the protease PCSK9). Mutations in LDLR are the most common cause of FH (95%), and over 1,000 different pathogenic mutations have been described in the LDLR. Mutations in the three different genes give essentially the same clinical picture of heterozygous FH, with elevated LDL-cholesterol (6–12 mmol/l), about a ten-fold increased risk for myocardial infarction at a young age, and cholesterol depositions in the form of tendon xanthomata. In its homozygous form the disease is very severe with very high cholesterol levels in plasma and ischaemic heart disease before the age of 20 years, often much earlier (41).

More recently, mutations associated with low LDL have shown a reduced risk for CVD (42, 43). A loss of function of the PCSK9 gene was associated with a 28% reduction in LDL and a reduction in CVD of 88% (44). Similarly, a loss of function of Niemann–Pick protein was associated with reduction in LDL and reduction in CVD (45).

Finally, an overwhelming number of controlled clinical trials with lipid-lowering drugs have shown a reduction in CVD (46–48). Most of the trials are with HMG-CoA reductase inhibitors (statins), but also other LDL-lowering drugs show reduced CVD in a dose-dependent manner (49–51).

In conclusion it can today be stated that LDL is a causative risk factor for atherosclerotic cardiovascular disease.

Small, dense LDL is a subfraction of LDL, which is accumulated in patients with high triglycerides and low HDL. The concentration of small, dense LDL has been suggested as an additional risk marker. Whether this is casual

or a marker for the general dyslipidaemia associated with metabolic syndrome or diabetes, has not been definitely clarified (52, 53).

Triglycerides

Whether or not plasma TG level is an independent risk factor for cardiovascular disease has been discussed for decades. A large number of studies show a very strong correlation between MI-risk and plasma TG in univariate analysis. However in multivariate analysis the strong correlation is often lost or attenuated (54). In more recent meta-analyses the evidence for TG as an independent risk factor has accumulated (55, 56). The reason for the loss of significance of TG in multivariate analysis is the strong correlation between TG and a number of other risk factors such as low HDL, obesity, insulin resistance, and low degree of physical activity. Thus, high TG is a major risk factor for patients with the metabolic syndrome or diabetes type 2 (57).

A causative role for TG has also been supported by genetic studies using Genome Wide Association Study (GWAS) (58) or Mendelian randomization (59–61). Related to the TG-rich lipoproteins are the remnant particles, representing partially degraded TG-rich lipoproteins (10, 18, 62). As suggested by Mendelian randomization studies, these particles may also play a key role in atherosclerosis.

Related to TG is the observation that high apoCIII is a strong risk marker. There is a strong correlation between TG and apoC-III mediated by the role of apoCIII in TG metabolism (63). In addition to the observation that apoC-III is a risk marker, recent Mendelian randomization studies suggest that apoC-III may have a causative role for the development of CVD (64, 65).

Non-HDL cholesterol

Non-HDL cholesterol can be calculated from total plasma cholesterol minus HDL. Non-HDL cholesterol has the advantage of including all atherogenic lipoproteins (VLDL, IDL, and LDL). Especially in hypertriglyceridaemia, a substantial proportion of plasma cholesterol may be carried in VLDL-IDL fractions, and thus not analysed as LDL. This is especially true in patients with high TG, since LDL will not include an important fraction of atherogenic lipoproteins. Non-HDL has been shown to be an independent risk factor (54), and to have a similar or even better predictive power to other lipoproteins (66–68).

HDL

Low plasma concentration of HDL-cholesterol has, in a large number of studies, been shown to be an independent risk factor for the development of atherosclerotic cardiovascular disease. Low HDL is often seen in conditions known to be associated with CVD, such as metabolic syndrome and diabetes (28, 69). HDL is included in most of the used risk estimation algorithms and has been shown to significantly add to the accuracy of risk estimation.

Whether low HDL is also causative to atherosclerosis has recently been questioned in a number of studies. Studies using different drugs to increase HDL have not shown any positive results. The CETP inhibitors that increase HDL-C have, in clinical trials, not resulted in reduction in CVD (70). Similarly studies using niacin have failed to reduce CVD (71).

In addition to the clinical trials with HDL-increasing drugs, Mendelian randomization studies suggest that HDL is not causative to CVD, but rather a marker for increased risk mediated by other factors, such as high TG or insulin resistance (59, 72, 73).

In recent studies, other aspects of HDL have been studied. From these studies it is suggested that reduced cholesterol efflux capacity of HDL may cause atherosclerosis (74).

Lipoprotein(a) (Lp(a))

Lp(a) is a subfraction of LDL, characterized by the additional apolipoprotein apo(a) (26). Lp(a) has been proposed as an additional risk factor for CVD since the early 1970s but has only recently been established as an important independent risk factor, most probably causal to atherosclerosis. Plasma concentration of Lp(a) in the population has a highly skewed distribution. The relation to cardiovascular risk is curvilinear, increasing more steeply after a plasma concentration above about 50 mg/100 ml. This is about the 80th percentile for Lp(a) concentration in the population, and 50 mg/100 ml is suggested as an upper limit for an acceptable Lp(a) level. In addition to the epidemiological data, recent studies utilizing Mendelian randomization have shown that Lp(a) is not only a marker for CVD risk, but is also most probably an important causal factor for CVD. The mechanism for Lp(a) in atherogenesis is not known but a number of mechanisms have been suggested (75). The homology between apo(a) and plasminogen may suggest interference with fibrinolysis. Experimental data suggest increased retention of LDL in the arterial intima or other prothrombotic mechanisms. Plasma Lp(a) level is, to a very large extent, genetically determined. The main genetic factor is the number of cringle four repeats in apo(a), but also other common gene variants have been identified that affect plasma Lp(a). High Lp(a) may be an important contributor to the presence of CVD in families, even in the absence of other risk factors (27).

Mechanisms of lipids in atherosclerosis

Lipoproteins in the circulation normally flux into and out of the arterial wall by transcytosis, a transport system in which LDL and other macromolecules are transported across the cell in specialized clathrin-coated vesicles, and ejected on the other side (76). The capacity of the transport is surprisingly high, and it has been estimated that about 2,500 clathrin-coated vesicles leave the plasma membrane of a cultured fibroblast every minute. Therefore, it is not the influx of lipoproteins into the artery wall that is rate-limiting, and thus determines the concentration of atherogenic lipoproteins in the artery wall, but rather the selective retention of lipoproteins in the artery wall.

Thus, atherogenicity of lipoproteins depends mainly on two factors: their ability to enter in to the artery wall, and their ability to become retained. The clathrin-coated transport vesicles are about 100 nm in diameter. Therefore, only lipoproteins up to approximately 70 nm in diameter can cross an intact endothelium (➔ Fig. 11.5). Thus, chylomicrons and large VLDL cannot transverse the endothelium (10, 14). This explains why patients with lipoprotein lipase deficiency do not develop atherosclerosis, despite very high plasma levels of triglyceride-rich lipoproteins. In contrast, LDL can easily flux into the artery wall. Because of their size, most remnant particles cannot cross the endothelium as efficiently as smaller LDL particles, but since each remnant particle contains approximately 40 times more cholesterol compared with LDL, elevated levels of remnants may lead to accelerated atherosclerosis and CVD (14).

The mechanism for how LDL and other atherogenic lipoproteins induce atherosclerosis has for many years been debated, and several hypotheses have been articulated. One of the earliest, the *response-to-injury hypothesis*, stated that endothelial injury leads to an inflammatory response as a part of a healing process in the arterial wall. Subsequently, the *response-to-oxidation hypothesis* proposed that lipoprotein oxidation is the important link in atherosclerosis. In 1995, it was suggested that selective retention of lipoproteins in the artery wall is the initiating step in atherogenesis (77, 78). This so-called *response-to-retention hypothesis* was based on pioneering work in the 1970s and 1980s showing that lipoproteins can interact with the arterial wall (79, 80). While these hypotheses are not mutually exclusive, and may even be considered mutually compatible with differences in emphasis, it is now generally accepted that subendothelial accumulation of atherogenic lipoproteins mediated by selective binding of the atherogenic lipoproteins to the extracellular matrix in the artery wall is critical for atherogenesis. Strong evidence indicates that proteoglycans, in particular, appear to play an important role. There are several vascular proteoglycans, including decorin, biglycan, perlecan, versican, and syndecan. They are all composed of a core protein and one or more covalently attached glycosaminoglycans (GAGs), which are linear polysaccharides consisting of repeating disaccharide units. There are four types of proteoglycan GAGs: heparin sulphate, chondroitin sulphate, dermatan sulphate, and keratan sulphate. Proteoglycans are negatively charged due to the acidic sugar groups in the GAG polysaccharide backbone and the addition of negatively charged sulphate groups. This is important since the negatively charged sugar groups bind to positively charged amino acid residues in apoB100, the principal protein of VLDL and LDL lipoproteins. Although most proteoglycans bind to lipoproteins *in vitro*, biglycan seems to be of particular importance for lipoprotein retention in human vessels, as histological studies of human arteries show strong co-localization between apoB100-containing lipoproteins and biglycan. However, the arterial composition of proteoglycans differs between animal species and, for example, perlecan has been shown to be the predominant proteoglycan in atherosclerotic lesions in mice (81). Studies with genetically modified mice expressing either proteoglycan-binding deficient LDL (i.e. LDL that fails to bind proteoglycans, since a few positively charged amino acid residues in apoB100 that are critical for proteoglycan-binding had been replaced with neutral amino acid residues) (82–84), or heparin sulphate-deficient perlecan (i.e. perlecan where the heparin sulphate chains have been removed) (81), have proven that subendothelial retention of atherogenic lipoproteins is the initiating event in atherogenesis.

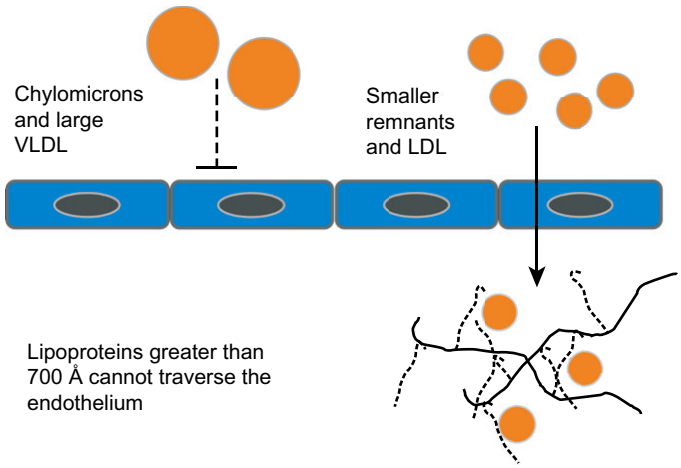

Fig. 11.5 Retention of lipoproteins in the arterial intima by binding to proteoglycans is the initial step in atherosclerosis.

As discussed, chylomicron remnants are atherogenic and can thus stick to artery wall proteoglycans. This probably explains why nonfasting (postprandial) triglyceride concentrations are a better risk predictor for cardiovascular disease than fasting triglycerides (85–87).

These particles contain apoB48 and the principal proteoglycan-binding site in apoB100 is therefore not present on chylomicron remnant (88, 89). However, remnant particles contain numerous apoE that has a proteoglycan-binding domain that is almost identical to the proteoglycan-binding sequence in apoB100. Thus, apoE-containing lipoproteins bind with high affinity to artery wall proteoglycans.

Following retention of LDL and other atherogenic lipoproteins by proteoglycans, the lipoproteins have been shown *in vitro* to undergo several modifications with important biological consequences (77, 78). For example, proteoglycan-bound LDL *in vitro* forms aggregates that resemble material seen *in vivo*, and retained lipoproteins are more susceptible to oxidative and non-oxidative modifications. The retained and modified lipoproteins are avidly taken up by macrophages leading to foam cell formation. In addition, retained and modified lipoproteins induce an inflammatory response in the artery wall that can accelerate further retention of atherogenic lipoproteins by increased expression of accessory pro-retentive molecules, notably lipoprotein lipase, sphingomyelinase, and phospholipases.

Much attention has focused on the inflammatory response and its role in atherogenesis, and many interesting mouse models have in detailed clarified important mechanisms and pathways. However, even if future therapies directed at the inflammatory, endothelial, or oxidative components of lesion progression may prove successful, no such treatment has yet proven efficacious in humans. The explanation for this is likely that inflammation is a consequence of apoB-lipoprotein retention, not a *de novo* initiating factor. For example, Cybulsky and coworkers demonstrated that while NFκB may be 'primed' in susceptible regions of the arterial tree of atherosclerosis susceptible *Ldlr*-deficient mice, NFκB *activation* occurred only in the setting of hypercholesterolaemia (90). Similar results were found in a study examining NFκB-induced endothelial inflammatory markers in normolipidaemic vs. hyperlipidaemic mice (91).

What makes the emphasis on retained lipoproteins as the key initiating step in atherogenesis so important? The answer lies in the concept that understanding the root cause of a disease provides the foundation for the most effective therapy and lowering the plasma concentration of atherogenic lipoproteins is so far the only successful pharmacological strategy for prevention of CVD.

Genetics of lipids and atherosclerosis

A number of common gene variants affecting lipoprotein metabolism and lipoprotein levels have been shown to be associated with CVD risk. In addition to the polygenic influence of common allelic variants on CVD risk, a number of monogenic disorders are causing severe forms of dyslipidaemia associated with a very high risk for CVD in young age.

Familial hypercholesterolaemia (FH)

The most common of the monogenic disorders is FH (40). The disease is caused by mutations in three different genes: LDL receptor (LDLR) gene, the apoB gene, or the PCSK9 gene. Most frequent is mutations in the LDLR. More than 1,000 different mutations in the LDLR have been identified causing FH. The mutations may cause a complete absence of functional LDL-receptors or different degrees of dysfunctional LDLR. Less common is mutations in the apoB gene causing a structural change in apoB blocking its binding to the LDLR, and thereby blocking LDL catabolism. The third mutation causing FH is a gain of function mutation in the PCSK9 gene. PCSK9 is an enzyme regulating LDLR catabolism and with a gain of function the number of LDLR is reduced.

The most severe disease is found in the absence of LDLR, while dysfunctional LDLR may cause varying severity of disease. The incidence of heterozygous FH in the population is 0.2–0.5%. The clinical phenotype is presence of hypercholesterolaemia and early cardiovascular disease in the family; in severe cases tendon xanthoma. Plasma cholesterol level usually is above 8 mmol/l and often up to 12 mmol/l. However, FH with mutation may also be present with lower plasma cholesterol, especially in the young. Diagnostic criterion in children usually is LDL above 4 mmol/l. Heterozygous FH usually responds to statin treatment with LDL reduction of 50% or more. To reach treatment targets, combination therapy is usually required, most commonly statin combined with Ezetrol (a cholesterol-absorption inhibitor).

Homozygous FH (41) is a severe disease with clinical symptoms already in childhood. Plasma cholesterol level is around 20 mmol/l. Affected children usually develop xanthoma early and develop cardiovascular disease in the teens, and in most cases die before 20 years of age. Treatment so far has been LDL apheresis, which temporarily reduces plasma LDL levels. More recently other drugs have been developed targeting apoB synthesis or the assembly of lipoproteins in the liver.

Dysbetalipoproteinaemia

Dysbetalipoproteinaemia is an uncommon disease characterized by severe combined hyperlipidaemia and early development of atherosclerosis, in the heart and in peripheral arteries, most commonly in the legs. The plasma lipid levels typically are within the range of 8–10 mmol/l, both for cholesterol and triglycerides. The hyperlipidaemia is caused by an accumulation of TG-rich remnant particles. The disease is caused by homozygosity for the apoE2 isoform of apoE. However, the genotype is not enough for the development of disease—an additional metabolic disturbance is required. Thus, dysbetalipoproteinaemia is most commonly associated with obesity, diabetes, or other components of the metabolic syndrome. Patients with dysbetalipoproteinaemia often have typical palmar xanthomas. The disease often responds well to lifestyle changes or pharmacological treatment.

Familial combined hyperlipidaemia

This is a common disorder (92) characterized by a variable dyslipidaemia, that might appear as hypertriglyceridaemia combined hyperlipidaemia or hypercholesterolaemia. The phenotype of the disease varies within one patient but also within the family. The genetic background is not clarified and the disease probably has a heterogeneous genetic background. The disease is commonly associated with the metabolic disturbances associated with the metabolic syndrome or diabetes type 2.

GWAS and Mendelian randomization

Recently a number of studies have utilized modern genetic technologies to study the genetic background to the association between lipid disorders and cardiovascular disease (93). These studies have identified common gene variants with established associations with TG and LDL-C to increased risk for CVD (58, 59, 94). The contribution of these gene variants to total risk, however, is still small in relation to established phenotypic risk factors. Genetic risk scoring therefore has so far not been used in clinical practice or in cardiovascular prevention.

Treatment of lipid disorders in atherosclerotic vascular disease

Lipid-lowering treatment is a cornerstone for the prevention and treatment of atherosclerotic cardiovascular disease. ApoB-containing lipoproteins is the target for life-style changes, as well as for pharmacological therapy.

The drug of choice in most instances is a HMG CoA reductase inhibitor (statin). These drugs act through a competitive inhibition of the key regulatory enzyme in the cholesterol synthesis. The main site of action is in the hepatocyte where the drug reduces cholesterol synthesis and increases the expression of LDL-receptors on the cellular surface, causing an increased LDL uptake and catabolism in the liver. A number of different statins have been developed with similar mechanism of action but with somewhat different pharmacodynamics and efficiency in cholesterol reduction.

Several randomized controlled trials with statins have shown a reduction in cardiovascular risk with treatment (46, 48, 95). The relative risk reduction is similar for studied subgroups, such as patients with diabetes, male or female, and subjects with FH. The absolute risk reduction is greater for patients with high risk. Thus the most absolute benefit from statin treatment is found in secondary prevention and in patients with high risk due to high risk factor burden (96). In guidelines for cardiovascular prevention the intensity of treatment is usually stratified according to risk, with the more intense treatment in patients at high risk.

Statins are generally well tolerated, with a low frequency of side-effects. A severe, but uncommon, side-effect is rhabdomyolysis, causing advanced muscular pain, myoglobinuria, and renal failure. Other side-effects are non-specific muscular pain and elevated liver transaminases (97). The transaminase elevation has not been associated with liver disease.

Ezetimibe is a cholesterol-absorption inhibitor blocking the NPC1L1 receptor in the gut, thus blocking uptake of nutritional cholesterol, as well as cholesterol in the enterohepatic circulation. Ezetimibe reduces LDL-cholesterol by 15–20% and, in combination with a statin, it potentiates a LDL reduction of about 20%. The number of studies with clinical end-points is limited but at least one trial has shown that the addition of ezetimibe to a statin can reduce the risk for CVD (51).

Bile acid sequestrants is another group of LDL-lowering drugs. The mechanism of action is that binding of bile acids in the gut blocks the enterohepatic circulation, leading to secretion of bile acids via faeces. Secondary to this, the uptake and degradation of cholesterol in the liver is increased. Bile acid sequestrants have been shown to reduce cardiovascular morbidity (98). The drugs have gastrointestinal side-effects and their primary use is as second or third drug in severe hypercholesterolaemia.

The proof for clinical benefit of targeting triglyceride-rich particles is weaker and is based on *post hoc* analyses,

subgroup analyses in studies, and meta-analyses, primarily with fibrates. Fibrates is a group of drugs with mechanisms of action mediated by a stimulation of the nuclear receptor PPAR-α in the liver. The reduction of TG is variable but often in the range of 20–40%. Available data suggest that fibrate may reduce the risk for CVD in patients with elevated TG and/or low HDL (99).

A recent development is drugs blocking the protease PCSK9. A number of monoclonal antibodies against PCSK9 are being developed and will be on the market during 2015 (43). These compounds may reduce LDL by 40–50%, even on top of a statin. At present no clinical end-point studies have been finished and the recommended use of these drugs is in severe hypercholesterolaemia or in patients who do not tolerate statins.

Conclusion

The causative role of plasma lipids in cardiovascular disease has been shown in a large number of studies: epidemiological, genetic, experimental, and through clinical intervention. The mechanisms for the development of atherosclerosis have been clarified in experimental studies, showing the importance of LDL retention—the response to retention hypothesis. Intense research is ongoing to establish the role for other lipoproteins in the development of atherosclerosis. Available therapies targeting LDL dramatically reduce the risk for cardiovascular disease. A number of new drugs targeting LDL, as well as other lipoproteins, are being developed to further reduce the morbidity and mortality of cardiovascular disease.

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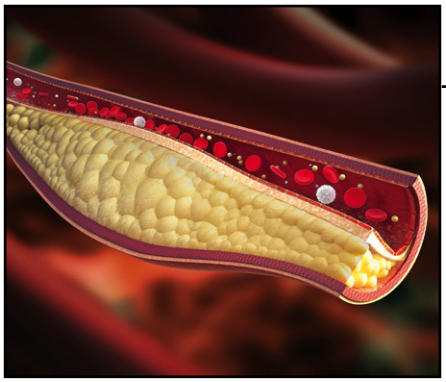

CHAPTER 12

Biomechanical theories of atherosclerosis

Jolanda J. Wentzel, Ethan M. Rowland, Peter D. Weinberg, and Robert Krams

Content

Introduction

Atherosclerosis is predominantly seen as an inflammatory disease driven by lipid accumulation within the arterial wall. The endothelium plays a key role in regulating the uptake of lipids and inflammatory cells. It is often overlooked that the disease develops at certain predilection sites and this suggests that localizing factors regulate the critical endothelial properties. These localizing factors have been studied extensively over the last hundred years; biomechanical factors have emerged as key candidates and are the subject of a number of reviews (1–4).

This chapter will critically evaluate the role of biomechanical factors in atherosclerosis, and in order to assist readers with a less pronounced background in biomechanics, we start by defining biomechanical parameters.

Biomechanical definitions

Mechanics is the study of the behaviour of materials under a mechanical load, and biomechanics is the sub-speciality of biological materials. A load is defined as the force acting on a perpendicularly oriented surface (➔ Fig. 12.1a). To normalize for dimensions, load is converted to stress, which is force divided by the area over which it acts. Material deforms on the application of stress and again a correction is made for the dimensions of the material, for example by dividing deformation by initial length—this is the strain (➔ Fig. 12.1a). The stress required to produce a certain strain is determined by tissue properties and for isotropic, non-viscous material can be summarized by tissue stiffness or Young's modulus (E; ➔ Fig. 12.1b).

Under normal physiological conditions the artery wall is constantly subject to mechanical loads. The primary externally-applied loads are caused by blood pressure and flow (➔ Fig. 12.1c). For vessels, being circular in cross section by nature, some forces balance out and blood pressure results in circumferential stress in the vessel wall, which can be calculated from Laplace's law:

$$\sigma^{\theta} = P \times r / h$$

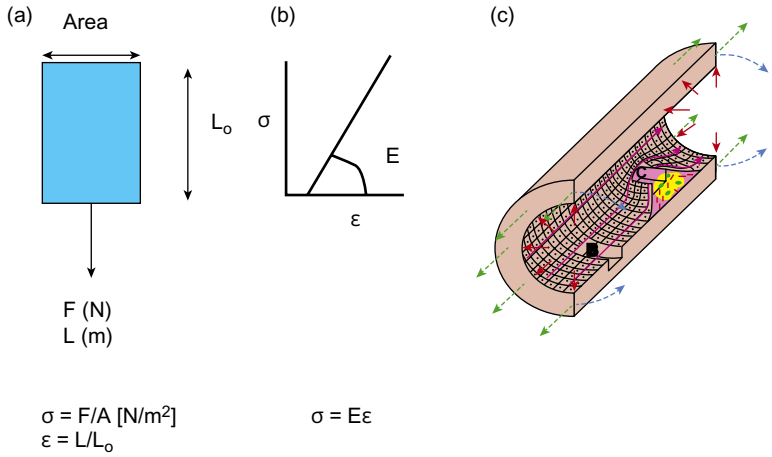

Fig. 12.1 Definitions of mechanical metrics are displayed in this figure. (a) The normalization of force by area to obtain a stress (σ N/m^2), and of deformation (L[m]) by initial length (L_o[m]) to obtain strain (ε). The slope of the stress-strain relationship is tissue stiffness (Young's modulus: b). For blood vessels curvature has to be taken into account (c). (See text for further details.)

where P is blood pressure, r is lumen radius, h is vessel wall thickness and σ^{θ} is circumferential stress. A typical value of P is 13.3 kPa and that of the resultant circumferential wall stress is 100–150 kPa. In addition to the normal stress of blood pressure, the luminal surface of the artery also experiences a tangential externally-applied stress caused by friction from the flowing blood—this is termed haemodynamic wall shear stress. This shear stress is normally denoted by tau (τ, Pa), and a typical value of shear stress is ~1 Pa, which is four orders of magnitude lower than blood pressure. Thus, while shear stress impacts on numerous aspects of vascular mechanobiology, it is usually of insufficient magnitude to alter arterial wall integrity directly. Most arteries also experience external loads due to the surrounding tissue and/or due to the motion of the tissues to which they are attached; for example, epicardial coronary arteries experience stress related to torsion of the heart during cardiac contraction, which may also influence atherosclerosis progression (5).

Finally, it is important to note that the internal stress of an artery wall is not only dependent on the externally applied loads, but also on residual stresses of its constituents. Residual stress is defined as the stress that exists within a material body in the absence of an externally applied load.

For an elastic material such as the artery wall, the presence of stress (or, more generally, a load) entails the presence of strain (or, more generally, a deformation), which is dependent on the stiffness of the material. Strain is a function of, but not equivalent to, stretch (for instance, in one-dimension, Green strain, $E = \frac{1}{2}(\lambda^2 - 1)$, where λ is stretch and stiffness is a mechanical property of a material that describes the resistance to deformation for a given load (defined as $d\sigma/dE$).

Stress and strain are used in biomechanics instead of force and deformation for the following reasons: when one compares two similar materials (same Young's modulus) but with one having twice the width of the other, then in order to deform the thicker material similarly one has to apply twice the force, while the stress in both materials is similar. When one compares two similar materials but now one is twice as long as the other, a similar force will induce twice the deformation, but strain will be similar. As a consequence, when one calculates stress over strain for an unknown material, then their ratio is the stiffness of that material. Importantly, stress, strain, and stiffness can all be defined point-wise (i.e. locally) within a material body. This latter phenomenon has led to the concept of stress or strain fields which can be estimated nowadays from finite element methods.

Several theories aim to provide an explanation for the observed predilection sites in atherosclerosis. These theories may be divided into transport theories, shear stress theories, and mechanical strain theories.

Transport theories

History of transport theories

The term atherosclerosis derives from two Greek words, ἀθήρα, meaning gruel, and σκλήρωσις, meaning hardening. Gruel refers to the lipid-rich material at the core of the lesions. As emphasized by Rindfleisch (6), histological studies show that this material lies within the arterial wall rather than on it. Where does the lipid come from? Although some lipid may be synthesized by the wall itself and a fraction of the lipid in advanced plaques may derive from the membranes of red blood cells, there is overwhelming evidence that the majority of the lipid in atheromata derives from lipoproteins, particularly low density lipoprotein (LDL), circulating in the blood.

This 'insudation theory', sometimes called the 'lipid hypothesis', was developed in its modern form by Anitschkow

(7); it was stimulated by his observation that an atherosclerosis-like disease occurred in rabbits made hyperlipidaemic by adding cholesterol to their diet. Indirect and direct support for the concept has accumulated over the years, and may be summarized as follows:

- Tracer and immunofluorescence studies show that circulating LDL does enter the arterial wall (8).
- The proportion of different fatty acids in early lesions is similar to their proportion in LDL (9).
- Patients with heritable hyperlipoproteinaemias, and hence markedly increased concentrations of lipid in their plasma, have an accelerated form of the disease (10).
- Even in the normal population, plasma concentrations of total cholesterol and of LDL are highly correlated with the risk of cardiovascular disease (11).
- Statins lower plasma cholesterol and LDL concentrations, and reduce the disease (12).
- Atherosclerosis-like diseases are induced in animals made hyperlipidaemic by feeding them cholesterol, and disease progression is stopped or reversed by removing the dietary supplements (13).
- Genetically hyperlipidaemic animals (Watanabe Heritable Hyperlipidaemic rabbits, and Apo-lipoprotein E or LDL-receptor knockout mice) develop atherosclerosis-like disease even on a normal diet (14, 15).

Perhaps the most important evidence, studied for over 100 years, is the association between anatomical variation in the arterial wall uptake of plasma macromolecules and anatomical variation in lesion prevalence. If plasma concentrations of macromolecules are uniform, this association suggests that transport properties of the wall may themselves play a key role in how quickly disease develops at a particular site. Variations in uptake around branch points are of particular interest because they have led to a debate about the transport properties that predispose to disease.

Anitschkow drew particular attention to the fact that diet-induced lesions develop in an arrowhead-shaped region surrounding the downstream half of branch ostia in the rabbit aorta. Experiments with intravital dyes such as Evans' Blue and its isomer, Trypan blue, have suggested that such regions are particularly permeable to circulating macromolecules, before the disease develops. When injected intravenously, these dyes bind to plasma proteins, some of which enter the wall and stain it blue. The intensity of staining is highest downstream of branch mouths in pigs (16), supporting the idea that high permeability to macromolecules predisposes to disease. However, Mitchell and Schwartz (176) showed that the distribution of fatty streaks in adult human aortas was different from those in rabbits: regions downstream of branches were spared the disease rather than being the most susceptible. Since the experiments with intravital dyes seemed to show that such regions were highly permeable to macromolecules, Caro et al. (17) suggested that disease in people is caused by a restricted egress across the endothelium of material made or modified within the wall, rather than an enhanced influx. Although this hypothesis could explain why human lesions seemed to occur in regions of low rather than high permeability, it is hard to reconcile with the strong relation between plasma LDL concentration and disease prevalence.

An alternative explanation for the discrepancy between rabbit and human disease is that lesion patterns change with age; according to this view, the discrepancy arises because immature rabbits have inappropriately been compared with mature people (18). Animals tend to be used when young, for reasons of cost, whilst post-mortem arteries are more often available from mature than immature people. When *mature* rabbits are fed cholesterol, the regions downstream of aortic branch points are spared disease (19); conversely, in *immature* human aortas, the downstream regions are susceptible to lipid deposition (20). Furthermore, although regions downstream of branch points have high permeability in immature aortas, that is not the case in mature vessels (21). Hence, when age is taken into account, there is a good spatial correlation between rabbit lesions, human disease, and regions of high uptake; it becomes possible to apply the insudation theory/lipid hypothesis to people.

A final point is that as well as determining where lesions develop, the level of uptake of plasma macromolecules by the wall may also determine the nature of the disease. Two types of lesion can be induced in hyperlipidaemic mice by placing a tapered cuff around their carotid arteries: lesions resembling stable human plaques develop at the narrower, downstream end of the cuff; whereas lesions resembling the unstable thin-cap fibroatheroma (TCFA) develop at the wider, upstream end (22). If the same cuff is used in normocholesterolaemic mice, to avoid complications caused by the development of disease, uptake of plasma macromolecules is elevated at both ends of the cuff, but significantly more so at the upstream end, where the TCFA develops (23). This result is consistent with a particularly high uptake of circulating lipoproteins leading to the development of the TCFA, perhaps by causing the formation of the large lipid-rich core that is characteristic of these lesions.

Mechanisms of shear-dependent transport

Given the key roles of arterial wall transport properties suggested by these studies, it is important to understand the

underlying transport processes; however, our knowledge is surprisingly incomplete in several areas. Studies in which LDL was labelled in both its protein and lipid moieties, and in which labelled, non-metabolized analogues of LDL were used, have shown that the particle enters the wall intact (8). There is a continuous transport of water and solutes from the lumen to the adventitia; this transport involves both convection and diffusion, as pressure is lower in the adventitial microcirculation and concentrations are lower in adventitial lymphatics than in the arterial lumen. The entry of LDL is dependent on pressure; there appears to be a strong influence of increased stretch of the wall (24). Interestingly, there may be a limit on the upper size of molecules that are able to penetrate the entire thickness of the wall. Labelled albumin, introduced into the lumen of perfused arteries, can later be detected on the adventitial side (25), but there has not been a similar demonstration for particles of the size of LDL.

The endothelial cell layer appears to be a major barrier to transport. Evidence for this includes increased transport in the vicinity of individual endothelial cells undergoing mitosis (26). Unfortunately, the dominant route for LDL transport across the endothelium has not been established. Solute transport occurs through the endothelial cell (transcellular) and through the space between cells (paracellular). Paracellular transport is regulated by the formation between cells of tight junctions, which consist of a series of molecules: junctional adhesion molecule (JAM 1 and 2), platelet endothelial adhesion molecule (PECAM 1), vascular endothelial (VE)-cadherin, occludin, and claudins. They are tightly controlled and their relative expression varies between the different barriers in our body (endothelial, epithelial, and blood–brain barriers). For instance, E-cadherin's are expressed in epithelial cells, while VE-cadherin is expressed predominantly in endothelial cells. Stable adherens' junctions are thought to be required for the formation of tight junction and wall shear stress plays an important role in their formation. Endothelial cells under shear stress reorient their cytoskeleton and form 'stress fibres': thick-cabled cytoskeletal fibres oriented in the direction of force. Stress fibres have been shown to be coupled to adherens' junctions and through that mechanism stabilize tight junctions (27, 28).

A more molecular explanation of how shear stress affects tight junctions has also been developed. The phosphoinositide-3 kinase (PI3-K)-protein kinase B/Akt pathway is known to be under the control of shear sensitive G-PCR and RTK-receptors in endothelial cells (29–31). After their activation, PI3Ks activate phosphatase and tensin homologue (PTEN), leading to Akt phosphorylation. In the presence of β-catenin, VE-cadherin induces forkhead box factor 1 (FoxO1), allowing it to activate expression of the junctional protein claudin-5. Interestingly, the PI3K–Akt pathway is regulated by at least two mechanosensors, each activated by different shear stress patterns. The mechanosensitive RTK receptor is predominantly activated by disturbed shear stress, while the G-PCR is activated by high shear stress (29–31). As a consequence, high shear stress leads to stabilization of tight junctions, while low and disturbed shear stress leads to apoptosis, cell turnover, and remodelling of junctions and lipid uptake.

Shear stress also activates Wnt signalling, translocation of β-catenin into the nucleus of endothelial cells, and expression of the junctional protein claudin-3 (32–34). In addition, Troy and Dr6, which are downstream targets of the Wnt signalling cascade, may be involved in regulating expression of Zonula Occludin (ZO-1) in endothelial cells. Other factors such as the alternative Frizzled-4 ligand Norrin, which is a co-activator of the canonical Wnt/β-catenin signalling, also contribute to the induction of junctional molecules such as claudin-5 (32–34)

The transport of water across endothelium is about 100-fold faster than the transport of LDL. This will lead to concentration polarization—LDL will have a higher concentration at the endothelial surface than in the blood as a whole. Convective transport of LDL towards the endothelium, and the relatively low permeability of the endothelium to LDL, lead to a build-up of the solute at the surface; this increased concentration leads to diffusion of LDL *away* from the wall, until an equilibrium is reached. The LDL concentration near the wall at this equilibrium is a matter of debate. Some simulations have suggested increases as large as 10–20%, and have also shown that the degree of elevation depends on local blood flow characteristics, perhaps accounting for local variation of LDL transport into the wall (35). However, studies that take into account the fact that water enters the wall predominantly between endothelial cells, rather than uniformly across them, have contradicted this finding when using physiologically realistic parameters (36). Furthermore, although the glycocalyx layer that coats the endothelium will restrict diffusion, leading to increased concentration polarization, it also shields the polarized layer from the flow, making it less likely that this mechanism can account for local variations in transport (36).

Clinical evidence for transport theories in atherosclerosis

Some research groups have studied LDL transport into the vessel wall as an underlying cause of atherosclerosis through computational studies. It is hard to decide on the

correct parameters in these simulations. Often a fluid-wall, single-layered model incorporating shear-dependent transport parameters like hydraulic conductivity and permeability for macromolecules is used. Further assumptions relate to the flow across the arterial wall, for which Darcy's law is employed. The mass balance of LDL can be governed by the convection-diffusion mechanism. Reaction of the LDL with particles in the vessel wall should be considered.

Simulations performed in 3D reconstructions of human coronary arteries showed co-localization between macromolecule accumulation and low shear stress regions (37, 38). Although LDL concentrations are correlated to low shear stress, regions of high luminal surface concentration do not always co-locate with the sites of lowest WSS (38). The degree of elevation of luminal surface LDL concentration is affected by water flux into the vessel wall. Pulsatility of flow leads to mixing of LDL with the blood and thus has also a great influence on the LDL transport (37).

Application of these models also explains the higher prevalence of plaque with hypertension. Hypertension is associated with higher transmural pressure, and leads to global elevation of LDL concentration in the arterial wall by facilitating the passage of LDL through wall layers (37).

Shear stress theories

History of shear stress theories

Anitschkow (7) suggested that mechanical forces might explain the development of fatty streaks downstream of aortic branch points in cholesterol-fed rabbits, but it was left to Fry (39) to propose a haemodynamic mechanism—he suggested that disease occurs in regions of high WSS. The elevated stress was thought to damage endothelial cells, accounting for the higher permeability seen in these regions. This intuitively satisfying theory was contradicted by the discovery that in the human aorta, regions downstream of branch points are spared disease. Caro et al. (17) proposed that high WSS is protective and that disease occurs instead in regions of low WSS. A subsequent study by Ku et al. (40) compared the distribution of wall thickness in the human carotid bifurcation with WSS measurements in transparent models of the bifurcation and found that the wall was thickest in areas where blood flow changed direction to the greatest extent during the cardiac cycle. A new index, the Oscillatory Shear Index (OSI) was developed to capture this pattern of stresses (40). Because high OSI is associated with low average WSS, the latter appearing in the denominator of the former, the theories of Caro et al. and of Ku et al. have become combined to some extent.

In addition to high, low, and oscillatory WSS, a large number of other WSS metrics have been proposed, including transverse WSS (41), the WSS spatial gradient (42), the WSS angle gradient (43), the WSS angle deviation (44), and the peak WSS temporal gradient. Related suggestions include the dominant harmonic (45), the relative residence time (46), and the Low Shear Index (47). Correlations exist between many of these metrics (48, 49), implying that they capture different features of essentially the same type of flow; the phrase 'disturbed flow' has been widely employed to describe it, but the term is imprecise and so cannot be related to any specific biomechanical mechanism.

Mechanisms underlying shear stress theories

The effect of shear stress on endothelial cell genotype and phenotype has been studied extensively and is the subject of a variety of reviews (2, 50–52). On the basis of a recent genome-wide study, we have identified 24 pathways, which for clarity are organized in three categories: inflammation, oxygen-radical formation, and apoptosis. (Mechanisms underlying lipid uptake and fluid flux into the vessel wall have already been discussed in transport theories.)

Shear stress and inflammation

A few pathways are specifically upregulated in low and disturbed shear stress (NFkB, Akt, and MAPK signalling pathways). Some signalling pathways that are associated with atheroprotection exert a lesser effect. The most important is the G-PCR-MAPK5 pathway, which has been shown to play a crucial role in controlling the KLF2 and KLF4 transcription factors via MEF2c. Both transcription factors play an essential role in anti-atherosclerotic gene expression, and are increased under high shear stresses (53–55).

The nuclear factor kappa B (NFkB) pathway is known to be affected by shear stress: it is upregulated under both low, and low, disturbed shear stress and down regulated at high shear stress (➔ Fig. 12.2) (56–59). The pathway is activated through the tyrosine kinase (TRK)–IP3K–Akt pathway and through PECAM1 (60–62). The IKK–NFkB pathway is known to be sensitive to spatial and temporal gradients of shear stress (56–59). While PECAM1 has been shown to be involved in the reaction to pulsatile shear stress, it is currently unknown how spatial gradients are detected. One possibility is that the RTK-receptor could form heterodimers (Tie1-Tie2) that activate NFkB under spatial shear gradients (62–64).

NFkB is under the control of IKK and amongst its target genes are IkBα and Cézanne, which inhibit the formation of NFkB and IKK, respectively (65–68). Negative-feedback

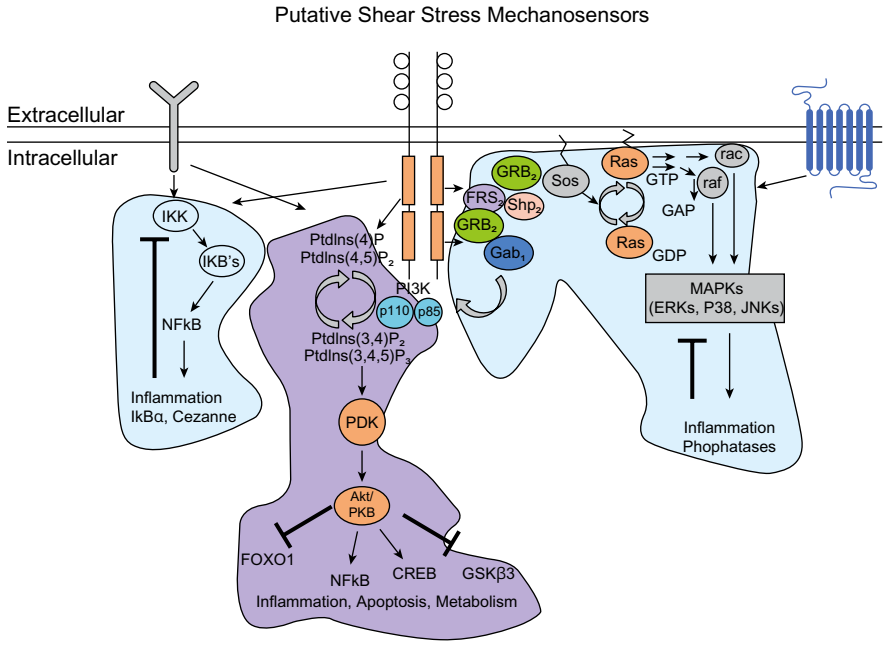

Fig. 12.2 Three major signalling pathways involved in the regulation of inflammation by shear stress. On the left side is the IKK–IkB–NFkB pathway, which is regulated by low shear stress and pulsatile shear stress. After activation by PECAM1, a complex is formed of VEGFR2 and VE-cadherin, which activates the kinase (IKK). IKK ubiquinates IkB in the NFkB–IkB complex, thereby releasing NFkB in the nucleus. NFkB encodes for ~200 inflammatory genes, including IkBα, thereby initiating a feedback loop. In the middle section is the IP3–Akt pathway. The phosphoinositide-3 kinase (PI3-K)-protein kinase B/Akt pathway is known to be under the control of shear sensitive G-PCR and RTK-receptors in endothelial cells. After their activation, PI3Ks activate PTEN, leading to Akt phosphorylation. Akt inhibits FOXO1, activates NFkB, eNOS, NRF2, and cellular metabolism. Another important, shear-controlled proinflammatory pathway is depicted on the right side: the mitogen-activated protein kinases or MAPK-pathway. The MAPK-pathway is activated by RTKs and G-PCR and after Ras-Raf activates a three-tier phosphorylation cascade, where each kinase needs to be double phosphorylated. Phosphatases dephosphorylate the cascade and, as they are produced by the transcription factors regulated by these kinases, one or more negative-feedback loops exists in these pathways

loops make the pathway less sensitive to external influences, but might lead to oscillations (65–68). Indeed, oscillations have been reported both after ligand and shear stress stimulation, and the oscillation frequency seems to be regulated by the level of shear stress. As the frequency of oscillations affects target gene expression, this pathway is a truly frequency modulated signalling pathway. The target genes of the NFkB pathway regulate cytokines/chemokines, cell adhesion factors, acute phase proteins, and cell surface receptors. Consequently, the pathway plays a central role in attracting and capturing inflammatory cells, but also in their differentiation in the subendothelial space.

Other important, shear-controlled proinflammatory pathways include the mitogen-activated protein kinases or MAPK-pathways. The MAPK-pathways consists of a three-tier phosphorylation cascade, where each kinase needs to be double phosphorylated. Phosphatases dephosphorylate the cascade and, as they are produced by the transcription factors regulated by these kinases, one or more negative-feedback loops exists in these pathways (69–72). Theoretical studies have confirmed that this cascade can react gradually, in an oscillatory mode or in a switch-like response, depending on the degree of stimulation (69–72). The complexity of the dynamics of the MAPK-pathways makes it difficult to interpret single time-point measurements of single molecules. Indeed, increased activation of members of the signalling pathway has been reported both after low and high shear stresses (29, 59, 69, 73–78). More specifically, studies have shown that ERK1/2, ERK5, Jun, p38, are upregulated in response to (high) shear stress, while Jun and p38 are known proinflammatory molecules, which become phosphorylated at higher shear stress values. Other studies have indicated that low shear stress and/or prolonged shear stress activate the MAPK pathways (75, 79, 80). These apparently contradictory findings may be reconciled by the dynamic behaviour of the MAPK pathway to shear stress. Another explanation for the discrepancy may reside in the fact that the MAPK pathways regulate proinflammatory (JNK) and anti-inflammatory (KLF2-KLF4) transcription factors. It is known that proinflammatory transcription is regulated by low shear stress, while anti-atherogenic pathways are regulated by high shear stress.

A few pathways were recently identified, after combining microarray studies from several sources, thereby increasing the power of the statistics. The NOD-like pathways were involved, regulating the inflammasome and chemokines like

CXCL1, MIP3, and CCL2 (69). Interestingly, Toll-like receptors were shown to react to shear stress and through their activation (CD14) stimulated AKT-dependent NFkB activation and production of IP-10 and IFN-α (69).

Shear stress and oxygen radicals

Endothelial cells possess an intricate mechanism to control formation of reactive oxygen species (ROS). ROS is a collective term that refers to oxygen radicals such as superoxide, O_2^-, and the hydroxyl radical, OH, and to non-radical derivatives of O_2, including hydrogen peroxide (H_2O_2) and ozone (O_3). ROS are determined by the activity of a variety of sources, including NADPH-oxidases, xanthine oxidase, mitochondria, and uncoupled eNOS (81–84), but also by inhibition of anti-oxidants such as superoxide dismutase, catalase, glutathione peroxidase, thioredoxin, peroxiredoxins, and heam oxygenase-1. Note that reactive nitrogen species (RNS), such as nitric oxide (NO), nitrogen dioxide (NO_2^-), peroxynitrite ($OONO^-$), dinitrogen trioxide (N_2O_3), nitrous acid (HNO_2), etc., also play a complex role in endothelial disorders.

Oxygen radical production in endothelial cells seem to follow a model of balance, where low, disturbed shear stress is associated with increased ROS-production and high shear stress with decreased ROS production. The precise mechanisms are depicted in Fig. 12.3.

In resting and dividing endothelial cells, ROS are mainly produced by mitochondria, but as shear stress regulates NADPH-oxidases and anti-oxidant system expression and activation, the 'redox state' is drastically changed after exposure to shear stress. During sustained high shear stress, the NADPH-oxidases, NOX-1, NOX-2, and NOX-4, are downregulated, and consequently ROS production is downregulated (81–84). In addition, after sustained high shear stress, KLF-2 and KLF-4 transcription factors are upregulated, thereby increasing eNOS expression (85–87). In parallel, the IP3K–Akt pathway is activated by shear stress, which enhances phosphorylation of eNOS. As a consequence, NO production—a ROS scavenger—is increased and peroxynitrite is formed. Peroxynitrite is thought to exert physiological functions in low concentrations and pro-atherogenic functions at high concentrations (Fig. 12.3) (87).

The transcription factor nuclear factor erythroid 2-related factor 2 (NRF-2) is the most important regulator of the expression of molecules that have anti-oxidant functions within the cell (88–90). Under resting conditions, NRF-2 is constitutively bound by the Kelch-like ECH-associated protein 1 (KEAP1)–Cullin 3 (CUL3) E3 ligase complex. NRF-2 is the sole controller of the enzymes that are responsible for producing glutathione (GSH), which is the most abundant anti-oxidant cofactor within the cell, but it also upregulates other anti-oxidant regulators, like TXN production, quinone detoxification, iron sequestration, and GSH production. Sustained high shear stress upregulates NRF-2, thereby increasing anti-oxidant genes and proteins, and thus reducing ROS. As sustained high shear stress also reduces ROS production this combined effect is a strong stimulus for controlling oxygen radical concentrations (Fig. 12.3) (88–90).

Sustained disturbed flow reduces KLF-2 and KLF-4 levels, and thereby eNOS expression and NO production. On the other hand, it increases NOX-1 and NOX-2, and enhances ROS production. As NRF-2 is reduced in regions of disturbed shear stress, its regulated genes (HO-1, thioredoxin reductase-1 (TrxR1)) are reduced, thereby reducing ROS scavenging. Sustained high ROS concentration in cells will enhance expression of adhesion molecules (VCAM-1 and ICAM-1) through activation of NFkB and the MAPK

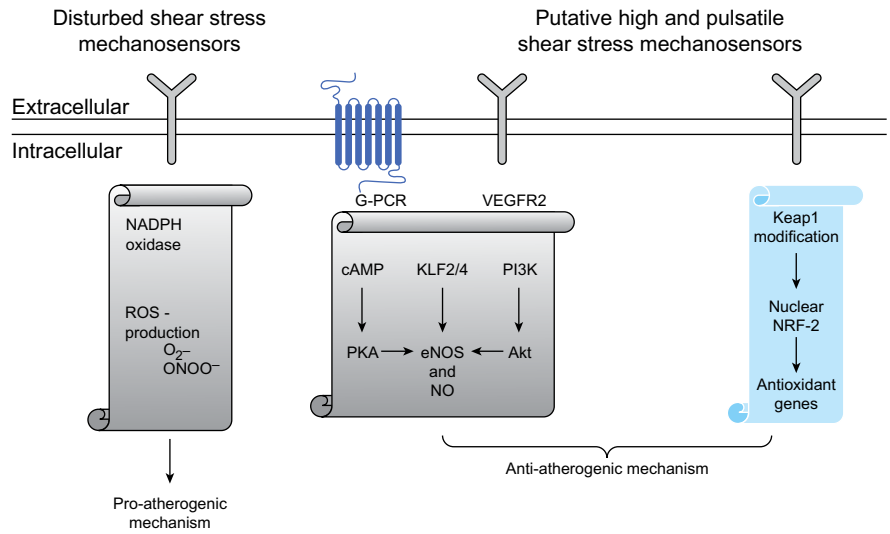

Fig. 12.3 Shear stress also regulates oxygen radical production by controlling all aspects of their production (NADPH-oxidases), binding (eNOS and NO), and regulation (NRF2).

pathways and it will enhance the oxidation of LDL in the subendothelium, thereby creating a milieu where macrophage phenotype is directed towards M1 and foam cell formation. At high concentrations, ROS will induce sustained DNA damage and endothelial cell apoptosis, and promotes leakage of lipids into the vessel wall.

Shear stress, apoptosis, and cell turnover

Several pathways determine endothelial cell turnover. The PI3K–Akt pathway plays an essential role in this process, especially when stimulated through Tie1-Tie2 (62, 91–94). Newly identified pathways are the JAK–STAT and the WNT pathways, which may play an indirect/direct role in endothelial cell turnover. High ROS production also facilitates endothelial cell apoptosis. It has been suggested that these pathways regulate death-associated protein (DAPK) as a final protein (95, 96). We found evidence for shear stress regulation of the TGF-β signalling, which also plays a role in apoptosis (69). Another interesting finding was the activation of the JAK–STAT pathway by shear stress (69). As these conditions occur at sites of flow disturbance induced at or near side branches, it has been postulated that local endothelial cell apoptosis may enhance LDL uptake and thereby initiate and sustain atherosclerosis at these sites (95–97).

Clinical evidence of shear stress theories

De novo early plaque and plaque progression

Early observations on the relationship between shear stress and atherosclerotic plaque localization were based on autopsy material (17, 98–100), and consequently no information on the influence of shear stress on plaque progression over time was available. Application of computational fluid dynamics in 3D reconstructions of coronary arteries of humans and laboratory animals have tended to confirm the earlier observed co-localization between low shear stress regions and *de novo* atherosclerotic plaque (101–103), although a recent review has challenged this concensus (104). Plaque progression was also shown to be related to low or oscillatory shear stress and this relationship was modulated by the level of hypocholesteraemia (100, 105, 106). Interestingly, a combination of shear stress, plaque phenotype, and plaque burden was shown to have a better predictive value for plaque progression than each of these parameters alone (107, 108). In a very large clinical trial (500+) of the Japanese population, lumen narrowing distal to the stenosis and plaque burden, but not plaque growth, were related to low shear stress (<1Pa). The combination of plaque burden (>58%) and low shear stress increased the positive predictive value from 25% to 41% (109, 110).

Plaque composition

Shear stress is not only involved in plaque initiation and progression, but also in modulating the composition of the atherosclerotic plaques. Early studies using histopathology for plaque characterization showed that regions presumed to experience low shear stress were associated with advanced plaques features like inflammation, a large lipid-rich necrotic core, a thin fibrous cap, and positive outward remodelling (111, 112), while presumed high shear stress regions were associated with a more stable plaque phenotype. Later studies confirmed this observation by VH-IVUS (113) or OCT (114) in combination with biplane angiography. Also, an increase in plaque vulnerability was observed at the low shear stress regions, reflected by an increased size of the necrotic core.

The causative role of low shear stress in vulnerable plaque formation was elegantly shown in several animal studies of plaque development in pre-defined low shear stress regions (115, 116). For that reason a tapered cuff was developed that creates a well-defined haemodynamic environment: low shear stress upstream from the cast and low oscillating shear stress downstream of the cast. Plaques with characteristics of plaque vulnerability were observed particularly in the low shear stress region (115–117).

Vascular remodelling

Vascular remodelling occurs during development of arteries and organs, and is beneficial in maintaining sufficient blood flow to the organs during growth. Early studies showed a convincing relationship between shear stress and vessel growth. More recent studies have revealed the role of shear stress during plaque remodelling (118). During plaque development arteries can undergo compensatory expansive remodelling, presumably to maintain the local shear stress at a constant level (119–121). In general, outward remodelling will lead to a persistence of shear stress in straight vessel segments, and to low shear stress in the inner curvature of curved vessel segments. The persistence of a low shear stress region may be associated with a continuous uptake of lipids and inflammatory cells, which may contribute to further development of vulnerable plaques. These postulates predict that plaques with a large necrotic core are found at low shear stress locations, which was recently confirmed (111, 113, 122–124). Plaques may also overcompensate for plaque growth, the so called 'excessive outward remodelling' (111), leading to a lumen radius increase. This type of remodelling is observed in a small percentage of arteries (125) and might lead to a persistence of low shear stress or may even lead to a viscous circle in which low shear stress leads to even lower shear stress (126). The opposite reaction of the vessel wall has also

been observed during atherosclerotic plaque formation. The precise mechanisms of vascular shrinkage are unknown, but scar formation has been proposed. Unfortunately, information regarding the relationship between vascular shrinkage and shear stress is currently lacking.

In the later phases of the disease, positive remodelling seems not to be sufficiently effective to compensate for plaque growth, resulting in narrowing of the vessel lumen. In general, lumen narrowing initiates when plaque burden exceeds 40% (120). Although the precise mechanism limiting outward remodelling is unknown, intraplaque bleeding (127, 128), multiple plaque ruptures (129), and a circumferential extension of endothelial dysfunction at the side of the plaque have been put forward as possible explanations (120, 130). Once atherosclerotic plaques encroach into the lumen, ECs experience a change in local shear stress, i.e. high shear stress at the upstream part and low/oscillatory shear stress at the downstream side of the plaque, where initially low shear stress was present (110). It is, however, unclear whether ECs covering the advanced atherosclerotic lesion remain responsive to changes in local shear stress, as the shear stress-dependent transcription factor KLF2 seems downregulated, cross-talk between ECs via connexins is diminished, and eNOS expression is decreased at plaques (132, 133). In contrast, ECs overlapping stented arteries retain their ability to respond to flow (134, 135).

In more advanced stages of the disease the necrotic core can be found at high shear stress locations (124). The fact that plaque ruptures/ulcerations are often observed at the upstream side of advanced plaques has strengthened the idea that high shear stress is involved in upstream plaque destabilization (112, 136–138). Plaque composition at the upstream side of the plaque is markedly different from the downstream side. The region upstream of the plaques is often associated with more macrophage accumulation and apoptosis, lipid accumulation, intraplaque haemorrhage, and thinner fibrous caps (69, 112, 137). Interestingly, upstream plaque regions exposed to high shear stress show an increased strain—a local measure for plaque weakness—implying that those regions are more vulnerable to rupture (139). A recent study in human coronary arteries confirmed an increased vulnerability of the plaque regions after exposure to high, but also to low, shear stress (113). However, more detailed analysis on the association between shear stress and plaque rupture indicated a linear relationship between shear stress and plaque rupture (140). As studies have reported low and high shear stress as predictors for plaque vulnerability, further studies are needed to investigate the potential causative role of shear stress in plaque destabilization.

Strain theories

History of strain theories

Strain is induced when a stress is applied to a mechanical body (see section "Biomechanical definitions" for details). In a simplified geometry, such as a cylindrical artery, circumferential strain is in equilibrium with blood pressure. However, in realistic geometries, strain would also vary as a result of a non-uniform curvature (bends, side branches, bifurcations, and taper), changes in wall composition (such as the switch from a more elastic to a more muscular architecture on proceeding peripherally), and through the influence of supporting structures—the passage of vertebral arteries through the foramen transversarium of cervical vertebrae is an extreme example of the latter but, more commonly, actions of muscles and effects of tethering are also likely to be important.

Strain is much less discussed than WSS in relation to arterial disease. Drawing on classical pressure vessel stress analysis, Thubrikar has proposed several examples of sites where atherosclerotic lesions could be associated with locally elevated stress and strains (141, 142). Although pressure vessel theory is strictly only applicable to stiff, isotropic and linearly elastic materials, it is possible to gain qualitative insight into regional strains; arteries display nonlinear stress-strain responses, but within the physiological pressure range a linear approximation of the curve is reasonable (143).

The vertebral arteries exhibit a periodic distribution of atherosclerosis: lesions occur in the segments that are free to expand, but are absent where the artery passes through the bone canal (144, 145). Similarly, the internal carotid artery is less prone to atherosclerosis where it passes through the carotid canal at the base of the skull (146). At myocardial bridges, where an epicardial coronary artery passes under a band of heart muscle and then re-emerges, the tunnelled segment is generally free of atherosclerosis. And lesions abruptly cease where the coronaries enter the myocardium, even when the proximal segments are severely affected (147).

Experimental studies have provided additional evidence for the importance of intramural stress and strain. Thubrikar et al. (148) showed that lesion formation can be prevented in cholesterol-fed rabbits by restricting vessel expansion. Under anaesthetically induced hypotension, a liquid dental acrylic was poured around the junction between the left renal artery and aorta, and, upon setting, created a rigid perivascular cast; mean arterial pressure recovered post-surgery (148). The branch was spared of fatty streaks, whereas control ostia became diseased. Lesions did develop when casting was performed at normal blood pressure. Tropea et al. (149) induced

hypertension and hypercholesterolaemia in rabbits by aortic banding and a cholesterol-enhanced diet, respectively. Wall motion and intimal plaques were reduced proximal to the banding in regions of the aorta that had been loosely or firmly encircled with an external wrapping.

A problem with both the observational and interventional studies is that reducing wall motion will affect WSS, despite claims to the contrary. If cyclic strain is restricted, then luminal diameter averaged over the cardiac cycle will be reduced, so long as wall thickness is not drastically decreased. Even if wall thickness diminished sufficiently to maintain the normal average WSS, *variation* in WSS during the cardiac cycle would increase in the constant-diameter segment. For a given volumetric flow rate, WSS depends on diameter raised to the third power, so even small differences could have significant influences on shear stress. Similar issues may apply to data derived from patients with chronic aortic coarctation—blood pressure and lesion prevalence are increased proximal to the occlusion and decreased distal to it (150)—and to results from the numerous experimental studies that have confirmed this finding (151): the same volumetric flow rate must occur in both segments but pulse pressure differs and wall remodelling is likely to occur, so WSS may not be the same upstream and downstream of the restriction. The same criticism can also be applied to mechanistic studies in which cyclic strain is applied to endothelial cells by culturing them on an elastic membrane that is periodically stretched. The bulk of the overlying medium will remain stationary when the cells move, so they also experience fluid dynamic shear stresses. It would be necessary to control for these fluid effects before attributing changes in cell behaviour solely to mechanically induced strain. To date, effects of mechanical strain and fluid shear stresses may not have been separated sufficiently well for lesion development and pro-atherogenic mechanisms to be unambiguously attributed to the former.

Mechanism of strain theories

Far less is known about the effect of strain/stretch on signalling pathways than about the effect of haemodynamic wall shear stress. It is known that some of the mechanosensors—G-PCR, stretch-activated calcium channels, integrins, and PECAM-1—are sensitive to stretch (152–155). And it is known which signalling pathways are regulated by these sensors: MAPK, NFkB, the cytoskeleton, and cAMP, PI3K–Akt pathways. Indeed, several studies confirmed that members of these pathways react to stretch (156–158). This has led to the finding that endothelial cells after stretch increase COX-2 levels, activate eNOS, and secrete ET-1, IL-6, and MMP (156–158). Thus endothelial cells are 'primed' under physiological strain, as they are under shear stress.

Pathological strain, defined as either too high (>15%) or too low (< = 5%), is associated with an increase in NFkB signalling, reduced NO production, but increased ROS production and increased MAPK activity (➔ Fig. 12.4). These findings suggest that pathological strain could aggravate the atherogenic state induced by low shear stress (156–158). However, regions of pathological strain do not always coincide with regions of low shear stress and it is necessary to discriminate between side branches and curved segments.

Near bifurcations and side branches, there are regions where the near-wall blood velocity is low, and strongly changing in direction and strain is pathologically increased (159–161). Strain is also increased above a large necrotic core, but here the shear stress is high. At or near side branches, the PI3–Akt pathway is activated through mechanosensitive RTK

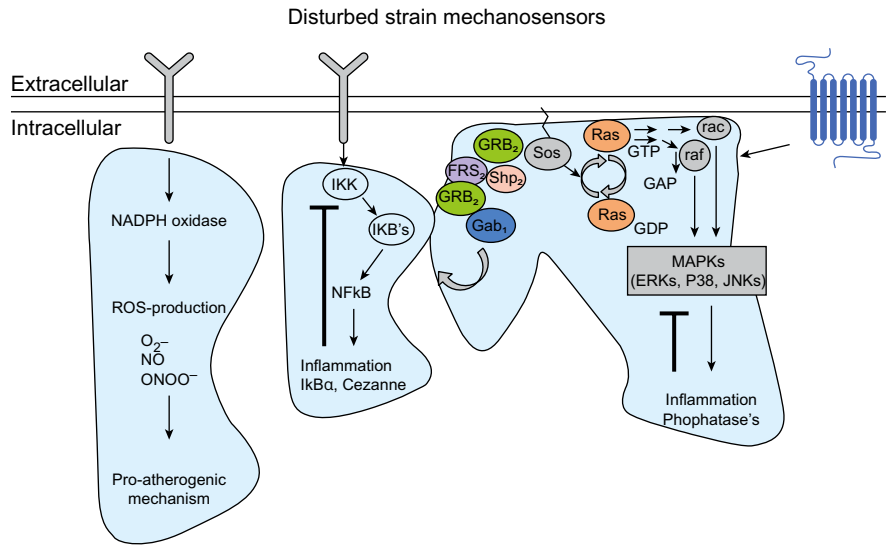

Fig. 12.4 Endothelial strain (for explanation see text), activates three important pathways: ROS, NFkB, and the MAPK. And while far less is known than for shear stress regulation, both high strain and low strain have been shown to regulate pro-atherogeneic signalling pathways. As high strain is present at predilection sites, emphasis is on the regulation of high strain signalling pathways. The three signalling pathways depicted here are similar to those identified for low shear stress and it seems if high strain regions coincide with low shear stress regions, the pro-atherogenic effects could be reinforced.

receptors (Tie1 and Tie2), and the resulting increased Akt kinase activity leads to formation of NFkB and, in combination with increased ER-stress, to NRF-2 activation, and HO-1 production, and ROS inhibition, which forms a negative-feedback loop to control ER-stress. NFkB is further activated by spatial and temporal shear stress gradients often occurring at or near bifurcations, which have a large angle with their mother vessel (T junctions). Here inhibition of Foxo1 is low (162, 163), and endothelial cells are driven to increased turnover, cobble stone formation, reduced tight and adherens' junctions, and increased permeability and lipid uptake into the vessel wall. During those conditions, Akt is also activated through VEGFR2, leading to a low-level chronic inflammation. This further favours lipid uptake and due to the persistence of vortices, the expression of adhesion factors, it also enhances inflammatory cell uptake into the vessel wall.

In the inner curvature of moderately curved vessel segments like the coronary arteries, sustained low, helical velocity patterns, and low shear stress is present; the shear stress vector varies minimally in direction, while strain is increased pathologically (➲ Fig. 12.5) (164). During these conditions, the dominant effects are those of a reduced anti-atherogenic to atherogenic balance. This imbalance is characterized by reduced KLF-2 and KLF-4 expression, leading to a reduced anti-atherogenic gene repertoire (1, 50), and a reduced NRF-2 expression and increased NOX expression, resulting in sustained ROS production. Furthermore, the reduced transit times and reduced stability of tight junctions leads to an increased lipid uptake, which will oxidize due to the increased ROS production. The increased ROS production in curved segments also activates NFkB, in contrast to the temporal and spatial gradients near side branches, leading to an increased adhesion factor expression (ICAM-1 and VCAM-1) and uptake of inflammatory cells. The more central role of ROS in curved vascular segments over side branches has recently been confirmed in genome-wide profiling studies (165).

Finally, we note that more direct molecular effects may be involved. An interesting concept championed by Stephens is that repeated cyclic strain vibration of the vessel wall leads to fatigue failure of fibrous proteins and hence to lesions (166).

Clinical evidence of strain theories

There has been less clinical interest in the evaluation of mechanical theories than in haemodynamic theories of atherosclerotic plaque formation. However, the recent developments in fluid-structure modelling have revitalized this interest and new clinical studies, including strain, have now been conducted. The majority of these studies have focused on studying the role of wall stress in plaque rupture.

Plaques ruptures if the local wall stress (i.e. stress within an atherosclerotic lesion) exceeds the strength of the fibrous cap (167). Stresses in the arterial wall are influenced

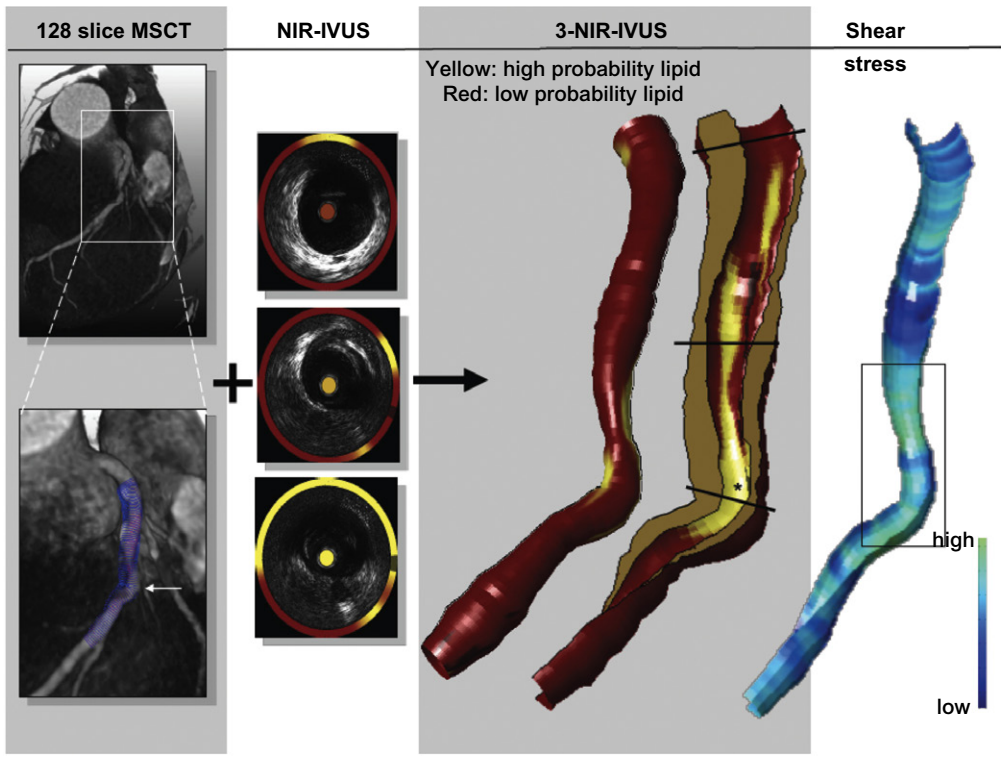

Fig. 12.5 Imaging of a coronary artery of a patient by a combination of non-invasive Multislice CT (MSCT) and NIR-IVUS. The NIR-IVUS provides the vascular composition while the lumen reconstruction based on X-rays permits computational fluid dynamics simulations and the prediction of wall shear stress. This novel approach allows decipherment of the role of haemodynamics in lipid accumulation.
(Reproduced from Wentzel, J., Van der Giessen, A., Garg, S, et al; In Vivo 3D distribution of lipid-core plaque in human coronary artery as assessed by fusion near infrared spectroscopy-intravascular ultrasound and multislice computed tomography scan, Circulation: Cardiovascular Imaging; Vol.3, No.6, (2010) with permission from Wolters Kluwer.)

by a variety of factors, including the blood pressure, local geometry, and the plaque composition (168). The wall stress is 10,000 times higher than the blood flow induced shear stress at the endothelium and, thus, it is hypothesized that shear stress modulates the plaque composition and that wall stress is responsible for the final plaque rupture (134). Interestingly, peak cap stresses in symptomatic patients are higher than those in asymptomatic patients, suggesting that plaques with higher stresses may be more prone to rupture, thus leading to cardiovascular events. Accordingly, if plaques are sorted according to plaque phenotype, plaques classified as thin cap fibroatheroma showed higher peak cap stress than plaques with pathological intimal thickening (169, 170).

Biomechanical stress metrics could, therefore, potentially be used to assess the risk of plaque rupture. However, the threshold value of wall stress that needs to be applied for risk prediction is currently under debate (171), since the cap strength might vary, depending on its collagen content. The highest wall stress is typically found (a) at the thinnest parts of the fibrous cap (172, 173), (b) in regions with increased macrophage density, (c) in regions with intraplaque haemorrhage (174), and (d) in the presence of local microcalcifications (175).

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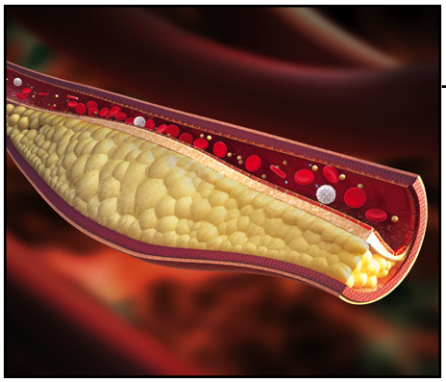

CHAPTER 13

Atherosclerosis: cellular mechanisms

Esther Lutgens, Marie-Luce Bochaton-Piallat, and Christian Weber

Content

Atherosclerosis

The first stage of atherosclerosis is characterized by activation, dysfunction, and structural alterations of endothelial cells leading to subendothelial retention of lipid components, such as low-density lipoproteins (LDL). Once trapped, LDL particles are subject to modification by oxygen radicals (reactive oxygen species) and enzymes (myeloperoxidases and lipoxygenases), resulting in the generation of modified LDL and other lipid products that initiate the inflammatory process. As a response to injury, the endothelium becomes activated and immune cells are recruited. Once the immune cells have infiltrated into the subendothelial space, taken up lipids, and differentiated and/or polarized into activated immune cell subtypes, atherosclerotic plaques arise. When immune cell infiltration progresses, and lipid infiltration continues, plaques grow and progress towards advanced, clinically relevant plaque stages via intertwined immunological interactions between immune cell subsets, endothelial cells, platelets, and smooth muscle cells, but also via extracellular matrix production, remodelling and degradation (1–3).

Cell types in atherosclerosis

Endothelial cells

The arterial endothelium is subjected to injurious stimuli such as shear stress, turbulent flow, and oxidative stress. Upon injury, an inflammatory response is initiated, and the endothelium starts expressing adhesion molecules like P- and E-selectin, ICAM-1, and VCAM-1, and secretes chemokines such as CCL2 to attract immune cells. The endothelial cell itself also becomes activated and expresses chemokines and proteases, thereby perpetuating the inflammatory response (4) (➔ Fig. 13.1).

Monocytes

One of the first immune cells that are attracted to the endothelium is the monocyte. These precursors of macrophages are short-lived mononuclear phagocytes. In humans, three major monocyte subsets exist. The 'classical' $CD14^{++}$ $CD16^{-}$ subset

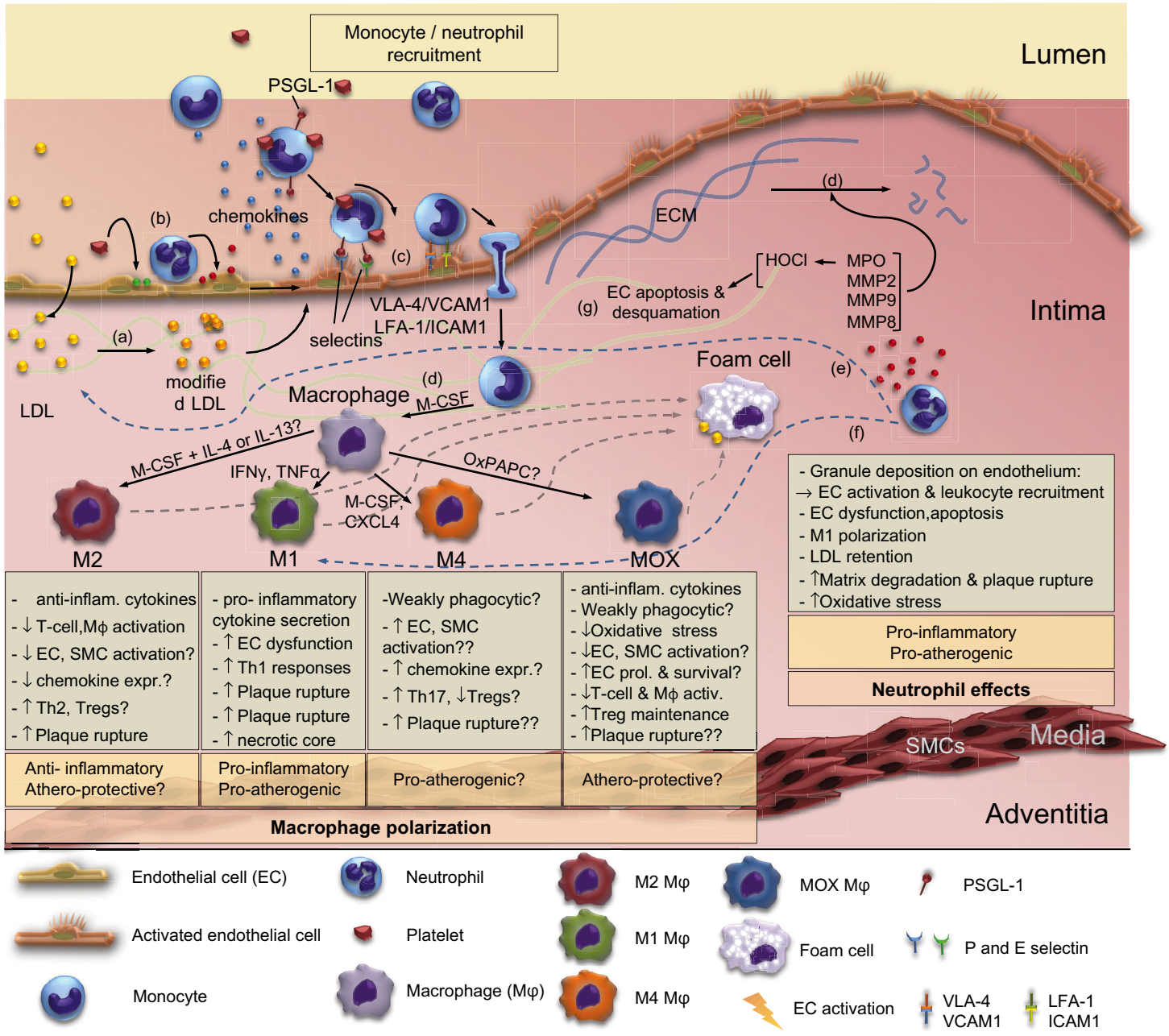

Fig. 13.1 The innate immune system in atherosclerosis. (a) Lipoproteins enter the intima, bind to proteoglycans, accumulate, become modified and activate the endothelium. (b) Platelets deposit C-C motif chemokine ligand 5 (CCL5) on the endothelium, promoting neutrophil recruitment to the vessel wall. Activated neutrophils secrete granule proteins, such as myeloperoxidase, azurocidin, and proteinase-3, that will enhance endothelial activation and dysfunction by inducing adhesion molecule expression, permeability changes, and limiting the bioavailability of nitric oxide. Moreover, granule proteins secreted or deposited on the endothelium induce adhesion and recruitment of inflammatory monocytes, but can also modify chemokines, enhancing their ability to attract monocytes. (c) Activated endothelial cells release chemokines, such as MCP-1, that attract circulating monocytes. Monocytes bind to P- and E-selectin on endothelial cells, roll, and finally come to arrest by adherence of their adhesion molecules (VLA-4, LFA-1) to VCAM-1 and ICAM1 on the endothelium. Platelets promote monocyte–endothelial interactions by expression of P-selectin, but can also form monocyte–platelet aggregates that further promote recruitment. Eventually, monocytes enter the intima through transendothelial diapedesis. (d) Infiltrated monocytes differentiate to macrophages, involving M-CSF, after which they polarize into various macrophage subsets (M1, M2, M4, or MOX) that exert numerous effects and can become foams cells (e). Plaque neutrophils trap LDL in the vessel wall by secretion of α-defensin that binds LDL. (f) Neutrophils promote M1 polarization of macrophages. (g) Neutrophil-derived MMPs and MPO-dependent oxidative stress induces apoptosis of endothelial cells and degradation of basement membrane, leading to endothelial desquamation. (h) Neutrophil MMPs can also degrade ECM components affecting plaque stability. ECM, extracellular matrix; MMP, matrix metalloproteinase; MPO, myeloperoxidase; LDL, low-density lipoprotein; M-CSF, macrophage colony stimulating factor; IFN, interferon; TNF, tumour necrosis factor; OxPAPC, oxidation products of 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphatidylcholine; EC, endothelial cell; HOCl, hypochlorous acid; PSGL-1, P-selectin glycoprotein ligand-1; VLA-4, very late antigen-4; VCAM-1, vascular cell adhesion molecule-1; LFA-1, leukocyte function-associated molecule 1 ;ICAM-1, intercellular adhesion molecule; SMC, smooth muscle cell. (Reproduced from Legein B, Temmerman L, Biessen EAL, Lutgens E. Inflammation and immune system interactions in atherosclerosis. Cell Mol Life Sci. 2013 Oct;70(20):3847–69 with permission from Springer.)

is an inflammatory subset and also highly expresses CCR2, whereas 'non-classical' patrolling $CD14^{+}$ $CD16^{++}$ monocytes express high levels of CX3CR1 and CCR5 but low levels of CCR2. Additionally, an 'intermediate' $CD14^{++}$ $CD16^{+}$ $CCR2^{+}$ subset can be distinguished. In mice, two monocyte subsets have been identified: the inflammatory monocyte, which is preferentially recruited to inflamed tissues and has a $Ly6C^{high}CX3CR1^{low}CCR2^{+}$ profile; and the resident or patrolling monocyte, that is characterized by CX3CR1-dependent homing to non-inflamed tissues and has a $Ly6C^{low}CX3CR1^{high}CCR2^{-}$ profile (5).

Hypercholesterolaemia induces monocytosis and especially increases the amount of the classical, inflammatory monocytes, which are more prone to enter the atherosclerotic plaque (6, 7). In mice, the increase in monocytes is caused by an increased proliferation and mobilization of haematopoietic stem and progenitor cells (HSPCs) in the bone marrow, which are also outsourced to the spleen and exert extra-medullary haematopoiesis, thereby generating a splenic reservoir of monocytes that are also able to 'feed' the atherosclerotic plaque (8, 9). In hyperlipidaemic humans, (18)F-FLT positron emission tomography computed tomography revealed an increase in haematopoietic activity compared to normolipidaemic individuals (10).

Besides a rise in monocyte numbers, atherosclerosis is also characterized by an increase in chemokine-dependent monocyte recruitment. Following chemotaxis, monocytes adhere to, and roll on, endothelial cells through interaction with selectins (such as E- and P-selectin). During rolling, monocytes upregulate integrins, like $\alpha_4\beta_1$, leading to firm adhesion, arrest, and subsequent diapedesis. Within the intima, monocytes secrete lipoprotein-binding proteoglycans resulting in increased accumulation of modified LDL, which sustains inflammation (11) (➔ Fig. 13.1).

Macrophages

Having entered the intima, monocytes differentiate into macrophages by differentiation factors such as macrophage-colony stimulating factor (M-CSF). Macrophages are the most abundant cell type in the atherosclerotic plaque, and their primary role is phagocytosis. However, macrophages can also instruct other immune cells by producing various immune effector molecules or by acting as antigen presenting cells (APCs) (12).

Within the intima/atherosclerotic plaque, macrophages start to take up LDL via scavenger receptors CD36 and SR(A) (13). When taken up, lipoproteins release entrapped cholesterol, which downregulates the expression of LDL receptors and decreases endogenous cholesterol synthesis. Intracellular free cholesterol undergoes re-esterification by ACAT (acyl-CoA cholesterol ester transferase) but can also traffic to the plasma membrane to become available for efflux (14). Removal of cholesterol from the cell occurs at the plasma membrane by passive diffusion or transfer to apolipoprotein A1 and HDL, a process involving ATP-binding cassette (ABC) transporters, in particular ABCA1 and ABCG1 (15). Impairment of efflux or ACAT function leads to cytotoxicity and macrophage death.

Macrophages express a myriad of receptors, including pattern recognition receptors (PRRs, e.g. TLRs, CLRs, NLRs, and scavenger receptors) and cytokine receptors (e.g. TNFRs, interleukin receptors, and growth factor receptors) through which they scan their environment for activation signals (e.g. PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; cytokines; and growth factors). Upon activation, macrophages/foam cells produce inflammatory cytokines and chemokines that enhance inflammation and further regulate monocyte/T-cell infiltration. Macrophages in the atherosclerotic plaque are capable of releasing a large repertoire of proinflammatory cytokines, including IL-1, IL-6, IL-12, IL-15, IL-18, TNF family members (such as TNFα), and MIF, as well as anti-inflammatory cytokines like IL-10 and TGF-β family members (TGF-β1, BMPs, and GDFs). In particular, TLR2 and TLR4 were shown to be important stimulators of macrophage cytokine production in atherosclerosis (12).

Besides PAMPs and DAMPs, macrophage activation in the plaque can also be initiated through exposure to crystalline material, like cholesterol crystals or increased oxidative stress, which lead to the formation of an inflammasome complex affecting protein maturation and secretion. Inflammasome formation leads to activation of caspase-1 that rapidly cleaves pro-IL1β and pro-IL18 into their mature forms, which are both pathogenic inflammatory cytokines (16). In atherosclerosis, the NLRP3/ASC inflammasome plays a major role (16).

Within the atherosclerotic plaque, sustained inflammation, growth factor deprivation, and oxidative stress, accompanied by prolonged activation of endoplasmic reticulum (ER) stress pathways, result in macrophage apoptosis and necrosis. The unfolded protein response (UPR), with factors like C/EBP homologous protein, $Ca2^{+}$/calmodulin-dependent protein kinase II, STAT1 and NOX, play a major role in this process. Necrosis and apoptosis, and the subsequent defective efferocytosis of macrophage cell debris, result in the formation of a necrotic lipid core within the plaque, and can induce a plaque prone to cause clinical symptoms (12, 17).

Besides producing inflammatory mediators, macrophages, as well as SMCs and neutrophils, produce proteases, such as

matrixmetalloproteases, tPA, uPA, elastases, and cathepsins, capable of degrading extracellular matrix components. These proteases significantly contribute to thinning of the fibrous cap, making atherosclerotic plaques more vulnerable for rupture (18, 19).

As in the monocyte population, macrophages also come in different flavours. Macrophages are a heterogeneous cell population that can be divided into classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages enhance and sustain inflammatory responses via production of TNFα, IL-6, IL-1β, and IL-12, and produce killing agents like iNOS. Sustained M1 activation results in tissue damage and eventually impaired wound healing. In contrast, M2 macrophages secrete anti-inflammatory cytokines, such as IL-10 and TGFβ, stimulate angiogenesis, scavenge debris, and promote the resolution of inflammation by means of, for example, clearance of apoptotic cells (efferocytosis) and dampening of immune responses, hence promoting tissue repair and healing (20). M1/M2 macrophages can switch phenotype, depending on their microenvironment, and both subsets are present at different stages of human atherosclerotic plaque development.

The concept of M1 and M2 macrophages in atherosclerosis may, however, not be so clear-cut (21). Both M1 and M2 subsets are present in human atherosclerotic plaques in all plaque stages, with M1 macrophages present at sites of plaque rupture, and M2 macrophages far from the lipid core and in the adventitia (22) (➔ Fig. 13.1).

Neutrophils

Neutrophils are among the first cell types to respond to invading micro-organisms or tissue damage by inducing rapid neutralization and clearance of pathogens via endocytosis of foreign material and production of reactive oxygen species, myeloperoxidase (MPO), and proteolytic enzymes. Although there are only few neutrophils present in the atherosclerotic plaque, the degree of hypercholesterolaemia-induced neutrophilia is positively correlated with atherosclerotic plaque size in experimental models, and associated with plaque vulnerability in humans (23). Neutrophils may enter the atherosclerotic plaque by means of either diapedesis or being conveyed as part of the blood components constituting intraplaque haemorrhages.

Much of the neutrophil proinflammatory activity can be attributed to the release of granule proteins, matrixmetalloproteinases and cathepsins. MPO, azurocidin, LL-37, α-defensins, and NGAL, as well as MMP8 and the different cathepsins, have been identified inside human atherosclerotic lesions and are potent determinants of atherogenesis in experimental animal models (19, 24) (➔ Fig. 13.1).

Dendritic cells

Dendritic cells (DCs) are professional APCs that play a critical role in innate, but also in regulation of, adaptive immune responses. Although DCs were discovered in 1973 by Steinman and Cohn (25), it took until 1995 before DCs were described in the aorta (26). Few DCs are present in the normal aorta, where they preferentially reside in the adventitia, apart from a few scattered intimal DCs. In the intima, DCs are mainly found at sites prone to develop atherosclerosis, such as the lesser curvature and branch points of the aortic arch (27). DC numbers dramatically increase in both intima and adventitia during atherosclerosis, where they cluster with T cells and localize in the plaque shoulder and rupture-prone regions of plaques (28). In patients with angina pectoris or acute myocardial infarction, blood-derived DC precursors are reduced, while in CAD patients, blood-DC numbers are down, which might be explained by increased recruitment to plaques (29).

In atherosclerotic plaques, T cells are found in close proximity with DCs, implying DC–T-cell interactions. Several studies indicated that oxLDL induces several changes that are characteristic for DC maturation, including enhanced expression of co-stimulatory molecules and increased ability to stimulate T cells (30). Moreover, deficiency of co-stimulatory molecules involved in antigen-loading, immunological synapse formation and T-cell activation (CD80, CD86, and CD40) all led to reduced atherosclerosis in experimental models (31). Several studies using DC transfer, depletion, or modulation, indicated that DCs are capable of skewing immune responses in atherosclerosis either towards an atheroprotective or promoting profile. It is likely that under atherosclerotic conditions, DCs take up atherosclerosis-specific antigens, become locally activated and migrate out of the plaque towards either local draining or distant lymph nodes, where they induce T-cell activation and proliferation (32).

Dendritic cells have the ability to produce various anti- and proinflammatory cytokines. TLR engagement, for example, can lead to the production of proinflammatory cytokines, including TNF, IL-6 and IL-12, all of which have been shown to be atherogenic, but TLR induction can also lead to IL-10 production, which is atheroprotective. Under homeostatic conditions DCs are known to have a tolerogenic effect. In the normal artery wall, resident DCs are thought to promote tolerance to antigen by silencing T cells. However, the inflammatory atherosclerotic microenvironment can activate DCs to switch from tolerance to activation of the immune system (32). Inducing tolerance to atherosclerosis-specific antigens might be a promising therapeutic target for the treatment of atherosclerosis (➔ Fig. 13.2).

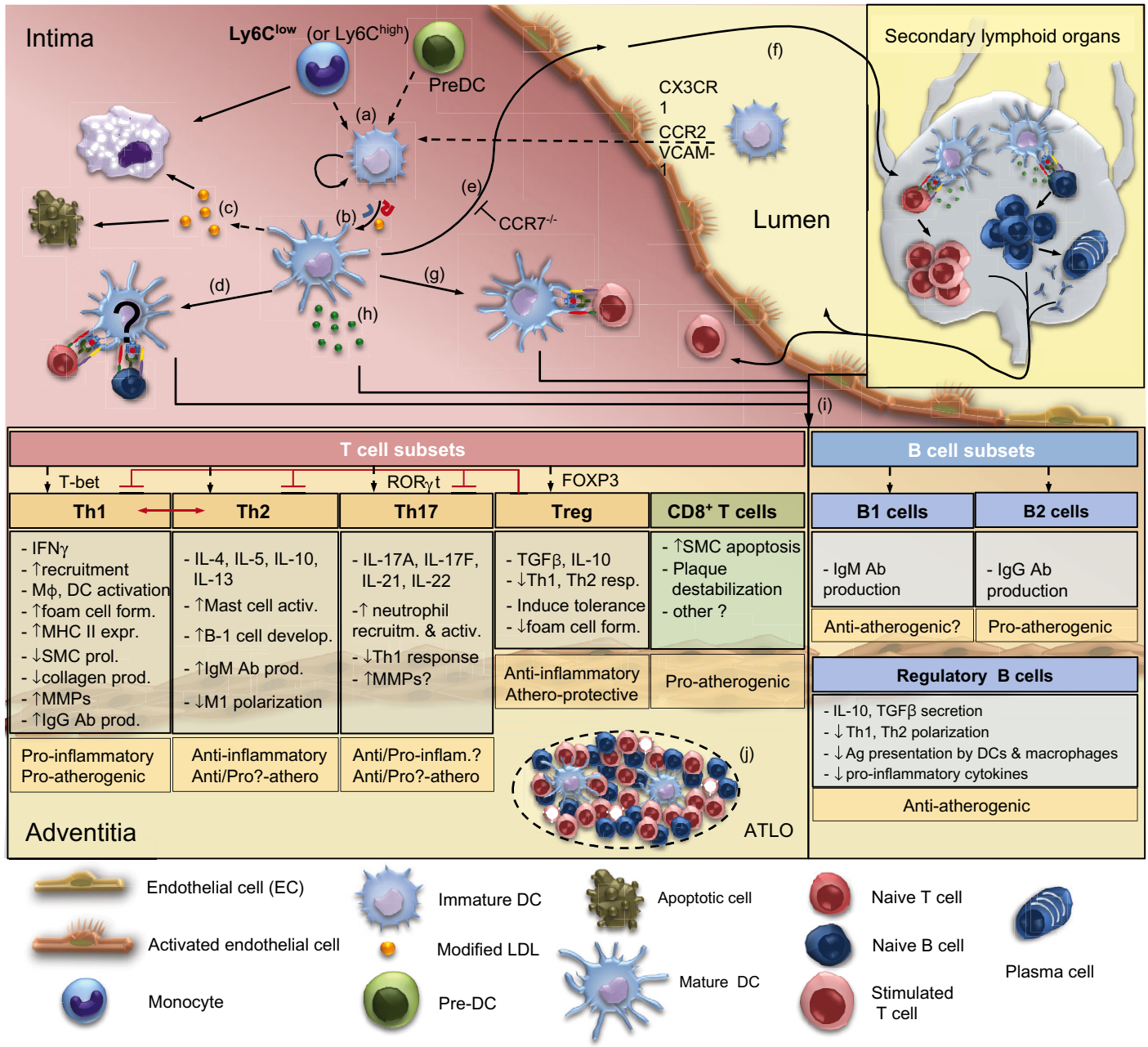

Fig. 13.2 The adaptive immune system in atherosclerosis. (a) Dendritic cells (DC) accumulate in the plaque through direct recruitment from the lumen, local proliferation, and differentiation from either monocytes (preferentially Ly6C^{low}) or DC precursors. Recruitment of DCs from the plaque to the lumen is CX3CR1, CCR2, and VCAM-1 dependent. (b) Plaque DCs take up (atherosclerosis-specific) antigens, become activated, and mature. (c) DCs take up oxLDL and can become foam cells. OxLDL induces DC maturation, but can also trigger DC apoptosis that might contribute to necrotic core formation. (d) Mature DCs, but also macrophages, are professional antigen-presenting cells; however, whether direct antigen presentation occurs in the plaque is not known. (e) Immune cells can emigrate from the plaque into the lumen, a process that is inhibited by both CCR7 deficiency and dyslipidaemia. Dendritic cells can also emigrate from the plaque via lymphatics. (f) Emigrated DCs migrate towards secondary lymphoid organs (spleen and lymph nodes), where they present the antigens to T and B lymphocytes. T cells become activated and clonally expand, after which they enter the bloodstream and are attracted to the plaque. After DC antigen presentation, B cells divide and eventually differentiate into plasma cells. Plasma cells produce various types of immunoglobulin antibodies that will end up in the blood and affect immune responses. Stimulated T (and B cells) can enter the plaque where they exert different effector functions, either promoting or reducing atherosclerosis. (g) Dendritic cells inside the plaque can restimulate primed T cells entering the plaque, boosting immune responses. (h) Dendritic cells secrete several chemokines that influence leukocyte recruitment to the plaque. Most DC-derived chemokines, like CCL17 and CCL22, are involved in T-cell recruitment. Dendritic cells also secrete various proinflammatory (e.g. TNFα, IFNγ, IL-6, IL-12) and anti-inflammatory (e.g. IL-10) cytokines that either stimulate or dampen immune responses. (i) Antigen presentation and cytokine production directly activate various B- and T-cell subsets that all affect atherosclerosis in specific ways. (j) DCs also contribute to the formation of arterial tertiary lymphoid organs (ATLOs), which affect plaque development remotely. MMP, matrix metalloproteinase; LDL, low-density lipoprotein; EC, endothelial cell; VCAM-1, vascular cell adhesion molecule-1; pre-DC, DC precursor; Ig, immunoglobin; SMC, smooth muscle cell; MΦ, macrophage, MHC major histocompatibility; TGF, transforming growth factor.

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T cells

T cells were first detected in human plaques in the 1980s, followed by the observations that HLA/MHCII and the typical T-cell cytokines, such as IFNγ, were present as well (33). The detection of antibodies and T cells specific for oxLDL, combined with the presence of oligoclonal T-cell populations in lesions, confirmed a role for adaptive immunity in atherosclerosis.

T cells are recruited to the vessel wall in parallel with macrophages, but in less quantity. Mechanisms involved are similar to monocyte recruitment. In the arterial wall, T cells become activated in response to antigens and start to produce proinflammatory mediators (e.g. IFNγ). Different T-cell subsets exist that can influence atherosclerosis in various ways, both at early plaque stages and at advanced lesions (32, 34). $CD4^+$ T cells and, to a lesser extent, $CD8^+$ and γδ T cells are present in plaques of atherosclerotic mice. Knockout, depleting antibodies, and cell transfer experiments in animal models suggest an overall pro-atherogenic role for $CD4^+$ and $CD8^+$ T cells, starting early during atherosclerotic disease progression (1).

Classically, T-cell responses are initiated by APCs (DCs, macrophages, and B cells), but can also be antigen independent. After antigen presentation, T-cell activation occurs through simultaneous engagement of the TCR with peptide antigen on MHC class complexes and co-stimulatory molecules with their ligands. In atherosclerosis, the antigen that triggers the immune response and induces T-cell proliferation and polarization is still not completely identified. However, recent evidence points towards atherosclerosis-specific antigens such as (the ApoB100 part of) LDL, and postulate that intimal DCs present these in draining or even distant lymph nodes (35). As the plaque itself contains classical as well as non-classical APCs (e.g. SMCs and endothelial cells), effector T cells immigrating into the lesion can be (re) activated by antigen presentation inside lesions (1–3, 32).

The majority of $CD4^+$ T cells in atherosclerosis are of the Th1 profile, and the pro-atherogenic role of the Th1 subset is well characterized. Th1 cells produce high levels of IFNγ, that promotes the recruitment of T cells and macrophages to the plaques, contributing to plaque growth, augments macrophage uptake of lipids leading to the formation of foam cells, increases the activation of APCs, and enhances the secretion of Th1-promoting cytokines. These events lead to an expansion of atherosclerotic plaque burden and perpetuation of the pathogenic Th1 response. Studies deleting IFNγ or its receptors report reduced atherosclerosis, while injection of recombinant IFNγ leads to increased lesion size. Besides their role in T-cell activation by antigen presentation, DCs and macrophages are instrumental in Th1 differentiation through secretion of IL-12. IL-12 activates Th1 transcription factors (such as STAT4 and T-bet), upregulates IFNγ expression, while downregulating IL-4 and IL-5 in T cells. Patients suffering from CVD show prominent Th1 activation (1, 4, 32, 34).

Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and provide help for antibody production by B cells. Th2 cells are rare in atherosclerotic lesions, although their number is increased in hyperlipidaemia. IL-4 drives Th2 cell differentiation by activation of the transcription factor GATA3 (through STAT6), leading to an increase in IL-4 and IL-5 production and a decrease in IFNγ. Th2 cells were thought to be atheroprotective as they oppose the pro-atherogenic Th1 differentiation. However, the role of Th2 cells in atherosclerosis is still controversial and depends on the site and stage of the lesions, as well as on the experimental model used. Studies on IL-4, the prototypic Th2 cytokine, report either no or pro-atherogenic effects, whereas IL-5 and IL-13 clearly protect against atherosclerosis (1–3, 32, 34).

Regulatory T cells (Tregs) maintain self-tolerance and prevent autoimmunity by suppression of immune responses, such as Th1 and Th2 responses. Natural Tregs develop in the thymus and recognize specific self-antigens. However, Treg cells can also be generated in the periphery in the presence of TGFβ or IL-10, the so-called induced Tregs (iTregs). Regulatory T cells are present in plaques and their depletion in atherosclerotic mice results in increased lesion size. Regulatory T cells are known to produce large amounts of TGFβ and IL-10, and thereby protect against atherosclerosis (36, 37).

IL-17-producing helper T cells (Th17 cells) are protective against fungal and bacterial infections, but are also involved in the development of some autoimmune diseases. Th17 cells mainly produce IL-17A and IL-17F, as well as IL-21 and IL-22. Although Th17 cells are present in both murine and human atherosclerotic lesions, their role remains controversial as both atherogenic and atheroprotective effects have been reported (38).

Follicular helper T cells (Tfh) play a crucial role in B-cell activation and differentiation into memory cells and plasma cells. In experimental atherosclerosis, the Tfh population expands with increasing plaque progression. $CD8^+$ Tregs tightly control the Tfh population. During atherogenesis $CD8^+$ Tregs-mediated control mechanisms fail, and the increase in Tfh cells induced accelerated atherosclerosis (39).

$CD8^+$ T cells are important in cell-mediated immunity, capable of inducing death in infected or dysfunctional somatic cells. $CD8^+$ T cells express T-cell receptors that

recognize specific antigens presented on MHC class I molecules, present on all nucleated cells. As MHCI molecules mainly present cytosolic peptides, this represents an effective mechanism for clearing viruses and other intracellular pathogens. Once activated, $CD8^+$ T cells induce apoptosis in their target cells by releasing cytotoxins, like perforin, granzymes, and granulysin. However, $CD8^+$ T cells also secrete cytokines such as IFNγ and TNFα. $CD8^+$ T cells are present in both murine and human plaques (40, 41). Although $CD8^+$ T cells are only present in low numbers in early lesions, they appear to be the dominating T-cell type in advanced human lesions. Experimental studies in mice revealed a predominant pro-atherogenic effect of $CD8^+$ T cells (42).

Unlike conventional T cells, which recognize peptide antigens presented by MHC molecules, NKT cells recognize a variety of (glyco)lipid antigens presented by a unique TCR on CD1d molecules, APCs. Upon activation, NKT cells secrete both proinflammatory cytokines, such as IFN, and anti-inflammatory cytokines, like IL-4, IL-10, and IL-13. Activated NKT cells can interact in a CD1d-dependent manner with other immune cells, promoting DC maturation and monocyte activation and can induce tolerance by communicating with Tregs. NKT cells are present in human atherosclerotic plaques, and mice lacking NKT-cells show reduced atherosclerosis, proving that NKT-cells are able to contribute to atherogenesis (43).

The interplay and imbalances between the different T-cell types and subsets can mediate the progression of atherosclerosis. An imbalance in Th1/Th2 towards the Th1 response or an imbalance in $CD4^+$ Tregs/Th1 cells or $CD8^+$ Tregs/Tfh cells promotes the progression of atherosclerosis (➔ Fig. 13.2).

B cells

B cells are characterized by the presence of a B-cell receptor and are classically known for their ability to produce antibodies important for the clearance of antigens. B cells possess antigen-presenting capacities, activating both $CD4^+$ and $CD8^+$ T cells. In addition, they can secrete a variety of cytokines (e.g. IFN-γ, IL-2, IL-12, IL-4, IL-6, and IL-10) and promote chemokine production (e.g. CXCL12, CXCL13, CCL19, and CCL21), key players in modulating chronic immune responses by promoting leukocyte recruitment and polarizing T cells. Mature B cells can be categorized into B1 cells, innate like T-cell independent B-cells that are capable of producing natural IgM antibodies, and conventional B2-cells, that are T-cell dependent and are important in adaptive immunity by production of specific IgG antibodies to their cognate antigen.

OxLDL is highly immunogenic and anti-oxLDL antibodies can be detected in atherosclerotic plaques, as well as in the circulation of mice and men. OxLDL-specific IgM titers, produced by natural antibodies, are associated with protection against atherosclerosis (44). In experimental animal models, this protective role of natural anti-oxLDL antibodies produced by B1 cells was found to be mediated by IL-5 (45).

In contrast to B1 cells, B2 cells promote atherosclerosis. Studies in atherosclerotic mouse models taught us that when B2 cells are depleted using anti-CD20, atherosclerosis decreases, and when B2 cells are transferred to atherosclerotic mice, atherosclerosis increases (46). This also is consistent with the observation that anti-oxLDL IgG antibodies, derived from B2 cells correlate with the presence of CVD (44, 47).

Another subset of B-cells is the regulatory B cell, characterized by the production of IL10, which dampens immune responses. Until now, the role of this subset in atherosclerosis is not yet clear; in one report, regulatory B cells protect against atherosclerosis, whereas in another report, no effects on atherosclerosis were observed.

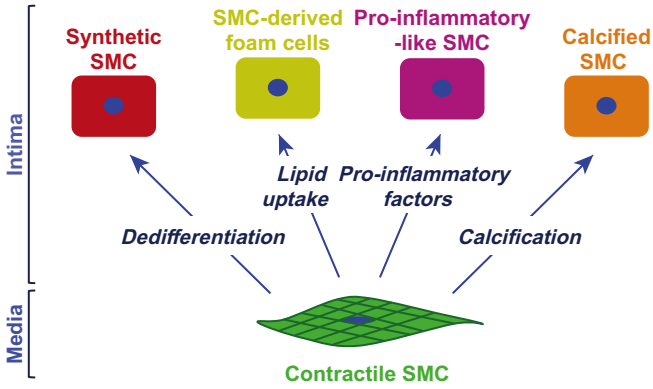
Thus, different B-cell subtypes are involved in atherosclerosis immunity, complicating the role of B cells in the disease (➔ Fig. 13.2).

Mast cells

Mast cells are only present in low numbers in arterial tissue, but increase in atherosclerosis. Mediators of activated mast cells, such as histamine, chemokines, cytokines, and proteases directly contribute to activation of other (immune) cell types, thereby propagating atherosclerosis (48, 49).

Smooth muscle cells

SMCs are the most abundant cell type in atherosclerotic plaques, as well as restenotic lesions following angioplasty or stent implantation in humans (50). SMCs that accumulate into the intimal space migrate from the media. During this process, SMCs undergo phenotypic modulation, i.e. switch from a contractile to a synthetic phenotype that refers to structural and functional changes (➔ Fig. 13.3). This phenomenon includes a process of cell dedifferentiation characterized by an altered expression of contractile proteins, proliferation, and migration, as well as increased production of extracellular matrix components. The SMC phenotypic modulation is described in detail in Chapter 7. SMC accumulation in the intima is triggered by oxLDL, as well as chemokines and growth factors released by the dysfunctional endothelium, platelets, and inflammatory cells (51–53). Many of these factors are well characterized, such as PDGF-BB, FGF-2, IGF, TGF-β, angiotensin II,



SMC plasticity: *Depending on their environmental cues/signals, SMCs switch from a contractile phenotype in the media to diverse phenotypes in the intima: synthetic SMCs, foam cells pro-inflammatory-like SMCs and calcified SMCs.*

Fig. 13.3 SMC plasticity: depending on their environmental cues/signals, SMCs switch from a contractile phenotype in the media to diverse phenotypes in the intima: synthetic SMCs, foam cells, proinflammatory-like SMCs and calcified SMCs.

endothelin-1, interleukins, and ROS. SMCs exhibit a remarkable plasticity and hence play diverse roles in early stage and progression of atherosclerosis depending on environmental cues/signals.

The synthetic SMCs are considered as beneficial players in atherosclerotic plaque development by essentially contributing to fibrous cap formation that protects the plaque from rupture. In this context, intimal SMCs synthesize extracellular matrix components, as well as metalloproteinases that play a crucial role in SMC migration and proliferation (for details see ➔ Chapter 10).

It is also well accepted that SMC-derived foam cells are present in the atherosclerotic plaque. Simultaneous staining for SMC markers and lipids has shown that a large proportion of intimal foam cells in human coronary artery are derived from SMCs (at least 50% of total intimal foam cells) (54). Like macrophages, SMCs can express scavenger and lipid receptors that are essential for their conversion into foam cells within atherosclerotic lesions. In particular, treatment with atherogenic growth factors and cytokines (e.g. IL-1, and TNF-α) SMCs express receptors promoting the uptake and storage of excess lipids, including LDL receptor, VLDL receptor, CD36, and type I and type II scavenger receptors (55–58). LOX-1 (scavenger receptor of ox-LDL), which is the most important receptor in the induction of endothelial dysfunction and the formation of atheromatous plaque, is also present in SMCs (59, 60).

Spindle-shaped SMCs isolated from rat aorta, as well as medial SMCs from human coronary artery (both exhibiting a contractile phenotype), express the reverse transport pathway, including the ATP-binding cassette (ABC) transporter, ABCA1, and interact with ApoA-I to form HDL (61); their expression is downregulated in rat epithelioid SMCs and human intimal SMCs (both exhibiting a synthetic phenotype). Moreover, ABCA1 expression is decreased in intimal SMCs of human coronary artery when compared with myeloid-derived cells (61). These findings suggest that cholesterol efflux is impaired in intimal SMCs.

Several studies have demonstrated that cholesterol loading of SMCs *in vitro* led to transdifferentiation of SMCs toward a macrophage-like state, with a decrease in the expression of SMC differentiation markers and an increase in the expression of macrophage-related genes (62), as well as CD68, a marker of macrophages (54, 63). It is noteworthy that SMC-derived cells exhibiting macrophage-like properties can be undetectable with the classical SMC phenotypic markers, as shown recently by tracing SMC lineage in atherosclerosis-prone ApoE$^{-/-}$ mice (64). The contribution of SMC-derived macrophage-like cells in atherosclerotic plaque formation has been likely underestimated (55, 64).

Besides their role in fibrous cap and foam cell formation, SMCs have the capacity to acquire inflammatory cell markers and to release proinflammatory factors. When addressed, this process is associated with an SMC phenotypic switch toward a synthetic phenotype (52, 57). Cytokines such as TNF-α or IL1β induce expression of inflammatory molecules such as ICAM-1 and VCAM-1, and release of MMP-9, a MMP related to plaque vulnerability (65). Other factors such as ox-LDL, oxidized phospholipid (proinflammatory mediators generated in apoptotic and necrotic cells), high mobility group box 1 (HMGB1, released by necrotic cells), or angiotensin II are also able to induce a proinflammatory-like SMC phenotype. TLR2 and TLR4 and the receptor for advanced glycated-end product (RAGE) are involved in this process, leading to production of inflammatory cytokine such as MCP-1 and IL-6 through MAPK and NFKB pathways (52, 57).

SMCs are the main source of cells contributing to atherosclerotic plaque calcification (66). SMCs release calcifying vesicles and calcification involved SMC apoptosis (52, 56, 67, 68). Calcified SMCs exhibit high expression of osteogenic markers, such as bone-morphogenic protein-2 (BMP-2), osteopontin, osteonectin, or the transcription factor Runx2, and decreased expression of calcification inhibitors, such as matrix Gla protein. SMC modulation to osteoblast-like cells promotes signalling pathways that induce bone matrix deposition in the vessel. Exposition of SMCs to high phosphate concentration increases osteogenic gene expression, which is associated with a SMC switch toward a synthetic

phenotype. *In vivo*, the matrix Gla protein-deficient mice show severe vessel calcification. This is associated with downregulation of myocardin (transcriptional coactivator of the serum response factor involved in SMC differentiation marker expression) and upregulation of Runx2 (69). Mechanisms of cell calcification are described in detail in Chapter 15.

It is well accepted that SMCs within the intima migrate from the media. However, studies have suggested the role of adult vascular progenitor cells in the development of atherosclerosis and vein graft atherosclerosis (70, 71). SMC progenitor cells have been mainly identified in the bone marrow (multipotent vascular stem cell progenitors and mesenchymal stem cells), in the circulating blood, and in the adventitia (resident SMC progenitor cells). These progenitor cells have the ability to differentiate into SMCs (72, 73). Additionally SMCs can transdifferentiate from endothelial cells (73).

Fibroblasts

Fibroblasts are located in the adventitia of the vessels and play a pivotal role during the negative, i.e. constrictive, remodelling that occurs in balloon-injured arteries, where it has been well described. A similar process can take place in complicated atherosclerotic vessels. It is characterized by the formation of a thickened adventitia that leads to vessel stenosis (74, 75). This phenomenon closely resembles wound healing and is characterized by the modulation of fibroblasts into myofibroblasts. The myofibroblast was first described in experimental wound healing as a fibroblastic cell located within the granulation tissue and exhibiting bundles of microfilaments (76). Since then, it has been shown to be a key player in different pathological conditions, such as hypertrophic scars, fibromatoses, systemic sclerosis, organ fibrosis, and stroma reaction to epithelial tumours. Myofibroblast differentiation is characterized by the *de novo* expression of α-SMA (the actin isoform typical of SMCs), which is upregulated through TGF-β1 produced by local inflammatory cells (77). Unlike SMCs characterized by a rapid and transient contraction, myofibroblasts exhibit a long-lasting isometric tension resulting in a slow, irreversible retraction (78); such contraction mechanism should also take place in vascular fibrosis.

Platelets

Besides thrombus formation, platelets also play a profound role in immunology. Platelets can promote monocyte–endothelial cell interactions by their expression of P-selectin. Repeated injections of P-selectin-deficient platelets into ApoE$^{-/-}$ mice resulted in smaller lesions compared to mice injected with P-selectin-expressing platelets (79). Platelet P-selectin is important in the formation of platelet–leukocyte aggregates, which promote the release of chemokines, such as CCL2, CCL5, and cytokines, like IL-1β, enhancing endothelial activation, leukocyte recruitment, rolling, and transmigration. In addition, platelets can deposit chemokines, like CCL5, on activated endothelium, which enhances monocyte recruitment and adhesion to the vascular wall (80).

Cell–cell communication in atherosclerosis

The communication between immune cells, as well as their interaction is crucial to initiate, maintain, dampen, and resolve immune responses and inflammation. Also in atherosclerosis, cell–cell communication between (different) immune and non-immune cell types is highly regulated. Here we will describe three important classes of regulators: chemokines, cytokines, and co-stimulatory molecules.

Chemokines

Chemokines, classified in subgroups based on the position of the N-terminal cysteine residues (CC, CXC, CX3C, and XC) are a family of chemotactic cytokines that plays a pivotal role throughout atherogenesis. Chemokines not only mediate immune cell recruitment, but also control cell homeostasis and activation of the different immune cell types and subsets (2).

During the first steps of atherogenesis (➔ Fig. 13.4), CXCL1 is released from the endothelium by lysophosphatidic acid, a component of LDL, and recruits neutrophils and classical monocytes via CXCR2 in the arterial wall. Together with macrophage-derived CCL2, the CXCL1–CXCR2 axis forms a positive-feedback mechanism for monocyte recruitment (81). CXCL1 also mediates the hypercholesterolaemia-induced mobilization of neutrophils and classical monocytes from the bone marrow, and facilitates their subsequent recruitment into the arterial wall (81). Classical monocytes express the chemokine receptors CCR1, CCR2, CCR5, and CX3CR1, but only use CCR1 and CCR5 to enter the arterial wall (82) (➔ Fig. 13.2). Recruitment of neutrophils has been shown to be especially important in plaque initiation and besides CXCR2 is mediated via CCR1, CCR2, and CCR5 (23).

Another chemokine receptor-ligand pair crucial in atherosclerosis is the CCR7-CCL19/CCL21 dyad, which can mediate macrophage egress under certain conditions, or mediate T-cell recruitment to the arterial wall (83).

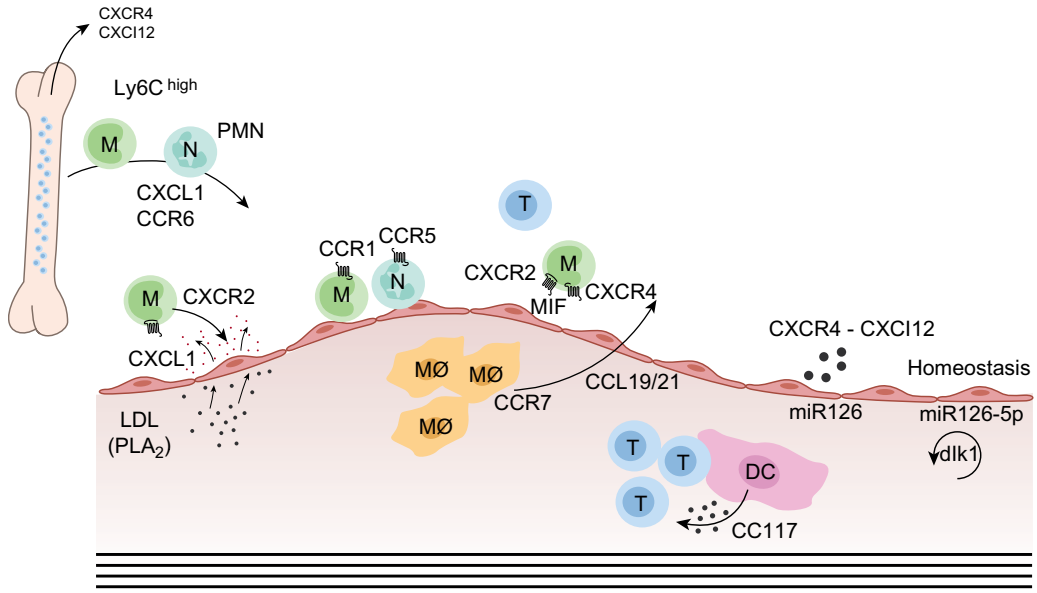


Fig. 13.4 Chemokines and their receptors in atherosclerosis. Induction of CXCL1 release from the endothelium via lysophosphatic acid (LPA) results in recruitment of (Ly6C^{high}) monocytes and neutrophils in the arterial wall and stimulates the mobilization of myeloid cells from the bone marrow. Ly6C^{high} monocytes enter the arterial wall via CCR1 and CCR5. The interaction between MIF and CXCR2 and CXCR4 further recruits monocytes/macrophages and T cells into the plaque. Egress of plaque macrophages might be facilitated by CCR-7/CCL19/21 interactions, but seems to occur only in selective conditions. Within the plaques, the balance between CD4$^+$ and Treg cells is regulated by CCL17-expressing dendritic cells. CXCR4–CXCL12 interactions are important to induce regeneration and mobilize HSPCs to counteract apoptosis. MiR126 is present in apoptotic bodies of endothelial cells and signals via RGP16 to upregulate CXCR4, which promotes surviving cells to produce CXCL12. Moreover, MiR126-5p is responsible for maintaining a proliferative reserve of endothelial cells via dlk-1.
(Reproduced from Bäck M, Weber C, Lutgens E. Regulation of atherosclerotic plaque inflammation. J Intern Med. 2015; 278(5): 462–82 with permission from John Wiley and Sons.)

Chemokines not only control immune cell mobilization and recruitment, but are also key in controlling the interplay between innate and adaptive immune cells. For example, the DC-derived chemokine CCL17 drives atherosclerosis by mediating the recruitment of T cells to the aorta, and by regulating homeostasis of regulatory T cells (84).

CXCL12 and its receptor CXCR4 are powerful mediators for the homeostasis of inflammation. CXCL12–CXCR4 interactions counteract apoptosis by mobilizing stem cells. In atherosclerosis, the CXCL12–CXCR4 axis is protective by controlling neutrophil homeostasis and promoting endothelial regeneration (85) (➔ Fig. 13.4).

The chemokine family has the unique property to confer heterophilic interactions. The interactions between platelet chemokines CXCL4 and CCL5 synergistically aggravated atherosclerosis (86). These findings epitomize the concept of a functional interactome, constituted by haemophilic and heterophilic chemokine interactions that integrate signals conferred by individual chemokines for the combinatorial control of immune responses.

Cytokines

Cytokines are a family of small proteins (5–20 kDa) that consists of interleukins, lymphokines, interferons, and tumour necrosis factors (TNF). Like chemokines, cytokines play a role in (inter)cellular communication in the immune system and thereby mediate activation and resolution of inflammation. Cytokines play a crucial role in the initiation and propagation of atherosclerosis and, combined with their receptors, have also been found in atherosclerotic plaques where they regulate immune reactions and inflammation (87).

Interleukins

Interleukin 1 is part of the Interleukin 1 family, which consists of 11 members. Of these members, IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-18, IL-33, and IL-37 are important in atherosclerosis(1). IL-1α and IL1β exacerbate atherosclerosis and the IL-1-receptor antagonist (IL-1ra), which inhibits the effects exerted by IL-1α and IL-1β, has an atheroprotective role (88, 89). Recently it was shown that the cardiovascular system in RA patients also benefits from Anakinra (a recombinant IL-1ra) treatment, with a reduction in oxidative stress, endothelin and IL-6 production, and improved left ventricular function (90). Currently, a clinical trial is underway investigating the effects of canakinumab treatment on recurrent cardiovascular events in stable post-myocardial infarction patients (91, 92). In

innate immune cells, the NLRP3 inflammasome plays a key role in the maturation of IL-1β and IL-18 by caspase-1 dependent cleavage of their pro-forms, and inactivation of IL-33 (16). Cytokines of the interleukin 1 family have been found to play a vital role in the initiation and propagation of atherosclerosis and could potentially provide targets for future treatment.

Interleukin 2 is required for the growth, proliferation, and differentiation of (thymic) T cells to effector cells, and is required for the maturation of regulatory T cells. In humans, serum IL-2 levels correlate with carotid intima-media thickness, and thus with atherosclerosis (93). If IL-2 is blocked in experimental atherosclerosis models, plaque burden is decreased, and the pool of regulatory T cells expands (94). IL-2 thus plays a role in T-cell mediated inflammation through both T-helper cells and regulatory T cells.

As described, IL-4 is secreted by T helper 2 (Th2) cells and induces T- and B-cell proliferation (32). In vascular disease, IL-4 plays an ambivalent role. Since the Th2 response is considered anti-atherogenic, IL-4 was long believed to have anti-inflammatory properties. However, experimental studies have revealed a more complex role for IL-4 in the pathogenesis of atherosclerosis. IL-4 increases VCAM-1 expression in endothelial cells and increases endothelial cell turnover. Furthermore, IL-4 induces the synthesis of MCP-1 and IL-6, and is able to induce endothelial damage via the generation of reactive oxygen species (ROS) (95). Accordingly, experimental atherosclerosis studies showed both pro- and anti-atherogenic properties for IL-4 (96–98).

Interleukin 5, the other Th2 cytokine (but also secreted by mast cells), is clearly protective in atherosclerosis. B cells express the IL-5 receptor and IL-5 plays a role in B-cell proliferation and differentiation, immunoglobulin class switching, and the production and secretion of IgM and IgA (45). In humans, a single nucleotide polymorphism (SNP) for IL-5 has been associated with CVD (99).

IL-6 and its receptor, gp130, exhibit both pro- and anti-inflammatory functions. IL-6 is secreted by a plethora of cells, including T cells, monocytes, and macrophages, but also endothelial cells, B cells, adipocytes, fibroblasts, smooth muscle cells, and hepatocytes. IL-6 signalling induces cell migration and proliferation, MMP production, B-cell differentiation, and reduces regulatory T-cell formation (100). In atherosclerosis, IL-6 is produced locally in atherosclerotic lesions, as well as systemically. It can aggravate atherosclerosis through endothelial dysfunction, the recruitment as well as activation of inflammatory cells, and the migration and proliferation of smooth muscle cells (SMCs). Finally, IL-6 is able to exert effects on the receptors responsible for the uptake of LDL, the scavenger receptors SR-A and CD36 (100). Although these findings point towards a clear proinflammatory role of IL-6 in atherosclerosis, animal experiments indicate a more complex system, i.e. that IL-6 can either increase or inhibit atherosclerosis, suggesting dual role of IL-6 in atherosclerosis (101–103).

IL-10 is one of the most intensively studied anti-inflammatory cytokines in atherosclerosis. IL-10 is able to inhibit the presentation of antigens, block the secretion of cytokines, and the expression of costimulatory molecules, as well as MHC-II. It is also able to modify chemokine secretion and chemokine receptor expression. IL-10 is produced by macrophages, but also by regulatory T cells (104). As expected, the results of animal studies have universally shown an anti-atherogenic role for IL-10 (105, 106).

IL-12, a cytokine consisting out of a 35-kDa light chain and a 40-kDa heavy chain, forms a bridge between the innate and adaptive immune response and promotes a Th1 based pro-atherogenic phenotype. It is secreted by activated monocytes, macrophages, neutrophils, and dendritic cells. Furthermore, in combination with IL-18, it drives the production of IFNγ by T cells (87). In concert with its proposed polarization towards a Th1-based response, animal studies have universally shown a proinflammatory role for IL-12 (107, 108).

Similar to IL-4, IL-13 is predominantly produced by Th2 T cells. Although intracellular signalling occurs via similar pathways as IL-4, IL-13 is believed to possess not just overlapping, but also unique, effects, including the production of TGF-β by macrophages, and has a clear protective role in atherosclerosis (109).

IL-17 is predominantly produced by T helper 17 (Th17) cells, which also produce IL-12 and IL-22 (1, 87). As described, IL-17 is believed to have both atherogenic as well as atheroprotective effects. It is currently believed that the specific effects IL-17 has on atherosclerosis are dependent on the region and microenvironment in which IL-17 is released (38).

Tumour necrosis factor, interferons, and TGF-β

TNF, formerly known as TNFα, plays a role in many inflammatory diseases. Monocytes and macrophages are the main source of TNF. TNF binds two receptors, p55 and p75, of which p55 (TNFRSF1A) controls the major functions of TNF. In experimental models, TNF promotes atherosclerosis (110, 111), and elevated levels of TNF have been observed in patients suffering from CVD (112).

IFNγ plays a crucial role in immune responses and is secreted by many cells in the atherosclerotic plaque, including T cells, monocytes, macrophages, and natural killer cells (113). Despite its strong proinflammatory effects, IFNγ also exhibits anti-inflammatory characteristics and, accordingly, is able to manipulate the secretion of both pro- as well as anti-inflammatory signalling molecules. In atherosclerosis, IFNγ promotes plaque growth by enhancing monocyte infiltration and differentiation into macrophages, foam cell formation, and induces a Th1-driven response by the adaptive immune system (114, 115).

IFNβ, a member of the interferon family, is produced primarily by fibroblasts, but also by macrophages and plays an important role in inflammation. IFNβ induces chemotactic signals from macrophages and adhesion of leukocytes. In experimental animal studies of atherosclerosis, the role of IFNβ is ambivalent (116–118)

TGF-β is produced by a multitude of cells, including macrophages and regulatory T cells. It plays an important role in many physiological processes, e.g. embryonic development and proliferation, differentiation, migration, adhesion, and apoptosis of cells. TGF-β has also been implicated in many diseases, including autoimmune diseases, cancer, and cardiovascular disease. In atherosclerosis, TGF-β exerts an anti-inflammatory role (119–121).

Immune checkpoint regulators: co-stimulatory molecules

Co-stimulatory molecules play a central role in fine-tuning immunological reactions, predominantly in lymphoid tissues, but also in other tissues. After binding of the T-cell receptor (TCR) to the MHC II molecule, co-stimulatory molecules provide additional stimuli that license the T cell and APC to elicit a proper immune response (122). However, co-stimulatory molecules are also expressed on a variety of other immune cells, such as neutrophils, macrophages, and mast cells, and non-immune cells, including platelets, endothelial cells, smooth muscle cells, adipocytes, and hepatocytes, but also on different epithelial cells, where they regulate inflammation (123). Interactions between co-stimulatory molecules result in the activation of the respective cell types and promote, but can also dampen, inflammatory responses. Hence, co-stimulatory interactions mediate a broad crosstalk between innate and adaptive immunity, also in atherosclerosis. Most co-stimulatory molecules belong to either the B7- or TNF-superfamily of co-stimulatory molecules.

B7 superfamily

The most well-known members of the B7 superfamily of co-stimulatory molecules are CD28 (T cells) and B7.1 (CD80) and B7.2 (CD86) (APCs). Upon binding, T-cell activation and survival is promoted. Besides CD28, B7 family members bind to the competing co-inhibitory protein CTLA-4, which is also upregulated on activated T cells. CD80/86-CTLA-4 interactions dampen the on-going process of CD28-mediated T-cell activation by promoting endocytosis of CD28, thereby decreasing T-cell priming and proliferation. Other members of the B7 superfamily are the co-stimulatory ICOSligand–ICOS, dyad and the co-inhibitory PD1/2–PD-L dyads. In atherosclerosis, many studies point towards a pro-atherogenic role for CD80 and CD86. Monocyte-derived DCs of patients suffering from cardiovascular disease display an increased expression of CD80 and CD86 (124, 125), and experimental models in which CD80/CD86 is blocked reveal a decrease in atherosclerosis (126). The ICOSligand–ICOS pathway dampens atherosclerosis, due to the involvement of this dyad in Treg development and function (127, 128) (➔ Fig. 13.5).

The co-inhibitory molecules CTLA4, but also PD1/2-PD-L dampen immune responses, and also decrease atherosclerosis (129–131) (➔ Fig. 13.5).

TNF superfamily

The second large family of co-stimulatory molecules is the TNF-TNFR superfamily. To date, the family contains 19 ligands and 30 receptors of which ox40L-ox40, CD40L-CD40; CD137-CD137L; CD27-CD70; glucocorticoid induced TNF-R like protein (GITR)-GITRL, and LIGHT-LTβR are the most common.

The CD40/CD40L axis is one of the best-studied co-stimulatory pathways of the TNF(R) superfamily, and was originally described to be crucial for T-cell activation, proliferation, and cytokine production, and for humoral B-cell responses, such as proliferation and Ig isotype switching (31). Nowadays, CD40–CD40L interactions are known to be important in other cell types as well. CD40L (CD154, TNFSF5) is expressed $CD4^+$ T cells and platelets, but also on $CD8^+$ T cells, endothelial cells, and vascular smooth muscle cells (VSMCs), and even epithelial cells. CD40, its receptor, is expressed on antigen-presenting cells (B cells and DCs), but also on T cells, platelets, macrophages, smooth muscle cells, epithelial cells, adipocyte, and hepatocytes (31). CD40 has no direct signalling capacity, but signals via adaptor molecules: the TNF-receptor associated factors or TRAFs. CD40 has two distinct binding sites for TRAF: a proximal site for TRAF1/2/3/5 and a distal site for TRAF6 binding (123).

In atherosclerotic plaques, CD40L and CD40 are expressed on numerous plaque-related cells, and are associated with

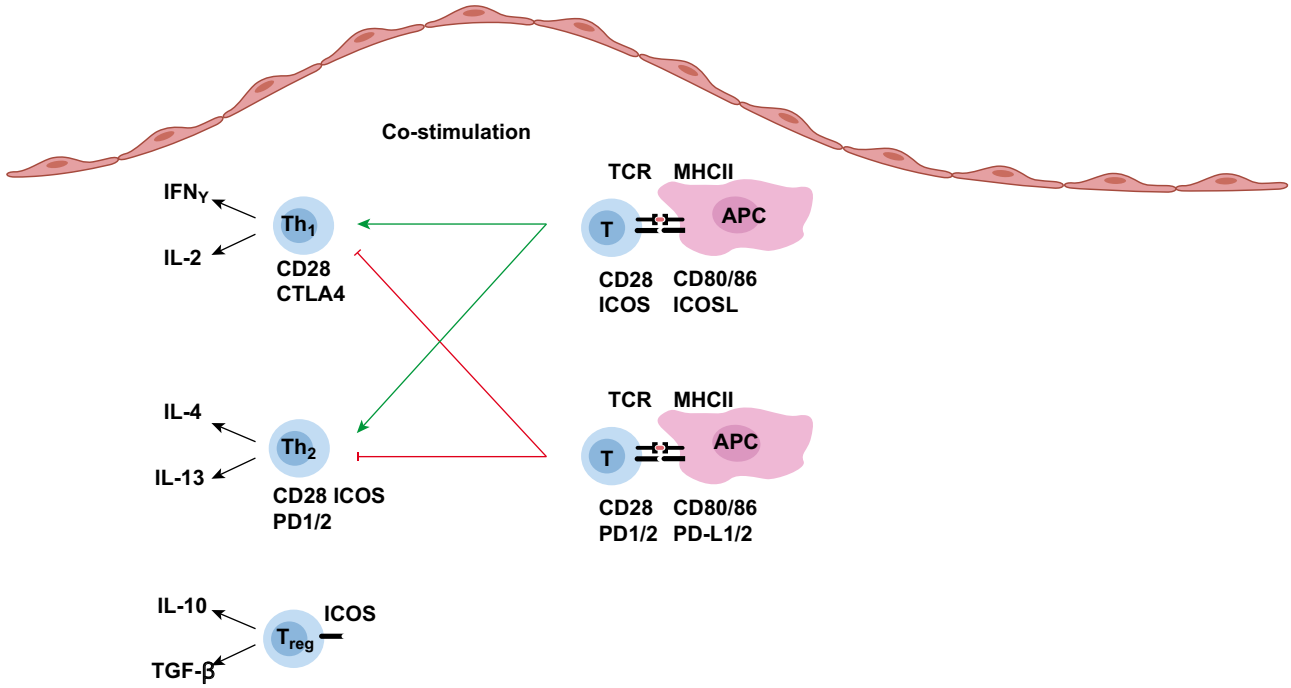


Fig. 13.5 The B7 family of co-stimulatory molecules. Co-stimulation is required for efficient propagation of the immune response, whereas co-inhibition limits the immune response. In the atherosclerotic plaque, both the dendritic cell and the macrophage can act as APC. The B7 family of co-stimulatory molecules comprises CD28–CD80/86 and ICOS–ICOSL interactions. Upon interaction between MHCII and the TCR, co-stimulation via B7 family members results in T-cell proliferation of Th1 and Th2 cells, and production of the Th1 and Th2 cytokines. Co-inhibition via CTLA4–CD80/86 and PD1/2–PD-L1/2 results in inhibition of Th1 and Th2 cell proliferation. ICOS–ICOSL interactions are required for Treg induction.
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plaque vulnerability. Increased (soluble) CD40L levels in plasma or in microparticles are related to an increased risk for (recurrent) CVD (132). Experimental studies showed that inhibition of either CD40L or CD40 results in a strong decrease in atherosclerosis, and in a favourable, stable atherosclerotic plaque phenotype that contains only few inflammatory cells and an increase in fibrosis (133–136). Especially the interaction between CD40 and TRAF6 appeared crucial in driving atherogenesis (137).

Ox40L was found to be on the *ath* locus and thus a susceptibility gene for atherosclerosis in mice, and inhibition of ox40L decreased atherosclerosis (138, 139). Experimental studies also showed that the CD137-CD137L and the CD30-CD30L dyads are prominent drivers of atherosclerosis (140, 141) (➔ Fig. 13.6).

Conclusion

The formation of an atherosclerotic plaque is due to a complex interplay between lipids, immune cells, and non-immune cells. This interplay is tightly regulated by extensive cell–cell communication by chemokines, cytokines, and co-stimulatory molecules. By understanding the interplay between the different cell types, and the effects on atherogenesis, treatment strategies beyond lipid lowering are just on their way.

Further reading

Allahverdian S, Pannu PS, Francis GA. Contribution of monocyte-derived macrophages and smooth muscle cells to arterial foam cell formation. Cardiovasc Res. 2012;95(2):165–72.

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Tabas I, Garcia-Cardena G, Owens GK. Recent insights into the cellular biology of atherosclerosis. J Cell Biol. 2015;209(1): 13–22.

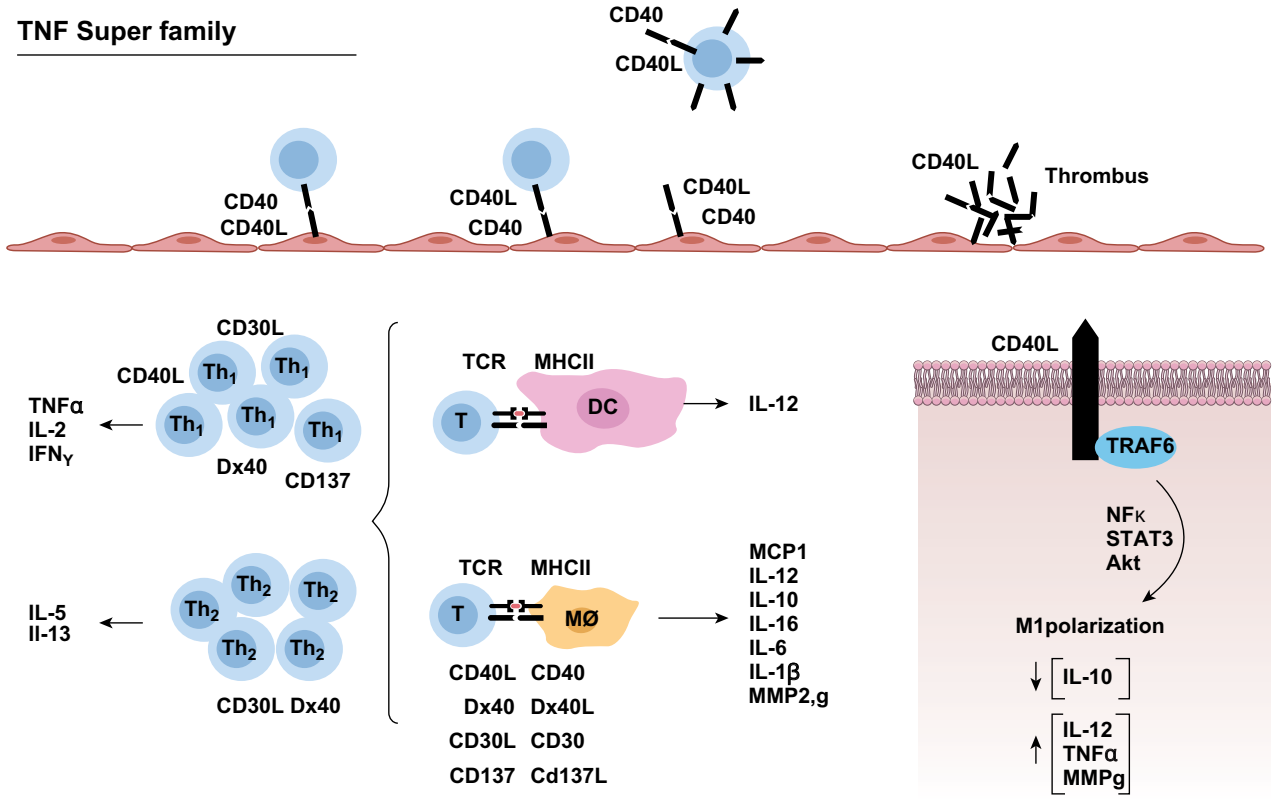

Fig. 13.6 The TNF superfamily of co-stimulatory molecules: focus on CD40–CD40L interactions. CD40 and CD40L are expressed on numerous plaque-associated cell-types, and contribute to the different stages of atherosclerosis. CD40 on monocytes, dendritic cells, neutrophils, and B cells can bind to CD40L on the endothelium, upon which recruitment via cell-adhesion molecules is initiated. The same is true for T lymphocytes and platelets expressing CD40L: both can be recruited via CD40 on the endothelium. Platelet CD40L can form aggregates with CD40-expressing leukocytes, thereby forming platelet–leukocyte aggregates that can produce cytokines like IL1β. Platelet CD40L is important for thrombus stability. Upon interaction between MHCII and the TCR, co-stimulation via TNF family members results in T-cell proliferation of Th1 and Th2 cells, and production of the Th1 and Th2 cytokines, as well as cytokines released by the antigen-presenting DC or macrophage. CD40 requires adaptor molecules to initiate signalling, and signals via TRAF6 in macrophages, which, via NFKB, STAT3, and Akt, induce M1 polarization, as well as the release of proinflammatory cytokines and proteolytic enzymes, such as MMP-9.
(Reproduced from Bäck M, Weber C, Lutgens E. Regulation of atherosclerotic plaque inflammation. J Intern Med. 2015; 278(5): 462–82 with permission from John Wiley and Sons.)

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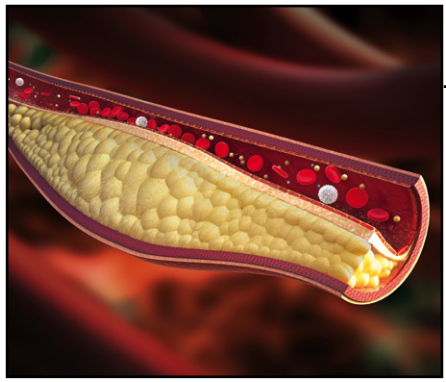

CHAPTER 14

Molecular mechanisms

Claudia Monaco and Giuseppina Caligiuri

Content

Introduction

Molecules that have a major role in host defence and inflammation play a significant role in the development of atherosclerotic lesions. During the initiation of atherogenesis the interaction between blood cells and the endothelium via adhesion molecules takes centre stage. Inflammatory signalling is activated within the vessel wall with the participation of pathways involved in innate and adaptive immunity, and also of lipid mediators. This chapter will take you through these fundamental steps in the development of the atherosclerotic plaque by summarizing very recent knowledge in the field and highlighting recent or ongoing clinical trials that may enrich our ability to target cardiovascular disease in the future.

Molecular regulation of the cross-talk at the blood/vessel interface

Atherosclerosis takes place at the interface between the endothelial cells lining the inner side of the arterial wall and those contained in the flowing blood (erythrocytes, leukocytes, and platelets). Under physiological conditions, endothelial cells constitute a functional 'barrier' that prevents platelet adhesion and leukocyte extravasation. The integrity of the endothelial barrier is essential to protect the vascular wall from the inappropriate infiltration and action of the molecules and cells contained in the flowing blood, thus preventing the development of atherosclerosis. In spite of the continuous interaction between endothelial and blood cells while blood flows, the latter neither adhere to nor pass through the endothelium and do not become activated within the circulation.

Atherosclerotic lesions are characterized by the perturbation of the endothelial barrier function and the resulting accumulation and activation of leukocytes at the inner side of the arterial wall. A number of molecules are involved in the regulation of the blood/vessel interface. Inducible, stress-related adhesion molecules, such as ICAM-1 and VCAM-1, trigger leukocyte adhesion and transmigration, whereas constitutive adhesive regulatory receptors (such as PECAM-1) are crucial in preventing these phenomena when inappropriate. An imbalance between the expressions of the former and

the latter may favour the adherence of circulating leukocytes and platelets to the endothelium at sites prone to atherogenesis (1).

Molecules involved in leukocyte–platelet–endothelial cell–cell interaction and atherosclerosis

The upregulation of molecules involved in leukocyte rolling, adhesion, and extravasation is an important hallmark of endothelial cells at sites of human atherosclerosis. Selectins (P, E, and L) and adhesion molecules (ICAM-1 and VCAM-1) are strong and independent predictors of all-cause and cardiovascular mortality in patients with stable carotid atherosclerosis (2). In the past, leukocytes were thought to enter the arterial wall exclusively from its inner endothelial layer. More recently, the topological pattern of these molecules in human studies suggests that leukocyte–endothelial cell interactions involved in atherosclerosis have been additionally mapped to plaque neovessels (3) and adventitial vasa vasorum, of which human arteries are rich (4). This is in agreement with a basic immunology paradigm whereby leukocytes reach inflammatory sites by crossing the post-capillary venules (5).

Leukocyte rolling on endothelial cells is supported by an intermediary cell–cell cross-talk between leukocytes, platelets, and endothelium. During rolling, carbohydrate ligands on the circulating leukocytes bind to selectin molecules on the endothelium forming transitory bonds, making the leukocytes slow down and roll on the endothelium surface. Activated endothelial cells express E-selectin and P-selectin, the former mediates leukocyte–endothelial interactions, the latter triggers platelet arrest by binding to GPIbα and PSGL-1. Subsequently, platelets through β_3 integrins firmly adhere and become activated. Activated platelets in turn express P-selectin and further activate the endothelium through the release of CD40L and IL-1β, providing pro-atherogenic alterations of endothelium (for a review see (6)). Endothelial and platelet P-selectins are all actively involved in atherosclerosis (7) and point at P-selectin counter-receptors as potential therapeutic targets (8).

Meanwhile, the release of chemokines induces surface integrin molecules to switch from the default low-affinity state to the high-affinity state. Experimental studies in hypercholesterolaemic mice suggest that both ICAM-1 and VCAM-1 mediate leukocyte–endothelial cell–cell interaction and atherosclerosis (9–11). VCAM-1 would play a more important role in the early stages (12) and novel therapeutic strategies for atherosclerosis include the use of blocking antibodies directed to VCAM-1 (13).

Platelet endothelial cell adhesion molecule (PECAM-1) trans-homophilic cell–cell interactions regulate the cross-talk at the blood/vessel interface

Because of its structural similarity to known 'CAMs', PECAM-1 (also known as CD31) was initially categorized as yet another endothelial adhesion molecule. Growing evidence suggests a broader physiological function of PECAM-1 in vascular biology (14), in particular in maintaining homeostasis within the circulation (➔ Fig. 14.1) by raising the threshold of activation in leukocytes and platelets, and by releasing signals required for the integrity of the endothelial barrier (15). The endothelial barrier function is regulated by a number of proteins exhibiting homophilic adhesive and signalling activities at the 'inter-endothelial contacts', which is where the endothelial cells establish a cell–cell cross-talk by the so-called adherens junctions. This involves vascular endothelial-cadherin, which recruits the cytoplasmic proteins, β-catenin and p120-catenin, and PECAM-1 at the site of contact between adjacent cells. In their resting state, the adherens' junction components are organized in *linear* structures along the actin-rich intercellular contacts and *reticular* domains that contain low levels of actin. The latter are localized in areas where neighbouring cells overlap and encompass the cell adhesion receptor PECAM-1 (16). At the endothelial cell intercellular borders, PECAM-1 serine phosphorylation is necessary to maintain the barrier function by sustaining the interplay between catenins and the cytoskeleton (17). The loss of endothelial PECAM-1 function, in response to proinflammatory stimuli (16) or due to deprivation stress (18), does not limit leukocyte extravasation (19) but rather drives endothelial cell apoptosis (19) and loss of the barrier function (15).

Since PECAM-1 is also expressed by the platelets and the leukocytes of the flowing blood and it acts as a homophilic co-receptor interacting with molecules of the same kind with both inhibitory and stimulatory functions, it is difficult to specifically establish the role of endothelial cell-specific PECAM-1 in atherosclerosis. Whereas its mechanosensory functions may account for an increased expression of PECAM-1 at atheroprone endothelial sites, such as arterial branching (20), the development of mature atherosclerotic plaques in hypercholesterolaemic mice is rather associated with the disappearance of endothelial PECAM-1 (21). The role of PECAM-1 in atherogenesis is controversial, as studies in PECAM-1 knockout mice show opposing results supporting both pro-atherogenic (22) and atheroprotective (23) functions for PECAM-1. However, studies in patients have shown that reduced expression of PECAM-1 on circulating lymphocytes in patients with atherothrombotic

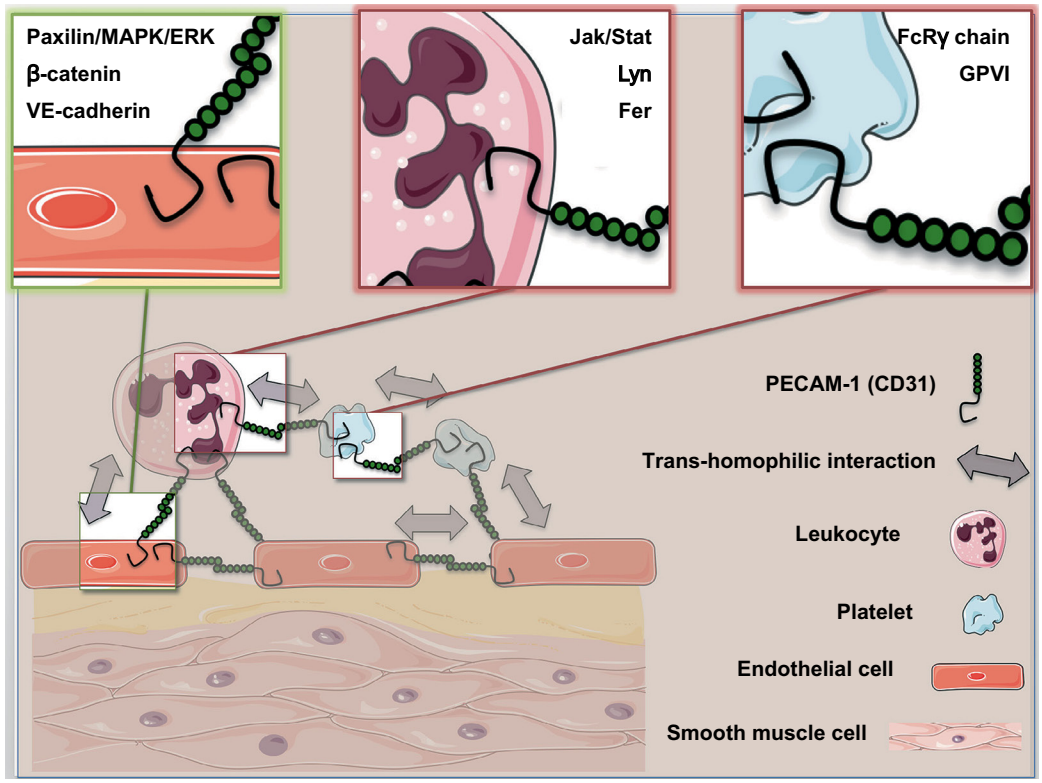

Fig 14.1 PECAM-1 is a trans-homophilic receptor constitutively expressed by all endothelial cells, platelets, and leukocytes. The trans-homophilic CD31-CD31 engagement on interacting cells drives the recruitment and activation of intracellular SH-2 phosphatases, a crucial process involved in the maintenance of the homeostasis at the blood–vessel interface. The intracellular signalling induced by phosphatases raises the threshold of cell activation due to tyrosine kinases, such as those involved in the activation of leukocytes (Jak/Stats, Lyn, and Fer) and platelets (FcRg chain and GPVI), whereas it releases the signalling pathways involved in endothelial cell survival (paxilin/MAPK/ERK) and barrier function (b-catenin and VE-Cadherin).

aneurysm (24) or acute coronary syndromes (25) may lead to increased T-cell extravasation (26) and accumulation at local sites of arterial inflammation. Furthermore, the use of a soluble recombinant protein or a synthetic peptide (27, 28) agonist of PECAM-1 prevent the progression of atherosclerosis, as well as its acute complications (27), in preclinical interventional studies.

Intracellular signalling pathways relevant to the pathogenesis of atherosclerosis and inflammation

How is inflammation established and maintained within an atherosclerotic plaque? Under physiological conditions, inflammation is an ancient self-limiting protective mechanism from invading pathogens. It relies on two arms: innate and adaptive immunity. Innate immunity is activated immediately after the encounter with the pathogen and is carried out primarily by myeloid cells with the participation of some 'innate' lymphocyte subpopulations. The adaptive is a second line of defence that is based on the generation of antigen-specific recognition tools at the cellular (T-cell receptor) and humoral (antibodies) levels.

In the past decade it has become apparent that the innate arm of the inflammatory immune response is not merely involved in phagocytosis. Rather it is the main orchestrator of the adaptive responses and that it senses pathogen-associated molecular patterns with a specificity that was previously unsuspected. The most represented cell types within the atherosclerotic plaque are innate immune cells, such as monocytes, macrophages, dendritic cells (DCs), and mast cells.

The potential for innate immunity to regulate the development of atherosclerosis has emerged in the last 5 years. Significant progress has been made recently in the field linking innate immune sensors to the recognition of cholesterol crystals and modified lipoproteins. Diverse innate immune signalling pathways cooperate to induce and maintain inflammation upon exposure to endogenous and exogenous molecular patterns.

Each extracellular regulatory molecule acts via its own cellular receptor, but all ultimately work by controlling protein phosphorylation, which is the major mechanism through which external stimuli regulate intracellular processes. Generally this involves activation of protein kinases in the cell, and the specificity of action of the stimulus is determined by the nature, pattern, and duration of the enzymes induced. The target proteins phosphorylated by the kinases are diverse (e.g. enzymes, transcription factors, receptors, and cytoskeletal proteins). Sometimes the substrates are themselves kinases, leading to cascades of

phosphorylation that in turn diversify and amplify a signal leading to the appropriate cell response. Herein we will summarize the prototypical inflammatory signalling pathways that are important in the pathogenesis of atherosclerosis as examples of intracellular signalling.

Host pattern recognition receptors

Innate immunity is the body's first line of defence against pathogens. The activation of the innate immune response is dependent on a set of highly conserved receptors broadly referred to as pattern recognition receptors (PRRs) and which allow a rapid response to danger signals. PRRs are the strongest known inflammatory catalysts and potent activators of the NFκB pathway. PRRs comprise a large family encompassing over 50 members that survey both the intracellular and extracellular compartments of a cell. Three groups of PRRs have been described: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).

Toll-like receptor (TLR) signalling

TLRs are the most studied of the PRRs and to date at least 13 have been identified in mammals. The members of the interleukin (IL)-1/TLR family share their cytoplasmic Toll/interleukin-1 receptor (TIR) domain with interleukin-1-receptor (IL-1R) that is required for signal transduction. Homophilic interactions between the common TIR domains of the receptors and adapters induce downstream signalling. The extracellular regions contain arrangement in tandem leucine rich repeats (LRR) forming a horseshoe-shaped solenoid structure (29). These receptors are membrane-spanning and thus either survey the extracellular space or the lumen of intracellular vesicles, including endosomes and lysosomes.

Each TLR is specialized to detect a defined type of pathogen-associated molecular pattern (PAMP). At the cell membrane, TLR2 detects bacterial lipoproteins and lipoteichoic acid, TLR4 recognizes bacterial endotoxin, and TLR5 is activated by bacterial flagellin. TLR3, TLR7, TLR8, and TLR9 detect nucleic acids of bacterial or viral origin from their location on the membrane of the endoplasmic reticulum (ER), endosomes, and lysosomes. Whereas most TLRs form homodimers following ligand binding, TLR2 heterodimerizes with TLR1 or TLR6 (30). TLR family members in the endosomal compartment are specialized in the induction of the antiviral type I IFNs. TLR3 signalling activates the transcription factors interferon regulatory factor 3 (IRF3) and NFκB via the adapter molecule TRIF in the MyD88-independent pathway. Conversely, the induction of type I IFNs by TLR7 and TLR9 depends entirely on MyD88 (➔ Fig. 14.2) (for a review see (31)).

Five TLR adaptor molecules bridge the 13 TLRs to downstream signalling: myeloid differentiation protein 88 (MyD88) and MyD88 adaptor-like (Mal) or TIRAP (TIR domain-containing adaptor protein), Toll–interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF) or TICAM-1, TRIF-related adaptor molecule (TRAM) or TICAM-2, and sterile alpha and HEAT/Armadillo motif (SARM) (30) (➔ Fig. 14.2). TLR-4 is unique amongst all other TLRs in its ability to activate simultaneously MyD88-dependent and TRIF-dependent signalling (32) (33).

MyD88-dependent signalling leads to NFκB activation through the canonical IκB kinase (IKK) complex and production of proinflammatory cytokines (➔ Fig. 14.2) (for a review see (30)). In the vast majority of cells, NFκB is sequestered in the cytoplasm in an inactive form complexed with inhibitor of NFκB (IκB) proteins. The IκB family consists of the classical regulators IκBα, IκBβ, IκBε, and atypical regulators, and is characterized by the presence of several ankyrin-like motifs important for the interaction with NFκB, and a C-terminal PEST motif important for the regulation of IκB stability and protease sensitivity (for a review see (34)). NFκB is rapidly activated by a large spectrum of pathways, including TLRs, cytokines such as TNFα and IL-1, and antigen receptors. Activation involves the phosphorylation, polyubiquitination, and subsequent degradation of IκB by the 26S proteasome, a major pathway for the degradation of intracellular proteins in eukaryotic cells (35, 36). The amino acid residues Ser32 and Ser36 of IκBα were identified as essential for the phosphorylation process (36), whereas it was Lys21 and Lys22 for the ubiquitination process. IκB degradation leads to the exposure of a nuclear translocation sequence of the NFκB dimer, allowing its nuclear translocation and DNA binding (37).

TLRs as extracellular sensors in atherosclerosis

The first suggestion that the TLR/IL-1 family is involved in atherosclerosis came from two landmark studies in 2004 (38, 39), demonstrating that genetically modified mice lacking MyD88 had reduced atherosclerotic lesions and lower levels of macrophage accumulation, even in the presence of hypercholesterolaemia. Similar results were obtained in hypercholesterolaemic mice lacking TLR2 and TLR4 (29, 40–43). Similarly, in arterial injury models, MyD88, TLR2, and TLR4 deficiency were protective (44–46).

Conflicting results have emerged from studies examining the effect of TLR gene polymorphisms in cardiovascular disease (for a review see (47)). Expression of TLR2 and TLR4

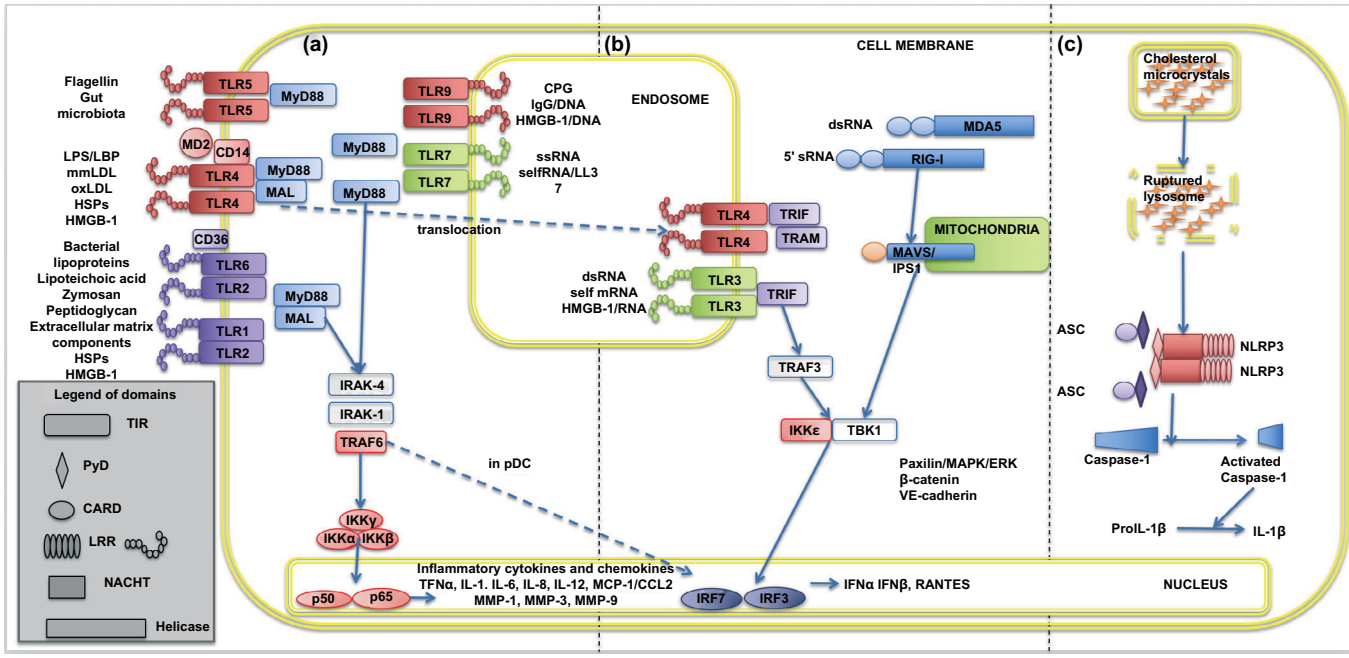

Fig. 14.2 Pattern recognition receptor signalling in atherosclerosis. (a) MyD88-dependent signalling. IL-1R, IL-18R, and all TLRs—with the exception of TLR3—recruit the adaptor MyD88. MyD88 recruits IRAK-1 and IRAK-4 to its death domain (DD), and in turn they recruit TRAF6. IRAK-1 and TRAF6 associate with TAK1 Tab1, and Tab2 (not shown). This complex leads to the activation of TAK1, which activates the canonical IκB kinase (IKK) complex and the mitogen-activated protein kinase pathway (not shown). NFκB activates multiple proinflammatory cytokine genes, including TNFa, IL-6, and IL-1. Mal/TIRAP functions as a bridging adaptor that recruits MyD88 to TLR4 and TLR2. The MyD88 and TRIF-dependent pathways are both activated by TLR4. MyD88 and Mal bind to TLR4 soon after LPS binding to induce NFκB activation and proinflammatory cytokine production. TLR4-induced production of Type I IFN is entirely dependent on TRIF and TRAM. After binding MyD88 and Mal, TLR4 is endocytosed in a dynamin-dependent manner and—after releasing the MyD88–Mal complex—it translocates to the endosome where it binds TRIF–TRAM inducing type I IFN (see b). (b) TRIF-dependent signalling. TRIF interacts with the non-canonical IKKs TBK-1 and IKKe (or inducible IKK-IKKi) through TRAF3, which mediates phosphorylation of IRF3 and induces expression of IFNs. Receptor-interacting protein-1 (RIP1) binds the C terminus of TRIF via a Rip homotypic interaction motif and mediates NFκB activation. There are cell-type specific differences in IFN responses. The induction of type I IFNs by TLR7 and TLR9 depends entirely on MyD88 in plasmocitoid dendritic cells (pDCs). IRF7—a transcription factor expressed constitutively in pDCs—forms a signalling complex with MyD88 and TRAF6 in the cytoplasm. MAVS-dependent signalling. As in the TRIF-dependent pathway, RIG-I and MDA5 interaction with MAVS results in the activation of TBK1 and the phosphorylation of IRF3 and IRF7 on specific serine residues, resulting in their homo- or heterodimerization. The dimers then translocate to the nucleus and activate the transcription of type I IFN genes. (c) ASC-dependent signalling. NLRP3 activates the CARD-containing adaptor ASC through PYD–PYD homophilic interactions. Subsequently, the CARD domain of ASC interacts with the CARD domain of caspase-1 and mediates its activation. These inflammasomes involve an adapter—apoptosis-associated speck-like protein containing a CARD (ASC)—that links these NLRs to caspase-1. The activation of caspase-1 results in the post-translational modification and, ultimately the secretion of IL1 protein.

Abbreviations: myeloid differentiation protein 88 (MyD88); MyD88 adaptor-like (Mal); Toll–interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF); TRIF-related adaptor molecule (TRAM); IL-1R-associated kinase (IRAK)-1; tumour necrosis factor receptor-associated factor 6 (TRAF6); TANK (tumour-necrosis-factor-receptor-associated factor (TRAF)-family-member-associated nuclear factor-B (NF-B) activator)-binding kinase (TBK)-1; transforming growth factor-activated kinase (TAK1); TAK1-binding proteins 1 (Tab1) and 2 (Tab2); IFN-regulatory factor (IRF); mitochondrial antiviral signalling protein (MAVS); NOD-like receptor family, pryin domain containing 3 (NLRP3); pyrin domain (PYD); caspase activation and recruitment domain (CARD); apoptosis-associated speck-like protein-containing CARD (ASC).

in human atherosclerotic plaques is co-localized with the nuclear translocation of the p65 NFκB family member in lesional endothelial cells and macrophages, suggesting the functional importance of these two receptors (48). In an *ex vivo* model of human atheroma based on the short-term culture of live cells from surgical carotid endarterectomies, comprising a mixed population representing the major cell types resident in human atherosclerotic plaques (e.g. macrophages, smooth muscle cell, and T lymphocytes) (49), blockade of TLR2 and MyD88 signalling almost abolished NFκB activation, the production of the inflammatory molecules CCL2/MCP-1, IL-6, CXCL8/IL-8, and the generation of the matrix-degrading enzymes MMP-1, -2, -3, and -9. Conversely, TLR4 and its adaptor TRAM were not required for cytokine production, but had a selective role in MMP-1 and -3 production (50).

TLR2 forms heterodimers with either TLR1 or TLR6, and the specificity of each heterodimer is different for specific PAMPs. The different specificities are relevant for atherogenesis. The intraperitoneal administration (a common systemic delivery route in murine models) of either TLR1/2 or TLR2/6 synthetic agonists mimicking bacterial PAMPs enhanced lesion formation in the abdominal aorta (51). This augmentation was lost in mice lacking TLR1 and TLR6 (51),

as well as in TLR2-deficient mice (52), indicating that TLR1/2 and TLR2/6 heterodimers are necessary for enhancing atherogenesis in the presence of bacterial PAMPs. Yet remarkably, when no exogenous agent was administered to the athero-prone mice, TLR1 and TLR6 were redundant for atherogenesis, suggesting that exogenous bacterial ligands and endogenous agonists generated during hypercholesterolaemia act through different pathways (51).

The identity of hyperlipidaemia-derived endogenous ligands in human pathology is unknown but some hints as to their nature have been recently generated. High-density lipoproteins (HDL) from patients with chronic kidney dysfunction markedly reduced nitric oxide bioavailability in human aortic endothelial cells. This resulted in an increase of systemic arterial blood pressure in wild-type mice via TLR2 in a TLR1- or TLR6-independent pathway, suggesting that HDL from patients with kidney disease can induce TLR activation (53).

Pattern recognition is often achieved via signalling complexes rather than single receptors in isolation. The first complex to be characterized was that of TLR4 that includes several accessory receptors aiding in the detection of lipopolysaccharide (LPS)—also called endotoxin. According to the current model of endotoxin recognition, serum LPS-binding protein (LBP) transfers LPS to CD14 that delivers it to the co-receptor MD2 and initiates TLR4 signalling (54). Cells that do not express CD14—e.g. endothelial cells—are relatively unresponsive compared to $CD14^+$ monocytes (55). To a lesser extent, CD14 also enhances TLR2–TLR6-induced cellular activation (56, 57) and TLR3 signal transduction (58). CD36—a class B scavenger receptor that recognizes oxidized phosphatidylcholine contained within oxidized LDL and membranes of apoptotic cells—participates in the recognition of diacylated lipopeptide from *Mycoplasma* macrophage-activating lipopeptide-2 (MALP-2) and LTA but not triacylated lipopeptides or zymosan (59).

The cooperation between scavenger receptors and TLRs is particularly relevant to endogenous agonist-elicited responses. Oxidized phospholipids, oxidized LDL, saturated fatty acids (SFAs), and lipoprotein(a) trigger apoptosis in ER-stressed macrophages through a mechanism requiring both CD36 and Toll-like receptor 2 (TLR2) (60). Moreover, oxidized LDL induced the formation of a unique complex, including CD36, TLR4, and TLR6, with an intracellular localization (61). TLR4–TLR6 dimerization and signalling was dependent on Tyr463 of CD36 and an intact endocytic pathway. This pathway is also shared by amyloid-β in microglia indicating a common pathogenic pathway for atherosclerosis and Alzheimer's disease (61).

Importantly, the effect of TLR signalling on atherosclerosis is strongly cell-type dependent. Surprisingly, TLR2 and TLR4 expression on bone marrow-derived cells is not involved in atherogenesis (52) (62). Also, the haematopoietic deficiency of the TLR4 adapter TRAM, but not MAL, reduces atherosclerosis without affecting cholesterol metabolism, via attenuation of systemic and vessel inflammation (63). These studies undermine the natural assumption that the pro-atherogenic role of TLRs is linked solely to their expression in haematopoietic cells. In support of this interpretation, a recent study has shown that when MyD88 expression is lost selectively in cells expressing CD11c (usually expressed by dendritic cells and some macrophages), atherosclerotic lesion formation increases—rather than decreases, as in whole body deficiency of MyD88 (38). This is due to the loss of formation of T regulatory cells and of their protective effect on lesion formation via transforming growth factor (TGF)β-mediated monocyte chemotactic protein (MCP)-1 reduction (64).

Intracellular TLRs in atherosclerosis

In the past 2 years, the spotlight has been placed on the role of endosomal TLR signalling as a modulator of atherosclerosis. One of the key features of endosomal TLRs is the activation of the interferon pathway, as well as cytokine pathways (➔ Fig. 14.2). Earlier studies had already shown that TLR9 stimulation with a synthetic oligonucleotide carrying unmethylated CpG-containing sequences (usually contained in bacterial DNA) induced IFNα production from segments of human carotid plaques in culture, presumably due to activation of plasmacytoid dendritic cells, that are strong producers of IFNα upon viral infections (65). Interferons can also, in turn, enhance the expression of intracellular PRRs in vascular tissues. IFNγ induces expression of the endosomal RNA sensor TLR3, and the members of the RLR family, MDA5 and RIG-I, in non-atherosclerotic human coronary artery rings (66). Indeed, TLR3 expression is significantly increased on smooth muscle cells from diseased tissue (AthSMC) compared to control cells. This increased TLR3 expression in AthSMC led to a 40-fold enhancement of TLR3 signalling and generation of both pro- and anti-inflammatory cytokines (67).

In a murine model of arterial injury, chronic intraperitoneal administration of the synthetic TLR3 ligand Poly(I:C)—which mimics viral, long, double-stranded RNA—attenuates neointima formation and also reduces injury-induced medial damage. In addition, in hyperlipidaemic mice genetically deficient in $TLR3^{-/-}$, the development of atherosclerotic lesions was accelerated, confirming that TLR3 has protective functions in the vessel wall (67, 68).

This enhancement of atherogenesis in TLR3$^{-/-}$ mice was observed in the absence of exogenous TLR3 stimulation, suggesting that protective endogenous agonists for TLR3 may be generated during atherogenesis. As yet, the endogenous TLR3 ligands involved in atherosclerosis are unknown, but mRNA from necrotic cells and stathmin, a microtubule regulatory protein have both been identified as potential endogenous TLR3 ligands (69, 70).

The outcome of TLR3 activation in the context of vascular responses is, however, not unequivocal. Intravenous administration of Poly(I:C) can augment endothelial dysfunction and impair rendothelialization (71). Finally, increased lesion development in high-fat fed ApoE$^{-/-}$ mice was observed after Poly(I:C) stimulation (71). Echoing these results, a recent link has been demonstrated between activation of endosomal TLR signalling with arterial and gestational hypertension (72–74). In particular, TLR3 activation induces the production of the vasoconstrictor peptide endothelin-1 in pulmonary arterial hypertension (75). The divergence of effect of TLR3 on atherosclerotic lesion formation (67, 68, 71) is difficult to explain fully, yet it is potentially dependent on the different doses and administration routes, as well as the presence or absence of a high-fat diet in the experimental setting, indicating that the effect of TLR stimulation is context-dependent. There is also indication that TLR3 signalling outcome is dependent on the cell type carrying it. LDLR-deficient mice carrying TLR3-deficient bone marrow were protected from lesion development, suggesting TLR3 activation on haematopoietic cells is detrimental to lesion development (63). This observation is in keeping with earlier studies showing that elective deficiency of IFNβ in bone marrow decreases atherosclerotic lesion formation (76). The detrimental role of myeloid TLR3 seems to be mediated through MMP2 (77), suggesting a role for TLR3 in degrading extracellular matrix in lesions. Accordingly, haematopoietic deficiency of the TLR3 and TLR4 adapter TRIF reduces atherosclerosis without affecting cholesterol metabolism, via attenuation of systemic and vessel inflammation (63, 68). Due to the protective overall effect of whole body TLR3 deficiency, it is possible that TRIF signalling is predominantly engaged by TLR4 in atherosclerosis. Collectively this body of information indicates that the outcome of TLR3 stimulation is dependent on the overall contribution of the different cell types bearing this receptor. These studies indicate that TLR signalling is a complex balance of heterogeneous cellular responses that need to be taken into account when designing therapeutics to address this area of pharmacology.

A protective role for TLR7 in atherosclerosis has also been recently described in atherosclerotic mice lacking TLR7 (78). These mice displayed increased lesion development and increased macrophages and lipids, with reduced smooth muscle cell content within the lesions. Reduced production of proinflammatory cytokines and chemokines was associated with TLR7 in human plaques (78). Like TLR3, TLR7 is surrounded by some controversy. In a femoral artery cuff model, blockade with a TLR7/9 antagonist reduced neointimal thickening, macrophage infiltration and reduced cytoplasmic HMGB1 expression, which indicates reduced cell stress (79). Given the protective role of TLR7 observed in ApoE$^{-/-}$ mice, it is possible to speculate that the antagonist mediates its beneficial effects by acting through TLR9. Indeed, in a transverse aortic constriction model of heart failure, it was recently shown that TLR9$^{-/-}$ mice had a better outcome, including improved cardiac function and less macrophage infiltration, than control mice (80). However, in a rabbit model of collar-induced injury, administration of the synthetic exogenous TLR7 agonist imiquimod augmented lesion formation, cytokine release, and plaque infiltration (81). Thus, whereas TLR7 appears to confer atheroprotective functions in the setting of unperturbed hypercholesterolaemia, administration of TLR7 agonists may have the reverse effect. It is possible that, similar to TLR2 and TLR3, the effects of TLR7 activation are different depending on exogenous versus endogenous ligand activation.

NOD-like receptor (NLR) signalling

NLRs belong to a large family of soluble proteins that survey the cytoplasm for the presence of exogenous and endogenous intracellular PAMPs and DAMPs. There are 23 NLR genes in humans and 34 in mice. From a phylogenetic point of view, NLRs can be grouped in three distinct subfamilies: (a) NODs, (b) NLRPs (or NALPs), and (c) IL-1β-converting enzyme (ICE)-protease activating factor (IPAF). NLRs are formed of three distinct domains: a C-terminal ligand-sensing leucine-rich repeat (LRR) domain, a central nucleotide-binding and oligomerization (NACHT) domain, which is responsible for oligomerization, and a N-terminal effector pyrin domain (PYD), caspase recruitment domain family (CARD), or baculoviral IAP repeat (BIR)-mediating homophilic interactions in the downstream signalling. NLRs lead to the release of the IL-1 family of inflammatory cytokines, including IL-1β and IL-18, through the formation of large cytoplasmic complexes known as 'inflammasomes', which involves caspase-1. Caspase-1 mediates the cleavage of the pro-form of these cytokines into mature forms, which results in the secretion of bioactive cytokines. Inflammasomes are distinguished into three main complexes according to the NLR involved: the NLRP3/NALP3 inflammasome, the NLRP1/NALP1 inflammasome, and the IPAF/NLRC4 inflammasome.

The NLRP3 inflammasome is currently the most fully characterized and consists of the NLRP3 scaffold, the apoptosis-associated speck-like protein-containing CARD (ASC) adaptor, and caspase-1. NODs drive the activation of mitogen-activated protein kinases (MAPK) and NFκB via interaction with the serine–threonine kinase RICK with the subsequent activation of the kinase TAK1. This leads to the induction of chemokines, cytokines, and defensins, which mediate the antimicrobial responses (for a review see (82)).

The role of NLRs in atherosclerosis has recently emerged for the first time in experimental models. LDLR-deficient mice transplanted with NLRP3- or ASC-deficient bone marrow and fed a high-cholesterol diet had markedly decreased early atherosclerosis and inflammasome-dependent IL18 levels (83). ASC deficiency also attenuated neointimal formation after vascular injury via reduced expression of IL-1β and IL-18 in neointimal lesions, resulting in a decrease of vascular inflammation. Neointimal formation was significantly decreased in wild-type mice in $ASC^{-/-}$ bone marrow chimeras (84). However, the effect of the inflammasome on atherosclerosis could be dependent on the genetic background of the mice or on the cell type, as when a different strain of hypercholesterolaemic mice ($ApoE^{-/-}$) were crossed with NALP3 or ASC-deficient mice, no effect was observed on atherogenesis and lesion phenotype (85).

Also NODs, and in particular NOD2, have a role in atherosclerosis. The administration of muramyl dipeptide (MDP), the NOD2 cognate ligand, aggravated atherogenesis and increased the size of lipid-rich necrotic cores in $LDLR^{-/-}$ mice. Myeloid-specific ablation of NOD2 protected from these effects on the vessel wall, potentially through the induction of lipid mediators (see Bioactive lipids and atherosclerosis) (86, 87).

The inflammasome activation is highly integrated. A good example of the interplay between TLR and NLR pathways is the requirement of priming with a TLR agonist or a proinflammatory cytokine, such as IL-1, or tumour necrosis factor-α (TNF-α) for inflammasome activation. The resultant NFκB activation leads to pro-IL1β synthesis, as well as inflammasome components such as caspase 11 and NLRP3. The second signal, which activates the caspase 1, allowing the conversion of pro-IL1β to IL1β, includes activation by ATP of the P2X7 purinergic receptor with potassium efflux. The second signal may also be achieved by reactive oxygen species (ROS), bacterial toxins, PAMPs, crystallized particles, and ultraviolet light (82). How is the NLRP3 inflammasome activated in atherosclerosis? Cholesterol crystals are a known presence in human atherosclerosis. Usually thought to be a rather late occurrence in the disease, their identification in murine lesions has been more elusive. Small cholesterol crystals accumulate as early as 2 weeks after the start of an atherogenic diet and correlate with the appearance of macrophages (83). Moreover, cholesterol crystals are able to activate IL-1b release, suggesting their strong role in atherosclerosis. In conclusion, a current model of activation of innate immunity in atherosclerosis is that of priming of the inflammatory response via modified lipoproteins via TLRs followed by cholesterol crystal-induction of NLRP3 inflammasome. Currently the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial is testing the efficacy of IL-1 blockade in 17,200 patients with coronary artery disease and previous myocardial infarction patients with persistent elevation of hsCRP (88).

Bioactive lipids and atherosclerosis

Bioactive lipids are produced from fatty acids, mainly from pre-existing membrane phospholipids, through specific biosynthetic pathways in response to extracellular stimuli. Their action is spatially restricted, because they are rapidly sequestered by dedicated processes on local cells. A growing body of evidence links the action of several such lipid mediators in atherosclerosis. The local generation of bioactive lipids is prompted by the dense clustering of different cell types in atherosclerotic plaques, which presents a unique situation for lipid handling. Similar to their protein counterparts, bioactive lipids are also a means of cross-talk between cells of different origin at the blood/vessel interface. In particular, the production of bioactive lipids is orchestrated by enzyme pathways that involve multiple cells (transcellular biosynthesis).

Unlike proteins, the information of bioactive lipids acting in the atherosclerotic plaque cannot be obtained from the genome since they are not genome-encoded. However, we can manipulate the genes encoding the set of enzymes and receptors that are essential for their actions. Most of the bioactive lipids involved in atherosclerosis derive from the hydrolysis of the phospholipids from cellular membranes and lipoproteins, but some (resolvins) are synthetized from dietary ω-3 fatty acids.

Products of secretory phospholipase A2

Secretory phospholipase A2 (sPLA2) is a member of a family of ten isoenzymes that hydrolyse phospholipids from cellular membranes and lipoprotein. Seven (out of ten) isoenzymes of sPLA2 have been detected in human atherosclerotic lesions (89) and compelling data point at a pathogenic role for the sPLA2 groups IIA, V, and X. In particular, experimental work in transgenic mice overexpressing

groups IIA, V, and X shows increased atherosclerosis formation, whereas mice deficient in these sPLA2 isoenzymes or atherosclerosis-prone mice treated with a sPLA2 antagonist (varespladib) show reduced atherosclerosis formation, suggesting a potential role for sPLA2 inhibitors as anti-atherosclerotic drugs (90). However, the inhibitor varespladib failed to provide a significant reduction in adverse events in patients with coronary artery disease and was terminated early (112), indicating that sPLA2 may have more complex functions in vascular biology.

In addition to the generation of bioactive lipids by sPLA2, due to their hydrolytic action on the phospholipids, sPLA2 are endowed with pleiotropic properties. Group IIA sPLA2 binds to proteoglycans and integrins αvβ3 and α4β1, at a site that is distinct from the catalytic site, and promotes leukocyte activation (91), whereas groups V and X sPLA2 drive a remodelling of the lipoproteins that enhances their binding to proteoglycans. This in turn favours foam-cell formation by promoting lipoprotein oxidation and internalization by tissue macrophages and stromal cells. The hydrolytic action of sPLA2 on the sn-2 ester of glycerophospholipids generates free fatty acids and lysophospholipids. The latter trigger specific cell-signalling pathways by binding to cognate G protein-coupled receptors (GPCR) on various cell types. Similarly, the eicosanoids generated from the oxidation of arachidonic acid, a free fatty acid produced by the hydrolysis of phospholipids, act as signalling lipids by binding to specific cell surface receptors on various cell types.

Lysophospholipids

Platelet-activating factor (PAF)

PAF, as well as other lysophospholipids formed by oxidative fragmentation of the polyunsaturated acyl group of the parent phosphatidylcholine from low density lipoproteins or cell plasma membrane, exert pro-atherogenic actions through the activation of PAF receptor on vascular smooth muscle cells (92). Moreover, they can trigger the PAF receptor of platelets (93) and explain, at least in part, the hyperaggregability of platelets in patients with acute coronary syndromes (94).

Lysophosphatidic acid (LPA)

LPA is present in the lipid-rich core of human atherosclerotic plaques where it may play a role in platelet activation after plaque rupture (95). LPA is generated by the hydrolysis of lysophosphatidylcholine by autotaxin (a member of the ectonucleotide pyrophosphatase/phosphodiesterase family with a unique lysophospholipase D activity) (96). It exerts potent pro-atherogenic activities by acting on specific GPCR on blood cells (platelets and monocytes), as well as on cells of the vessel wall (endothelial cells, smooth muscle cells, and macrophages). Both circulating and intestinal LPA appear to exert pro-atherogenic effects in experimental models (for a review see (97)).

Sphingosine 1-phosphate (S1P)

S1P, also known as lysosphingolipid, is generated within the cell by the phosphorylation of sphingosine (derived from ceramides) by two distinct sphingosine kinases (SK1 and SK2) and is transported to the extracellular space where it can activate cell-specific signalling pathways by binding to seven transmembrane span G-protein coupled receptors. Human studies have shown that S1P binds to apolipoprotein M and suggest that it may contribute to the atheroprotective effects of HDL (98). On the other hand, experimental studies show that S1P can have both pro- and anti-atherosclerotic effects, depending on the targeted cell-specific S1P receptors. Whereas S1P1 agonism reduces atherosclerosis (99), S1P2 and S1P3 receptors appear to have a pro-atherosclerotic role (100, 101).

Biaoctive lipids deriving from arachidonic acid

Arachidonic acid is the precursor of potent bioactive lipids comprising the prostaglandins, thromboxanes, leukotrienes, lipoxins, resolvins, and protectins. All mammalian cells, except erythrocytes, synthesize eicosanoids. All eicosanoids function locally at the site of synthesis, through specific GPCR. Two main pathways are involved in the biosynthesis of eicosanoids: the prostaglandins and thromboxanes are synthesized by the cyclic pathway; the leukotrienes by the linear pathway.

Products of COX

The cyclic pathway is catalysed by the cyclo-oxygenases (COX). Of the two COX isoforms, COX-1 is, in most cases, constitutively expressed, whereas COX-2 is an immediate early gene that is rapidly upregulated in response to proinflammatory stimuli. Both COX-1 and COX-2 convert arachidonic acid to an intermediate, PGH2, which is subsequently processed by specific enzymes to produce different types of prostaglandins (PG). COX-2 is more efficient than COX-1 for the generation of prostacyclin (PGI2) and PGE2. The different PGs bind specific receptors and exert pleiotropic effects. PGI2 and PGE2, via their binding to the IP and the EP2/EP4 receptors, respectively, exert anti-inflammatory and anti-thrombotic effects by driving the activation of adenyl cyclase and the release of cytoplasmic cAMP. In platelets, the elevation of cAMP, induced by PGI2,

suppresses their activation and thromboxane synthesis. In monocytes/macrophages, cAMP signalling inhibits cell adherence and migration, scavenger receptor endocytosis, phagocytosis and killing of bacteria, and the synthesis of proinflammatory cytokines such as TNF-α and IL-1β. In the context of atherosclerosis, exposure of platelets, monocytes, and macrophages to the COX-2 products, PGI2 and PGE2, should therefore be protective. Experimental atherosclerosis is accelerated in the absence of the IP receptor, supporting an atheroprotective role for PGI2. Interestingly, in agreement with the opposing role of ICAM-1 and PECAM-1 discussed earlier, the endothelium overlying the plaques of $IP^{-/-}$ atherosclerotic mice showed increased expression of ICAM-1 and decreased expression of PECAM-1 (102).

The role of thromboxane A2, another product of arachidonic acid by the cyclic pathway, is more complex. In atherosclerotic mice lacking the thromboxane receptor, or treated with COX/thromboxane inhibitors, atherosclerotic lesion size is reduced but lesions display a less stable plaque phenotype (103), in agreement with the adverse cardiovascular effects of potent COX inhibitors in atherosclerotic patients. Atherosclerotic patients are commonly treated with aspirin, which inhibits both isoforms of COX, whereas the potent COX-2 inhibitors (coxibs) that have been associated with adverse cardiovascular events selectively reduce vascular PGI2 synthesis without disrupting COX-1-derived thromboxane synthesis in platelets (104).

Products of LOX

The linear pathway is driven by the action of lypo-oxygenase (LOX) enzymes on arachidonic acid and gives rise to the leukotrienes (LTs). Leukocytes are major producers of LTs through the member of the LOX family, arachidonate 5-lipoxygenase, also known as 5-lipoxygenase (5-LOX). There are two types of LTs, LTB4 and the cysteinyl LTs, LTC4, LTD4, and LTE4. LTB4 has been extensively studied in the field of atherosclerosis (105, 106). It is a chemoattractant and a proinflammatory mediator able to trigger the activation of all types of leukocytes and the upregulation of proinflammatory genes in the target cells. The cysteinyl LTs act on non-leukocyte cells, such as epithelial, endothelial, and vascular smooth muscle cells.

Genetic and experimental studies suggest that LTs have a pathogenic role in atherosclerosis and its acute complications (myocardial infarction and stroke). Consequently, LT-targeting agents are gaining attention for the treatment of patients with atherosclerosis (107). However, inhibitors of leukotriene synthesis or of leukotriene receptor in experimental atherosclerosis have generated contradictory results so far (for a review see (108)).

A complex and specific transcellular LOX pathway gives rise to the production of lipoxins, a newly discovered class of signalling lipids also derived by the linear pathway of arachidonic acid but playing an essential role in resolving inflammation. The first step is the formation of a LT (LTA4), which can be favoured by the use of aspirin because aspirin inhibits solely the COX pathway leaving more arachidonic acid free for LOX enzymes. The production of LTA4 involves the combined action of 5-LOX and of the 5-LOX-activating protein (FLAP) to produce 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which is further metabolized by 5-LOX. These enzymes are all present in leukocytes. Subsequently, adherent platelets can convert LTA4, donated by leukocytes, to lipoxin (LX)A4 and LXB4, via the catalytic action of their 12-LOX (109). Interestingly, aspirin-triggered lipoxin are significantly lower in patients with atherosclerosis (110).

Resolvins

Resolvins (Rv) are endogenous lipid mediators biosynthesized from the major ω-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), denoted E series (RvE) and D series (RvD) resolvins, respectively. Like LX, Rv can be produced through transcellular cooperation, initiated by enzymes in endothelial cells and completed by adjacent leukocytes. The substrates EPA and DHA are released from membrane phospholipids, metabolized in a transcellular fashion, and secreted in amounts sufficient to reverse the course of inflammation, once the initial proinflammatory stimulus has been completely eliminated. Likely, the EPA-poor 'Western diet' may lead to reduced capacity to produce RvE and experimental studies have shown that Rv reduce the inflammatory process linked to atherogenesis and exert anti-atherosclerotic activities (111).

Conclusions

The induction of signalling in host defence is tightly regulated in physiological conditions to achieve the maximum effect on pathogens with minimal tissue destruction. In chronic inflammatory conditions, including atherosclerosis, the inflammatory processes are chronically engaged, even in the absence of pathogens, either due to persistence of the proinflammatory stimuli (such as modified lipoproteins) or due to the failure of regulatory mechanisms (e.g. bioactive lipids). This chronic activation enhances pathogenic cellular cross-talks, enhancing cell recruitment within the vessel wall, as well as the production of adhesion molecules and proinflammatory mediators such as cytokines and leukotrienes. The activation of inflammatory signalling in the

context of metabolic diseases such as atherosclerosis differs from inflammation induced by canonical pathogen recognition during host defence. This is accomplished by the selective use of specific innate immune receptors and the participation of scavenger receptors, as highlighted in this chapter. The challenge for the future is to devise therapeutics that are able to target atherogenic events leaving the host response unimpaired. Moreover, the most studied pathways in atherogenesis are hyperlipidaemia-dependent, while human disease is multifactorial, posing the challenge of understanding in detail how signalling is initiated and maintained in human disease.

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SECTION IV

Pathophysiology of other cardiovascular diseases

Section introduction

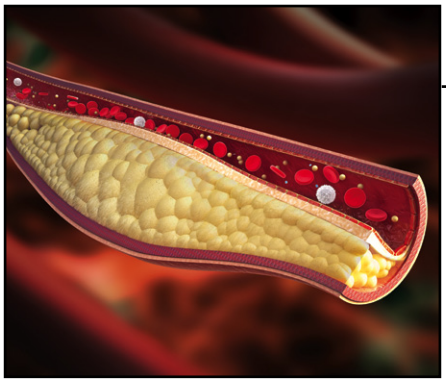
Esther Lutgens

In the previous sections of this text book, the structure, physiology, and biology of the normal vasculature, as well as the changes that occur during the most prevalent disease of the vasculature, atherosclerosis, have been discussed in detail. However, although the most prevalent, atherosclerosis is not the only disease of the vasculature.

This section of the book reports on the other important, prevalent diseases of the vasculature. In Chapter 15, Drs Kovanen and Bäck have done an excellent job in describing the pathogenesis of valvular heart disease. In Chapter 16, Dr Michel provides us with all the ins and outs on the pathophysiology of aneurysm formation and arterial dissection. Lastly, in Chapter 17, Drs Masson and Tombetti have minutely outlined the different vasculitides and their pathophysiology.

This important section of the textbook will provide great knowledge on the other diseases of the vasculature. After reading this section, I hope there will be an increased awareness that the vasculature can be affected by a plethora of diseases, and that recognition and understanding of the pathophysiology of these vascular maladies is of the utmost importance in developing proper treatment regimens for patients suffering from vascular disease.

I hope you'll read the next section with great interest.



CHAPTER 15

Valvular heart disease

Petri T. Kovanen and Magnus Bäck

Content

Introduction

Heart valves

The four heart valves open and close with every heartbeat, that is about 100,000 times per day, to maintain a unidirectional flow of the cardiac blood to the pulmonary and systemic circulations. The atrioventricular valves are referred to as the tricuspid and mitral valves, situated between the right atrium and ventricle, and the left atrium and ventricle, respectively. The pulmonary valve ensures the right ventricular outflow, whereas the ejection from the left ventricle into the aorta is maintained through the aortic valve.

The aortic valve consists of three leaflets (called cusps), corresponding to the physiological dilatations of the aortic root, which are referred to as the sinuses of Valsalva. The left and right cusps correspond to the aortic departure of the left and right coronary artery, whereas the posterior aortic valve leaflet is referred to as the non-coronary cusp. In addition to this common tricuspid anatomy of the aortic valve, a congenital bicuspid valve is found in approximately 0.5–2% of the general population, giving rise to differential biomechanical forces both on the valve and the aortic wall (1).

The mitral valve is made up of an anterior and a posterior leaflet, the edges of which are attached to the cardiac papillary muscles by thread-like bands called chordae tendineae.

Valvular heart disease

Valvular heart disease refers to valvular dysfunction due to congenital and/or acquired causes and can be divided into either stenotic or regurgitant lesions. In stenosis, there is a narrowing of the valve opening causing an obstruction of the valvular flow, whereas regurgitation refers to a back-flow through the valve. Valvular regurgitation may occur as a result of either changes to the structure to which the valve leaflets are inserted (secondary or functional regurgitation), or as a result of a malfunctioning of one or more valve leaflets (primary or organic regurgitation). The displacement of part of the valve leaflet below the level of coaptation is a referred to as valve prolapse, and is one cause of, for example, mitral regurgitation. Of note, valve stenosis and regurgitation may co-exist in the same valve and in addition, more than one heart valve may be affected at the same time.

In the Euro Heart Survey, which was performed in 2001 and included 5,000 individuals from 25 European countries, aortic stenosis was the most frequent

native valve disease and mitral regurgitation the second most common, followed by aortic regurgitation and mitral stenosis, which were observed with similar frequency (2). This chapter will specifically address the pathophysiology of aortic stenosis, and also provide a brief outline of the pathogenesis of mitral valve prolapse.

Aortic valve stenosis

Diagnosis and clinical presentation

The thickening and calcification of the aortic valve leaflets, which is referred to as aortic sclerosis, precedes the development of haemodynamically significant aortic stenosis, causing outflow obstruction from the left ventricle (➔ Fig. 15.1). Aortic valve stenosis is severe when the valve opening area falls below 1.0 cm^2 (or 0.6 cm^2/m^2, when normalized to body surface area; ➔ Table 15.1) (3). The haemodynamic consequences of the reduced valve opening can be measured in terms of pressure differences (gradients) over the stenotic valve by invasive catheterization, or calculated from the transvalvular velocities obtained by Doppler echocardiography (➔ Fig. 15.1). A haemodynamically severe aortic stenosis is present when the mean pressure gradient is above 40 mmHg and the maximal velocity is above 4.0 m/s (➔ Table 15.1) (3). In addition to morphological aortic valve analysis and quantitative assessment of stenosis

Table 15.1 Diagnostic criteria for the definition of severe aortic stenosis

Aortic valve area (AVA)	<1.0 cm^2
Indexed AVA (AVA/BSA)	<0.6 cm^2/m^2 body surface area
Mean gradient (Pmean)	>40 mmHg
Maximum jet velocity (Vmax)	>4 m/s
Velocity ratio	<0.25

Source data from Vahanian A, Alfieri O, Andreotti F, Antunes MJ, Baron-Esquivias G, Baumgartner H, Borger MA, Carrel TP, De Bonis M, Evangelista A, Falk V, Lung B, Lancellotti P, Pierard L, Price S, Schafers HJ, Schuler G, Stepinska J, Swedberg K, Takkenberg J, Von Oppell UO, Windecker S, Zamorano JL and Zembala M. Guidelines on the management of valvular heart disease (version 2012). Eur Heart J. 2012;33:2451–96.

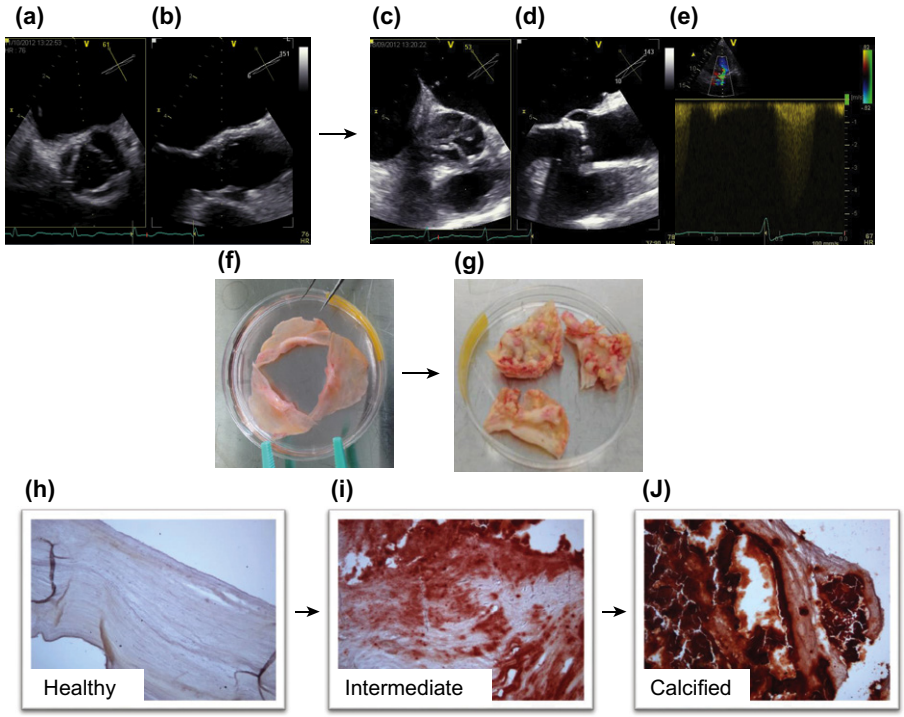


Fig. 15.1 Aortic valve stenosis. Top panels show echocardiographic images of a normal aortic valve: (a) short axis, (b) long axis; and of a stenotic valve with calcification and reduced opening (c) short axis, (d) long axis. (e) The velocity–time tracing of the systolic flow over a stenotic aortic valve using continuous wave Doppler. Note the maximum velocity above 4 m/s (cf. ➔ Table 15.1). The pressure difference between the left ventricle and the aorta (the transvalvular gradient) can be calculated using the Bernoulli equation in which the pressure difference equals four times the velocity squared ($4*v^2$). Middle panels show normal (f) and stenotic (g) human aortic valve derived from cardiac surgery (courtesy of Professor Anders Franco-Cereceda, Department of Thoracic Surgery, Karolinska University Hospital). Bottom panels show Alizarin red-stained histological sections for evaluation of calcium-rich deposits in aortic valves: sections of normal (h), intermediate (i), and calcified (j) human aortic valve tissue.

(Reproduced from Nagy E, Andersson DC, Caidahl K, Eriksson MJ, Eriksson P, Franco-Cereceda A, Hansson GK and Bäck M. Upregulation of the 5-lipoxygenase pathway in human aortic valves correlates with severity of stenosis and leads to leukotriene-induced effects on valvular myofibroblasts. Circulation. 2011;123:1316–25 with permission from Wolters Kluwer.)

severity by echocardiography, CT measurement of aortic valve calcification represents an important part of aortic stenosis evaluation (4), especially when other measures are not congruent (3).

The cardinal symptoms of aortic stenosis are exercise-induced chest pain (angina), dyspnoea on exertion and, at later stages, at rest, and exercise-induced syncope. On physical examination, a systolic murmur is auscultated over the aortic valve. At advanced stages, patients with aortic stenosis present with reduced left ventricular function and ensuing heart failure. The onset of reduced left ventricular function and/or signs and symptoms of overt heart failure is a sign for poor outcome and constitutes the indication for either surgical or transcatheter aortic valve replacement (3).

Epidemiology

Few studies have assessed the prevalence of valvular heart disease in European populations by echocardiography. In an initial study, performed in Helsinki in 1990, of a randomly chosen cohort of >500 individuals (ages 55 to 86 years), echocardiography revealed some degree of calcification in 53% of the subjects, with a significant increase with age, being present in 75% of subjects over 84 years old (5). The prevalence of severe aortic stenosis in this study was 3% and the prevalence of at least moderate aortic stenosis was approximately 5% in the age group between 75 and 86 years old (5). The Tromsø study used data from repeated echocardiographic examinations of >3,200 subjects during 1994 to 2008, and confirmed the increased prevalence of aortic stenosis with age (6). The reported aortic stenosis prevalence ranged from 0.2% in the 50–59-year cohort to almost 10% in the 80–89-year cohort (6). Moreover, the latter study established an incidence rate of 5%/year for aortic stenosis in the whole studied population. Similar figures have been reported in American cohorts (7, 8), and the data support the notion that aortic stenosis preferentially affects adults of advanced age. A recent meta-analysis integrating echocardiographic and CT measures of aortic valve calcification underlined the age-dependency and also reported an increase in morbidity and mortality associated with aortic valve calcification (9). It should, however, be noted that aortic stenosis on a congenital bicuspid valve is the dominating aetiology in younger age groups.

Risk factors

As mentioned, age is a dominating determinant for the risk of aortic valve calcification and stenosis (5–7), which in addition has a male predominance (7, 10). Furthermore, aortic valve stenosis shares several risk factors with atherosclerosis, e.g. hyperlipidaemia, hypertension, smoking, and diabetes (7, 10–13). In contrast, body mass index (BMI) has been reported to be inversely correlated with aortic valve calcification (11). Finally, bone mineral density is negatively associated with aortic valve calcification (14), which underlines the complexity of valvular calcification, a topic further discussed later in the chapter.

Whereas impaired kidney function is only modestly associated with aortic valve calcification (15), several studies of patients with end-stage chronic kidney disease and haemodialysis have identified a higher prevalence of aortic valve calcification and aortic stenosis, which actually occurs in these patients 10–20 years earlier than in the general population (16). In further support of a disturbed calcium and phosphate balance as a risk factor, parathyroid hormone and vitamin D levels have also been associated with the prevalence of aortic valve calcification (11) and aortic stenosis progression (17), suggesting that secondary hyperparathyroidism in chronic kidney disease may further accelerate aortic valve calcification.

Aortic valve calcification

Initially regarded as a passive development, aortic valve calcification involves several active processes. Two forms of active calcification have been identified in the human aortic valve: dystrophic calcification and heterotopic ossification (18, 19).

Dystrophic calcification

By far the most prevailing form of calcification in human stenotic aortic valves is dystrophic calcification (18), which refers to valvular deposition of calcium and phosphate complexes with an apatite structure. Nucleating structures, such as apoptotic cells, may serve as a starting point for apatite formation, and the valvular interstitial cells actively participate in this process by alterations of the extracellular matrix and by producing a number of non-collagenous matrix proteins, which act as regulators of biomineralization (cf. *infra*). Activated valvular interstitial cells, as well as infiltrating leukocytes, are a source of proteases, e.g. matrix metalloproteinases (MMPs) (20) and cathepsins (21), which induce an adverse extracellular matrix remodelling. One of the initiators of dystrophic calcification in the valve is the elastin degradation by gelatinases, such as MMP-9, which is gradually upregulated in the *continuum* of aortic stenosis development (22), and increased in aortic stenosis compared with other pathologies of the aortic valve (23).

The importance of the calcium and phosphate balance in dystrophic calcification has been raised based on the increased valvular calcification in chronic kidney disease, and supported by the calcification of valvular interstitial cells derived from different species in the presence of high

concentrations of either organic or inorganic phosphates. The *in vitro* calcification of human valvular interstitial cells induced by β-glycerophosphate is dependent on alkaline phosphatase activity (24) (Fig. 15.2). Studies of explanted human aortic valves have located alkaline phosphatase near calcified areas (25) and revealed increased levels in calcified, compared with normal, valves (26), with the highest levels in patients with a haemodynamically more severe aortic stenosis (25). Inorganic phosphates generated by alkaline phosphatases can be taken up by valvular interstitial cells through the sodium-dependent phosphate transporter Pit-1 (Fig. 15.2). Dimeric phosphates, referred to as pyrophosphates, act as inhibitors of calcification and are generated through ATP hydrolysis by membrane-bound enzymes such as ectonucleotide pyrophosphatase 1 (ENPP1) (27). Subsequent pyrophosphate metabolism by alkaline phosphatases will, however, increase the phosphate-to-pyrophosphate ratio, hence favouring valvular calcification.

Membrane matrix vesicles are secreted by many cell types through a budding process and have been identified in human aortic valves. Matrix vesicles derived from a mineralizing cell will exhibit the membrane characteristics and carry content derived from the parent cell. The participation of matrix vesicles in valvular dystrophic calcification may be both by favouring nucleation of hydroxyapatite deposition and by regulating phosphate and calcium homeostasis (28).

Heterotopic ossification

The transdifferentiation of valvular interstitial cells towards an osteogenic phenotype will induce the formation of an osteoid matrix in the valve, similar to mature lamellar bone formation with haematopoietic elements and active bone remodelling. An osteoblast-like cellular phenotype has indeed been identified in calcified human aortic valves (26). Heterotopic ossification, however, contributes to a smaller proportion of aortic valve calcification, and was present in only 13% of the valves in the initial characterization (18).

Although the exact mechanism today remains largely unknown, transdifferentiation of valvular interstitial cells may involve epigenetic changes (29), as well as several signalling pathways, which will be further discussed. The two transcription factors, runt-related transcription factor 2 (Runx2/Cbfa1) and osteorix (SP7), are central regulators of osteoblastogenesis and have been detected in calcified human aortic valves (26, 30, 31).

The valvular osteoblastic cell types secrete osteogenic proteins, such as bone morphogenetic proteins (BMP) and osteocalcin, which participate in valvular calcification

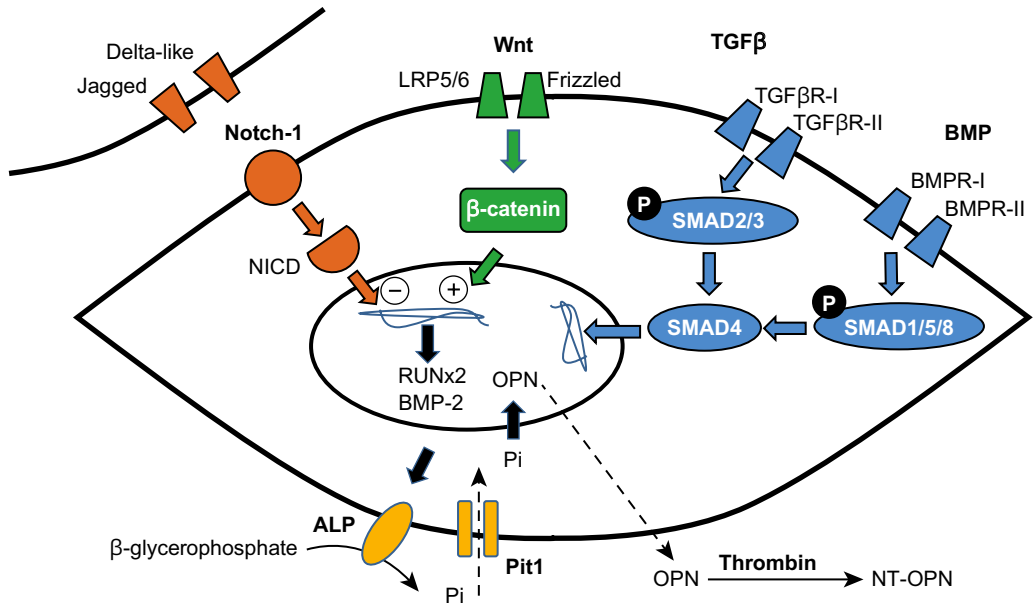


Fig. 15.2 Signalling pathways of calcification in valvular interstitial cells. Notch-1 binding to delta-like or jagged ligands on neighbouring cells liberates its intracellular domain (NICD), which translocates into the nucleus and negatively regulates gene expression. The canonical wingless (Wnt) pathway is initiated when Wnt agonists bind LRP 5 and 6, with Frizzled proteins as co-receptors, which will lead to the accumulation of β-catenin, which translocates to the nucleus and regulates expression of target genes, including BMP-2. TGFβ, and BMPs, activate canonical pathways by phosphorylation of the receptor-regulated SMAD-2/3, and SMAD-1/5/8, respectively. Inorganic phosphates generated by ALP can be taken up by valvular interstitial cells through the sodium-dependent phosphate transporter Pit-1. See text for detailed description of the pathways and their interactions.

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenic protein; LRP, LDL receptor related protein; NICD, notch intracellular domain; NT, N-terminal; OPN, osteopontin; Pi, inorganic phosphate; Pit1, sodium-dependent phosphate transporter; RUNx2, runt-related transcription factor 2; TGF, transforming growth factor.

(cf. *infra*). Furthermore, biomechanical stresses can induce microfractures in the heterotopic ossification of the aortic valve (1, 18). Osteoclasts are formed by the fusion of mononuclear circulating precursors, and participate in the process of bone remodelling. Osteoclast-derived proteases, such as MMP-9, cathepsin K, and tartrate-resistant acid phosphatase (TRAP), may further aggravate the valvular calcification (18, 30).

Signalling pathways in valvular calcification

Wnt signalling

Signalling through the canonical wingless (Wnt) pathway, also referred to as the Wnt/β-catenin pathway, regulates a number of cellular and developmental processes. Wnt agonists bind the low-density lipoprotein-receptor-related proteins (LRP) 5 and 6, with Frizzled proteins as co-receptors, which will lead to the accumulation of β-catenin in the cytoplasm (➔ Fig. 15.2). The subsequent β-catenin translocation to the nucleus regulates expression of target genes, including BMP-2 (19). In support of an active Wnt/β-catenin pathway in aortic valve calcification, Wnt3, LPR5, and β-catenin are upregulated in calcified tricuspid aortic valves (32).

Notch1 signalling

The *NOTCH1* gene encodes a single transmembrane protein that upon binding to delta-like or jagged ligands on neighbouring cells is cleaved, after which the liberated intracellular domain translocates into the nucleus and negatively regulates gene expression (➔ Fig. 15.2). Notch signalling plays an important role in cardiac development, and *NOTCH1* mutations have been associated with valvular calcification and familial clustering of bicuspid aortic valve (33). The mechanisms by which Notch1 regulates valvular calcification include repression of Runx2 (33) and, as recently demonstrated, also regulation of MGP expression in endothelial cells (34).

SMAD signalling

Transforming growth factor (TGF) β and bone morphogenic proteins (BMPs) are members of the TGF-β ligand superfamily. TGFβ binding to the TGFβ receptor type I and II will activate its canonical pathway by phosphorylation of the receptor-regulated SMAD-2/3, whereas the canonical BMP signalling pathway is coupled to SMAD-1/5/8 (35) (➔ Fig. 15.2). For both pathways, the type II receptor phosphorylates and activates the type I receptor, which in turn phosphorylates the downstream SMADs. The TGFβ and BMP pathways converge when the respective phosphorylated SMADs bind to co-SMAD-4 and translocate into the nucleus to induce gene expression (➔ Fig. 15.2). In contrast, SMAD-6 acts as an inhibitor of BMP signalling by preventing the formation of the SMAD1-SMAD4 complex (35).

TGFβ is upregulated in calcified aortic valves (36), and stimulation of valvular interstitial cells with TFGβ induces a myofibroblastic phenotype with alpha-smooth muscle actin expression (37). In addition, TGF-β may also activate SMAD-independent Wnt/β-catenin signalling to differentiate valvular interstitial cells (38).

BMP-2 and BMP-4 are upregulated in calcified areas of stenotic aortic valves and increase alkaline phosphatase expression (22, 39, 40), indicating a BMP-induced osteogenic differentiation. This is further supported by the BMP-2-induced Runx2 and osteopontin expression in aortic valve interstitial cells through the SMAD1 pathway (40).

Regulators of calcification

Gla proteins

Proteins containing glutamate residues, which by vitamin K-dependent carboxylation form γ-carboxyglutamate, are referred to as Gla proteins. Among the extrahepatic vitamin K-dependent proteins, the bone Gla protein (BGP or BGLAP) osteocalcin, the matrix Gla protein (MGP), and the Gla-rich protein (GRP) have been detected in human aortic valves (26, 34, 41). Whereas osteocalcin is an osteoblast marker, which stimulates the calcification of bone matrix, the γ-carboxylated form of matrix Gla protein (MGP) sequesters BMP-2 to protect non-osseous tissues from calcification. The notion of Gla proteins in valvular calcification originates from the co-administration of warfarin and vitamin K (to specifically inhibit extrahepatic γ-carboxylation), which induces calcification of the elastic lamellae of rodent aortic valves, ascribed to decreased MGP carboxylation. Consequently, mice lacking MGP exhibit extensive cardiovascular calcifications (19, 42, 43). Finally, warfarin use has been associated with increased aortic valve calcification in haemodialysis patients (44).

Non-collagenous matrix proteins

Glycoproteins appear both as structural and secreted components of calcifiable matrices and may bind calcium and/or phosphate, as well as modulate phosphatase activity (45). Likewise, collagen-interactive phosphoproteins secreted into the extracellular matrix may nucleate mineralization by interacting with collagen fibrils (46).

The glycophosphoprotein osteopontin is increased in calcified, compared with healthy, aortic valve tissue (30) and its expression correlates with valvular macrophage infiltration (47). Interestingly, whereas osteopontin mediates anti-calcification, its cleavage by thrombin generates an

N-terminal osteopontin fragment that, in contrast, appears to stimulate calcification in aortic valves (48). Examples of other glycoproteins that have been implicated in aortic valve calcification are osteonectin and bone sialoprotein (26, 39). In contrast to those pro-calcifying glycoproteins, fetuin-A, which belongs to the cystatin superfamily of cysteine protease inhibitors, is a negative regulator of biomineralization. Circulating fetuin-A levels are decreased in chronic kidney disease, as well as in subjects with aortic stenosis (49), and mice lacking fetuin-A exhibit increased ectopic calcification (50). These findings support that the negative regulation of biomineralization by fetuin-A is involved in the regulation of aortic valve calcification.

OPG/RANKL/RANK pathway

Activation of RANK (receptor activator of nuclear factor kappa B), which is expressed on the surface of osteoclasts, by the RANK ligand (RANKL) is critical for osteoclast differentiation. In contrast, osteoprotegerin (OPG), a soluble receptor that is part of the TNF receptor superfamily, binds to RANKL and hence blocks its interaction with RANK to prevent bone destruction. The expression of the RANKL/RANK/OPG pathway has been demonstrated in human aortic valves (18, 30, 51).

In contrast to the effects in osseous tissue, RANKL induces calcification of human aortic valve interstitial cells *in vitro*, which is associated with an increased alkaline phosphatase and osteocalcin expression (51). In line with those findings, the RANKL/OPG-ratio is highest in calcified regions of stenotic aortic valves (30). Moreover, it has been observed that addition of high-density lipoproteins (HDL) to cultured valvular interstitial cells increased OPG-secretion without increasing RANKL secretion, so lowering the RANKL/OPG-ratio and suggesting that HDL could inhibit valvular calcification (52).

Inflammation

Aortic valve stenosis is a chronic inflammatory disease and, in terms of its pathogenesis, it can be considered to be an active atheroinflammatory process in the affected leaflets (53). As in atherosclerosis, the key drivers in the calcific aortic valve disease stenosis are lipid accumulation, infiltration of inflammatory cells, fibrosis, calcification, and neoangiogenesis. Thus, it is not surprising that many cellular and molecular components are operative in both diseases, although their relative importance may differ and be disease-specific. An atherosclerotic plaque is called a fibroatheroma, which refers to its two main components, fibrosis and atheros (Greek; *ather* = gruel; named because of the soft lipid), i.e. two components characterizing also a diseased aortic leaflet. However, there is one key feature that separates atherosclerosis and aortic valve stenosis—the calcification. Thus, while in atherogenesis tissue calcification is a rather late event, in aortic valve stenosis it is present throughout the duration of the valve disease, and increasingly dominates its pathology as the disease progresses (54). Interestingly, however, calcification of an atherosclerotic plaque has been conceptualized as a convergence of bone biology and vascular inflammation pathobiology (55), and determination of the extent and degree of coronary artery calcification has been incorporated into clinical practice as an indicator of advanced coronary atherosclerosis and as a robust marker of coronary artery disease risk (56). Likewise, valvular calcification measured by CT is associated with increased risk of myocardial infarction, hence reinforcing the link between valvular and coronary heart diseases (57).

Immune cells in aortic stenosis

A normal aortic leaflet contains only few scattered macrophages and mast cells, but apparently no T lymphocytes (58–60). The stenotic process starts by thickening of focal areas on the aortic side of the leaflets. In these mildly raised leaflet areas, the numbers of macrophages and T lymphocytes are increased, whereas in advanced thickenings associated with clinically significant stenosis, high numbers of all three inflammatory cell types are present (58–60) (➔ Fig. 15.3). Moreover, the numbers of valvular mast cells strongly associate with the degree of aortic stenosis (61). In another study of surgically excised aortic valves displaying end-stage disease, mast cells were especially prominent in the atheromatous regions of the valves (18).

The typical location of an inflammatory cell within the leaflet depends on the cell type and on the stage of the disease. Thus, in the thin, healthy leaflets, the macrophages are diffusely scattered within the tissue stroma, while the mast cells reside subendothelially, i.e. close to the endothelial cells (59, 60). In stenotic leaflets, all three cell types are widely distributed throughout the leaflet, the T lymphocytes and mast cells being associated especially with the calcific deposits, and so providing a link between inflammation and calcification at the cellular level (58–60).

Macrophages belong to the system of innate immunity and T lymphocytes to the system of adaptive immunity, while mast cells stand at the interface between the two systems. Thus, the inflammatory reaction in a diseased valve includes cellular responses involving both the innate and the adaptive immune system (62, 63). Importantly, all three inflammatory cell types in the diseased valves show signs of activity as a sign of an ongoing inflammatory

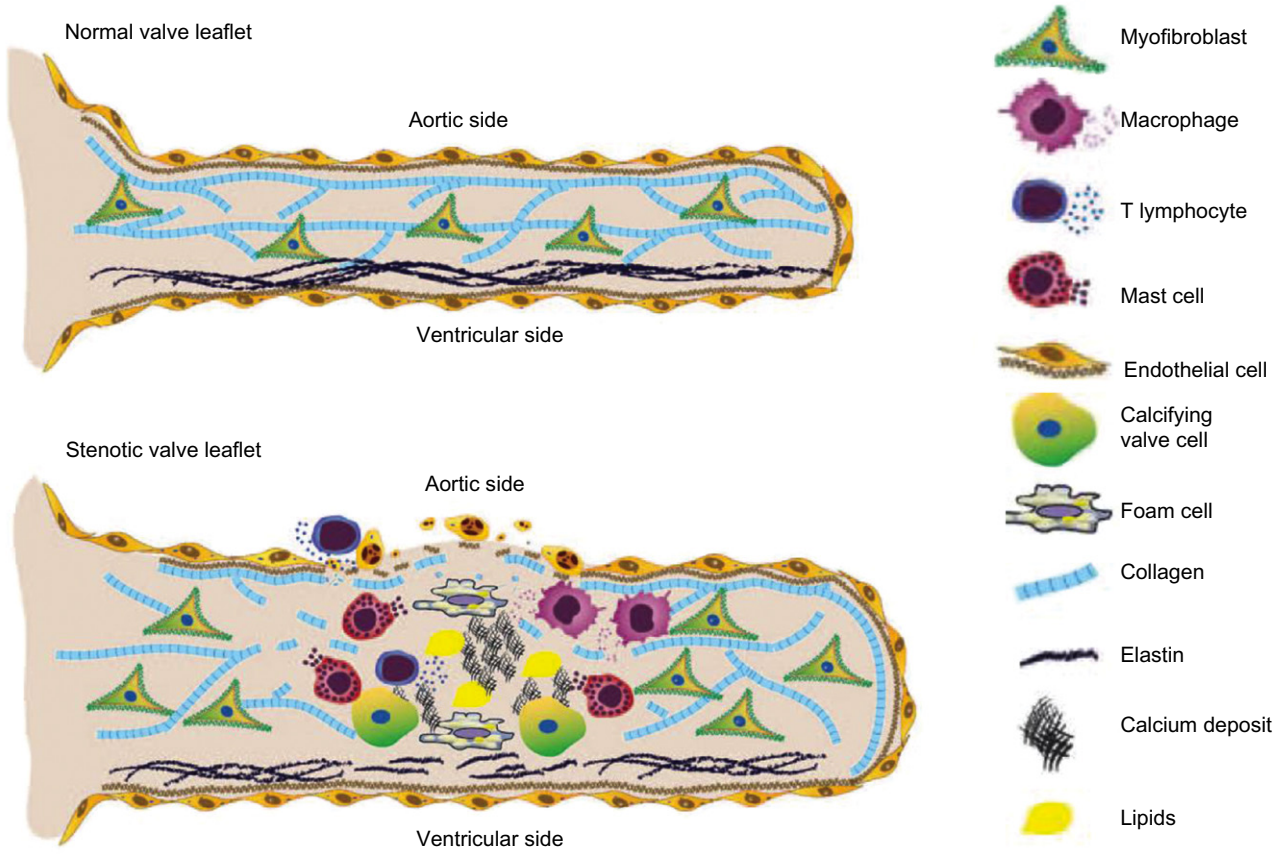


Fig. 15.3 Pathophysiological changes in aortic valve stenosis. Schematic cross-sections of a normal (top) and stenotic (bottom) aortic valve leaflet. The normal aortic leaflet is composed of three layers, with the collagen-rich fibrosa towards the aortic surface, the elastin-rich ventricularis towards the left ventricle, and the spongiosa in between these layers. In the stenotic leaflet, the interstitial cells undergo a phenotypic transdifferentiation towards a myofibroblast or osteoblast phenotype (calcifying valve cell). On the aortic side, the endothelial cells are dysfunctional and express adhesion molecules. Moreover, they are leaky and show erosions. Lipoproteins and inflammatory cells (monocytes, T lymphocytes, and mast cell progenitors) enter the leaflet from the aortic side. Subendothelially, the monocytes differentiate into macrophages and the mast cell progenitors differentiate into mature mast cells filled with secretory granules. The pathophysiological changes in the stenotic valve are characterized by accumulation of intracellular lipids (macrophage foam cells) and extracellular lipids, activation of the inflammatory cells, calcification, and collagenous fibrotic thickening. In addition, the extracellular matrix becomes remodelled so that the collagen/elastin ratio increases. Continuous calcification and matrix remodelling together thicken the valve, render is stiff, leading to reduced valvular opening and a progressive obstruction of the ventricular outflow.
(Reproduced from Helske S, Kupari M, Lindstedt KA and Kovanen PT. Aortic valve stenosis: an active atheroinflammatory process. Curr Opin Lipidol. 2007;18:483–91 with permission from Wolters Kluwer.)

process. For example, already in the earliest focal lesions, a significant fraction of macrophages are filled with cytoplasmic lipid droplets, i.e. they have actively ingested extracellular lipids (58). Furthermore, in contrast to the T lymphocytes in control valves, a fraction of them in stenotic valves express interleukin-2 (IL-2) receptors as a sign of long-term activation (59). The findings on mast cells in aortic valves well agree with the functional status of the macrophages and T lymphocytes. Accordingly, in normal valves the few mast cells present are resting, but in stenotic leaflets most of the numerous mast cells have been stimulated to degranulate, as evidenced by the presence of extracellular cytoplasmic secretory granules in their immediate vicinity (60).

Immuno-activators

Among the potential activators of inflammatory cells in the diseased leaflets, the complement system is a good candidate. Thus, the system is activated in stenotic aortic valves (64), and its effector molecules C3a and/or C5a are capable of activating macrophages (65), lymphocytes (66), and mast cells (67). Regarding the persistent activation of T lymphocytes, it was suggested that it results from an immunological reaction to local antigens, e.g. injured matrix components (59). Also, oxidatively modified plasma lipoproteins in the stenotic lesion, like oxidized low-density lipoproteins (oxLDL), could serve as a persistent local immunogen (68). Indeed, oxLDL is highly immunogenic, as reflected by the presence of IgG anti-oxLDL antibodies in the circulation and in atherosclerotic

lesions of patients with atherosclerotic vascular disease (69, 70). Moreover, oxLDL-IgG immune complexes deposit in atherosclerotic plaques (70) and they can activate macrophages (71), mast cells (72), and the classical complement pathway (73), so providing the immune complexes potentially important roles also in the chronic activation of both innate and adaptive immune systems in diseased aortic valves.

Leukotrienes are lipid mediators of inflammation derived from arachidonic acid metabolism by the enzyme 5-lipoxygenase (5-LO), and exert proinflammatory effects by means of specific receptors expressed on several immunological cell types (74, 75). Macrophages in human stenotic aortic valves express 5-LO and other enzymatic components of leukotriene biosynthesis (22). The mRNA levels of 5-LO are gradually upregulated in human aortic valve tissue classified as healthy, thickened, and calcified, and they significantly associate with stenosis severity as determined by echocardiography (22). In addition, leukotriene release into conditioned media derived from human aortic valves correlates with the degree of valvular calcification (76). Besides the immune cells, valvular interstitial cells also express leukotriene receptors and exhibit leukotriene-induced effects in terms of, for example, increased oxidative stress and induction of calcification pathways (22, 77), suggesting that leukotrienes may both activate the inflammatory response and directly contribute to calcification in stenotic aortic valves (78). Leukotriene receptor antagonists clinically used in the treatment of asthma have been associated with a decreased cardiovascular risk (79) and with a non-significant reduction of aortic stenosis incidence (80) in retrospective pharmacoepidemiological studies.

Endothelial activation

Since the early aortic valve lesions are characterized by infiltration of inflammatory cells and calcification in the subendothelial layers on the aortic ('upper') side of the leaflets, it is likely that endothelial activation with ensuing dysfunction, or even endothelial injury on the aortic side of the leaflets as a response to side-specific fluid shear mechanical stress, are early events in aortic valve stenosis (1, 81) (➲ Fig. 15.3). Indeed, morphological evidence of endothelial damage is found already in early valvular lesions, which are uniformly located on the aortic side of the leaflet, and characterized by loss of individual endothelial cells and by disruptions of the endothelial basement membrane (58). The loss of endothelial cells leads to exposure of subendothelial thrombogenic and procoagulant tissue and results in local activation of platelets and formation of microthrombi, as also observed in the advanced stage of aortic stenosis (82). Activated platelets secrete growth factors, notably the platelet-derived growth factor (PDGF), capable of promoting proliferation of smooth muscle cells and inducing synthesis of the various components of extracellular matrix by them. In analogy, loss of endothelium (erosion) in the early valvular lesions could lead to activation of the valvular interstitial cells with ensuing fibroproliferative response and valvular thickening by the platelet-dependent mechanisms. Of great interest are the findings demonstrating that adhesion molecules, such as the intracellular cell-adhesion molecule 1 (ICAM-1), vascular cell-adhesion molecule 1 (VCAM-1), and E-selectin, all are expressed on the endothelium of stenotic but not normal aortic leaflets (83). Thus, the adhesion molecules expressed by activated endothelial cells are likely to both initiate and facilitate the early recruitment of inflammatory cells into the subendothelial regions on the aortic side of disease-prone valves.

Obviously, the very initial proinflammatory trigger of endothelial activation in human leaflets remains unknown. Since infiltration of plasma lipoproteins, notably of the low-density lipoproteins (LDL), is an early event in the disease-prone leaflets, it is reasonable to hypothesize that their local oxidation with ensuing generation of the proinflammatory oxLDL particles capable of activating the endothelial cells (84) is at least one of the starting events triggering the long process of calcific aortic valve stenosis.

Lipid accumulation

Aortic valve disease in animal models of hypercholesterolaemia

As mentioned, oxLDL has received prominence as being a candidate for the initial activation of endothelial cells. In addition, the endothelial activation and dysfunction allow the plasma lipoproteins to enter the subendothelial space and to be retained within the matrix (85). The notion of lipid-induced valvular changes has received support from animal studies. For example, genetically hypercholesterolaemic mice exhibit endothelial activation in the aortic valve commissures, which represent regions of high mechanical valvular stress (86). In addition, LDL receptor-deficient mice with high levels of apoB100-containing LDL ($Ldlr^{-/-}Apob^{100/100}$ mice) develop valvular calcification and, with increasing age, also aortic transvalvular gradients similar to human aortic valve stenosis (87). Since those genetically engineered mice show extreme hypercholesterolaemia since birth, the results may be applicable only to patients with such a condition, like the extremely rare patients with homozygous familial hypercholesterolaemia, who develop aortic stenosis in their teenage years (88) Also, cholesterol-fed rabbits develop extreme hypercholesterolaemia, and

accumulation of lipoprotein-derived lipid particles in the aortic valves is observed (89). However, these highly non-physiological animal models only show that it is possible to induce valvular thickening by forcing the development of atherosclerotic lesions also in the aortic valves.

Lipids and lipoproteins in human aortic valves

In human valves, lipoprotein-derived lipids start to accumulate in the affected leaflets, and early valvular lesions and coronary lesions may develop in the presence of mild hypercholesterolaemia, or even in the absence of hypercholesterolaemia (58). This can be explained by the multifactorial aetiology of both arterial diseases, and by the fact that the defined 'normal' range for LDL-cholesterol level in humans is significantly higher than the range of physiological levels (90).

The lipids in the aortic valve leaflets have been detected with Oil-Red O, which stains neutral lipids, i.e. cholesterol esters and triglycerides (58). Both intracellular and extracellular neutral lipids are observed in stenotic aortic valves (➔ Fig. 15.3). In the early lesions, numerous macrophage foam cells are visible subendothelially, while deeper in the lesions, where only few macrophages are present, lipids accumulate extracellularly, often along collagen fibres (58). This finding can be explained, when considering that apoB-100, which normally does not bind to collagen, can be linked to collagen via the small proteoglycan decorin, which co-localizes with collagen in the valves (91, 92). Another small proteoglycan, biglycan, localizes chiefly to the subendothelial layer and, importantly, both in normal valves and stenotic valves it associates with apoB-100 and apoE, so providing an additional mechanism for the site-specific accumulation of the atherogenic lipoproteins in the valves (52). The cause of accumulation of extracellular lipid particles is considered to be the binding of infiltrated apoB-100-containing lipoproteins to the subendothelial proteoglycans in the disease-prone areas of the arterial system, a phenomenon called 'retention' of lipoproteins (93). This mechanism also applies to lipid accumulation in the disease-prone stenotic aortic valves. These findings reveal that the macrophages have been actively ingesting extracellular lipids and have been converted to foam cells. In addition, the macrophages co-localize with oxidized lipids, which also trigger an inflammatory reaction in the tissue. In this sense, lipid accumulation and its link to local inflammation in the aortic valves may be similar to what has been observed in atherosclerotic lesions.

Lipoproteins

The excess valvular lipids are derived from circulating lipoproteins, rather than being synthesized locally. Thus, the immunohistological studies that have revealed the presence of apoB-100, and to some extent also of apoE in aortic valves, actually demonstrate infiltration of the atherogenic lipoproteins, notably LDL, and to some extent of the triglyceride-rich intermediate-density lipoprotein (IDL), which can also carry cholesterol into the valvular lesions (94). On the other hand, the amount of apoA-I is reduced in stenotic valves, probably reflecting degradation of this anti-atherogenic and anti-inflammatory protein component of the HDL particles by extracellularly located proteases in the inflamed leaflets (52).

The diseased valves also show positive immunostaining for the anti-apo(a) antibody, which reveals the presence of Lp(a) (94). Lp(a) is an LDL-like lipoprotein, which contains, in addition to apoB-100, apo(a), which possesses structural homology with plasminogen and plasmin, but lacks fibrinolytic activity (95). Initial studies brought the attention to aortic valve calcification being associated with serum levels of Lp(a) (7, 96). In a genome-wide association study (GWAS), a single nucleotide polymorphism (SNP) in the gene encoding Lp(a) was significantly associated with aortic valve calcification (as determined by CT), as well as with incident aortic stenosis (97). Subsequent reports replicated this observation, and also associated elevated Lp(a) levels and corresponding genotypes with increased risk of aortic stenosis (98, 99). In addition, Lp(a) can be linked to the circulating lipoprotein-associated phospholipase (Lp-PLA_2). This enzyme is also secreted by tissue macrophages and shows an increased expression in stenotic aortic valves, where it has been considered to possess multiple activities, notably an ability to lower oxidative stress by hydrolysing oxidized phospholipids, and also to promote local inflammatory activity and mineralization via generation of lysophospholipids, such as lysophosphatidylcholine (LPC) (100). More specifically, LPC can trigger valvular interstitial cells to induce calcification in association with increased expression of ENPP1, alkaline phosphatase, and the phosphate transporter Pit-1 (100).

Lipoprotein modification

Interestingly, the accumulation of lipids in aortic valves has been considered to trigger valvular calcification (58, 101). It is of importance, however, to recognize that native lipids derived from the infiltrating lipoproteins are not bioactive, and that local modification of the lipoprotein lipids is likely to be necessary for the initiation of various cellular reactions relevant to the pathogenesis of thickening and calcification of the aortic leaflets. Thus, proteolytic and lipolytic enzymes, and oxidative agents released into the extracellular fluid, particularly by macrophages and mast cells, are capable

of extracellularly modifying the proteoglycan-bound, i.e. retained, lipoproteins (102). The modified lipoproteins can aggregate and fuse, and such lipoprotein-derived large lipid particles have been found to accumulate extracellularly in human stenotic aortic valves (102). Importantly, oxLDL in stenotic valve leaflets co-localizes with inflammatory cells and calcium deposits (68), and, moreover, oxLDL has been shown to activate vascular biomineralization and vascular osteogenic signalling processes (103, 104).

The LPC is a particularly interesting bioactive product generated by lipoprotein modification, which, in addition to the already mentioned Lp-PLA_2, is generated by oxidation or by the action of another phospholipase, the type IIA secretory PLA_2. This enzyme is present in stenotic aortic valves (105), and when bound to proteoglycans, is capable of hydrolysing LDL particles (106) and of inducing their fusion to generate extracellular lipid droplets, such as is found in human aortic valves (102, 107).

Another interesting bioactive molecule in extracellularly modified LDL particles is unesterified cholesterol (UC), which may crystallize and then cause a strong inflammasome-dependent inflammatory response in macrophages (108, 109). Interestingly, in hypercholesterolaemic rabbits, the first sign of lipid accumulation in aortic valves is the appearance of extracellular lipid particles rich in UC (89). UC can be generated by the action of lysosomal acid lipase, an enzyme secreted by activated macrophages, and also released from dying macrophages (110), i.e. from cells abundantly present in stenotic aortic valves. Thus, modified LDL particles can activate not only the structural cells, but also the valvular inflammatory cells, which, by secreting a multitude of cytokines and other bioactive molecules can accelerate valvular fibrosis and calcification (➔ Fig. 15.3). Once lipoprotein particles are retained and modified in the aortic valve, the continuous generation of proinflammatory lipid species may trigger self-perpetuating positive-feedback loops, which cannot be controlled any more by the currently available pharmacotherapies.

Valvular foam cells and scavenger receptors

The oxidized and otherwise modified forms of LDL particles bind to scavenger receptors (111), which, by mediating uptake of the modified lipoproteins, induce conversion of macrophages into lipid-filled foam cells, a pathogenic process well characterized in atherosclerosis (109), and also identified in stenotic aortic valves (58). Among the scavenger receptors, SR-A1 is involved in foam-cell formation, and its expression level was found to be increased in macrophages of stenotic aortic valves, while the expression of the two other major scavenger receptors, CD36 and LOX-1, was confined to endothelial cells and interstitial cells in the diseased valves (112). Moreover, cultured valvular interstitial cells derived from stenotic valves accumulated more oxLDL compared with those derived from healthy valves. These findings revealed an enhanced foam cell-forming potential of valvular interstitial cells in the diseased valves, which may alter the role of these cells in the key processes of fibrosis and calcification. Although the role of endothelial LOX-1 in aortic valve stenosis remains to be established, we can envision that, by mediating binding and uptake of oxLDL it contributes to endothelial dysfunction (113).

Extracellular matrix remodelling

An aortic valve leaflet thickens as a result of fibrosis, i.e. an increase in the amount of collagen. The fibrotic change is associated with remodelling of the extracellular matrix, which results from a strong increase in the amount of leaflet collagen and a concomitant decrease in the amount of elastin. Such increase in the valvular collagen/elastin ratio provides a key pathogenic mechanism to the slowly progressing disease, involving both thickening and loss of elasticity of the leaflets. Indeed, the increase in the collagen/elastin ratio, together with an increase in calcification, jointly leads to stiffening of the leaflet and restriction of the cusp movement. Thus, these pathological processes, and particularly their intensity and speed, are the very determining factors that dictate the progression of valvular stenosis into a clinically significant obstruction of the ventricular outflow.

Valvular collagen and elastin are continuously synthesized and degraded, although their turnover is likely to be very slow. It appears that in the thickening leaflets, both synthesis and degradation of collagen are increased, the former more than the latter, so resulting in net collagen synthesis (60, 114). In contrast to the complex regulation of collagen metabolism, the mechanisms leading to loss of elastin involve primarily, even if not solely, activation of elastin-degrading pathways and a decrease in elastin amounts (60, 114). The molecular mechanisms behind the net accumulation of collagen involve activation of profibrotic mechanisms and inactivation of anti-fibrotic mechanisms. During adverse matrix remodelling, collagen not only accumulates, but its composition and architecture (fibre density, structure, orientation, cross-linking, etc.) are also altered. Such compositional changes of collagen, which alter its structural mechanical properties and lead to stiffening, are the result of both the increased synthesis and increased degradation of collagen. The collagen-degradation pathways involve the activity of various collagenolytic MMPs, the

synthesis, activation, and inhibition are tightly controlled (20, 23, 115–117). Such control is nicely exemplified by the overexpression of the MMP inhibitor, TIMP-1, which accompanies the increased expression and activity of the MMPs in stenotic valves (23). Some of the regulatory pathways leading to collagen accumulation and elastin loss are presented later in the chapter.

Angiotensin

It is reasonable to assume that a major driver of fibrosis in stenotic aortic valves is angiotensin II (Ang II) (➔ Fig. 15.4). The presence of the angiotensin-converting enzyme (ACE), the generator of Ang II, in stenotic valves was initially demonstrated by immunohistochemistry, which also revealed a co-localization with apoB-100 (118). As some circulating LDL particles also contain ACE, it was concluded that ACE may enter the valves together with LDL particles. Later, it was demonstrated that enzymatically active ACE is also locally produced in aortic valves, and that its expression is augmented in stenotic valves (60).

Besides ACE, other enzymes are also capable of generating Ang II, notably the mast cell-derived neutral proteases, chymase and cathepsin G (➔ Fig. 15.4). These alternative pathways of Ang II generation may actually contribute to Ang II formation in stenotic valves, since such valves contain increased numbers of chymase- and cathepsin G-containing mast cells, which are activated to secrete these proteases into their microenvironment (114). In support of this notion, the levels of cathepsin G mRNAs were found to be increased in stenotic valves, and positively correlated with the mRNAs of collagen I and III, and also with the mRNA of the profibrotic molecule TGF-β1. Moreover, stenotic valves show upregulation of the profibrotic angiotensin II type 1 receptors (AT-1Rs), but fail to express the anti-fibrotic AT-2Rs (60). In summary, an upregulation of all three Ang II-generating systems, together with a profibrotic balance of Ang II receptors in stenotic valves, support the notion that Ang II significantly contributes to the fibrotic thickening of the valves in aortic stenosis.

Bradykinin

Components of the kinin system have also been detected in aortic valves (119). Like the Ang II system, this system acts via two antagonistic receptors. Thus, bradykinin activates the anti-fibrotic bradykinin type 2 receptor (BK-2R), while a cleavage product of bradykinin, the bradykinin-(1-8) (BK-(1-8)), activates the profibrotic bradykinin type 1 receptor (BK-1R). Like Ang II, also bradykinin is degraded by ACE, chymase, and cathepsin G (120). Since the activities of all three enzymes are elevated in stenotic aortic valves (60, 114), a

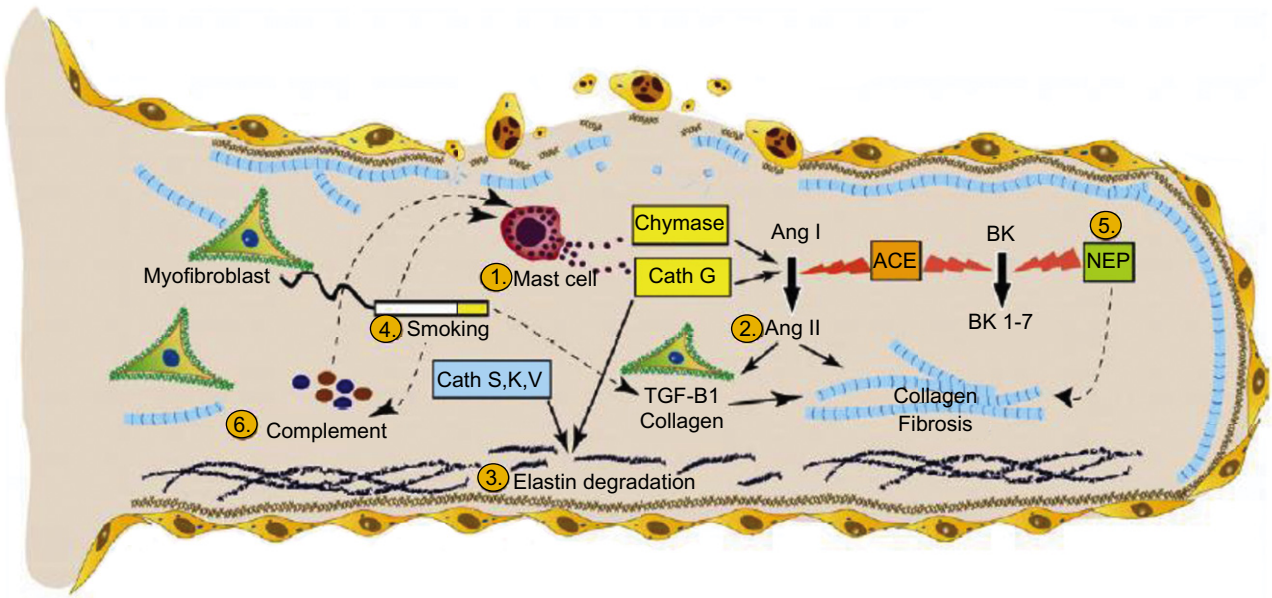


Fig. 15.4 Extracellular matrix remodelling and fibrosis pathways in aortic valve stenosis. Activated mast cells release chymase and cathepsin G (Cath G; 1), which, along with the angiotensin-converting enzyme (ACE), generate profibrotic angiotensin II (Ang II; 2). Cathepsin G and the other elastolytic cathepsins S, K, and V degrade elastin fibres in valves (3). Cigarette smoke activates mast cells and induces TGF-β1 expression in valvular interstitial cells with a myofibroblast phenotype (4). ACE and neutral endopeptidase (NEP) inactivate bradykinin (BK; 5). The complement system, a potent stimulator of mast cells, is activated in stenotic valves (6), hence contributing to promoting fibrosis in aortic valves. (Reproduced from S. Helske: Pathobiological aspects of nonhreumatic aortic valve stenosis. http://ethesis.helsinki.fi Yliopistopaino 2007.)

shortage of the protease-sensitive anti-fibrotic bradykinin in the diseased valves may ensue. Moreover, the activity of the neutral endopeptidase (NEP), a fourth bradykinin-inactivating enzyme, is increased in stenotic valves (119) (➔ Fig. 15.4). Regarding BK-R expression, a substantial upregulation of the profibrotic BK-1Rs and a smaller upregulation of the anti-fibrotic BK-2Rs was observed in stenotic aortic valves, supporting a combined profibrotic effect of this kinin-dependent receptor system (119). Thus, the balances of the pro- and anti-proliferative kinin ligands and their receptors appear to favour fibrosis in stenotic valves.

Degradation of elastin

In healthy aortic valves, the level of elastin degradation is below the detection limit, while in stenotic valves it is markedly increased (21, 114). The enzymes potentially responsible for degradation of elastin fibres in the valves include various elastolytic cathepsins, among them the mast cell-derived cathepsin G (➔ Fig. 15.4). In the stenotic valves, activated cathepsin G-positive mast cells accumulated in areas in which elastin fibres were degraded (114). Supportive evidence for the role of cathepsin G in actual degradation of valvular elastin was obtained by incubating thin sections of normal aortic valves with cathepsin G. Such incubation resulted in loss of intactness of the elastin fibres and resulted in a disarray similar to that observed in stenotic valves. Of potential clinical relevance was the observation that nicotine and acetaldehyde contained in cigarette smoke, itself a well-recognized independent risk factor of aortic stenosis, activated mast cells to release cathepsin G (114).

In addition to cathepsin G, the expression and activity of the elastolytic cathepsins S, K, and V are markedly increased in stenotic valves (21). Interestingly, the expression of the inhibitor of these cathepsins, cystatin C, was also increased in the diseased valves. A striking microscopic observation was made demonstrating that cystatin C is present in areas with preserved elastic fibres, but absent from areas in which the elastic fibres are not well-preserved (114). This observation can be understood to mean that an enhanced cystatin C expression in diseased valves inhibits cathepsin-mediated degradation of elastic fibres, but only incompletely. Moreover, the observation substantiates the notion that an increased expression and activity of elastolytic cathepsins in the stenotic valves actually contributes to the degradation of elastic fibres in the diseased valves. The ability of the elastolytic cathepsin S and K to also degrade collagen (121) renders these two enzymes potentially truly powerful matrix-remodelling enzymes in stenotic aortic valves. Also, the interstitial collagenase MMP-1, which is upregulated in the stenotic valves in areas in which macrophages express TNF-α, is actively regulating matrix remodelling in calcific aortic stenosis (115).

In summary, several proteolytic enzymes are released into the extracellular fluid by both macrophages and mast cells in stenotic aortic valves. These enzymes can act on collagen and elastin directly or indirectly by activating other matrix-degrading enzymes. The presence of a multitude of extracellularly active proteases in the diseased valves points to their key role in extracellular matrix remodelling in human aortic valve disease, a notion supported by molecular imaging studies in an experimental animal model, which revealed proteolytic activity of valvular macrophages already in early aortic valve disease (86).

Angiogenesis and lymphangiogenesis in stenotic aortic valves

Healthy aortic valve leaflets are avascular, i.e. they lack blood vessels and lymph vessels (122, 123). The lack of capillaries can be explained by the thinness of the leaflets (about 500 μm), which should be adequate for sufficient diffusion of oxygen to prevent hypoxia of cells located even in the middle of the leaflets. Indeed, since the leaflets are bathed by circulating blood on both sides, the maximal distance from the highly oxygenated blood to the centre of the leaflet is about 250 μm, a distance similar to the maximal diffusion distance of oxygen in tissues, which, depending on tissue ranges from 200 to 500 μm (124).

As the fibrotic thickening of leaflets increases, the diffusion distance for oxygen and the cells located in the middle of the leaflet begin to suffer from hypoxia. Indeed, in stenotic valve leaflets, neovessels have been consistently detected (122, 125). A second factor increasing tissue hypoxia is the infiltration of macrophages, which are strong consumers of oxygen and therefore easily create hypoxic micro-regions around them. Interestingly, macrophages favour energy production by glycolysis even in a normoxic environment. Thus, both anaerobic and aerobic glycolysis are likely to become stimulated in the macrophages infiltrating the leaflets, resulting in increased production of lactate and protons, with ultimate extracellular acidification, such as observed in atherosclerotic lesions (126). Since acidity activates macrophages to secrete proinflammatory cytokines and to ingest modified LDL particles with ensuing foam cell formation, a multitude of disease-promoting processes in the leaflets may by aggravated by a local hypoxic and acidic environment. Nevertheless, extracellular acidification has also been shown to induce calcium resorption, and regression of aortic valve calcification (127).

By contributing to the generation of a hypoxic milieu in the thickening aortic leaflets, macrophages are important triggers of angiogenesis in the leaflets (128). Similarly, mast cells, which are considered as modulators *par excellence* of angiogenesis, may induce and maintain angiogenesis in aortic leaflets by multiple activities. The many proteolytic mechanisms involved in the continuous remodelling of the extracellular matrix provide auspicious microenvironments for angiogenic sprouting. Also, the endothelial cells at the aortic side of the leaflets, and the valvular interstitial cells show high angiogenic potential (129).

Regulators of angiogenesis

In stenotic aortic leaflets, a wealth of proangiogenic and anti-angiogenic players have been identified, such as osteonectin, TGF-β, thrombospondin-1, angiopoietin-1, chondromodulin, and endostatin. In a pioneering study by Soini and co-workers (122), vascular endothelial growth factor A (VEGF-A), the most important proangiogenic regulator, and its receptors VEGFR-1 and VEGFR-2 were detected in stenotic aortic valves. VEGF-A and its receptors were expressed preferentially in endothelial cells, and also to some extent in valvular interstitial cells (Fig. 15.5). Interestingly, increased VEGF-A secretion by cultured valvular interstitial cells was observed in response to hypoxia (123). In the cited study by Soini et al., the angiogenic response was strongest in moderate valve stenosis and associated positively with the degree of mononuclear inflammation consisting of macrophages and T lymphocytes (122). Also, an enhanced expression of eNOs in the endothelial cells lining the neovessels indicated VEGF-mediated stimulation of NO production as a possible angiogenic stimulus.

A variety of proteases participate in the modulation of periendothelial and extracellular matrices involved in angiogenic sprouting and vascular remodelling (130). The proteolytic degradation of extracellular matrix not only clears the way for the emerging angiogenic sprouts, but also modulates angiogenesis by releasing fragments from various components of the extracellular matrix, notably from collagen and elastin, and by inducing conformational changes in some proteins of the extracellular matrix (131).

Of particular importance are the macrophage-generated proteases, notably the various released MMPs. By degrading and remodelling the extracellular matrix, they provide a favourable environment for angiogenic sprouting (128). Moreover, the MMPs and cathepsins may exert either anti- or proangiogenic effects by proteolytic processing of various angiogenesis-regulating target molecules with ensuing generation of either pro- or anti-angiogenic peptides. An interesting source of such peptides is osteonectin (or SPARC), and among the proteases capable of generating angiogenesis-modulatory peptides from osteonectin are plasmin, cathepsins, and MMPs (132–134), i.e. three proteases known to be upregulated in stenotic valves. On the other hand, intact osteonectin can induce the expression of certain of the MMPs, including MMP-9, a finding with potential significance since osteonectin and MMP-9 co-localize in stenotic valves mainly in the neovascularized areas (135).

The proangiogenic environment in stenotic aortic valves also partly depends on the suppression of local anti-angiogenic factors. An example is chondromodulin-1, which is expressed strongly in normal avascular human cardiac leaflets, but expressed at markedly reduced levels in diseased valves in regions of new vessel formation that strongly expressed VEGF-A (136). Another anti-angiogenic molecule, the matrikine endostatin, which is generated by partial proteolysis of collagen XVIII by cathepsins and MMPs, and which inhibits VEGF-A-induced endothelial cell migration, is strongly induced in stenotic valve tissue, particularly in vessel-like channels with larger diameters (137). The presence of endostatin, which stabilizes newly formed endothelial tubes, implies that endogenous anti-angiogenic processes are also activated during the development of valvular stenosis and could contribute to stabilization and maturation of the neovascular sprouts.

The formation of organized arterioles in heavily calcified valves implies that valves that have reached the clinical end stage, i.e. are requiring surgical replacement, have also reached an advanced stage of angiogenic remodelling (123). Moreover, the densities of all neovessels, including small microvessels, medium-sized microvessels, and mature arterioles, were highest in the most advanced lesions, so revealing continuing formation and maturation of new vessels. Furthermore, the expression of the potent angiogenic factor VEGF-A by multiple valvular cells, including mast cells (123), points to a proangiogenic switch in the valvular microenvironment. At the same time, it is likely that the neovessels facilitate inflammatory cell infiltration into the aortic valves, thereby feeding the proinflammatory territory of the stenotic valves. This notion is supported by the finding that neovessels, like lymphatic vessels, typically resided in areas with abundant inflammatory cell infiltration (123).

Microvessels usually lack a well-developed basement membrane, and therefore in an inflamed tissue, such as in an atherosclerotic lesion, easily rupture and cause local microhaemorrhages (138, 139). Indeed, in a recent study, intraleaflet haemorrhages were frequently observed in end-stage aortic stenosis, and their presence was associated with rapid progression of stenosis (140). The microvessels are also leaky so allow lipoproteins to enter the neovascularized

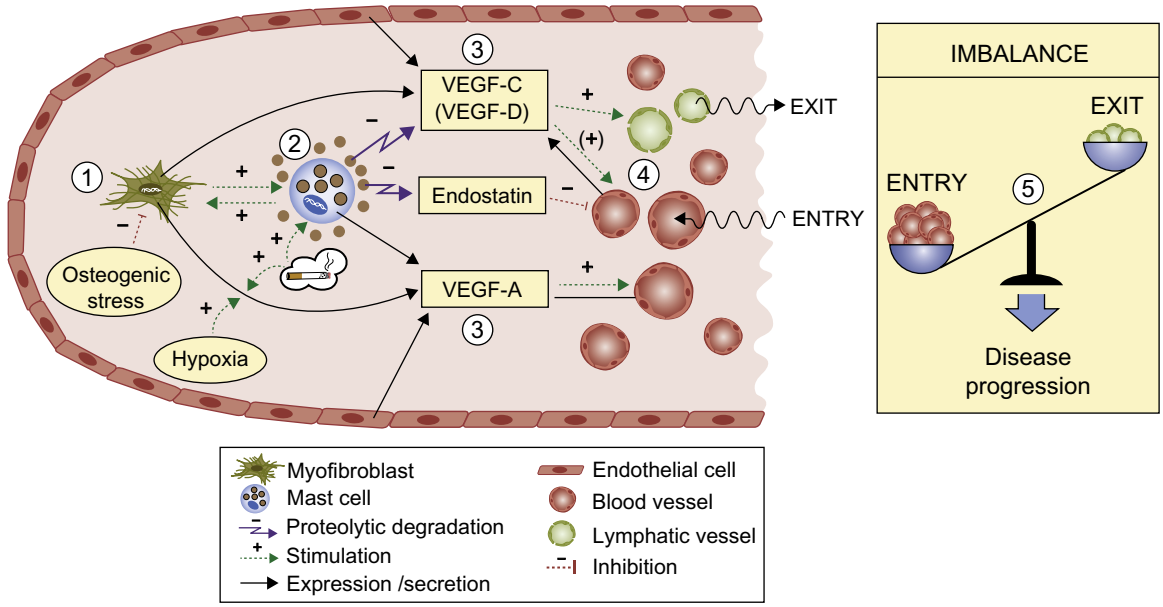


Fig. 15.5 Lymphangiogenic and angiogenic regulators in aortic valves. Valvular interstitial cells with a myofibroblast phenotype secrete VEGF-A, -C, and -D in response to mast cell-derived components and hypoxia (1). In turn, myofibroblast-derived factors induce VEGF-A secretion from the mast cells. (3) Endothelial cells lining the aortic valve produce VEGF-A, -C, and –D (3). Valvular lymphatic vessels may provide an exit route for lipids (carried in high-density lipoproteins) and for inflammatory cells, whereas abundant blood vessels could serve as entry routes for atherogenic lipoproteins (LDL) and inflammatory cells (4). The imbalance between these parallel processes may favour lipid accumulation, inflammation, valvular thickening, and fibrosis in the affected aortic leaflets, so leading to aortic valve stenosis progression (5). VEGF: vascular endothelial growth factor.
(Figure reproduced from Syväranta S, Helske S, Lappalainen J, Kupari M and Kovanen PT. Lymphangiogenesis in aortic valve stenosis--novel regulatory roles for valvular myofibroblasts and mast cells. Atherosclerosis. 2012;221:366–74 with permission from Elsevier.)

regions of the tissues and promote lipid accumulation. On the other hand, lymphatic vessels carry HDL particles containing cholesterol derived from macrophage foam cells back to the circulating blood (141). As HDL particles have been found in the stenotic leaflets (52), the development of lymph vessels should aid removal of lipids from the leaflets. However, since lipids continue to accumulate in the valve leaflets as the lesions progress and the degree of stenosis increases (94, 102), the compensatory actions of the lymphatic system appear to be insufficient for adequate removal of lipids from the leaflets. Indeed, the average proportion of lymphatic vessels compared to the number of blood vessels was only 1 in 20 (142). Considering that in the diseased valves, microvessels can serve as entry routes for lipids and inflammatory cells, while the much fewer lymphatic vessels provide an exit route for them, an obvious imbalance between entry and exit of these regulators of valvular thickening is obvious (➔ Fig. 15.5). Taken together, the imbalance between angiogenesis and lymphangiogenesis may promote lipid accumulation and maintain chronic inflammation in the stenotic aortic leaflets.

Finally, formation of new blood vessels is associated with calcified and ossified areas of leaflets in end-stage aortic valve stenosis (18). Since, VEGF-A may promote both endochondral and intramembraneous ossification (143), and VEGF-D promotes osteoblast maturation (144), these angiogenesis- and lymphangiogenesis-promoting growth factors expressed in stenotic valves have the potential to couple the two processes in the diseased leaflets.

Inflammation and angiogenesis

For maintenance of a dynamic architecture of neovascularization, a large number of both proangiogenic and anti-angiogenic factors, and their coordinated actions, are required. As already noted, thickening of the leaflet is the mandatory prerequisite for the induction of angiogenesis, but inflammation clearly dictates the process at all stages of the valvular disease. Of major interest are the findings demonstrating that all three types of inflammatory cell, i.e. macrophages, T lymphocytes, and mast cells, are able to induce angiogenesis by multiple mechanisms (139). Actually, because of their high numbers in the diseased leaflets, the contribution of macrophages to angiogenesis may be dominating. However, the joint impact of interacting inflammatory cells is likely to surpass the contribution of any single type of cell.

The cellular interactions involved in angiogenesis appear to also involve cross-talk between the inflammatory cells

and the structural cells of the valves, i.e. the interstitial cells. Thus, the joint contribution of activated mast cells and activated valvular interstitial cells appears to include regulation angiogenesis and lymphangiogenesis. Indeed, such coordinated action provides an illustrative example of potential interaction of two types of cell, one bone marrow-derived and the other structural, in the diseased aortic valves. Thus, initial immunohistochemical observations had shown that valvular mast cells produce VEGF-A, but not VEGF-C, and so suggested an angiogenic but not a lymphangiogenic role for the mast cells (123). This impression was reinforced by the observations made in cell culture experiments in which preconditioned medium obtained from interstitial cells derived from stenotic aortic valves increased secretion of VEGF-A by cultured human mast cells (123). Similarly, addition of components released by activated mast cells to the valvular interstitial cell cultures significantly increased VEGF-A secretion by these cells above the level of constitutive secretion. Surprisingly, however, when valvular interstitial cells were exposed to compounds released by activated mast cells, the mRNA expression of VEGF-C increased, but no secretion of VEGF-C protein by these cells into the incubation medium could be detected (142). This apparent paradox was explained when it was found that the mast cell-derived neutral proteases tryptase, chymase, and cathepsin G degraded the secreted VEGF-C protein, but left the secreted VEGF-A intact. Thus, a specific mechanism was identified in which activated mast cells and valvular interstitial cells could coordinately induce VEGF-A-dependent angiogenesis in a mutually stimulating fashion, while the activated mast cells would tend to suppress induction of VEGF-C-dependent lymphangiogenesis by interstitial cells in stenotic aortic valves (112, 123, 142).

Treatment

The currently available therapeutic options for aortic stenosis are aortic valve replacement surgery (SAVR) and transcatheter aortic valve implantation (TAVI) (3). In contrast, no medical treatment has proven to be effective in slowing the process of aortic valve calcification and stenosis. The poor prognosis and increased mortality associated with aortic stenosis after the onset of symptoms in the absence of either SAVR or TAVI stresses the importance of seeking medical treatment, which would slow down the disease progression. Applying the understanding of the molecular and cellular pathophysiology of calcific aortic stenosis, outlined above, the following putative targets have been proposed for their potential therapeutic properties in the treatment of aortic stenosis.

Lipids

Observational studies supported the hypothesis that lipid-lowering therapy may retard aortic stenosis progression, similar to its progression-retarding effects on atherosclerosis (145, 146). In addition, a non-randomized and open-label trial in which subjects who had an indication for statin treatment (because of elevated LDL) and received rosuvastatin treatment, showed decreased progression in echocardiographic parameters of aortic stenosis severity (147). However, three large prospective studies failed to show any significant differences between statin treatment and placebo regarding progression of aortic stenosis (148–150). Taken together, there is currently no convincing evidence in support of the treatment of aortic stenosis with statins being efficacious. However, an exciting novel therapeutic option for the prevention of aortic stenosis can be envisioned for patients with elevated Lp(a), since the newly introduced powerful LDL-cholesterol-lowering drugs, PCSK9 inhibitors, unlike statins, also lower Lp(a) level (151, 152).

Since a low proportion of plasma HDL cholesterol to plasma total cholesterol is associated with an increased rate of progression of aortic valve stenosis (153), HDL-based therapies for attenuation of lipid accumulation and inflammation in the diseased aortic valves are a reasonable, but still a remote, therapeutic option for the treatment of human aortic stenosis. Unfortunately, however, all HDL-cholesterol-raising pharmacotherapies, with the ultimate aim of reducing inflammation and lipid load in atherosclerotic lesions and ensuing reduction in cardiovascular morbidity and mortality, have so far been unsuccessful (154). Another opportunity for HDL-based therapy is intravenous infusion of HDL mimetics consisting of the major apolipoprotein of HDL, the apoA-I, or of apoA-I mimetic peptides, either in lipid-free form or complexed with phospholipids (155). Importantly, apoA-I has anti-atherosclerotic activities by increasing cholesterol removal from foam cells, and also possesses anti-inflammatory, antioxidant, and endothelial protective properties. Such HDL-mimetic therapies have already been shown to induce rapid improvement of coronary atherosclerosis in patients suffering from coronary heart disease (156, 157).

ApoA-I is also able to stimulate OPG secretion by cultured human aortic valve interstitial cells (52), a finding suggesting that apoA-I may have an anti-calcifying effect in human aortic valves. Considering the many similarities in the pathobiology of an atherosclerotic plaque and stenotic aortic valves, and the ability of apoA-I to potentially combat both pathologies, HDL-based therapies could also be considered potentially in patients with aortic valve stenosis. Such a proposal is supported by the findings demonstrating

that apoA-I mimetic therapy has resulted in regression of experimental aortic valve stenosis in rabbits receiving a cholesterol-enriched diet and vitamin D2, and also in mice receiving a high-fat diet and lacking apoE or having Werner progeria gene deletion (158, 159).

As discussed earlier in this chapter, one causal pathway mediating aortic valve stenosis may involve three closely linked potential effectors of the disease, and they are Lp(a), Lp-PLA2, and oxPL. Based on the available biochemical and clinical data, Drs Hung, Witztum, and Tsimikas (110) recently concluded that the Lp(a)–Lp-PLA2–oxPL axis provides a plausible mechanism through which this pathway may mediate aortic valvular stenosis, as has been earlier suggested for atherosclerosis and cardiovascular disease (160). In particular, two recent observations have suggested that targeting the axis may be a viable approach in mitigating aortic valve stenosis (98, 100), which remains to be validated in experimental models and ultimately in appropriate clinical trials (99).

Fibrosis

Since a wealth of data show that fibrosis in aortic stenosis is driven by a combination of increased profibrotic and decreased anti-fibrotic mechanisms, a randomized and placebo-controlled study was started in patients referred for consideration of valve replacement surgery, with the aim to assess whether inhibition of the RAS system with candesartan, an AT-1R blocker, could attenuate the Ang II-dependent pathobiological processes in stenotic aortic valves (161). Blocking the AT-1R, rather than inhibiting the ACE, was chosen as the strategy, since blocking AT-1R prevents the effects of Ang II irrespective of the pathway of its generation. This is of particular importance, since the activities of all three Ang II-generating enzymes, ACE, chymase, and cathepsin G, are increased in the diseased valves (60, 114), and inhibiting only one of them would leave the two others active. It remains to be seen whether such systemic pharmacological inhibition of the renin–angiotensin–aldosterone system (RAAS) will also act locally in the diseased valves during the final months (5 months, on average) that were available before the stenotic valves were surgically removed.

Calcification

Bisphosphonates are analogues of pyrophosphate prescribed to prevent and treat osteoporosis. Their mechanisms of action are complex and involve direct inhibition of osteoclasts. Although initial observational studies (162–164) have indicated that bisphosphonate use was associated with a decreased aortic stenosis progression, the most recent and largest cohort did not reveal any significant differences in echocardiographic aortic stenosis progression, valve replacement surgery, or overall survival between bisphosphonate treated and non-treated subjects with mild to moderate aortic stenosis (165). Furthermore, based on data from a large CT screening cohort, bisphosphonate use was associated with increased aortic valve calcification in women less than 65 years of age, whereas a trend towards lower prevalence of aortic valve calcification was reported in women ≥65 years (166).

Another osteoporosis medication is denosumab, which is a fully human monoclonal antibody against RANKL (167). Denosumab may be of therapeutic interest in preventing aortic valve calcification, given the apparent pro-calcifying effects of RANKL (51) *in vitro*, and the gradually increased RANKL expression as aortic valves progress from healthy through thickened to calcified tissue (30).

Mitral valve prolapse

Definition and clinical presentation

Fibromyxomatous changes of the mitral valve leaflets may cause a prolapse, defined as the displacement of one or both mitral valve leaflets into the left atrium. There may, in addition, be an eversion of the leaflet tip into the left atrium, which is referred to as a flail. Prolapse is one cause of organic (or primary) mitral regurgitation, which may be asymptomatic or associated with symptoms of heart failure.

The aetiology of mitral valve prolapse can be divided into myxomatous degeneration (Barlow's disease) and fibroelastic deficiency. The classical clinical signs of mitral valve prolapse involve a systolic murmur preferably auscultated at the apex, and a mid-systolic click. The diagnosis is made on echocardiography, which additionally allows the quantification of the regurgitant blood volume.

Myxomatous degeneration

Myxomatous mitral valve degeneration refers to a myxoid infiltration resulting in a thickened valve with redundant valve tissue and prolapse affecting a large portion of the mitral valve. Myxomatous degeneration may also be associated with mitral annular dilatation or with elongated chordae. Valvular interstitial cells derived from myxomatous mitral valves exhibit increased expression of extracellular matrix-degrading enzymes, such as MMPs and cathepsins, whereas the collagen-synthesizing capacity is unaltered (168). In addition, there is an accumulation of glycosaminoglycans in myxomatous, as compared with normal, mitral valves, a difference that is even more pronounced in the subvalvular apparatus, i.e. the chordae (169). The myxomatous

chordae of prolapsed valves contain, for example, high levels of hyaluronan and chondroitin-6-sulphate (169).

Fibroelastic deficiency

Whereas Barlow's disease represents the presence of excess connective tissue, mitral valve prolapse due to fibroelastic deficiency is characterized by a thinning of the mitral leaflet due to a deficiency of collagen, elastin, and proteoglycans (170). Fibroelastic deficiency usually results in a single prolapsing mitral valve segment, typically in association with chordal rupture.

Conclusions

Among the valvular heart diseases, aortic valve stenosis and mitral valve prolapse are the most frequent pathologies. If severe, symptomatic, and left untreated, they are associated with increased mortality.

The pathophysiological changes taking place in aortic valve stenosis and mitral valve prolapse exhibit similarities as well as fundamental differences. Thus, initiation and progression of aortic valve stenosis is characterized by lipid accumulation, inflammation, calcification, fibrosis, extracellular matrix remodelling, and angiogenesis, all of which culminate in fibrotic thickening and calcified valvular leaflets. In contrast, the pathobiology of mitral regurgitation is dominated by myxomatous degeneration and/or fibroelastic deficiency of the leaflets and their supporting chordae, a process ultimately resulting in prolapse of the valves. Obviously, however, it is not possible to learn in humans about the very sequence of events during the transition of a healthy valve leaflet into a diseased one. Moreover, since valvular heart disease is multifactorial, animal models mimicking the human risk-factor profile are not easy to construct. An understanding of the molecular and cellular pathophysiology is necessary to identify novel therapeutic targets for the treatment of valvular heart diseases.

Further reading

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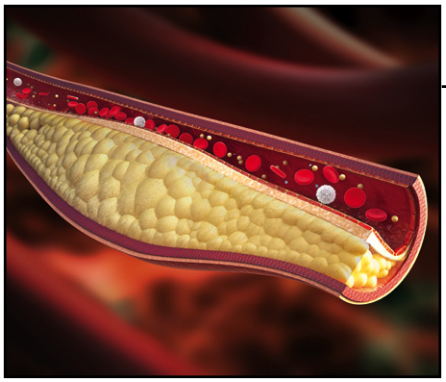

CHAPTER 16

Biology of vascular wall dilation and rupture

Jean-Baptiste Michel

Content

Introduction

Nothing makes sense in biology except in the light of evolution

T. Dobzhansky (1973)

Parallel phylogenesis of the circulatory function and arterial wall structure

Phylogenetically, the circulatory system evolved from the simple diffusion of extracellular fluid in invertebrates via a low-pressure circulating system animated by an archaic heart in fish (kinetic energy), to a highly organized system with organ-regulated directional blood flow (vasomotricity) propelled through the conductance arterial tree with a defined wall structure, by the pumping action of the mammalian heart. Anatomically, this evolution is associated with a branched model of the circulatory system, including conductance arteries, not directly in contact with the organs and resistance arteries and arterioles directly or indirectly coupled to organ function and energetic demand. Therefore, in this concept, the evolutionary teleonomy of the circulation is to assist the specification and metabolic autonomization of organ function. In this phylogeny, peripheral resistance to blood flow is the most recent biological acquisition in the circulatory system, generating arterial blood pressure (potential energy). Arterial resistance to blood flow is an evolutionary requirement for regulating diversified blood supply to organs through local/regional organ-adapted inhibition of arterial tone, spatially and temporally coupling specific localized function with local arterial vasomotricity. Without arterial tone, regulation of local blood supply to metabolic demand is not possible. Functionally dependent vasodilatator signals within metabolically active tissue, as well as endothelial-dependent vasodilatation in conductance arteries, provide local inhibition of arterial tone with no significant change in pressure. This functional evolution is associated with the gradual development of vascular anatomy into a system where organs are blood-supplied by multi-branched conductance arteries. In contrast, the multi-branched pulmonary arterial circulation, in which haematosis does not necessitate localized adaptation of flow, did not evolve toward a system involving arterial resistance. Indeed, the shift toward arterial resistance is highly pathologic in the pulmonary circulation.

In parallel, wall topophysiology and structure also progressively changed. The conductance arteries, in order to respond to the pressure load, evolved from a thin cellular structure to a thick matrix-rich structure, in which the specific stromal cells, vascular smooth muscle cells (vSMCs), assume both the contractile function and the synthesis of the extracellular matrix and its maturation, providing a hydrophobic network of insoluble, highly crosslinked fibres.

Finally, at the mammalian stage of species' evolution, arterial haemodynamics are summarized by three variables, the heart beat generating phasic flow and pressure, which may be concomittant in the systemic circulation, but dissociated in the coronary circulation, and the interactions between them and the wall (for more details see ➔ Chapters 3 and 12). In an ideal conducting system, the phasic flow would be laminar, generating a regular shear stress at the wall interface, and pressure, a phasic wall tension. Aortic wall tensile stress is proportional to pressure and radius and inversely proportional to wall thickness (Laplace law: T = P.r/2h). Since progressive physiological dilation of the aorta is observed with age in animals (1) and humans (2), tensile wall stress increases with ageing, independently of pressure. Moreover, the arterial tree of conductance arteries is not ideal, and each morphological/structural change—bifurcation, dilation, and stenosis—modifies the interactions between the different components (plasma and particulate components) of the blood (haemorheology) and the relationship between the blood and the arterial wall, creating new localized 'hot spots' of impedance, transforming blood kinetic energy into potential energy (3).

Spatial organization of the arterial wall

With regard to haemodynamics, the wall structure of conductance arteries, including the aorta, is spatially organized in three layers, from inside to outside: intima, media, and adventitia. The intima is physiologically a virtual, endothelial-covered space inside the internal elastic lamina. The media is the principal structural/functional layer of the arterial wall, comprising matrix and vSMCs; and the external adventitia is composed of loose connective tissue. The medial layer displays spatial and functional connectivity between vSMCs and extracellular matrix, assuming the function of supporting the haemodynamic load (the content) within the arterial system (the container) (4). vSMC differentiation and survival is dependent on cell adhesion (5), creating tensegrity (6) within the cell, via extracellular matrix and intracellular cytoskeletal molecular interactions, largely dependent on local phasic (systole/diastole) haemodynamic parameters. The blood-containing function of the arterial wall mainly depends on the extracellular matrix (ECM) synthesized by, and matured in close vicinity to, the vSMCs. Lysyl oxidase, synthesized and secreted by the vSMCs, is the main enzyme involved in maturation of fibrillar structures by promoting covalent crosslinking between tropo-elastin molecules and between tropocollagen molecules. The crosslinked fibrillar and hydrophobic nature of the ECM of conductance arteries renders it insoluble. Therefore, degradation of the ECM is largely due to the biological action of proteases. In the arterial wall environment, elastic network damage is preferentially associated with progressive dilation, whereas collagen damage leads to rupture.

Physiologically, the medial layer of the arterial wall, structured by its elastic laminae, is an avascular tissue (except for its external part in human aorta) devoid of any microcirculation. It is also an immune-privileged tissue, poorly accessible to circulating leukocytes due to this absence of capillaries and veinules, the specific sites of leukocyte rolling and tissue migration. In contrast, the loose connective tissue of the adventitia is rich in nerve endings, arterioles, capillaries, veins, and lymphatics, allowing the migration and extravasation of circulating leukocytes, including monocytes and lymphocytes (4, 7). Adventitial intersititial pressure is low: 10 mmHg. Therefore, an important transmural pressure gradient (100 mmHg) exists between intraluminal arterial blood pressure (130/80 mmHg) and adventitial interstitial pressure, creating unidirectional outward hydraulic conductance across the arterial wall. The medial layer thus acts as the parenchyma of the arterial tissue, whereas the adventitia is the stromal/mesenchymal part, in which the immune response takes place in relation to (i) outwardly convected signals, (ii) the ability of small vessels to translocate circulating leukocytes to interstitial tissue, and (iii) the intrinsic organizing capacity of the SMC. In later stages of arterial disease, inwardly directed neo-angiogenesis permits leukocyte extravasation into the media and intima (8, 9).

Outward convection of blood components through the wall

This hydraulic conductance is responsible for radial mass transport of soluble plasma molecules and macromolecules through the arterial wall. This biomechanical phenomenon is named 'outward convection' (➔ Fig. 16.1). Conversely, there is no inward retrodiffusion of soluble mediators from the wall into the blood. In contrast, mediators spontaneously or artificially generated in the adventitia could be inwardly retrotransported by vSMCs through intercellular connectivity within the media. This is the case in experimental models in which high concentrations of calcium applied to the adventitia are inwardly transported by the vSMCs, inducing the release and precipitation of calcium phosphate under the

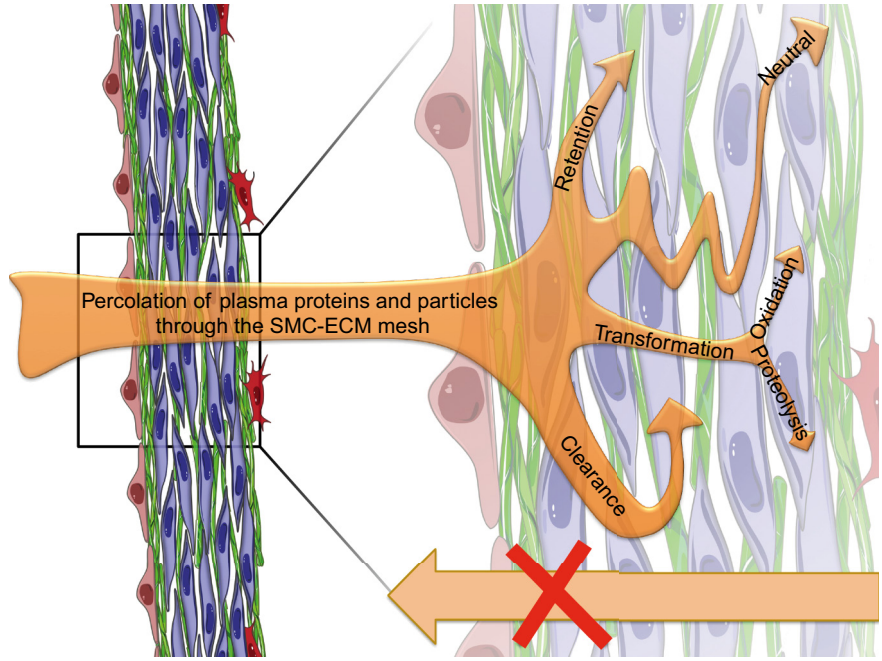

Fig. 16.1 Schematic representation of percolation of blood-borne components, particularly plasma proteins (but also lipoproteins, microparticles, etc.), through the wall in relation to the phenomenon of outward convection. During their outward transport across the wall, proteins can remain neutral or be transformed in relation to interactions with vSMCs and/or matrix, including proteolysis, oxidation, clearance by vSMCs, and retention by extracellular matrix. There is no inward retrodiffusion of soluble mediators through the media. This process of percolation, transformation, and retention of soluble plasma mediators is the most common denominator of aneurysmal pathologies.

effect of alkaline phosphatase, and the elastic network breakdown. Convection intensity is dependent, on the one hand, on haemodynamic factors, including pressure and shear (10, 11), local collision power of blood components on bifurcations (3) and haemorheology and, on the other hand, on the porosity of the arterial wall. In this paradigm, pressurized blood-borne components percolate through the wall, creating interactions with vSMCs and/or the extracellular matrix, leading to retention, proteolysis, oxydation, clearance, and metabolism of plasma proteins or blood particles, or to their exfiltration towards the adventitia for recycling and locally impacting the immune response (4, 12). For example, the difference between results of proteomic analysis, in which plasma proteins represent 40% of the arterial wall proteome, and those of transcriptomics, where 100% of the mRNA represent the wall cell genomic and in which mRNA of numerous plasma proteins are totally absent, provides evidence of plasma protein enrichment of the wall. Conversely, percolation of blood components through the arterial wall modifies the connections between cells and matrix within the wall. In this paradigm, blood-borne components can injure the arterial wall, but conversely, the arterial wall can metabolize systemic blood components, modifying their circulating concentration. Therefore, outward convection of blood-borne components is the largest common denominator of all arterial pathologies (13). In parallel, blood cell components also interact with the arterial wall, where they may be cleared by angiophagy, a property made possible by vSMC plasticity.

Biological definition of aneurysms

Whatever their localization, arterial aneurysms are defined anatomically as localized dilations of the arterial wall, as a progressive loss of blood-containing function (the capacity of the wall to support haemodynamic load) leading to rupture, and structurally, by thinning of the wall, corresponding to degradation of extracellular matrix and loss of vSMCs. Therefore, as compared to other pathologies of conductance arteries involving the intima, aneurysms are characterized by medial injury, mainly of a proteolytic nature. Aneurysms can develop all along the arterial tree, but are more frequent in the abdominal aorta, the ascending aorta, the cerebral arteries, and less frequent in the femoral and popliteal, renal, and/or splenic arteries. Since the insoluble extracellular matrix (elastin and collagen) of the arterial wall largely supports the haemodynamic load, the action of proteases, able to degrade the insoluble fibrillar matrix, is the most common denominator of aneurysms. In the context of outward convection, these proteases originate mainly from blood components released by circulating leukocytes, such as elastase or MMP-9 from neutrophils, or directly from

plasma zymogens, such as plasminogen, which are activated during their passage across the wall.

Limits of experimental models

In order to test new diagnostic and therapeutic approaches preclinically, and to support pathophysiological hypotheses, experimentators have tried to develop animal models of aneurysm formation. Numerous models have been tried and tested in small and large animals. For convenience, murine (rat and mouse) models are the most extensively developed and commonly used models. Four classical models predominate: the elastase model in rats (14), the angiotensin II model in apo $E^{-/-}$mice (15), the decellularized aortic xenograft in rats (16), and the calcium chloride model, initially developed in rabbits (17). All these models have their own respective advantages and limits, and the choice largely depends on the question raised. The elastase model consists of a pressurized intraluminal infusion of pancreatic elastase in a segment of the aorta. The model is characterized by an initial small dilation, the appearance of an intraluminal thrombus (ILT) at day 3 to 5, and the subsequent progression of dilation in relation to ILT biological activity (18). At 2 weeks, the ILT is the main source of proteases, including MMPs, leukocyte elastase, urokinase, and plasmin (19). The model aneurysm may either rupture early or never. The elastase model, terminally applied to the left common carotid artery in rabbits, is the most classical model of intracerebral sacciform aneurysm. This model in rabbits allows the use of intravascular interventional catetherisms.

The angiotensin II model initially described in $apo^{-/-}$ mice is essentially a model of intramural haematoma rather than a true dilation of the lumen (20). Mice are perfused with angiotensin II for 28 days using an osmotic minipump. Mice become moderately hypertensive and develop more or less repeated aortic transmural disruptions, usually localized at the ostia of the intercostal or lumbar arteries (21, 22). This model can also be used as a model of aortic dissection in mice with genetic defects in extracellular matrix or with TGF-β abnormalities. Dissection can also be pharmacologically induced by the use of β-aminopropionitryl (BAPN), an inhibitor of lysyl-oxidase (angiolathyrism) in mice and rats (23, 24).

The decellularized aortic xenograft model is based on the principle that the extracellular matrix is antigenic in xenografts but not in allografts, in which antigenicity is mainly due to MHC-I, carried by the cells. In this context, the decellularized aortic xenograft is the target of a lymphocyte reaction, including antibody synthesis, leading to extracellular matrix degradation and the progressive dilation of the arterial wall. This model is also associated with an ILT.

The calcium chloride model is induced by prolonged application of $CaCl_2$ onto the adventitia of an arterial segment (initially the carotid artery in rabbits, but the model has been largely extended to rats and mice). A high adventitial concentration of soluble $CaCl_2$ leads to Ca retrotransport by vSMCs across the media towards the lumen. Ionized Ca is transformed into calcium phosphate ($Ca_3(PO_4)_2$) under the action of alkaline phosphatase (furnishing phosphate from phosphorus) synthesized by vSMCs. The pathophysiology of the model remains unclear but both the cellular and the matrix components of the arterial wall are involved. The model is initially characterized by a patchy disappearance of endothelial cells and ILT formation. Calcium phosphate precipitates on the extracellular matrix and matrix-precipitated hydroxyapatite crystals lead to subsequent fragmentation of the elastic network. The model is associated with leukocyte extravasion in the adventitia, and finally by colonization of the ILT by vSMC proliferation and matrix production.

All these fusiform experimental models are characterized by stabilization of aneurysmal progression in relation to cessation of the initial stimulus (elastase, angiotensin II, xeno-antigens, and $CaCl_2$). In all cases, stabilization is associated with a healing process of intimal vSMCs proliferating and invading the ILT, a phenomenon not readily observed in large human aneurysms. This difference could be due to the leukocyte count inversion in murines, which have a low level of neutrophils (10–20% neutrophils), as compared to the human leukocyte count (70% neutrophils). This species' difference is due to deletion of the interleukin-8 gene in murines (25). Therefore, to prevent healing and to enhance dilation progression in animal models, it is important to promote neutrophil adhesion and activation in the ILT. For this purpose weak pathogens, like *Chlamidia pneumonia* (26) or *Porphyromonas gingivalis* (27), are repeatedly injected. The injected pathogens localize in the ILT, and have a powerful positive impact on neutrophil trapping. In contrast, this healing process is probably less active in sacciform aneurysms, in relation to a higher recirculating flow in the aneurysmal sac, inducing a highly active and long-lasting ILT (fibrinogenesis/fibinolysis).

Aneurysm of the abdominal aorta (AAA) in humans

AAA is the most frequent localization of aneurysmal disease, developing in 1 to 5% of the male population over 65 years of age. AAA is usually asymptomatic, incidentally discovered, or detected by mass screening in the exposed population. This preferential localization and dilating evolution of atherothrombosis is probably related to haemodynamic conditions, and particularly to wave

reflection at the iliac bifurcation. The role of haemodynamics in AAA development was exemplified by the remarkable clinical investigation performed by Vollmar and colleagues showing that AAAs are more frequent in men with above-knee amputation than in age-matched controls, and that the greater convexity of the AAA developed always on the opposite side of the non-amputated leg (28) suggesting that reflexion waves on bifurcations play an important role in the lateralization of the dilation.

From atherothrombotic nature to staccato evolution of AAA

Acquired aneurysm of the abdominal aorta is a site-specific, archetypal human model of atherothrombotic disease, involving risk factors similar to those of occlusive atherothrombosis (male gender, ageing, tabagism inducing antiprotease oxidation, hypercholesterolaemia, and LDL/HDL), with similar pathophysiology (haemodynamic role in localization, cholesterol crystals, and oxidative role of RBCs), and association with coronary and cerebral atheroma, etc. Diabetes is the major exception (29), since chronic hyperglycaemia induces covalent crosslinking in the extracellular matrix, rendering it more resistant to proteolysis, providing evidence of the predominant role of proteolytic injury of the extracellular matrix in aneurysmal pathogenesis. Specific genetic susceptibility may be involved in aneurysm formation, promoting the disease by favouring proteolytic activities, vSMC loss, or extracellular matrix defects in familial cases. Nevertheless AAA is usually a multifactorial disease in which the genetic part is limited. Association of polymorphisms in CDKN2BAS, SORT1, LRP1, MMP3, AGTR1, ACE, and AOA1 with AAA frequency have been reported and reproduced (30). In particular, LRP1 is relevant, since this scavenger receptor is involved in the regulation of ECM breakdown by its ability to clear protease/antiprotease complexes by vSMCs (31).

The abdominal aorta is a site particularly sensitive to the development of atheroma, including initial fatty streaks and plaques. Due to the specific haemodynamics of the terminal aorta (reflexion on the iliac bifurcation), atheroma becomes rapidly circular, generating numerous asymptomatic plaque ruptures and formation of intramural clots that may, or may not, be healed by intimal fibrocellular cap formation. These mural thrombotic events potentially initiate the formation of a non-occlusive ILT (32), which becomes the main source of proteases (plasminogen, elastase, and MMP-9) and oxidative mediators that are outwardly convected across the media (superoxide anion, O_2^-, and oxygenated water, H_2O_2), directly or indirectly initiating the adventitial response (➔ Fig. 16.2). Abundance of autofluorescent ceroids in the wall provide evidence of these oxidative phenomena. The ILT progresses in relation to dilation-induced haemorheological changes (vortexing) in blood flow. As described, the ILT is a highly porous neotissue (33), with spatio-temporal biological dynamics, including luminal renewal at its interface with the circulating blood, intermediate biochemical transformation of the clot, and abluminal lysis at its interface with the arterial wall. Such biological dynamics lead to a multilayered morphology of the ILT corresponding to the different stages of its transformation: due to the enrichment in intact RBCs, the luminal layer is red, the intermediate layer is brown, corresponding to haemoglobin metabolism, and the abluminal layer is highly granular, providing evidence of fibrin degradation and oxidative reaggregation (fibrinolytic fragments can be reaggregated by oxidative covalent links). In addition to RBCs, the luminal layer is rich in aggregated platelets providing a platform for prothrombin activation and fibrin formation. Due to P-selectin exposure by aggregated platelets, the luminal ILT recruits circulating leukocytes, mainly neutrophils, which degranulate and die subsequent to tissue retention, releasing numerous components, including u-PA, L-elastase, and MMP-9 and MMP-8. Neutrophil components are partially and temporarily retained within the ILT by the formation of neutrophil extracellular traps (NETs), filamentous complexes of free DNA and histones, capable of retaining cytosolic molecules.

In addition to proteolysis, the ILT is also the main source of oxidation processes due to the powerful catalytic enhancement of oxidase activities, mainly myeloperoxidase of neutrophil origin, by haem-derived iron released by RBC degradation in the luminal layer (Fenton and Haber–Weiss reaction). Luminal RBC membranes also release unesterified cholesterol, which usually accumulates at the ILT/wall interface as solid cholesterol crystals (cholesterol cleft on deparaffinized formol fixed sections). The presence of large amounts of iron in the adventitia (Perl's reaction), provides evidence of RBC/haem degradation in the ILT, with subsequent iron release, outward transport to the adventitia, and endocytosis by phagocytes (➔ Fig. 16.3). Moreover, plasma components, including lipoproteins, which percolate through the ILT, can be oxidized during this mass transport. Due to tissue activation processes, the luminal layer of the ILT releases a large amount of microvesicles/particles of platelet, neutrophil, and RBC origin, able to outwardly convect numerous membrane-bound mediators such as exposed procoagulant phosphatidyl-serines, components of the fibrinolytic system, ADAM metalloproteinases, etc. The ILT may also be a site for deep bleeding, acutely enhancing ILT proteolytic activities. This high-attenuating 'crescent' sign observed in a CT scan has a prognostic value of rupture risk (34).

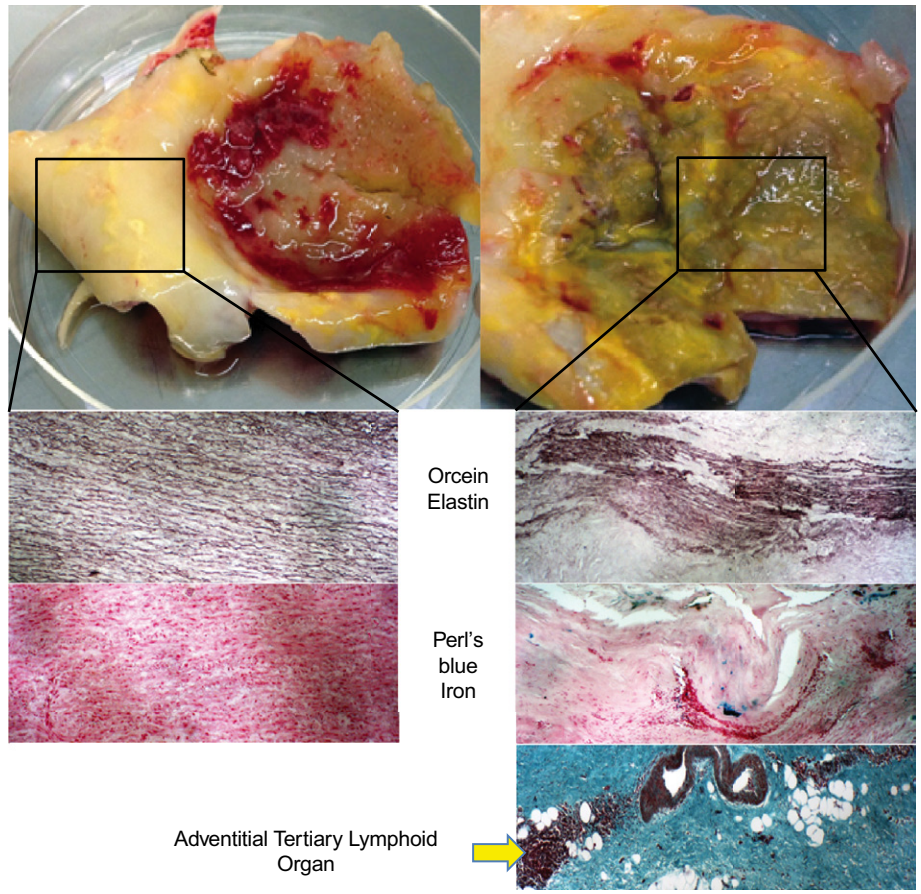

Fig. 16.2 Role of the non-occlusive intra-luminal thrombus in aneurysmal pathology. The area not covered by the thrombus (left part of the sample) appears macroscopically normal, with limited fatty streaks. Histologically, the wall appears healthy with numerous vSMCs and a well-conserved elastic network structure. The luminal clot is macroscopically normal, associating fibrin and RBCs. The area subjacent to the thrombus appears highly degenerative (right), with intense fragmentation of the elastic network, loss of vSMCs, presence of haem-derived iron and an intense angiogenic and immune adaptative response (ATLO) in the adventitia.

All the soluble or particulate components, conveyed from the blood or generated within the luminal part of the ILT, are transformed and outwardly convected towards the wall, interacting with the medial vSMCs and the extracellular matrix, and are finally recycled in the adventitia. This convection, mainly of proteases, causes injury to the medial layer, leading to vSMC disappearance and breakdown of collagen and elastic fibres, allowing progressive dilation and, finally, rupture.

As a result of convection across the media, numerous mediators reach the adventitia, including pro-oxidant mediators, where they directly or indirectly induce adventitial responses, including inwardly directed angiogenesis, immune maturation, and fibrosis. Since outward convection of mediators creates a growth factor gradient across the wall, the adventitia responds by an inward migration of endothelial cells, promoting centripetal sprouting of neovessels from the adventitia in atherothrombotic diseases (35). This can be easily observed in AAA (4, 36). Inward sprouting is initiated by lipid mediators, generated via membrane phospholipid metabolism by phospholipases, associated production of arachidonic acid, and transformation by cyclo-oxygenase. In particular, PGj_2 is able to stimulate PPAR-γ in vSMCs, promoting VEGF expression and secretion. Nevertheless, due to the highly proteolytic environment in which inward neovascularization develops, different to that of classical plaques, the centripetal neovascularization does not reach the ILT in AAA. In this context, neo-angiogenesis provides a gateway for leukocyte extravasation into the tissue, including monocytes, which undergo a phenotype shift to become macrophages capable of phagocytosis/endocytosis, and also mastocytes and lymphocytes. AAA is frequently characterized by the presence of adventitial tertiary lymphoid organs (ATLOs), the result of lymphoid neo-organization, in which the immune adaptive response takes place, including B-cell maturation and finally antibody production. In parallel, the presence of mastocytes has been reported in the adventitia. Nevertheless, the relationship between ATLOs, IgE production, and mastocyte activation remains to be established. Similarly the roles of this immune adaptive response in

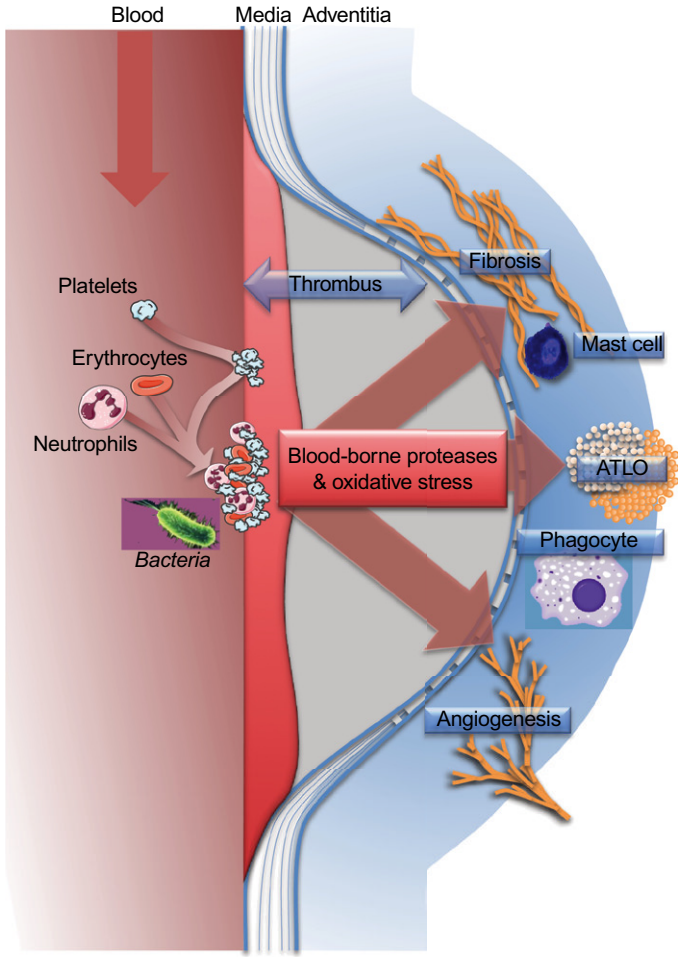

Fig. 16.3 Cellular and plasma components involved in ILT formation (fibrinogen, zymogens, platelets, neutrophils, RBCs, and, in some cases, weak bacteria) and their convected impact on the arterial wall: degradation of the media, oxidation of lipids, proteins and DNA, and adventitial response (phagocytosis, innate and adaptive immune response, and neo-angiogenesis).

AAA remain to be determined, including the mechanism of lymphoid tissue formation, the identification of the neo-antigens, and, lastly, the pathogenic consequences of B-cell proliferation and mastocyte activation. Finally, an intense fibrotic process may take place in the adventitia, in which the accumulation and organization of collagen fibres increase the resistance of the wall to rupture. In cases of 'inflammatory' AAA, characterized by an intense adventitial phagocytic and immune response, the adventitial fibrosis can extend to the retroperitoneum, a process mediated by ATLO, TGF-beta, and, possibly, IgG4.

One of the main characteristics of AAAs is that their evolution is rarely linear but staccato, in which periods of latency are followed by more evolutionary ones. Spontaneously, without exogenous stimuli, AAA lesions may heal. Since fibrin is the usual substrate of the healing process, involving stromal cell colonization of the fibrin network, healing in AAA corresponds to the disappearance of the ILT, due to its colonization by vSMC proliferation and migration, and the synthesis of extracellular matrix. In contrast, pathological evolution corresponds to the resumption of ILT biological activities, involving ILT formation and lysis associated with neutrophil trapping and activation, forming an oxidant and proteolytic impediment to vSMC recolonization. In this random clinical context, it is important to explore the mechanisms causing the resumption of ILT activity. Contamination of the ILT by weak pathogens, originating from dysbiotic changes in resident microbiomes, could be one of the major exogenous stimuli able to reinduce ILT biological activities, particularly by attracting neutrophils. Among the different biotopes, gingivo-dental weak pathogens could play an important role (27).

This pathophysiological view implicating the ILT in AAA initiation and progress towards rupture opens up new perspectives in the development of new diagnostic and preventive therapeutic approaches. In particular, markers of ILT turnover, platelet activation, and thrombin generation on the one hand, and of fibrinolysis and neutrophils on the other hand, could be used as biomarkers of AAA progression (37). In parallel, the currently developing functional and molecular imaging of the ILT should provide new opportunities to monitor its biological activities (38), including the intermediary clotting in endoleaks. In parallel, trials of the protective effect of platelet inhibition on AAA progression are in progress.

Blood/wall interactions in cerebral aneurysms

As compared to AAA, cerebral aneurysms are more frequently sacciform dilations that develop at bifurcation sites. Arterial bifurcations are highly sensitive to outward transwall mass transport of plasma components (39). Fusiform cerebral aneurysms also exist, but are mainly located in the basilar trunk. Familial forms are possible, providing evidence of genetic susceptibility (40), favouring haemodynamic/arterial wall pathological interactions, affecting arterial wall defects, particularly at the arterial bifurcations. Sacciform aneurysms, due to haemorrheologic vortexing, usually develop an ILT at the deepest part of the dilation. Therefore, intracranial aneurysms evolve in two stages: a first stage, in which genetic, congenital, or environmental factors make the arterial bifurcation susceptible to plasma-borne, haemodynamic-dependent, proteolytic injury, and a second, in which a haemorheologically induced intra-saccular thrombus promotes aneurysmal dilation and rupture (41).

Weak pathogens may also contaminate the intra-saccular thrombus in cerebral aneurysms (42). In this context neutrophil retention also plays an important role, as shown by the prognostic value of the plasma concentration of myeloperoxidase (43). The potential role of the ILT is also suggested by the risk of delayed rupture following flow-diversion. This treatment, by a highly porous stent, induces thrombosis of the sac, but not biological exclusion of the thrombus (41, 44) Therefore, understanding this pathophysiology should also lead to new developments in diagnostic and therapeutic prevention of rupture in cerebral aneurysms.

Aneurysm of the thoracic ascending aorta (TAA)

Contrasting with AAAs, TAAs are non-atheromatous aneurysmal diseases related to three main aetiologies: monogenic diseases, association with bicuspid aortic valves, and degenerative (45). TAAs are characterized by the usual absence of an ILT due to the powerful washing effect of left ventricular blood ejection. Therefore, TAA is a human model of the interaction between outwardly convected plasma components and vSMCs. In this context, syndromic or non-syndromic monogenic diseases (mutations of smooth muscle proteins (ACTA2, MYH11, PKG I) or proteins of the extracellular matrix (fibrillin in Marfan syndrome, *Col3A1* in vascular Ehler–Danlos, MFAP5, fibulin, microfibril associated protein 5, linked to elastin, lysyl-oxidase) and mutations on the TGF-β1 signalling pathway (TGF-β receptors, SMAD3, TGF-β2) (non-exhaustive) sensitize the extracellular matrix or the vSMCs to plasma-borne proteolytic injury (46), and bicuspid aortic valves to changes in haemodynamics at the aortic root. Degenerative forms are mainly linked to ageing. Usually in genetic aetiologies, TAA appears early and develops predominantly in the sinus of Valsalva. As described first by Leonardo da Vinci, the sinus of Valsalva is a site where physiological vortexing occurs during diastole, in relation to the closing of the aortic valve and coronary inflow. In contrast, in TAA associated with bicuspid aortic valves, haemodynamic modifications promote aneurysmal development on the outer curvature of the ascending aorta. Finally, degenerative forms can localize either in the sinus of Valsalva or in the aortic root. Whatever the aetiology, TAAs are characterized by a pathological process of alcianophilic mucoid degeneration associated with vSMC disappearance, and elastic and collagen fibre degradation. This pathological process can be, at least in part, compensated by intimal vSMC proliferation and by intramedial neo-angiogenesis coming from the adventitia. Intimal proliferation and intramedial neo-angiogenesis are more frequent in degenerative forms. In the absence of an ILT, no immune response takes place in the adventitia, except in rare aetiologies such as Takayasu disease or other forms of auto-immune aortitis. The mucoid degeneration is also a common pathological signature of TAAs and dissections.

As compared to AAA, the pathophysiology common to all forms of TAA is less well defined. Several hypotheses exist, mainly including the proteolytic pathways and the TGF-β pathways. There are many publications showing the involvement of proteases in TAA development and progression. In particular, matrix metalloproteinase (MMP) overexpression, retention in mucoid-degeneration areas (MMP-3 and MMP-7), diffusion (MMP-2 and MMP-9), and activation have been reported. Similarly to other arterial pathologies, outwardly convected plasma zymogens of serine-proteases, such as plasminogen and prothrombin, could play a major role in extracellular matrix degradation and vSMC loss, by their mass transport through the wall and their activation on molecular platforms exposed on vSMC membranes. Plasminogen has been particularly explored since it is converted to plasmin by vSMCs, and plasmin could produce cell loss and matrix injury by degrading fibronectin, fibrillin, and other adhesive intermediate glycoproteins. Plasmin also activates the TGF-β pathway by mobilizing TGF-β from its matrix storage sites (47). The pathogenic role of prothrombin activation by vSMC tissue factor expression remains to be explored. The second hypothesis is that an excess of TGF-β could be pathogenic in TAA, particularly in Marfan syndrome. This hypothesis was constructed from experimental data in fibrillin transgenic mice showing that treatment with anti-TGF-β antibodies protects against aneurysmal dilation. Since the canonical TGF-β pathway, including SMAD2 activation and nuclear translocation, initiates a healing process (increase in expression and secretion of extracellular matrix proteins, antiproteases, etc.), this hypothesis proposes the non-canonical JNK and ERK1/2 signalling pathway as a potential pathogenic and therapeutic target in TAA (48). In parallel, it was observed that p-SMAD translocation to the nuclei of vSMCs is a common feature to all forms of TAA, whatever the aetiology (49). Since this phenomenon persisted when vSMCs from TAA were cultured and was conserved after several passages of the cells, it was identified as an epigenetic phenomenon, related to histone acetylation and HAT/transcription factor activation on the promoter of the SMAD2 gene (50). The consequences of p-SMAD translocation to vSMC nuclei are an increase in the expression of genes controlled by the SMAD2 pathway, including the synthesis of CTGF, adhesive glycoproteins (fibronectin), collagens, antiproteases (serpins, TIMPs), anti-inflammatory mediators, and inhibition of lymphocyte proliferation. In this context,

SMAD overexpression and p-SMAD translocation are independent of TGF-β. Indeed, the dilatation (increased radius) in TAAs is associated with a decreased medial thickness. This leads to a dramatic increase in wall tension, at constant blood pressure, according to the law of Laplace, to which the remaining vSMCs are submitted. The nuclear envelope is coupled to mechanotransduction through vSMC adhesion to matrix, the coupling of integrins to actin, intermediate filaments and linkers of the nucleoskeleton to the cytoskeleton (LINC) (51, 52). It has been proposed that the biomechanical environment impacts the state of vSMC chromatin, thereby controlling vascular gene expression and function (53). It is, therefore, likely that the increased wall tension in TAAs leading to enhanced tensional mechanotransduction signalling between the matrix and the nucleus will have an impact on the chromatin remodelling in vSMCs. It was recently reported that TAAs associated with an ACTA2 nonsense mutation did not translocate p-SMAD suggesting that α-actin (54) is one of the necessary intermediates in the initiation of the vSMC chromatin remodelling associated with TAA.

Differences and common features of thoracic dissections and aneurysms

As compared to TAAs, characterized by a progressive dilation of the aorta, dissections are acute events defined by intramural rupture, with or without a subjacent aneurysm, usually of small dilation. The initial intimal tear, leading to blood leaks in the external part of the media, can take place in the ascending aorta (DeBakey type I dissection or Stanford type A) or just distal to the ostia of the left subclavian artery, in the descending thoracic aorta (DeBakey type III dissection or Stanford type B). Acute dissections and TAA share common aetiologies, including monogenic diseases, increased risk associated with bicuspid aortic valves, and degenerative forms. Similarly, the dissected tissue is also characterized by areas of mucoid degeneration, which pave the way for initial tears and haemorrhagic suffusion and diffusion within the wall. Tobacco, hypertension, and intensive physical effort (weightlifting), are risk factors for acute dissections. As compared to chronic dilation of TAA, acute dissections of the ascending aorta are characterized by similar activation of plasminogen, but in the absence of epigenetic modification of the SMAD2 pathway. In this context, due to SMAD2 translocation and autonomization in vSMCs from TAA, an overexpression of the tissue serpin protease nexin-1, able to block prothrombin, but also t-PA and plasmin, limiting *in situ* the activation of plasminogen into plasmin, was observed (50). Therefore, chromatin remodelling prevents vSMC anoikis in response to plasminogen (55). In contrast, in the absence of chromatin remodelling and SMAD2-dependent PN-1 overexpression, acute dissection (rupture) is promoted in relation to areas of mucoid degeneration (50) rather than chronic dilation (TAA) (➔ Fig. 16.4).

Dissections of the descending aorta are usually treated medically in an attempt to transform the acute event into a chronic dissection. Nevertheless, some morphological aspects of the dissection promote its detrimental evolution towards dilation and rupture (56). Usually, complete thrombosis of the false channel or a freely circulating false channel has a favourable evolution and does not require interventional correction. In contrast, partial thrombosis of the distal false channel, leading to formation of a recirculating

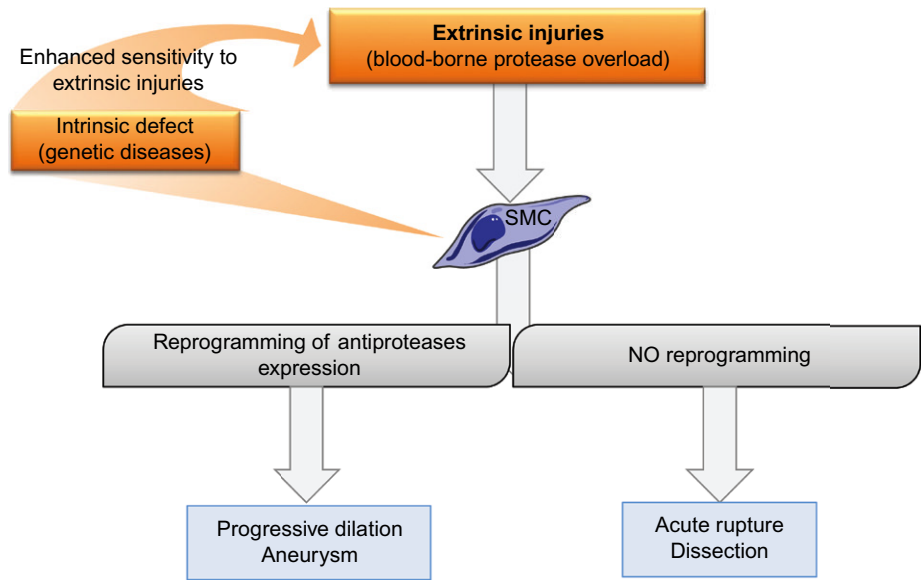

Fig. 16.4 Integrative diagram of the pathophysiology of acute (dissection) or chronic (progressive dilation) diseases of the ascending aorta.

pocket at the site of dissection entry, has a poor prognosis (56). Reflecting this partial thrombosis, markers of ILT formation (platelet activation, thrombi/antithrombin complexes) and degradation (fibrinolysis: D-dimers, plasmin/antiplasmin complexes) are circulating biomarkers of this unfavourable evolution (57). Moreover, in some cases, the biological activities of the ILT in the recirculating false lumen are so intense that they can produce a localized consumption coagulopathy with peripheral bleeding episodes, associating plasma fibrinogen and platelet consumption by the ILT with a high level of fibrinolysis (plasmin/antiplasmin complexes, D-dimers). In this situation, a curative intervention excluding the dissection, stops peripheral bleeding and reverses the consumption coagulopathy, restoring fibrinogen and platelet levels, and decreasing plasmin/antiplasmin and D-dimers (57).

Dilation in the venous system: varicosis

Contrasting with the arterial system, there is no process of outward convection in the low pressure, venous system. The interactions between blood components and the venous wall are essentially due to diffusion from blood stasis. Nevertheless, the venous endothelium is more adapted to leukocyte rolling than the arterial endothelium, particularly for neutrophils. The main determinant of acute (thrombosis) or chronic (varicose veins) venous pathologies is stasis. Venous stasis is rapidly associated with interactions between neutrophils and the endothelium, via P-selectin expression on the activated endothelium and PSGL-1 (P-selectin glycoprotein ligand-1 (CD162) a sulphated glycoprotein containing the *sialyl Lewis*^{X} tetrasaccharide) constitutively present on the neutrophils.

Saphenous varicose veins are characterized by a succession of dilated segments, in which the venous wall is atrophic, and more stenosing ones, in which vSMCs are hyperplasic, dissociated by bundles of collagen and elastolysis (58). Stasis is induced by blood reflux, due to valve incompetency, in the saphenous vein in the upright position, possibly aggravated by increased intra-abdominal pressure (pregnancy) and a female hormonal environment. In the supine position there is no reflux and the stasis disappears. The transitory character of the stimulus probably explains the succession of dilated, proteolytically injured segments and sclerotic segments corresponding to a localized healing process. Therefore, measurement of markers of neutrophil/venous wall interaction should be made locally (saphenous vein) and in an upright position, favouring reflux and stasis (59). In this condition, it is easy to observe a decrease in PO_2 and an increase in biomarkers of neutrophil activation, including MMP-9, sVCAM, sACE acute release (59), and others.

Conclusions

Aneurysms, whatever their localization, are important life-threatening arterial pathologies, whose diverse pathophysiological mechanisms are only beginning to be understood, but with a common denominator of outward mass transport of circulating components across the wall, and a predominant role of proteolysis. Nevertheless, the pathophysiology differs in part between AAA of atherothrombotic origin and aneurysms of the ascending aorta, where genetic susceptibility and interaction between convected plasma zymogens potentially play a predominant role in their evolution. Several questions remain unresolved, such as the mechanism of dissection initiation, but the recently acquired knowledge in the field paves the way for the development of diagnostic tools for monitoring the evolution of detected aneurysms, and new therapeutic approaches for preventing their evolution towards rupture.

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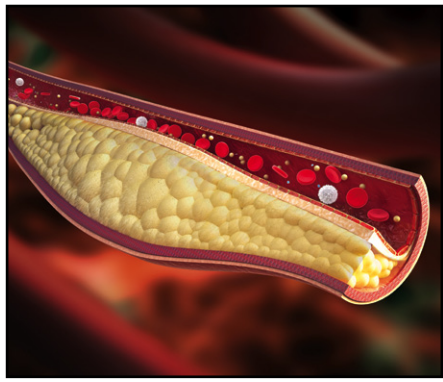

CHAPTER 17

Pathophysiology of vasculitis

Enrico Tombetti and Justin C. Mason

Content

Introduction

The circulatory system is essential in complex multicellular organisms for maintaining tissue homeostasis, metabolic requirements, leukocyte trafficking, removing waste products, carrying endocrine signals, and promoting tissue repair. However, pathogens and toxins may be blood borne. Thus the circulatory system must sense potential threats and react to danger signals. The central roles played by the vasculature in inflammatory, immune, and repair responses, while beneficial, may also represent a threat if deregulated. Hence, protective and homeostatic mechanisms evolved to maintain vascular integrity (1). Macrovessels, which are intrinsically less plastic than small vessels, have developed specific defence mechanisms, such as the creation and maintenance of an immunoprivileged niche within the arterial wall (2–4). Vasculitides occur when these protective mechanisms fail, and inflammation resulting in tissue injury ensues in the vascular wall.

The vasculitides are a group of disorders that affect blood vessels from capillaries to the aorta. The revised Chapel Hill consensus nomenclature is predicated upon division into large-, medium-, and small-vessel vasculitides (5) (➔ Fig. 17.1). These diseases may, in their various forms, affect all age groups, with, on occasion, devastating outcomes. Over the last three decades, treatment efficacy has improved remarkably, particularly for patients suffering from anti-neutrophil antibody-associated vasculitis (AAV). Thus granulomatosis with polyangiitis (GPA), once almost invariably fatal, is now managed effectively in the significant majority of patients. Although progress has been slower in the large-vessel vasculitides (LVVs), the outcome for patients with Takayasu arteritis (TA) has improved over the last decade (6, 7). Notwithstanding, the side-effect burden of therapy for many vasculitis patients remains high, morbidity significant, and lifespan often curtailed. Despite this, the outlook is good and, although much work remains to be done, the constant advances in our understanding of disease pathogenesis will lead to novel, targeted biological therapies. This optimism is supported by the emergence of B-cell depletion as part of standard therapy for refractory AAV, or in those for whom cyclophosphamide is contraindicated (8, 9). However, there remains an urgent need to minimize the dependence upon corticosteroids in all vasculitis treatment protocols. While multi-centre clinical trials have played a major role in the development of evidence-based treatment

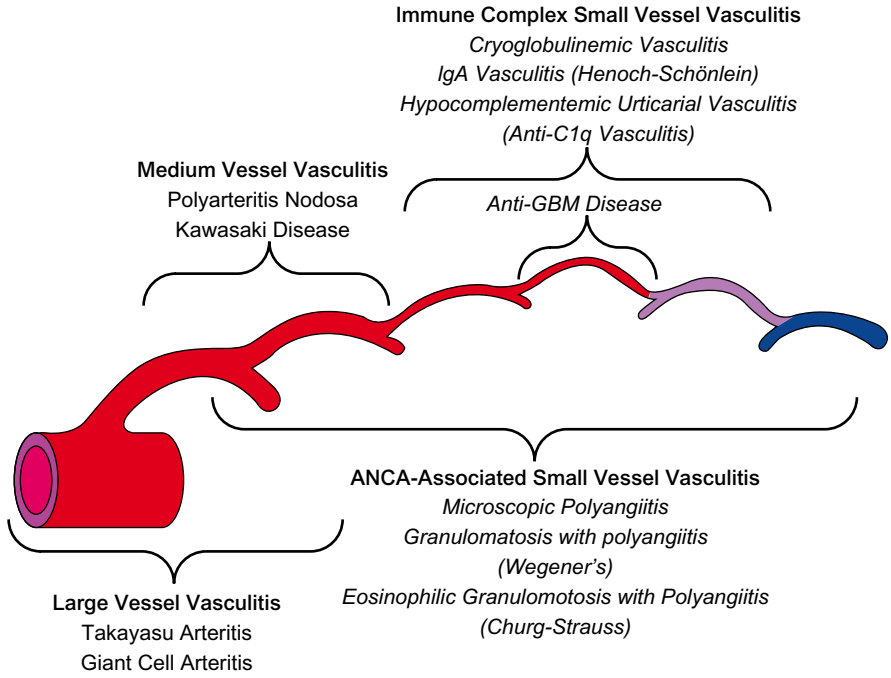

Fig. 17.1 Distribution of vessel involvement by large-vessel vasculitis, medium-vessel vasculitis, and small-vessel vasculitis. Note that there is substantial overlap with respect to arterial involvement, and an important concept is that all three major categories of vasculitis can affect any size artery. Large-vessel vasculitis affects large arteries more often than other vasculitides. Medium-vessel vasculitis predominantly affects medium arteries. Small-vessel vasculitis predominantly affects small vessels, but medium arteries and veins may be affected, although immune complex small-vessel vasculitis rarely affects arteries. Not shown is variable-vessel vasculitis, which can affect any type of vessel, from aorta to veins. The diagram depicts (from left to right) aorta, large artery, medium artery, small artery/arteriole, capillary, venule, and vein. Anti-GBM = anti-glomerular basement membrane; ANCA = anti-neutrophil cytoplasmic antibody.

(Reproduced from Jennette JC et al: 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides Arthritis and Rheumatology 65:1–11, 2013 with permission from John Wiley & Sons.)

protocols for AAV (10, 11), this is not yet the case for the LVVs giant cell arteritis (GCA) and TA (12), and new carefully designed clinical trials are required (13).

This chapter will focus on current understanding of the pathogenesis of AAV and LVV, their cardiovascular sequelae, and relevance to those involved in delivering cardiovascular healthcare.

ANCA-associated vasculitis

Clinical features

These pauci-immune vasculitides may affect multiple organ systems or remain limited and confined to a single organ, such as the upper airways, the lung, or the kidneys. Severity varies from mild through to life-threatening. Multi-disciplinary clinical input is required to achieve optimal patient outcomes. Organ involvement can be classified as granulomatous or vasculitic, with potential pathogenic differences. Frequently seen manifestations include a purpuric rash, ocular inflammation ± proptosis, deafness, upper airway inflammation ± nasal bridge collapse, glomerulonephritis, arthralgia/arthritis, mononeuritis multiplex, asthma, lung nodules, and pulmonary haemorrhage. Severe myocarditis may be seen, most commonly in patients with EGPA, while pericarditis, valvular heart disease, coronary arteritis, and aortitis have all been reported (14).

AAV have been carefully categorized in the 2012 revised Chapel Hill Consensus nomenclature (5) (➔ Fig. 17.1). Anti-neutrophil cytoplasmic antibodies (ANCA)-positive patients with granulomatosis, asthma, and blood eosinophilia are labelled eosinophilic granulomatosis with polyangiitis (EGPA; Churg–Strauss syndrome), those with granulomatosis, no asthma or blood eosinophilia are defined as granulomatosis with polyangiitis (GPA; Wegener's granulomatosis), and, finally, those with vasculitis and no evidence of granulomatosis or asthma are diagnosed as microscopic polyangiitis (MPA). In addition, the ANCA antigen specificity can be added to this label. Although myeloperoxidase (MPO) ANCA are commonly associated with MPA and proteinase-3 (PR3) ANCA with GPA this is by no means absolute. It is now recommended that patients are labelled as PR3-ANCA GPA, MPO-ANCA GPA, MPO-ANCA-MPA, or PR3-ANCA MPA, as appropriate (5).

Anti-neutrophil cytoplasmic antibodies

ANCA, first reported in 1985 (15), play a central pathogenic role in the AAVs. AAVs most commonly affect arterioles and small arteries, and on occasion medium/large arteries and the venous circulation. Two indirect immunofluorescent staining patterns for ANCA are described: perinuclear (pANCA) and cytoplasmic (cANCA) (➔ Fig. 17.2). To determine clinical significance, a positive ANCA test must be further validated by an enzyme-linked immunoabsorbent assay or an equivalent test. Antibodies against the two principle antigens, MPO and PR3, are sought. The pANCA staining pattern is most commonly associated with antibodies against MPO (16), and cANCA staining with anti-PR3 antibodies (17). Alternative ANCA targets include elastase, most frequently found in AAV associated with drug abuse, including the use of cocaine contaminated with levamisole (18). Although lysosomal-associated membrane protein-2 (LAMP-2) has been proposed as an alternative ANCA target antigen, the reported frequency in different series of AAV patients varies significantly, and anti-LAMP-2 ANCA has also been found in sera from patients with Henoch–Schönlein purpura and polyarteritis nodosa (19–21). A positive immunofluorescence ANCA test, typically with a pANCA pattern and without a positive MPO or PR3 ELISA test, may also be found in disorders such as inflammatory bowel disease.

The predominant role for ANCA detection is in diagnosis, where a positive ANCA and identification of the associated target antigen has >90% sensitivity and specificity for the diagnosis of GPA or MPA. ANCA positivity is less frequent in EGPA (≈40% of patients). However, the ANCA titre does not translate to accurate assessment of disease activity during follow-up, or for prediction of relapse (22). Additional data are also required to determine whether vascular injury and organ damage in AAV is initiated by ANCA alone or in conjunction with other immune mechanisms. Involvement of the latter is suggested by the observation of active disease in ANCA-negative patients (23, 24). This may to some extent reflect insensitivity of current assays, the presence of alternative autoimmune mechanisms, and that MPO and PR3 ANCA represent only part of a secondary response to an initial upstream insult. ANCA epitope specificity is a critical factor, determining not only pathogenicity, but also ease of detection (25).

Animal models

Although direct evidence in support of pathogenic ANCA in patients has not been obtained, two therapeutic approaches support their central role. First, plasma exchange has a beneficial effect (26), especially in those presenting with blood creatinine >500 μmol/l or with pulmonary haemorrhage. Second, B-cell depletion is an effective treatment for AAV. Rituximab, an anti-CD20 mAb, is effective for inducing remission at disease presentation and for gaining control in refractory relapsing disease (8, 9, 27). However, important gaps in our knowledge exist, including details of the mechanisms underpinning the generation and persistence of ANCA, and the precise roles of humoral and cellular immunity. In this regard, rodent models have been instrumental in expanding our understanding (28).

An important breakthrough was the report that immunization of *Mpo*$^{-/-}$ mice with murine MPO generated

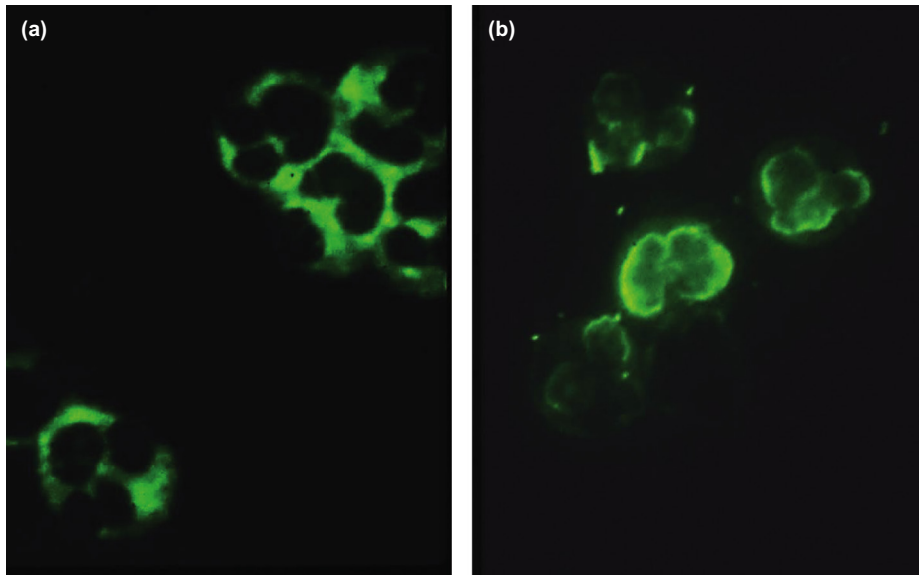

Fig. 17.2 Anti-neutrophil cytoplasmic antibody staining. Indirect immunofluorescence assay demonstrating: (a) cytoplasmic cANCA staining pattern and (b) perinuclear pANCA staining.

anti-MPO antibodies, which, when transferred to recipient wild-type or immunodeficient mice, were able to activate neutrophils and induce proteinuria, haematuria, and pauci-immune glomerulonephritis (29, 30). Subsequent studies using mice from different genetic backgrounds revealed variable sensitivity to MPO antibodies and dependence upon neutrophil numbers and the presence of B cells for disease development (31). Moreover, vasculitis was exacerbated by pre-treatment with granulocyte colony-stimulating factor and the addition of lipopolysaccharide to mimic an infectious insult (32).

Additional studies of the murine MPO model have shed further light on the pathogenesis of ANCA. First, using a bone marrow transplant technique, the necessity for MPO expression by haematopoietic cells for disease manifestation was revealed (33). Second, an unexpected finding has been the demonstration that, despite the pauci-immune nature of the glomerulonephritis, the alternative pathway of complement plays a role in the activation of neutrophils and disease pathogenesis (34). Furthermore, in transgenic mice expressing the human C5a receptor, a small molecule antagonist of the C5a receptor reduced glomerular disease (35). An important T-cell effector arm of MPO-associated AAV pathogenesis has also been identified. Immunization of C57Bl/6 mice with MPO plus adjuvant followed by low-dose anti-glomerular basement membrane serum, induced neutrophil recruitment to the glomerulus and deposition of MPO. The recognition of MPO by CD4+ T cells subsequently contributed to the evolution of nephritis (36, 37). Th17 cells also appear to play an important role. Their numbers are increased in the plasma of patients with AAV, along with serum IL-17 and IL-23 (38), while IL-17A-deficient mice exhibit resistance to anti-MPO-associated glomerulonephritis (39).

An alternative model of MPO-associated vasculitis has been developed in the WKY rat, a strain particularly prone to glomerulonephritis. WKY rats develop anti-MPO antibodies along with MPO-reactive effector T cells when immunized with human MPO plus adjuvant (40). The subsequent disease is characterized by a pauci-immune crescentic glomerulonephritis and some rats develop pulmonary capillaritis (41).

In contrast to MPO, developing models of PR3-associated AAV have proved more challenging (28). However, immunization of the autoimmune prone non-obese diabetic (NOD) mice with murine PR3 resulted in the development of PR3-ANCA generating splenocytes, which were then transferred to naïve NOD-severe combined immunodeficient recipient mice (NOD-SCID). These animals developed vasculitis and a necrotizing crescentic glomerulonephritis, while, of note, the immunized NOD mice developed PR3 ANCA without vasculitis. In a refinement, chimeric mice were generated by transfer of human haematopoietic stem cells into irradiated NOD-SCID-IL-2 receptor deficient mice. Subsequent infusion of human anti-PR3 antibodies resulted in a proportion of mice developing a mild glomerulonephritis, haematuria, and pulmonary haemorrhage (42).

Although rodent models continue to prove invaluable for the dissection of pathogenic mechanisms in AAV, the questions of how disease is initiated and how immune tolerance is broken in patients remain to be determined.

Generation of ANCA

A variety of theories for ANCA generation have been proposed (19, 43) (➔ Box 17.1). Initiation by infection, although difficult to prove, is likely to play a role at some level. Indeed, nasal carriage of *Staphylococcus aureus* is a risk factor for relapse in GPA that has been most commonly implicated, and co-trimoxazole is often used as part of maintenance therapy. In the case of LAMP-2 ANCA, molecular mimicry has been suggested as an explanation for pathogenic antibody development (20). The identification of low-titre non-pathogenic ANCA in healthy people may be evidence for ANCA being part of the natural antibody repertoire (44), with the onset of disease reflecting loss of immune regulation and generation of high-affinity pathogenic antibodies (45). It has been reported that epitope spreading is an important contributor to this switch (25). This may in turn reflect impaired T- or B-cell regulatory function. An intriguing alternative hypothesis suggests that autoimmunity may arise following an immune response to a peptide that has a complementary structure to the auto-antigen. A subsequent anti-idiotype response results in the development of antibodies that target the autoantigen (19, 46).

Box 17.1 Factors proposed to contribute to ANCA generation

- Precipitating infection (e.g. *S. aureus*, Ross River virus)
- Natural non-pathogenic ANCA
- Modulation of epitope specificity (epitope spreading)
- Neutrophil apoptosis
- NETosis
- Genetic variation in antigen recognition
- Immune response to an autoantigen complementary peptide
- Defective immunoregulation (B regs and T regs)
- Drug-induced ANCA (e.g. propylthiouracil, levamisole adulterated cocaine)

Mechanisms underlying vascular injury

In vitro studies have demonstrated that MPO and PR3 ANCA activate both neutrophils and monocytes. Activation of neutrophils by ANCA is dependent upon a variety of factors, including neutrophil priming. Proinflammatory factors (TNF-α and C5a) and microbial components (lipopolysaccharide) induce neutrophils to express MPO and PR3 on their surface, so exposing them to the immune system (47, 48). Engagement of Fcγ receptors also plays a central role in neutrophil and monocyte activation by antigen-bound ANCA. The release of cytokines and proteases, a resultant neutrophil respiratory burst, reactive oxygen species (ROS) generation, complement activation, and enhanced leukocyte adhesion to vascular endothelium also contribute to disruption of endothelial function and endothelial cell death (49–52). Moreover, activated monocytes secrete proinflammatory chemokines, including CCL-2 and interleukin-8, which further amplify the response via recruitment of additional monocytes and neutrophils (53).

Upon activation, neutrophils extrude chromatin fibres, referred to as neutrophil extracellular traps (NETs), the function of which is to engulf and kill pathogens. The ANCA-induced neutrophil respiratory burst is sufficient to induce NETosis. MPO and PR3 have been detected within NETs and can bind DNA (54). ANCA-induced NETs have been found both *in vitro* and in renal biopsies from AAV patients with nephritis. The formation of NETs is likely to contribute to vascular injury in AAV, similar to that observed in sepsis (55), and may also present PR3 and MPO to the host immune system (54).

Eosinophils play a central role in the asthma associated with stage 1 of EGPA, and in the second stage characterized by blood and tissue eosinophilia. In stage 3, eosinophils infiltrate the tissues. In the vasculature they target small arteries and veins (56). In contrast to MPA and GPA, a direct role for ANCA in EGPA is not established, despite ANCA being detected in 30–40% of patients.

B- and T-cell autoimmunity

An important T-cell contribution to AAV pathogenesis is supported by the presence of activated T cells in renal and pulmonary biopsy specimens (43). $CD4^+$ effector memory T cells are increased in the circulation of GPA patients in remission. They are decreased during a flare and present in increased numbers in the urinary sediment of patients with active nephritis (57). Further, evidence suggests that Th1 and Th17 cells contribute to pathogenesis. In biopsy tissue from AAV granulomatous lesions Th1 cells predominate (58), while Th17 cells appear to be increased in GPA, and when exposed to PR3 they secrete IL-17 (59). Indeed, both IL-23 and IL-17 levels are increased in the serum of patients with AAV (38).

Despite the efficacy associated with B-cell depletion in AAV (8, 9, 60), understanding of the role of B cells, above and beyond generation of ANCA, remains incomplete. B cells can present antigens to T cells, and an antibody-independent role in disease pathogenesis is suggested by the identification of activated B cells closely associated with $PR3^+$ cells and plasma cells in endo-nasal lesions and in GPA-associated glomerulonephritis (61, 62). Further, B-cell activity factor (BAFF) levels may be increased in patients with AAV (63, 64), and the activation state of peripheral blood B cells appears to be altered. B-cell CD38 and CTLA-4 expression is increased during active AAV (65), while CD86 and CD25 are raised on B cells from patients with corticosteroid-induced remission (66). Considerable interest has focused on the relative roles of regulatory B and T cells (43, 67). The former are identified as $CD24^{hi}$ $CD38^{hi}$ B cells and exhibit increased IL-10 secretion and an ability to modulate T-cell activity. Although studies are ongoing, B regs are reported to be reduced in active AAV when compared to healthy controls, while in a separate study numbers were comparable in patients with active disease and those in remission (68, 69). Todd and colleagues have suggested that an imbalance between B regs and B memory cells associated with reduced IL-10 and increased Th1 cell activation might be reflected in the high relapse rate seen in AAV, and particularly in PR3+ patients (70).

The understanding of the role of T regs in AAV is also evolving. Although research findings to date are not always concordant, the consensus suggests that T-reg function is diminished and that this predisposes to Th1- and Th17-dominated responses (43). Moreover, functional impairment in B regs with reduced IL-10 generation may exacerbate this phenotype.

Disease manifestations

The diverse clinical features encountered in patients with AAV reflect vascular and extravascular disease (see Figs 17.3–17.5). The vasculitis is typically pauci-immune and targets small arteries, arterioles, capillaries, and venules. The invading neutrophils are prone to apoptosis/necrosis and NETosis. The resultant injury to vessel walls may lead to haemorrhage in the lung in GPA and in the bowel in EGPA, and also predisposes to the development of fibrinoid necrosis (5). As the lesions become more chronic, monocytes and macrophages

Clinical Features associated with GPA

- Pulmonary nodules
- Pulmonary haemorrhage
- Tracheal stenosis
- Glomerulonephritis
- Cutaneous vasculitis
- Sinusitis and proptosis
- Orbital and nasal bridge collapse
- Conductive hearing loss
- Myalgia and arthralgia
- Mononeuritis multiplex
- Cranial nerve palsy
- Cardiac involvemet
- GI Tract involvement

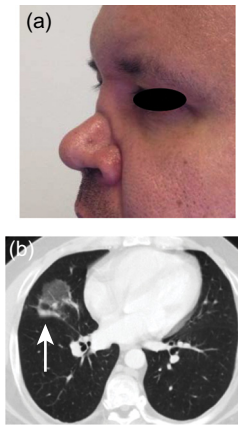

Fig. 17.3 Granulomatosis with polyangiitis (GPA). The clinical features of GPA are listed. (a) Characteristic nasal bridge collapse. (b) CT scan of the chest with a GPA-associated granulomatous mass lesion in the right lung (arrow).

Clinical Features associated with MPA

- Glomerulonephritis
- Myaligia and arthralgia
- Pulmonary capillaritis
- Pulmonary haemorrhage
- Interstitial lung disease
- Cutaneous vasculitis
- Mononeuritis multiplex
- Sensorimotor neuropathy
- Cranial nerve palsy
- Cardiac involvement
- GI Tract involvement

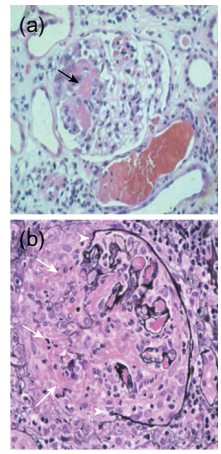

Fig. 17.4 Renal biopsies in microscopic polyangiitis. (a) Haematoxylin and eosin stain showing segmental fibrinoid necrosis (black arrow). (b) Haematoxylin and eosin with additional silver staining. The biopsy reveals crescentic nephritis (white arrow) with rupture of the Bowman's capsule (arrowheads).
(Images courtesy of Professor Terence Cook, Imperial College London.)

begin to dominate and T cells migrate to the sites of inflammation. In glomeruli this may manifest in the development of a crescentic nephritis (➔ Fig. 17.4). Although eosinophils may be present in these lesions in both MPA and GPA, they predominate in EGPA (➔ Fig. 17.5).

Extravascular granulomatosis with multinucleate giant cells is typically found at the site of pulmonary lesions in GPA and these may cavitate. Granulomatosis is the predominant lesion in the airways and may result in local tissue destruction leading to nasal bridge collapse, orbital erosion, and severe tracheal involvement (➔ Fig. 17.3). Pulmonary infiltrates are a characteristic early feature in EGPA. Eventually, vascular and extravascular lesions are prone to replacement by scar tissue and varying degrees of residual organ damage.

In the cardiovascular system perhaps the most serious complication in AAV is myocarditis, most frequently associated with EGPA. In addition to necrotizing vasculitis, eosinophils may invade the cardiovascular tissues resulting in eosinophilic myocarditis, pericarditis, endomyocardial fibrosis, or coronary artery occlusion and myocardial infarction. Although rare, coronary arteritis, myocarditis, and aortitis are recognized in GPA, while MPA has been associated with pericarditis and coronary artery micro-aneurysms and myocardial infarction (14).

Large-vessel vasculitides (LVVs)

Large-vessel vasculitides (LVVs) are rare conditions characterized by idiopathic inflammation within the wall of large-sized arteries (lumen >5mm) (71). Typical involvement of the inner arterial wall structures, such as the intima and media, distinguishes LVVs from peri-vasculitides.

Clinical Features associated with EGPA

- Asthma
- Pulmonary infiltrates
- Sinusitis, polyposis
- Eosinophilia with tissue infiltration
- Mononeuritis multiplex
- Sensorimotor neurophathy
- Cardiac involvement with myocarditis
- Cutaneous vasculitis
- GI tract involvement
- Arthralgia

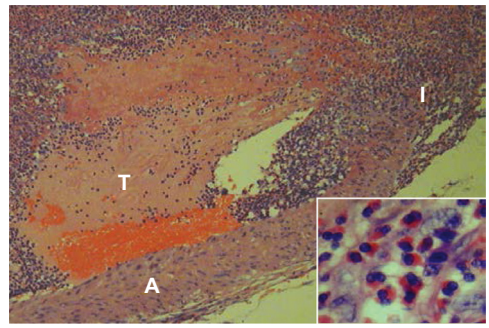

Fig. 17.5 Eosinophilic granulomatosis with polyangiitis The clinical features of EGPA are listed. The biopsy shows the wall of an arteriole (A) with thrombus (T) and a dense leukocytic infiltrate (I), consisting predominantly of eosinophils (inset high power view).

LVVs (Box 17.1) are mainly represented by GCA, affecting patients >50 years, and associated in ≈50% of subjects with polymyalgia rheumatica (PMR), and TA, which typically affects patients <40 years (5) (Fig. 17.1). Isolated aortitis may represent a localized variant of GCA and TA, and it is still unclear whether it should be considered a separate disease (72–74). Finally, large-sized arteries may be involved in other vasculitides, especially the 'variable-vessel vasculitides' (e.g. Beçhet's syndrome or Cogan's disease).

GCA and TA share many pathogenetic features and have been proposed to represent two extremes of the same disease spectrum, with a modified age-related phenotype (75–77). The essential features of LVVs are discrete arteritic lesions that typically involve all the three tunicas, resulting in wall thickening. Lesions predispose to steno-occlusions or ectasia/aneurysms, and have a patchy nature with a highly variable spatial distribution. Moreover, GCA can also involve medium-sized arteries, resulting in the common 'cephalic' phenotype, characterized by steno-occlusion of the extra-cranial branches of the external carotid arteries. A rarer, large-vessel, 'systemic' GCA (LV-GCA), sometimes indistinguishable from elderly-onset TA, has been recognized relatively recently (78). Cranial and systemic phenotypes may co-exist and combine with ascending aortitis. In TA and LV-GCA the pattern of arterial involvement is usually symmetric (79), with regional clustering of arterial lesions.

Understanding of LVV pathogenesis derives predominantly from GCA temporal artery biopsies. Active lesions reveal a lymphomonocytic infiltrate, sometimes structured in granulomas with giant-histiocytes (80–82). In GCA, inflammation is usually transmural, involving primarily the media–adventitia border and secondarily the intima–media junction, with fragmentation of the internal elastic lamina, giant histiocytes, and occasionally laminar necrosis (81). Vasa vasorum neoangiogenesis is frequent. Inflammatory infiltrates most commonly comprise monocyte/macrophages and $CD4^+$ T cells, followed by $CD8^+$ T cells and dendritic cells (DCs) (2, 81, 83, 84) (Fig. 17.6). Plasma cells and eosinophils are generally inconspicuous and neutrophils rare (81). Lumen occlusion is primarily due to myofibroblast intimal hyperplasia, whereas thrombosis is rare (81). The media is typically hypoplastic or scarred, with occasional leukocytic infiltrates. One-fifth of GCA biopsies reveal inflammation limited to the adventitia. These cases may represent disease variants less often associated with cranial/systemic symptoms or an acute-phase response (81). Histological findings in TA are similar, with the differences being a more severely scarred adventitia (72), a preponderance of γδT cells within infiltrating lymphocytes, and the presence, although not abundant, of NK-cells (85).

Aside from age, concurrent PMR and involvement of medium-sized cranial arteries, GCA also differs from TA because the disease more often follows a monophasic course

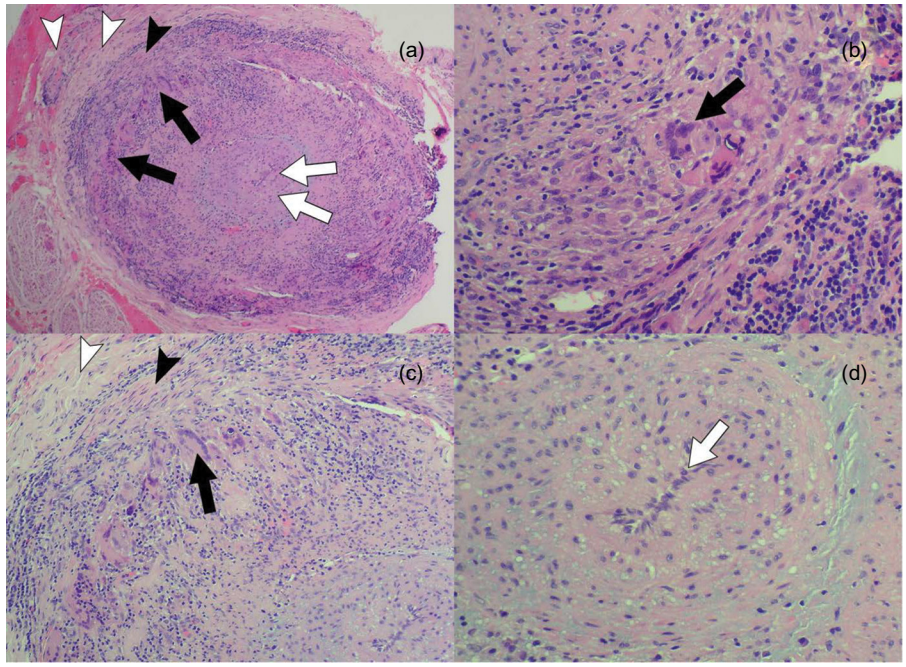

Fig. 17.6 Giant cell arteritis. (a)–(d) Haematoxylin and eosin stain of a temporal artery affected by giant cell arteritis. Note the mononuclear inflammatory infiltrate with the internal elastic lamina no longer identifiable. The adventitia is inflamed and with increased vasa vasora (white arrowheads). The medial smooth muscle is degenerate and scarcely recognizable (black arrowheads). Giant cells are present in the tunica media (black arrows) and lumen-occlusive intimal hyperplasia is evident (white arrows).

and displays a robust systemic inflammatory response, which is more closely associated to disease activity. In the large arteries, GCA is less stenotic and more aneurysmal, and is less responsive to DMARDs and tumour-necrosis factor-α (TNFα)-inhibitors.

Pathogenesis of LVVs: general considerations

LVVs represent a response to a heterogeneous combination of unrecognized environmental factors and genetic predisposition (see ➔ Genetic associations in vasculitis). Cellular immunity plays a central role. Foreign antigens, particularly infective in nature, have been implicated (86, 87); indeed, varicella zoster can cause cerebral arteriopathy and has been linked with GCA (88, 89). However, such observations are controversial, since the presence of microbes in normal arteries is increasingly acknowledged (90).

In the microcirculation, circulating leukocytes interact with the post-capillary endothelium, extravasate, and reach tissues or the arteriolar compartment guided by pericytes (91–93). In macrovessels, high flow velocities limit this mechanism. Indeed, infiltrating leukocytes localize close to vasa vasorum, suggesting that the latter may represent the portal of entry to the arterial wall. Vasa vasorum are limited to the adventitia in healthy human medium and large arteries (with the exception of the thoracic aorta, where they extend into the media). Unfortunately, representative rodent models of the LVVs are difficult to develop, as the arterial wall is thin enough to be supplied solely by diffusion from the lumen (71). This led to the generation of a chimera, where human temporal arteries are engrafted into immunodeficient mice (94).

Dendritic cells

Studies of human–mouse chimeras indicate that myeloid DCs are critical for the induction and the maintenance of vasculitis. Inactive DCs are tolerogenic, while activated myeloid DCs induce immune responses in naïve and memory T cells. DCs are the most efficient antigen-presenting cells (APCs) in the arterial wall, as shown by *in vitro* engineered models of medium-sized arteries (3).

Chemokines, different cellular stresses, or ligands of innate-immunity receptors (pathogen-associated molecular patterns, PAMPs; damage-associated molecular patterns, DAMPs) can trigger DC activation and maturation. Toll-like receptors (TLRs) are the best-known family of innate-immunity receptors. During a multi-step maturation process, DCs first express the chemokine receptor CCR7 and migrate to lymph nodes where they secrete T-cell trophic chemokines (e.g. CCL18, CCL19, and CCL21) and activate CCR7. Thus, they remain trapped in lymphoid structures and orchestrate cellular immunity.

Large- and medium-sized arteries normally lack leukocytes, except for a particular population of myeloid DCs at the adventitial side of the external elastic lamina: the vascular DCs (VDCs) (95). VDCs are most abundant in the aorta, followed by carotids and iliac arteries (96). A smaller population resides in the intima of the aorta and carotids (96). In active vasculitic lesions, DCs are activated, populate the intima–media and express the co-stimulatory molecule CD86 (2, 82). Otherwise, VDCs act as sentinels maintaining an inactive state and tolerance to vascular antigens. However, activation of VDCs by lipopolysaccharide (a TLR4 agonist), induces T-cell activation and arterial wall inflammation (2, 96). Interestingly, distinct VDC agonists are associated with different patterns of vasculitis that reflect the histologic variants of GCA (81): TLR4-agonists induce a transmural vasculitis, while TLR5-ligands promote an adventitial pattern (97).

These studies showed that (i) macrovessel immunoprivilege can be overcome by VDC activation, and (ii) DCs can locally prime immune responses, endowing macrovessels with secondary lymphoid organ properties. VDC activation represents an early step in LVV pathogenesis and appears to be necessary for maintenance of vasculitis (2). Given their gatekeeper function, VDCs have been implicated as potential factors in the tropism of large- and medium-vessel vasculitides (MVVs), the localization of inflammatory lesions and their heterogeneous spatial distribution (71). Different arterial vascular beds harbour distinct VDC populations, which express diverse TLRs and respond to different activating stimuli (96). The physiological function underlying this spatial compartmentalization of VDCs is not clear.

T-cell responses

T cells and macrophages are the most abundant leukocytes in LVV lesions. In GCA arteries, $CD4^+$ T-cell counts are two to three times greater than $CD8^+$, while γδT-cells are rare (98–100). In TA arteries, γδT-cells are abundant, while $CD4^+$ T cells, $CD8^+$ T cells, and NK-cells are equally represented (82). Priming of T-cell immune responses may occur either in lymph nodes or locally within the arterial wall. After maturation into effector cells, T cells circulate between blood and the arterial wall, where they are retained by specific antigens and local chemokines (100–103). In GCA T-cell activation, proliferation, and survival are also promoted by NOTCH1 receptor signalling, recognizing cognate ligands on DCs and stromal cells (104).

T-cell target antigens in LVVs remain to be determined. Candidates might be foreign antigens, such as microbes and inert materials, or auto-antigens in the arterial wall. One mechanism proposed to explain autoimmunity against vascular antigens is molecular mimicry triggered by antimicrobial responses (105). However, a common target antigen has not been identified, and this may reflect epitope spreading or disease heterogeneity.

In GCA, vasculitis-promoting $CD4^+$ T cells appear to polarize towards Th1, Th17, or overlapping Th1/Th17 phenotypes (98). In parallel, regulatory T-cell activity seems to be impaired (106, 107). Th1 and Th17 cells participate in type-1 and type-3 responses that protect against intracellular and extracellular microbes, respectively (108). Efficient type-1 immunity requires cooperation between Th1 cells and other lymphocytes, including cytotoxic $CD8^+$ T cells-1 (Tc1), NK-cells, and group-1 innate lymphoid cells (ILC1) (108). Th1 cells produce interferon-γ (IFNγ) and proinflammatory cytokines, such as TNFα and lymphotoxin-α. Moreover, they induce macrophage activation and are required for granuloma formation. The recently identified Th17 cells represent the best studied population responsible for type-3 immunity (108). Upon antigen recognition, they produce inflammatory cytokines, including IL-17A, IL-17F, IL-22, TNFα, IL6, and granulocyte-monocyte colony-stimulating factor, that recruit macrophages and neutrophils (108).

While Th1 cells typically originate from naïve T cells in the presence of DC-derived IL-12, differentiation of human Th17 cells is more complex. They may derive from naïve T cells and also from $CD161^+$ precursors, driven by different Th17-polarizing stimuli (108, 109). In response to Th1-polarizing stimuli, Th17 cells display plasticity, acquiring overlapping Th1/Th17 or even Th1-reminiscent phenotypes (108).

In GCA, arterial-infiltrating lymphocytes, including 50% of IFNγ-producing cells, express CD161 (107), suggesting that many infiltrating lymphocytes with a 'Th1-like' phenotype may actually derive from $CD161^+$ precursors. These cells may represent a potential therapeutic target. IL-21, a cytokine produced by IL6-activated T cells, is another potential target. IL-21 appears an important regulator of T-cell polarization in GCA, stimulating both Th1 and Th17 and suppressing T-reg differentiation (106).

Th1 cells appear to maintain subclinical inflammation in GCA patients treated with steroids (98), and may, therefore, be involved in disease flares. Th17 cells are likely responsible for the acute systemic inflammatory component of LVVs. Glucocorticoids readily suppress the Th17 but not the Th1 arm in GCA, possibly by reducing Th17-polarizing cytokines despite stable IL-12 concentrations (98). Additionally, Th1-cytokines influence wall remodelling: IFNγ favours lumen-occlusive intimal hyperplasia, as well as medial degeneration (➔ Table 17.1) (71). Notably, IFNγ levels predict the risk of ischaemic manifestations (71). In TA, Th1, and Th17, roles may be similar to GCA, although a different pattern of steroid-responsiveness has been reported (110).

Despite these advances in knowledge it remains unclear whether (i) Th1 and Th17 responses in GCA are sustained by separate APCs and/or by diverse stimuli, (ii) if relapses are accompanied by recurrent Th17 activity, (iii) if this is favoured by a persistent Th1 response, and (iv) how steroids can induce long-term medication-free remission in a significant percentage of patients while not controlling Th1 responses.

Takayasu lesions are particularly abundant in γδ-cells, which are CD4–CD8– T cells expressing the γδT-cell receptor (TCR), rather than the 'conventional' αβ TCR. Compared to αβ-cells, γδ-cells recognize a smaller number but wider variety of potential antigens (111). Since negative selection

Table 17.1 Mechanisms of vascular remodelling in LVVs

Pathologic event	Pathogenesis	Mechanisms of injury
Steno-occlusions	Intimal hyperplasia	Migratory and secretory phenotype of medial VSMCs IFNγ Macrophage-derived PDGF MMPs NOTCH ligands Macrophage-mediated angiogenesis VEGF Fibrotic scarring TGFβ
	Thrombosis	
	Adventitial thickening (Large-sized arteries)	? TGFβ ? PDGF
	? Inflammatory oedema	
Ectasia/aneurysms	Medial degeneration	ECM alteration Proteases (e.g. MMP2, MMP9) Leukocyte invasion ?Altered component synthesis VSMC toxicity Lipid peroxidation IFNγ Cytotoxicity by lymphocytes ?Autoantibodies
Restenosis post-revascularization	? Pauci-inflammatory intimal hyperplasia	PDGF (of both macrophage and non-macrophage origin) ? Others mechanisms

of self-reactive γδ-cells is less stringent, these cells frequently recognize auto-antigens (112). Given their reduced capability to react to random antigens, it is not surprising that γδ-cells tend to recognize specific patterns that are related to infections or deregulated self antigen (112). Some of the MHC-like ligands of γδ-TCRs, such as MIC-A/B or UL16-binding proteins (ULBPs), are stress- or infection-induced and recognized even without cargo antigens.

Naïve γδ-lymphocytes have peripheral patrolling functions and TCR specificity seems to influence γδ-cell homing (112). After activation, γδ-cells show less clonal expansion and faster onset of effector functions, including cytokine release and cytotoxicity (112). These features place γδ-cells between adaptive- and innate-immunity. Indeed, they express other activating receptors that recognize DAMPs and stress-associated proteins: MIC-A can also engage NKG2D (an activating receptor expressed by NK-, γδ-, $CD8^+$ T, and senescent $CD4^+$ T cells), and exposure to DAMPs of mitochondrial origin induces expression of TLR2 and TLR4 on murine γδ-cells (113). In turn, TLR4 can be activated by heat-shock protein 60 (HSP60), a housekeeping chaperone protein upregulated during cellular stress.

γδ-cells play an important role in the early phase of the immune response. They participate in peripheral surveillance and interact with DCs, thus influencing whether an immunogenic or tolerogenic response ensues. Active γδ-cells can also behave like efficient phagocytes and APCs. Ultimately, they have effector functions and participate in the efferent phase of the immune response (112). In TA lesions, γδ-cells are activated and cytotoxic (85). Circulating γδ-cells show increased cytotoxicity against aortic endothelial cell lines (114), suggesting either an enrichment in anti-aorta circulating γδ-cells in TA or the presence of non-specific γδ-activating stimuli. Indeed, cells in TA lesions are stressed, expressing HSP60 and MIC-A, possibly as a consequence of infections or the immune response itself.

γδ-cell activation does not require synchronous engagement of TCR, co-stimulatory and cytokine receptors (112). Therefore, stress-induced co-stimulatory molecules might locally activate γδ-cells recognizing either self or foreign antigens in the vascular wall. Finally, γδ-cell activation might be the inciting element for a self-reactive immune response by αβ-cells in genetically predisposed subjects (115). Indeed, specific anti-HSP60 responses are frequent in TA (116).

To summarize the current hypothesis external stressors on the arterial wall (e.g. infections and ROS) induce expression of stress molecules and VDC activation, thus allowing T-cell recruitment and activation of γδ- and NK-cells. Important regulatory cross-talk between γδ-cells and VDCs may occur. In genetically predisposed individuals these elements might favour the development of Th1 and Th17 immune responses against vascular antigens, which escape the resolution phase of inflammation and lead to overt LVV. Despite a lesser role for γδ- and NK-cells in GCA, stress-associated pathways may be involved in autoimmunity, as $CD4^+$- and $CD8^+$-cells upregulate NKG2D in GCA, and might receive costimulatory signals from MIC-A expressed in GCA arteries (117).

B cells and humoral responses

B cells are usually present at low frequency in LVV lesions. However, they may participate in vascular injury, as suggested by case reports of therapeutic responses to B-cell depletion with rituximab (118, 119). Mechanisms of B-cell pathogenicity may not be limited to autoantibodies, although they are a marker of B-cellular deregulation. Numerous autoantibodies have been associated with LVVs and PMR with variable accuracy in diagnosis or in identification of active disease. These include anti-endothelial antibodies (notably including anti-HSP60) (116, 120), anti-aorta including anti-14-3-3 (105), anti-phospholipid, anti-vinculin, anti-laminin A/C, anti-voltage-dependent anion-selective channel protein 2 (121), and anti-ferritin (122, 123). Some of these antibodies increased endothelial adhesive properties and induced endothelial apoptosis and cytokine production *in vitro* (116, 124), but their potential contribution to LVV pathogenesis remains to be determined.

Macrophages: the main effector cells in LVVs

Injury in LVV is focused on specific wall compartments, causing lumen-occlusive intimal hyperplasia, fragmentation of elastic membranes, and medial degeneration with loss of VSCMs and scarring (➔ Table 17.1). In large arteries, adventitial thickening also contributes to steno-occlusions (125). Infiltrating monocyte/macrophages represent the main leukocytic effectors of wall injury in LVVs, although lymphocyte cytotoxicity, tissue invasion, and chemokine secretion may participate, together with stromal cells.

Many different stimuli induce macrophages to become activated and differentiate. Traditionally, the activated macrophages are divided into two groups: classically activated, proinflammatory M1-macrophages, and, alternatively, activated M2-macrophages, with anti-inflammatory, reparative, and profibrotic properties. In fact, the activated macrophage phenotype shows high plasticity depending on the combination of inciting stimuli, and on the duration and intensity of exposure (126, 127). Accordingly, LVV lesions show

multiple macrophage populations, which do not fit into rigid phenotypes. Determinants of differentiation and tropism within the wall of these populations are largely to be elucidated, although lymphocytes and their cytokine and chemokines likely play a central role. Fifty-percent of LVV patients show multinucleated giant cells near the internal elastic lamina, generated in response to macrophage stimulation by lymphocyte-derived IFNγ. Beside giant cells, approximately 30% of the infiltrating macrophages produce TGF-β and reside in the adventitia, frequently co-expressing IL-1β and IL-6 (128, 129). A second population (≈50% of infiltrating macrophages) mainly localizes to the internal media, extending to the intima and elastic membranes. These cells have tissue-destructive properties, producing the metalloproteinases, MMP2 and MMP9, and ROS, causing peroxidation of lipids and necrosis of nearby stromal cells (128–131). Increased macrophage synthesis of platelet-derived growth factor (PDGF) and vascular-endothelial growth factor (VEGF) have been associated with intimal hyperplasia (132), neoangiogenesis and disruption of the internal elastic lamina (133).

Notably, ischaemic features in GCA were not associated with blood IL-6 levels and systemic inflammation but with intimal hyperplasia, presence of giant cells, disruption of the internal elastic membrane, intimal neovascularization, and high serum levels of IFNγ and VEGF (131–135). IL-33 exhibits nuclear expression in granulomas (136, 137) and may confer an abnormal reparative, profibrotic phenotype on these macrophages (138). Another macrophage population, whose pathogenetic role is still unclear, expresses inducible NO-synthase and shows preferential intimal tropism (129). In addition to this local role, monocyte/macrophages probably contribute to systemic inflammatory manifestations of GCA and PMR through synthesis IL-1β and IL-6 (128).

Infiltrating macrophages and giant cells also exert vasoprotective actions, including expression of the long pentraxin PTX3. The latter is a soluble, innate immunity protein that is constitutively expressed by endothelial cells and inducible on other stromal and myeloid cells by microbial or sterile inflammatory stimuli (139). PTX3 has immunomodulatory and vasoprotective properties, favouring the clearance of proinflammatory apoptotic debris and inhibiting fibroblast growth factor-2 (FGF2)-induced neoangiogenesis and intimal thickening after balloon angioplasty (139). Medial-infiltrating, likely pathogenetic macrophages co-localize at areas with high PTX3 expression in LVVs (134, 140), thus explaining its potential role as a biomarker of blindness in GCA and of vascular progression in TA (134, 141).

The use of biologic agents inhibiting TNFα and IL-6 in LVV patients, refractory to conventional therapies, is increasing. Observational studies have suggested that TNFα-blockers are efficacious in refractory TA patients (142); however, these results were not confirmed by a randomized clinical trial with infliximab in GCA (143), highlighting the differences between the two conditions. A smaller experience with the IL6-antagonist tocilizumab has been reported in TA (142), and a randomized trial in GCA is on-going (ClinicalTrials.gov NCT01791153). In TA, these regimens appear to control the disease without curing it (142). Although the systemic and local anti-inflammatory actions of these agents are obvious, it remains to be elucidated how and to what extent they inhibit the cellular populations responsible for arterial injury and remodelling.

In conclusion, macrophages appear to exert many effector and injurious actions in GCA, while little data is available for TA. Variable macrophage activation may direct the outcome of wall inflammation towards stenosis or aneurysms. This model might need refinement in light of a recent report (81), showing retinal ischaemia is equally frequent in clinical-histologic GCA subsets with or without intimal and medial infiltrates in the temporal arteries. However, it should be noted that no studies have compared inflammation patterns in temporal and posterior ciliary arteries.

Arterial stromal components

Arterial wall stromal components are not innocent bystanders in macrovessels. Indeed, they influence vascular immunity. They can sense microbial and sterile (including mechanical) stressors, produce cytokines and growth factors, and express co-stimulatory and adhesion molecules. Despite a higher threshold for PAMPs and DAMPs than myeloid cells, vascular stromal cells may also be important in this regard, given their density and their non-redundant response (4). Priming of immune responses is more efficient in arterial-like structures than in the fluid-phase (3). DCs and stromal cells interact bi-directionally with T cells via NOTCH–NOTCH ligand pathways, inhibition of which is potently anti-inflammatory. In addition, VSMCs probably cooperate to maintain medial immune-privilege and, following IFNγ stimulation, they express indoleamine 2,3-dioxygenase, which has a tolerogenic effect (4).

Stromal components may influence the tropism of LVVs and MVVs, as they differ in accordance with diameter and anatomical location. Vasa vasorum are limited to vessels with a diameter >2 mm, while large arteries have a higher concentration of elastic fibres and the embryonic origin of VSMCs varies across anatomical districts (144), influencing cellular responses, including the *ex vivo* response to TGF-β (145).

Stromal components are also actively engaged by leukocytes during repair, remodelling, and injury laying down the extracellular matrix and cooperating in the synthesis of effector cytokines, growth factors, and proteases (➔ Table 17.1). Maladaptive remodelling derives from chronic activation of tissue-reparative responses. Intimal thickening reflects medial VSMC mobilization, migration to the intima, and proliferation and secretion of matrix components. In contrast to atherosclerosis or post-angioplasty restenosis, LVV-associated intimal hyperplasia is orchestrated by macrophages and lymphocytes via TGF-β, PDGF, IFNγ, and possibly NOTCH ligands (71, 129, 132). IFNγ may additionally favour medial degeneration (➔ Table 17.1) (71). Despite this leukocytic drive, wall remodelling is not closely-associated with systemic inflammation in LVVs. The clinical detection of active vessel remodelling and its effective therapeutic targeting are major unresolved challenges in the management of LVVs (142, 146).

Another example of abnormal repair and vessel remodelling is restenosis after revascularization (147, 148). Mechanisms of restenosis-associated intimal hyperplasia post-revascularization are similar to those in non-revascularized LVV arteries (148–150) (➔ Table 17.1). However, a stromal origin for mediators of intimal hyperplasia is suspected in restenosis following revascularization. Interestingly, TA has higher restenosis rates than occlusive atherosclerosis (142). Histology data available for restenosis in TA are scarce and appearance is similar to non-vasculitic cases (146, 151, 152). Thus, the higher restenosis rate may reflect the micro-environment in LVVs and a particular role for leukocyte-derived mediators. Accordingly, restenosis rates are lower when revascularization is performed during remission and if pre- and post-interventional immunosuppressive therapy is employed (153, 154).

Thrombosis

Arterial thrombosis may be found in GCA temporal artery biopsies. Moreover, observational studies have reported a protective role for aspirin (155, 156). Thus, thrombosis represents an additional mechanism of injury in LVVs. As small and medium-sized molecules can diffuse bi-directionally across the arterial wall (4), inflammatory mediators in the deeper layers can diffuse and activate luminal endothelium and induce expression of adhesion molecules. In turn, circulating platelets can interact with inflamed endothelium, become activated, interact with neutrophils and monocytes, activate the coagulation cascade, and produce cytokines and growth factors such as PDGF. Factor X and Factor II activate proinflammatory and reparative/remodelling pathways via protease-activated receptors (157–160). Luminal thrombosis may also participate in the early phases of post-angioplasty restenosis (148).

Pathogenesis of clinical complications of LVVs

The clinical picture in LVVs is determined by: (i) vascular complications, (ii) extravascular involvement, and (iii) systemic inflammation.

Vascular complications are the direct consequence of arterial injury: steno-occlusions predispose to ischaemia, while ectasia/aneurysms may result in rupture, dissection, or aortic regurgitation (➔ Table 17.2). Indeed, the leading disease-related cause of death in GCA is aneurysm rupture. Blindness following posterior ciliary artery occlusion represents the major cause of morbidity (161, 162). Intracranial vessel involvement is rare in LVVs, except for the posterior circulation in GCA (163). Watershed territory stroke due to involvement of multiple brain-feeding vessels and

Table 17.2 Clinical complications of LVVs and respective pathogenesis

Complication	Pathogenic mechanism
Dissection	Increasing arterial dilatation Arterial wall fragility
Aneurysm rupture	Increasing arterial dilatation Arterial wall fragility
Local ischaemia	Steno-occlusion
Arterial hypertension	Renovascular (atypical coarctation/RAS) Reduced total arterial compliance Baroreceptor dysfunction Iatrogenic (corticosteroids)
Atherosclerosis (see ➔ below)	Increased traditional risk factors (obesity, hypertension, dyslipidaemia, corticosteroids) Non-traditional risk factors
CAD	Atherosclerotic Ostial coronary arteritis Non-ostial coronary arteritis
Stroke	Haemodynamic impairment from extracranial involvement Intracranial involvement (mainly posterior circle, predominantly GCA) Atherosclerosis
Chronic renal failure	Ischaemic Hypertensive Parenchymal inflammatory involvement (rare)
Pulmonary hypertension	Post-capillary (left heart disease) Hypertensive diastolic dysfunction Cardiomyopathy (AR, CAD) Precapillary (pulmonary artery involvement)

RAS: renal artery stenosis, AR: aortic regurgitation.

haemodynamic impairment is relatively frequent in our TA experience. Atheroembolism is not common, but can complicate concurrent atherosclerosis.

Peripheral ischaemia is characteristic of TA, most frequently involving the upper limbs and, secondly, the kidneys (164), with associated renovascular hypertension and/or chronic renal failure. Renal artery stenosis is most commonly caused by ostial stenosis. Aortic involvement most frequently results in ascending aorta/aortic root dilatation, which may be complicated by aortic regurgitation and secondary cardiomyopathy. Atypical aortic coarctation may also be seen, while symptomatic splanchnic ischaemia is uncommon, thanks to protective collateral circulation.

Arterial hypertension affects most TA patients and has multiple causes, including renovascular mechanisms, reduced total arterial compliance due to widespread wall thickening, baroreceptor dysfunction secondary to aortic arch and carotid disease, and iatrogenic corticosteroid effects (146).

Local and systemic inflammations exacerbate atherogenic mechanisms, including endothelial activation, lipoprotein modification, and leukocyte activation (165). Indeed, chronic inflammatory conditions are associated with accelerated atherosclerosis. Moreover, disruption of arterial immunoprivilege in large and medium-vessel vasculitides increases influx of activated leukocytes, local synthesis of inflammatory and remodelling mediators, and ROS generation. Interestingly, steroids may be protective rather than pro-atherogenic in patients with active inflammation, when used appropriately to control disease activity (166). Vasculitis patients also typically exhibit traditional atherosclerotic risk factors (165). However, it is unclear whether GCA has a significant impact on the progression of atherosclerosis (165, 167): cardiovascular risk is increased, but progressively reduces over time (168, 169). This suggests a major role for vasculitis *per se*, rather than atherosclerosis, in causing ischaemia. In TA, accelerated atherosclerosis is clearly documented (165, 170), and is particularly severe at sites of vasculitic involvement, suggesting the importance of local factors, inflammatory or haemodynamic in nature (171).

At least 50% of TA patients have coronary artery disease (CAD), with three types observed: (i) atherosclerosis, possibly accelerated in LVVs, (ii) ostial coronary arteritis, associated with ascending aortitis, and (iii) non-ostial coronary arteritis independent of ascending aortitis.

Pulmonary hypertension (PH) is reported in TA (172). In our experience, post-capillary PH due to hypertensive, ischaemic, or valvular left-heart disease is the most frequent occurrence, and precapillary PH associated with pulmonary artery involvement is rarer. It is unclear if TA-associated precapillary PH might associate with some degree of pulmonary vascular remodelling reminiscent of chronic thromboembolic PH or of pulmonary arterial hypertension, and may, therefore, be responsive to specific pulmonary vascular therapies.

Extravascular complications of LVVs may be partly dependent on therapies (e.g. steroid-associated side-effects) and inflammatory involvement, such as serositis, arthritis, and bursitis. The latter are particularly frequent in GCA, especially if PMR is present (173). Myocarditis is anecdotally described. Contrary to vascular complications, extravascular inflammatory involvement usually shows a strong association with systemic inflammatory responses.

Outline of MVV pathogenesis: Kawasaki disease

Kawasaki disease (KD) is a systemic inflammatory illness, characterized by a self-limited, typically single, episode of high fever and exanthema, enanthema, ocular inflammation, lymphadenopathy, redness and swelling of hands and feet, and vasculopathy, with a particular tropism for the coronary arteries. Inflammation may also involve the central nervous system, the myopericardium, and the digestive and the respiratory tracts (174).

KD, which primarily affects children from 6 months to 5 years, is the leading cause of childhood-acquired heart disease in developed countries (175). Rare adult cases have been described, frequently associated with HIV infection (176). Coronary artery (CA) involvement is the leading prognostic factor. Approximately 25% of patients develop fusiform or saccular CA aneurysms from the second week. These aneurysms may progressively enlarge, rupture, and thrombose causing ischaemia/infarction, or gradually and paradoxically develop steno-occlusive intimal hyperplasia (177).

Ubiquitous environmental factors, especially of an infective nature, are suspected to elicit abnormal immune responses in genetically predisposed individuals (177). This hypothesis would explain racial and familiar factors of KD, the age selectivity, observed epidemics, and seasonal incidence fluctuations, and the rarity of recurrence (177).

Genetic studies and the robust systemic inflammation in the first 10–15 days support the hypothesis of an abnormal immune response (177, 178). Expansion of Th1- and Th17-cells, and T-regulatory cell depletion has been observed (178). Histology reveals three major overlapping processes causing KD vasculopathy (179). The first, necrotizing

neutrophilic vasculitis of medium-sized arteries and especially the CA, is usually self-limiting by the third week after fever onset. The other two, subacute chronic vasculitis and intimal hyperplasia, which are closely associated, begin in the first two weeks, and can last for months/years. Necrotizing vasculitis apparently progresses from the lumen to the adventitia, suggesting disruption of arterial immune-privilege follows a different model to that described above. Necrosis extending to the media and adventitia has been associated with saccular aneurysms with low potential for reparative responses or steno-occlusive remodelling. On the contrary, subacute chronic vasculitis is associated with lymphocytes, plasma cells, and eosinophils in the adventitia, with possible extension towards the lumen. It is interesting to note that some features of subacute vasculitis recall those described for LVVs. Subacute vasculitis can result in fusiform aneurysms, with higher remodelling potential, as preserved VSMCs in the media represent the cellular source of the myofibroblasts responsible for intimal hyperplasia (177). Unfortunately, the events maintaining subacute vasculitis and remodelling after resolution of the acute episode are unknown.

Genetic associations in vasculitis

In addition to environmental factors there is likely to be a significant genetic component to AAV. Although understanding of this influence remains relatively limited, it has been significantly advanced by the publication of two genome-wide association studies (GWAS), The Vasculitis Clinical Research Consortium (VCRC) study of 1,020 GPA patients of European descent from the USA, and the European Vasculitis Genetic Consortium (EVGC) study of 2,687 Caucasian GPA and MPA patients (180, 181). The EVGC analysis demonstrated AAV association with *HLA-DP*, *PRTN3*, which encoded PR3, semaphorin 6A (*SEMA6A*), and *SERPINA1* encoding α1 anti-trypsin. Further analysis showed that MPO ANCA-related disease was associated with *HLA-DQ*, while PR3 ANCA-positive disease was the strongest factor associated with *PRTN3*, *HLA-DP*, *SEMA6A*, and *SERPINA1*. The findings of the VCRC study emphasized the important association with the MHC and additional loci were unearthed, *HLA-DPB1* and *HLA-DPA1*. These studies demonstrated that the genetic associations identified are more strongly linked to ANCA-specificity than to clinical features. The on-going challenges include collection of adequate numbers of both MPO- and PR3-positive patients alone, for future GWAS studies aimed at identifying new disease-specific associations. Furthermore, a first GWAS study of EGPA is needed (182).

Familial cases of vasculitis also highlight the role of genetic influences in the vascultides (183–185). The recent reports of loss of function mutations in the cat eye syndrome chromosome region candidate 1 (CERCR1) gene, resulting in adenosine deaminase 2 deficiency and development of a vasculitic illness, are particularly striking (186, 187). Intriguingly, a complete range of disease severity is apparent in the cases reported so far, along with a wide range in age at presentation. The patients with this disorder typically fulfil criteria for polyarteritis nodosa (PAN) or cutaneous PAN. These reports also illustrate the power of whole-exome sequencing in rare cases of childhood vasculitis that might be monogenic. Identification of specific gene mutations has the potential to reveal new pathogenic mechanisms and ultimately novel therapeutic targets.

Genetic studies support the immune-mediated nature of LVVs: class I MHC (in particular the HLA-B locus) and MHC class I chain-related (MIC)-A have been associated with TA (188), while class II MHC (especially the HLA-DRB locus) has been associated with GCA (189, 190). Non-MHC regions associated with TA encode IL12p40 (a subunit of IL12 and IL17), IL6, long non-coding RNAs, ribosomal protein S9, and the MHC1-recognizing protein LILPRB3 (188, 191). Non-MHC associations with GCA include IL17A, IL33 protein tyrosine phosphatase N22 (which regulates lymphocyte activation), and NLRP1 (a scaffold inflammasome protein), although these did not reach genome-wide significance level (189, 192). Clearly, these data show different genetic backgrounds between TA and GCA (188).

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SECTION V

Vascular-associated pathologies

Section introduction

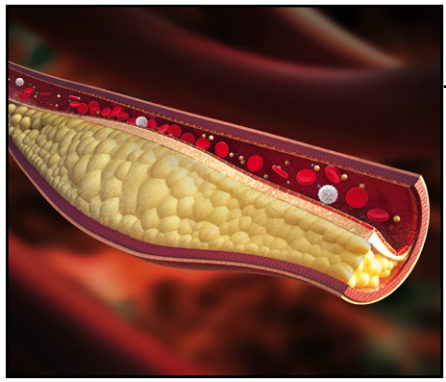
Giuseppina Caligiuri

This section deals with the pathophysiology of thrombosis, hypertension, and perivascular fat accumulation, three key pathologic processes that are intrinsically associated with the progression and clinical manifestations of vascular diseases.

Thrombosis, over a ruptured or eroded atherosclerotic plaque, is the ultimate process leading to ischaemia leading to the concept of 'atherothrombosis' as the clinically overt, complicated stage of atherosclerosis. However, the loss of the physiologic endothelial anti-thrombotic function occurring very early during atherogenesis may support a role for non-occlusive thrombi that can contribute to clinically silent plaque progression. Both soluble mediators (coagulation/fibrinolysis factors) and particulate elements (platelets) contribute to atherogenesis from the earliest stages of the disease.

Hypertension fosters a pathologic remodelling of the arterial tree and functionally contributes to atherogenic mechanisms, such as endothelial dysfunction and inflammation, through the activation of the renin–angiotensin system and direct action on both vascular and inflammatory cells.

Finally, the accumulation of *perivascular fat* is increasingly recognized as a critical component of vascular pathology due to the related effects on both vascular structure and function. The balance between adipose tissue-derived relaxing and contracting factors, and/or growth promoting and growth inhibiting factors, and/or between pro-inflammatory and anti-inflammatory factors is typically impaired in metabolic conditions associated with atherosclerosis such as obesity and diabetes.



CHAPTER 18

Pathophysiology of thrombosis

Lina Badimon, Felix C. Tanner, Giovanni G. Camici, and Gemma Vilahur

Content

Arterial thrombosis

Cardiovascular disease is estimated to be the leading cause of death and disability worldwide, with coronary ischaemic disease the most prevalent form of heart disease (1). The World Health Organization (WHO) reported in 2010 that cardiovascular disease represents around 30% of global deaths and estimated that by 2030 more than 23.3 million persons will die annually from cardiovascular disease (2). The pathological mechanism primarily responsible for the majority of acute coronary ischaemic syndromes is the superimposition of an occlusive thrombus on a culprit atherosclerotic plaque (i.e. atherothrombosis).

In the following section (The healthy endothelium) we will discuss the components, mechanisms, and signalling pathways that contribute to thrombus formation, growth, and stabilization.

The healthy endothelium: a critical feature for thromboresistance

The vascular endothelium is a semi-permeable barrier that controls multiple functions (➔ Fig. 18.1) (3, 4). One of the most important functions is related to the control of haemostasis and thrombosis. The endothelium synthesizes and releases molecules that modulate the coagulation cascade, platelet aggregation, and fibrinolysis. The anti-coagulant properties of the endothelium are conferred by the expression of two transmembrane proteins, thrombomodulin and heparin sulphate proteglycan, and the release of tissue factor pathway inhibitor (TFPI). Thrombomodulin serves as a binding site for thrombin to activate protein C, eventually disabling FVIII- and FV-activation (thrombin formation); heparin-like molecules act as cofactors for anti-thrombin III, and TFPI inhibits both factor Xa and the tissue-factor–factor VIIa complex. Endothelial cells also produce tissue-type plasminogen activator (TPA), which activates the fibrinolytic system. Finally, the antiplatelet properties of the endothelium are mainly regulated by the surface exposure of ecto-adenosine diphosphate (ADP)-ases CD39 and CD73, and the synthesis and release of nitric oxide (NO) and prostacyclin (PGI_2). Atherosclerosis development not only impairs these endothelium-conferred antithrombotic properties, but shifts them towards a prothrombotic profile.

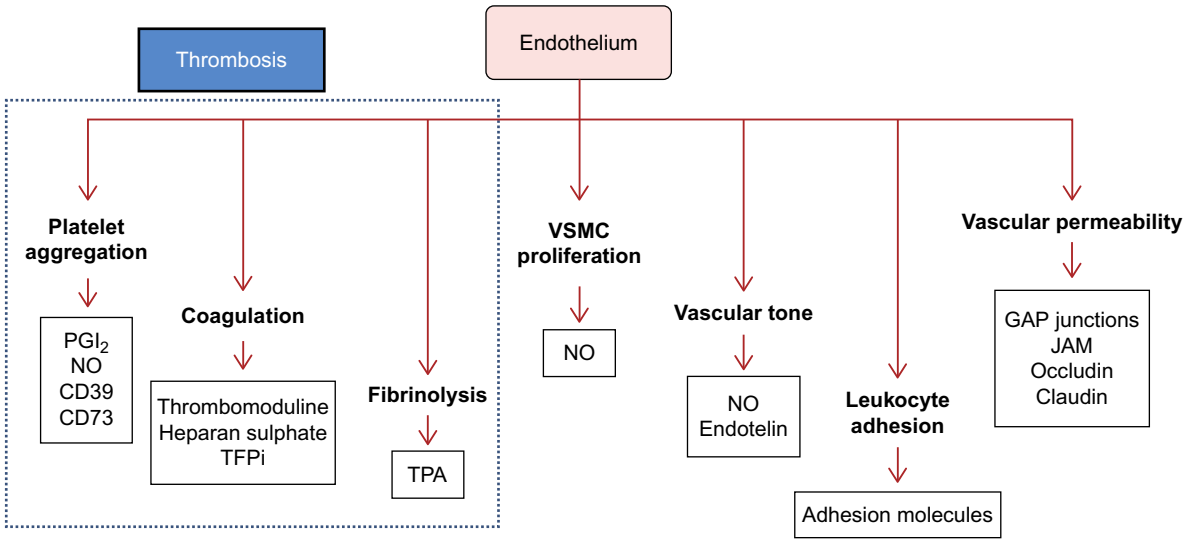


Fig. 18.1 Functions modulated by the endothelial layer and mechanisms/molecules involved.
PGI_2: prostacylin; NO: nitric oxide; TFPi: tissue factorpathway inhibitor; TPA: tissue plasminogen activator; JAM: junction adhesion molecule; VSMC: vascular smooth muscle cells; CD39/CD73: ecto-adenosine diphosphate (ADP)-ases.

The culprit atherosclerotic plaque

Atherosclerotic lesions with the potential to rupture, i.e. vulnerable plaques, are the primary cause of luminal thrombosis and consequent clinical symptoms. Post-mortem studies have revealed that the coronary artery plaque morphologies primarily responsible for thrombosis are plaque rupture and plaque erosion, with plaque rupture being the most common cause of acute myocardial infarction, especially in men (5). In contrast, women <50 years of age more frequently have erosion and from then onwards the frequency of rupture increases with each decade (6). Pathological and anatomic features of high-risk or vulnerable plaques have been reviewed extensively in the literature (7, 8). Briefly, high-risk plaques have a large, acellular, lipid-rich necrotic core with an overlying thin fibrous cap infiltrated by inflammatory cells, diffuse calcification, haemorrhage, and show a great outward (positive) remodelling (9). Yet, although it is well established that plaques with vulnerable features are responsible for most clinical episodes, not all plaques with such features lead to an acute event. It has been suggested that among plaques with features of vulnerability, those with a larger size (10) or a higher degree of progression have much greater chance to result in an acute coronary event (11).

Platelets as key players in haemostasis and thrombus formation on damaged vessels

Blood platelets are enucleated circulating cells that play a critical role in haemostasis and arterial thrombosis (12). The initial capture and firm adhesion of platelets to the injured vessel is mediated by the interaction between the surface platelet receptors glycoprotein (GP) Ib/IX/V complex and Von Willebrand Factor (VWF) immobilized to the exposed extracellular matrix (➔ Fig. 18.2). Platelet adhesion is further reinforced by the interaction of platelet receptor GPVI and fibrillar collagen at the vascular site (➔ Fig. 18.2). Interactions between these elements are largely influenced by flow. Besides platelet–collagen interaction, circulating agents such as epinephrine, serotonin, adenosine diphosphate (ADP; released from lysed erythrocytes at the site of injury and by aggregating platelets), and thrombin (generated upon atherosclerotic plaque exposure of TF) may also activate platelets via specific platelet surface receptors generating an inside-out signalling (➔ Fig. 18.2). Platelet activation is accompanied by a rise in the cytosolic Ca^{2+} concentration, a change in cell shape through rearrangement of the cytoskeleton, and the secretion of granule contents. The rise in intracellular Ca^{2+} concentration, a key event for platelet activation, is due to both the entry of extracellular Ca^{2+} through the plasma membrane and the release of intracellular Ca^{2+} through Ca^{2+} pools. Platelet shape change exposes a procoagulant surface that facilitates activation of the coagulation cascade. Release of granule content include the secretion of adhesive molecules (VWF and fibrinogen), growth factors, and inflammatory and angiogenic mediators, which play crucial roles in inflammation and atherosclerosis development, besides thrombus formation. However, platelets are not only stores of several bioactive molecules, but also generate lipid mediators, such as thromboxane A2 (TXA_2). Platelet activation induces phospholipase-A2 (PLA_2) activation that triggers arachidonic acid metabolism. Platelet cyclo-oxygenase 1 (COX-1) catalyses the conversion

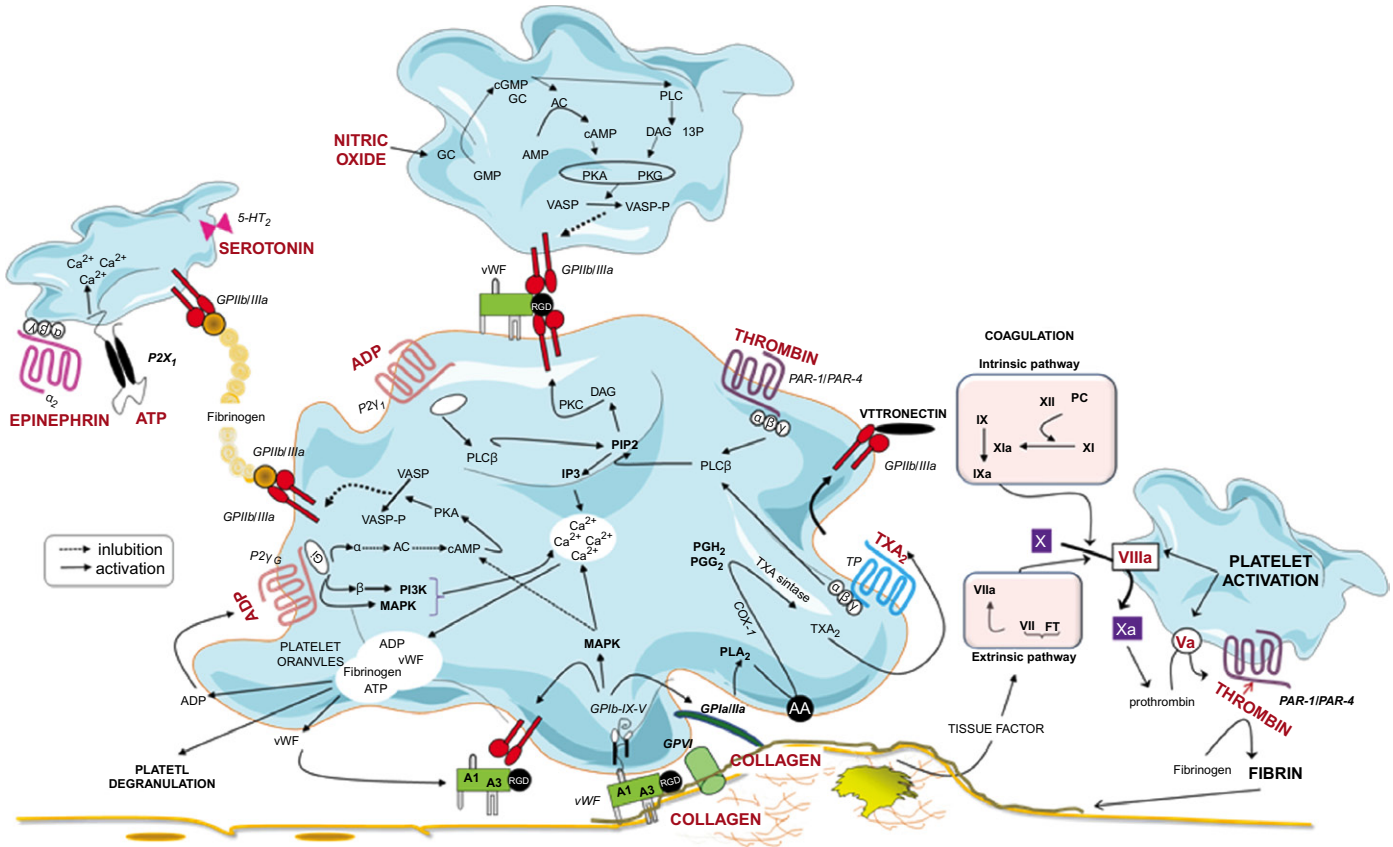


Fig. 18.2 Atherosclerotic plaque rupture-induced fibrin formation and platelet adhesion, activation and aggregation.
VWF: Von Willebrand factor; GP: glycoprotein; Fg: fibrinogen; AA: arachidonic acid; TXA_2: thromboxane-A2; TP: thromboxane receptor; ADP: adenosine diphosphate; PAR: proteinase activated receptor; TF: tissue factor; PK: protein kinase; DAG: diacylglycerol; IP3: inosytol-triphosphate; AMP: adenosine monophosphate; AC: adenylate cyclase; VASP: platelet reactivity index VASP; MAPK: Mitogen-activated protein kinases; ADP: adenosine di-phosphate; PLC: phospholipase C; PI3K: phosphoinositide 3-kinase; PG: Prostaglandin;

of arachidonic acid to prostaglandin G2/H2, and the latter is converted to TXA_2 (➔ Fig. 18.2). TXA_2 is released into the bloodstream, where it binds to TX receptors (TP) present on the surface of adjacent platelets where it activates phospholipase Cβ (PLCβ), resulting in the production of diacylglycerol (13) and inositol trisphosphate (IP3). DAG and IP3 activate protein kinase C and mobilize cytoplasmic Ca^{2+}, respectively (➔ Fig. 18.2) (14). Platelets also possess mitogen-activated protein (MAP) kinases (extracellular signal-regulated kinase 2 (ERK2), p38MAPK, and c-Jun NH2-terminal kinase 1 (JNK1)), which are activated by ADP and collagen (15–17) (➔ Fig. 18.2). All these events lead to a conformational change and consequent activation of GPIIb/IIIa, integrin alpha(IIb)beta(3), which mediates platelet–platelet interaction (platelet aggregation), mainly through its binding with fibrinogen and, to a lesser extent, to VWF (➔ Fig. 18.2).

Tissue factor and its key role in atherothrombosis

Tissue factor (TF) is highly expressed in deep vascular layers and atherosclerotic plaque components playing a key role in both haemostasis and atherothrombosis. In fact, the exposed TF is considered the initial event triggering arterial thrombosis because it induces thrombin and the formation of a fibrin monolayer covering the exposed surface, where flowing platelets are further recruited. (➔ Fig. 18.2) (7, 18).

Tissue factor function and structure

TF (thromboplastin, CD142) protein can be separated into three domains: an extracellular (219 amino acids), a membrane-spanning (23 amino acids), and a cytoplasmic (21 amino acids) region (19). It represents a class I integral membrane protein with an extracellular amino-terminus and a cytoplasmic carboxy-terminus. SDS gel mobility studies demonstrate that the molecular weight of the fully glycosylated protein is about 44 kDa. The extracellular domain contains two high-affinity sites for binding of factor VII to TF, which is crucial for induction of the conformal changes required to activate factor VII (see ➔ The critical role of tissue factor in blood coagulation) (20).

TF undergoes different post-translational modifications. As such, the extracellular domain of TF is glycosylated at several sites and this modification can influence proteolysis of factor X, while interaction with factor VIIa does not seem to be affected (21).

Membrane anchoring of the TF protein via its hydrophobic transmembrane domain has also been demonstrated to be essential for TF procoagulant activity; however, the nature of the anchoring domain does not seem to be important, since replacement of the cytoplasmic tail is not paralleled by an impaired TF procoagulant activity. Importantly, recent studies focusing on non-haemostatic functions have revealed that phosphorylation of the cytoplasmic tail activates intracellular signalling pathways and regulates transcription of numerous genes involved in cell migration, cell growth, apoptosis, and angiogenesis.(22)

Tissue factor distribution in the cardiovascular system

TF is not detected in endothelial cells under healthy conditions. However, the exposure to inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) (23) and/or interleukin-1β, induce TF expression. TF expression is regulated by the MAPK p38, ERK, and JNK leading to activation of transcription (nuclear factor kappa B (NFkB), activator protein-1 (AP1), or early growth-response (EGR) gene product-1), which bind to the TF promoter region (24, 25). Induction of TF expression occurs either via activation of all three MAP kinases (23) or a single pathway, depending on the particular stimulus (25). Protein kinase C and the Rhokinase pathway can also induce endothelial TF expression (25). While the MAP kinase, Rho-kinase, and protein kinase C pathways positively regulate TF expression, the phosphoinositide 3 kinase pathway exerts a negative effect (26). Downstream targets of phosphoinositide 3 kinase are able to regulate TF expression at the transcriptional and translational level. Akt and glycogen synthase kinase 3β modulate TF transcription, while the mammalian target of rapamycin (mTOR) and p70S6 kinase impair TF translation (26). An additional mechanism affecting the regulation of TF protein expression is stabilization of TF messenger RNA. A decreased TF promoter activity may be counteracted by an increased TF mRNA or protein stability resulting in an overall enhanced protein expression (23).

In contrast to endothelial cells, vascular smooth muscle cells (VSMC) constitutively express TF, representing an important haemostatic barrier after vascular injury (27, 28). However, TF expression in VSMC can be enhanced similarly to what occurs in endothelial cells. CD40 ligand, histamine, thrombin, bacterial endotoxin, oxidized LDL, and/or C-reactive protein are known to play a role in this context (29, 30). As for endothelial cells, MAP kinase and phosphoinositide 3–kinase pathways are also suggested to be involved in the regulation of VSMC-related TF expression.

Finally, as mentioned, TF is also highly expressed in atherosclerotic lesions. Foam macrophages and foamy VSMC expressing TF are detectable in coronary atherectomy specimens and in the culprit lesion from patients with unstable angina and myocardial infarction (31).

TF is also detectable, although at low levels, in plasma and urine, and increases in both fluids under inflammatory conditions such as atherosclerosis, diabetes, or sepsis. Monocytes constitutively express TF and represent the major source of plasmatic TF. Monocyte TF expression is induced by many inflammatory mediators (32, 33). In line with this, high plasma levels of TF are detectable in sepsis and may account for the thrombotic complications occurring in such patients (34). TF has also been detected in circulating granulocytes (eosinophils, neutrophils, and basophils) (35, 36). In fact, a prothrombotic state has been associated with the presence of hypereosinophilic syndromes. On the other hand, neutrophils, extracellular traps, or NETs (chromatin filaments containing different proteins attached, which are released by neutrophils upon cytokine or activated platelets' interaction) represent a new source of extracellular active TF. As such, thrombi from the culprit lesion in acute coronary syndromes contain NETs decorated with TF, which is thought to be involved in thrombus stabilization and/or propagation (37, 38). Platelets are another source of TF. Platelet-derived TF is either localized in the membrane, α-granules, and/or canalicular system (39, 40). Despite controversial data, it has been suggested that both megakaryocytes and platelets express TF mRNA, and that platelets can make *de novo* protein synthesis (41). Yet, the main mechanism thought to be responsible for the presence of TF within platelets is through the uptake of TF-positive microparticles (MP). TF containing MP from activated monocytes, leucocytes, or endothelial cells (42) fuse with the platelet membrane in a P-selectin-dependent manner and thereby deliver TF to platelets (40, 42).

TF containing MP in plasma have been well characterized. Besides vascular cells (35), platelets, monocytes, and macrophages are the main source of TF containing MP. MP are procoagulant because they exhibit TF and negatively charged phospholipids on their surface and, thereby, stimulate thrombin generation *in vitro* (43).

The full-length form of TF mRNA contains six exons and splicing of exon 5 results in a frame-shift mutation generating a soluble, alternatively spliced TF protein lacking the transmembrane domain and, therefore, is not anchored to the cell membrane (44). Apart from plasma, alternatively spliced TF has been found in several tissues and cell types, including the lung, placenta, vascular smooth muscle cells, and others. Yet its contribution to the TF procoagulant activity in plasma, as well as thrombus formation *in vivo*, is not well understood (45). Alternatively spliced TF released from

cytokine-stimulated endothelial cells may exert procoagulant activity (46). However, in the absence of full-length TF, alternatively spliced TF expressed during embryonic development has no detectable procoagulant activity (47).

The critical role of tissue factor in blood coagulation

Initiation of coagulation is thought to be triggered by two distinct pathways: the extrinsic or tissue factor-dependent pathway, and the intrinsic or contact activation pathway. Even though the two pathways are activated in an independent manner, they cannot be separated completely since they share a final common pathway and there is cross-talk between the two systems.

Extrinsic coagulation pathway

Exposure of the extracellular domain of TF to the flowing blood serves as the primary initiator of the coagulation cascade upon atherosclerotic plaque rupture. Reversible binding of the zymogen factor VII (FVII) or activated FVII (FVIIa) to membrane-bound TF is the initial step and leads to the formation of both TF:FVII and TF:VIIa complexes. Only the latter, however, possesses the enzymatic activity necessary for activation of downstream proteases. Once bound to TF, FVII is activated to FVIIa via limited proteolysis by plasma proteases, such as factors XIIa, Xa, IXa, or thrombin, the latter representing an important positive-feedback loop (48). The TF:FVIIa complex containing TF as a cofactor with regulatory functions and FVIIa as a catalytic cofactor is a potent activator of the coagulation cascade. It activates factor IX to IXa, which catalyses the conversion of factor X to Xa in association with VIIIa. Factor X is also converted directly to factor Xa by the TF:FVIIa complex. TF needs to undergo a post-translational modification, the so-called decryption, in order to express procoagulant activity (49). Some studies suggest that exposure of negatively charged phospholipids mediates decryption of TF procoagulant activity after cell stimulation (50), while others propose that thiol disulphide exchange reactions regulate TF activity. Human TF contains two disulphide bonds, which are conserved in mouse and many other species; the C-terminal bond shares similarities with other allosteric disulphide bonds and might thus be involved in regulation of TF activity.

Intrinsic coagulation pathway

The intrinsic pathway is activated when there is contact between blood or plasma and the subendothelial connective tissue or negatively charged surfaces. Contact with negatively charged surfaces activates factor XII, which is followed sequentially by activation of factor XI and factor IX, and releases bradykinin from high molecular weight kininogen via kallikrein (51). The intrinsic and extrinsic pathways converge at the level of factor X activation. Since bradykinin induces vessel dilatation and permeability, as well as neutrophil chemotaxis, this pathway exerts prothrombotic and proinflammatory properties. Subjects with partial or even severe deficiency in factor XII do not exhibit a clinically significant bleeding phenotype, although there is a marked prolongation of activated partial thromboplastin time. However, mice deficient in factor XII display severely impaired thrombus formation indicating an important role of this coagulation factor in pathological thrombosis *in vivo* (52). This effect is observed in both large arteries and the microcirculation. The importance of this pathway for normal haemostasis is not well understood, as congenital deficiency in the factors involved neither induces increased bleeding nor clinically apparent pathologies in humans. Yet, such observations suggest that factor XII may represent an attractive target for inhibition of thrombosis without concomitantly increased bleeding risk.

Common coagulation pathway

Factor Xa, in association with factor Va and divalent calcium, forms the so called prothrombinase complex cleaving prothrombin to thrombin (19). Thrombin is a central player in thrombus formation. It cleaves fibrinopeptides from fibrinogen, which permits polymerization of fibrin monomers into an insoluble fibrin network. It also activates factors V, VIII, XI, and XIII, representing an important auto-feedback loop. Besides its actions on coagulation factors and being a potent platelet activator, thrombin exerts numerous effects on the vessel wall such as: (i) it stimulates vascular remodelling by stimulating proliferation and migration of VSMC (53); (ii) it induces vasoconstriction by a direct action on VSMC and modulates its effect through the concomitant release of endothelium-derived NO; (iii) it increases vascular permeability; (iv) it positively regulates proliferation of endothelial cells and thereby modulates angiogenesis (54), and (v) it influences the interaction of tumour cells with platelets, endothelial cells, and extracellular matrix. Hence, thrombin constitutes a link between the coagulation system, vascular remodelling, angiogenesis, and tumour progression (55).

Thrombus progression and growth

Thrombus dynamics: cellular and molecular mechanisms

Thrombus aspiration, a newly recommended technique to facilitate thrombus removal from the culprit coronary artery at the time of percutaneous coronary intervention

(PCI), has allowed the study of human thrombosis *in vivo*, thereby providing new insights into thrombus composition and dynamics (56). Rittersma et al. (57) investigated around 200 aspirated intracoronary thrombi obtained from STEMI patients within 6 hours of symptom onset and described, upon histopathological examination, that thrombi were several days or weeks old in at least 51% of the patients with acute STEMI, reflecting the fact that plaque instability occurs a few days or weeks before occlusive thrombus formation occurs. These observations also indicate that plaque disruption is only part of the process, and suggests that the absence of adequate, complete healing of an ageing thrombus may play an important role in the occurrence of sudden occlusive coronary thrombosis. Recently, electron microscopy analysis of retrieved intracoronary thrombi from STEMI patients has reported on how thrombus composition evolves over time during the first 12 hours since pain onset (58). The authors found an increase in fibrin content for each ischaemic hour, whereas platelet content followed the opposite pattern. Similar observations on coronary thrombus composition have been reported by using histochemical techniques (59) or magnetic resonance imaging (60). We have also demonstrated, in retrieved thrombus from STEMI patients, a rapid (3 hours from onset of pain to PCI) recruitment of neutrophils and monocytes, and, later (6 hours from onset of pain to PCI), the appearance of T- and B-lymphocytes along with a few progenitor undifferentiated cells (59). It has been demonstrated that cytokines released at the site of the ruptured plaque (e.g. IL-6) can induce TF in vascular cells (61). Finally, the application of protein discovery approaches and advanced cellular microscopy have enabled researchers to identify novel proteins in thrombus formation and potential biomarkers of ischaemic damage, such as profiline-1, a cytoskeleton-associated protein probably released by thrombin-activated platelets at the atherosclerotic culprit site (59).

Microparticles: regulators of thrombus growth

Microparticles (MP) represent a heterogeneous population of vesicles (diameter 100–1,000 nm) that are released by budding of the plasma membrane and express antigens specific of their parental cells. Although MP formation represents a physiological phenomenon, MP are raised in a wide range of cardiovascular diseases, including atherosclerotic lesion progression, thrombosis, heart failure, arrhythmias, and inflammation-related vascular disease, as well as in conditions in which cardiovascular disease risk factors are not controlled (62). These observations suggest a potential correlation between the quantity of MP and the clinical severity of the disease. In addition, MP have recently emerged as potential bioactive paracrine effectors of cardiovascular disease. Indeed, there has been increasing interest in the contribution of the cross-talk between MP and cells involved in atherosclerosis progression and thrombus formation (63). Several reports have suggested that the role of platelets in atherothrombosis is mediated, in part, by local secretion of platelet-derived MP (pMP). In this regard, we have reported for the first time that both circulating and pMP enhance thrombosis either on human atherosclerotic plaques or in injured vessels under high shear rate conditions (64). pMP also have been shown to localize within the growing platelet thrombi upon collagen exposure, stimulating further platelet deposition and enhancing thrombus formation. In fact, it has been shown that MP are captured by thrombus-associated platelets through the interaction between MP-exposed P-selectin glycoprotein ligand 1 (PSGL-1) and P-selectin from platelets (65). Circulating MP (cMP) have also emerged as surrogate markers of pathological conditions. As such, we have reported that the prevalence of atherosclerotic plaque burden is related to the level of platelet-derived MP-associated TF levels and activity. The inclusion of these prothrombotic-MP to the currently used risk algorithm Framingham score has proved to add an incremental prediction value in patients at high cardiovascular risk (66). Finally, beyond pMP, we have recently pointed out that erythrocyte-derived MP may emerge as novel sensitive biomarkers of ongoing thrombosis in patients with complete thrombotic coronary occlusion (67).

Blood-borne tissue factor

Blood-borne TF may contribute to the propagation of thrombus formation; as soon as a thrombus is formed it prevents the initially formed TF:VIIa complexes from further interaction with circulating coagulation factors. This hypothesis is supported by the observation that the low levels of TF in the bloodstream are not able to trigger a coagulant response while TF concentrations reach functionally significant levels on the surface of a forming thrombus (44). A role of blood-borne TF in thrombus formation is underscored by the observation that sub-picomolar TF concentrations, such as those detected in blood, cause an increase in thrombin formation under flow, but not under no-flow conditions.

On the other hand, propagation of thrombus formation seems to be TF independent once thrombin has started to be formed (68). By abrogating TF activity with specific antibodies, three clotting phases can be distinguished: a first short (<10 s) period of absolute TF:VIIa dependence; a second period (10–240 s) of partial TF:VIIa dependence, decreasing in importance as thrombus formation progresses; and a final period that is TF:VIIa independent and only begins

after 2 min. In this *ex vivo* model, the initial activation of procoagulant proteases by the TF:VIIa complex seems to be sufficient for maintaining a pool of activated procoagulant factors able to maintain a sustained coagulation response in a TF-independent manner. However, more recent observations indicate that TF derived from MPs or NETs contributes to thrombus propagation *in vivo* (69, 70).

Stroke

The term stroke denotes a syndrome whereby a permanent or a transient neurological deficit is observed following an atherothrombotic event, a thromboembolism, a subarachnoid haemorrhage, an intracerebral haemorrhage, or other similar causes (71). Different types of stroke are broadly divided into two different classes: (i) ischaemic and (ii) haemorrhagic (see Fig. 18.3).

These typologies of strokes are characterized by opposite pathophysiological states. Ischaemic stroke represents 87% of all stroke events (72, 73) and is characterized by a reduced supply of blood, oxygen, and nutrients to the brain. Ischaemic strokes can be further subdivided into two principal categories: (a) thrombotic strokes and (b) embolic strokes (74–76). In counterpart, haemorrhagic stroke is an excess of blood found within the closed cranial cavity (77).

As recently announced at the 'world heart day' organized by the European Society of Cardiology in Europe, stroke is the second single cause of death responsible for almost 1.1 million deaths each year. More than 15% of women and 10% of men affected by this disease die. Epidemiological data indicate considerable differences in stroke-associated mortality between different European countries with various Eastern European countries displaying high and increasing stroke mortality rates, while most West and South European countries show low and decreasing rates (78). Noteworthy, differences in European countries were mainly associated to incidence of ischaemic stroke, while occurrence of haemorrhagic strokes was comparable amongst different countries (79).

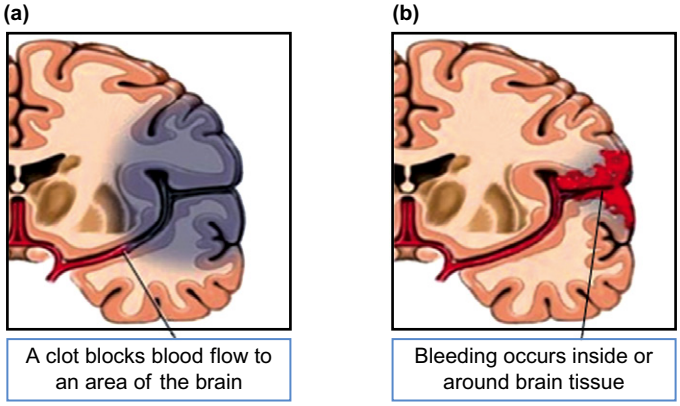


Fig. 18.3 (a) Broad classification of stroke types occurring in humans. Ischemic stroke whereby a clot formed locally or originating proximally occludes a cerebral artery. (b) Haemorrhagic stroke whereby rupture of a cerebral vessel causes a haemorrhage on the brain.

Thrombotic stroke

Thrombotic strokes account for the vast majority of strokes and take place in large-/medium-size cerebral arteries, often as the result of occlusions occurring '*in situ*', as a consequence of atherosclerotic plaque rupture in carotid, vertebrobasilar, or cerebral arteries, usually in close proximity to major branches. Diagnosis of a thrombotic stroke requires the presence of either occlusive or stenotic (at least 50% diameter reduction) vascular disease due to atherosclerosis (76, 80). The degree of stenosis is calculated by the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria (80). Ischaemic strokes may also result as a consequence of emboli originating from cardiac arteries. An embolus consists of platelet aggregates, thrombus, platelet-thrombi, cholesterol, calcium, bacteria, etc. Cardiogenic emboli are a common source of recurrent stroke and may account for up to 20% of acute strokes (74). Most cardiogenic emboli are caused by atrial fibrillation, whereby abnormal, rapid heartbeat causes the blood to pool, forming clots that can travel to the brain and cause a stroke (81).

Factors that modulate the risk for suffering thrombotic stroke

Risk factors similar to those that account for coronary of peripheral arterial disease (such as hypertension, diabetes, smoking, ethnicity, and family history) have been shown to increase the likelihood of developing thrombotic stroke (82). Generally, risk factors increase the odds of developing a thrombotic stroke by favouring the development of key biological processes that are instrumental to the pathogenesis of stroke. In particular, different risk factors were shown to promote production of free radicals (83), inflammation (84), and mitochondrial dysfunction (84), as well as acidosis (84).

Hypertension is the single main modifiable risk factor for ischaemic stroke (85); interestingly enough, however, the beneficial effects of blood pressure lowering after the onset of ischaemic stroke are still controversial (86). Most estimates for hypertension indicate a four-time increased relative risk of stroke when hypertension is defined as systolic blood pressure ≥160 mmHg and/or diastolic blood pressure

≥95 mmHg (87). A summary of seven independent studies determined a decreased risk at blood pressure 136/84 mmHg and an even lower risk at blood pressure 123/76 mmHg as compared to to borderline or mild hypertension (87). The exact mechanisms by which high blood pressure leads to an increased risk of developing ischaemic stroke are not fully understood; nonetheless, several pathological components underlying this effect have been described. Indeed, hypertension was shown to accelerate the development of atherosclerosis and to cause intracerebral vascular damage leading to increased blood–brain barrier permeability (88, 89). In hypertension, local cerebral changes in the endothelium and endothelium–blood cell interaction, followed by the release of substances that influence vascular tone and/or permeability, such as endothelin, nitric oxide, cytokines, and free radicals, are likely to play additional roles (90). In line with this, different factors, e.g. angiotensin II, involved in controlling blood pressure are being considered as potential therapeutic targets (91).

Ageing is the single most important non-modifiable predictor of stroke (92, 93). In fact, with ageing the brain undergoes different alterations (94) increasing its vulnerability to ischaemia (95); in line with this, each successive 10 years after the age of 55, rate of stroke more than doubles in both men and women (96). As a consequence, elderly people who experience a stroke show a worse prognosis compared to younger patients (97, 98). Furthermore, in the light of increasing life-expectancy observed over the last 100 years, and given the fact that ageing is associated with stroke (93), together with the progressively ageing population, it is estimated that the prevalence of stroke will increase by a fifth by 2030 (92). Several mechanisms were demonstrated to mediate the effect of age on stroke; amongst these, increasing production of free radicals was suggested to be a pivotal one. In line with this, the adaptor protein $p66^{Shc}$ and the pro-oxidant enzyme NAPDHoxidase were shown to play an important role (99). Stroke incidence and mortality also vary considerably between different racial groups. For instance, blacks are more than twice as likely to die of stroke as whites are. Indeed, between 45 and 55 years of age, mortality rates are four to five times greater for Afro-Americans than for whites; however, this difference decreases with ascending age (87). Irrespective of ethnical background, some families show an increased incidence of stroke. Potential reasons accounting for this are a hereditary tendency for stroke, a genetic predetermination for other risk factors for stroke, and an established familial exposure to specific environmental or lifestyle risks (87, 100). Early studies investigating this matter suggested an increased risk for men whose mothers died of stroke and women who had a family history of stroke. In the Framingham study an offspring analysis revealed that both paternal and maternal histories were associated with an increased risk of stroke (87, 101).

Cellular and molecular mechanisms involved in thrombotic stroke

The cellular and molecular mechanisms underlying thrombus formation in stroke are not well understood and this lack of knowledge is mirrored by the lack of a specific therapeutic strategy for its treatment. As a consequence, the drugs currently used to treat acute stroke or prevent its recurrence, e.g. recombinant tissue plasminogen activator, show only limited efficacy and can only be used in a limited time window without significantly increasing risks of bleeding (102).

The two main systems mediating thrombus formation in stroke are platelets and the coagulation cascade. Indeed, much effort is being made to elucidate the role of several platelet receptors, e.g. glycoprotein Ib (GPIb) and GPVI, and their signalling pathways with the goal of developing different strategies to interfere with their deranged function. Inhibition of these platelet receptors in different rodent models of stroke prevented infarctions without increasing the risk of intracerebral bleeding (103). Similarly, it is becoming increasingly evident that the intrinsic coagulation factors, FXII and FXI, play a crucial role in thrombus formation and stabilization during stroke. In line with this, their reduced expression or inhibition protects from cerebral ischaemia without increasing risks of bleeding (103).

Conclusion

Rupture of atherosclerotic plaques ultimately leads to occlusive arterial thrombosis. However, whether preceding non-occlusive luminal thrombi are responsible for the clinical event has yet to be fully determined. There is a need to better understand the complex mechanisms involved in platelet interaction with vascular surfaces and other circulating cells and proteins, as well as the timings by which these events occur in order to selectively inhibit the pathways most relevant to the pathological aspects of atherothrombosis. Moreover, further insights as to platelet biology should also focus on studying reticulocytes, as platelet activity might be determined early in the bone marrow (104).

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Recommended reading

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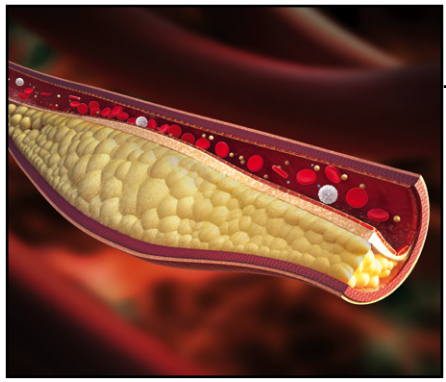

CHAPTER 19

Vascular pathophysiology of hypertension

Tomasz J. Guzik and Rhian M. Touyz

Content

Introduction

Hypertension is a highly prevalent multifactorial disease, affecting approximately 1 billion adults worldwide. It is observed in ≈30% of adults and an additional 30% of adults are at high risk of the disease (1, 2). The prevalence of hypertension increases with age, with 70% of adults older than 70 years being affected, which is particularly important in the face of an ageing society (3).The Global Burden of Disease Study identified elevated blood pressure as the leading risk factor, among 67 studied, for death and disability-adjusted life-years lost during 2010 (4). It is a major risk factor for many common chronic diseases, such as myocardial infarction, stroke, transient ischemic attacks (TIA), vascular dementia, heart failure, and chronic kidney disease (2).

Clinical evidence indicates that blood pressure lowering leads to reduced morbidity and mortality (5). Drug therapy for hypertension improved dramatically between 1975 and 1985, with the use of angiotensin-converting enzyme inhibitors, angiotensin-receptor blockers, diuretics, calcium-channel blockers, and beta blockers. Since the mid-1980s, however, progress in developing new therapies has become much slower. This is unfortunate, because many patients (up to 40%) continue to have elevated blood pressure despite the use of multiple anti-hypertensive agents. In some patients this is related to poor treatment compliance. It also highlights that our understanding of high blood pressure mechanisms and identification of treatment targets is sub-optimal (6). While a few cases of hypertension are due to single gene mutations (7), or underlying correctable causes such as renal artery stenosis, pheochromocytoma, or adrenal adenoma (8), these are uncommon and most cases of adult hypertension have no clearly identified aetiology. In these cases certain neurohumoral factors, such as angiotensin II (Ang II), play a key role, as drugs interfering with this pathway are effective anti-hypertensive agents. Systemic vascular resistance is generally elevated in hypertension (9–11), and vasodilators lower blood pressure. These observations would suggest that while renal, cardiac causes are important in hypertension, it is primarily a vascular disease (2).

Physiological maintenance of blood pressure depends on the force generated by the heart, the resistance in the vasculature, and the amount of intravascular fluid (2). The nineteenth-century French physician Poiseuille defined three primary factors that determine the resistance to blood flow within a single vessel: vessel diameter, its length, and viscosity of the blood. Vessel diameter is the most

important parameter, because the resistance is inversely proportional to its fourth power (r^4; in Poiseuille's equation). Vessel diameter in blood pressure regulation is dependent on the balance between its vasocontricting and vasorelaxing properties. Therefore, key cellular controllers of vascular resistance are vascular smooth muscle cells and endothelial cells, which, by releasing number of vasoactive paracrine factors (such as vasorelaxing nitric oxide, prostacyclin, and vasoconstricting endothelin 1, reactive oxygen species, or thromboxane), control vascular tone and resistance (12). Vascular resistance is regulated primarily at the arteriole (small arteries <300 μm in diameter) level. In accordance with Poiseuille's law, even moderate arteriolar constriction will disproportionally increase the resistance and, therefore, blood pressure. In order to achieve tight blood pressure regulation, the system of vascular resistance is controlled systemically (13). For example, the kidney contains key sensors that regulate the control of blood pressure. When the glomerular filtration rate (GFR) drops, the stretch receptors in the macula densa signal cells of the juxtaglomerular apparatus secrete renin, which induces generation of angiotensin II, which effects vasoconstriction, mainly in peripheral arterioles, thus increasing peripheral vascular resistance, thereby elevating blood pressure. In addition, renin stimulates release of aldosterone by adrenal cortical cells. Aldosterone affects transporters in distal renal tubules, increasing sodium reabsorption and potassium loss. Retention of sodium increases fluid in the vascular system to maintain pressure. Dysregulation of these systems is essential in hypertension pathobiology and is affected by numerous pathophysiological and environmental factors linked to hypertension, including age, body mass index, salt intake, diet, local inflammation, lifestyle, as well as genetic makeup predisposing to hypertension (2).

While arteriolar tone controls over 80% of vascular resistance, micro- and macrovascular remodelling are key features of sustained hypertension. It results from the stated pathogenetic mechanisms, but also from direct effects of increased tension in the vessel wall. Increased vascular stiffness characterized by reduced elasticity/distensibility of the vessel wall is caused by remodelling within the medial layer of the vessel and by vascular/perivascular fibrosis. It is defined clinically by increased pulse wave velocity and elevated central aortic pressure, both of which constitute important predictors of overall cardiovascular risk (14–16).

This process is important in various vascular beds. In particular, the capacitance property of the aorta normally blunts blood pressure elevation during systole and maintains diastolic pressure and tissue perfusion during diastole (17). This phenomenon has been termed the 'Windkessel effect' and its loss in the proximal aorta causes an increase in systolic pressure, a decline in diastolic pressure, and an increase in pulse wave velocity (17). The augmentation of systolic pressure caused by aortic stiffening increases the incidence of stroke, renal failure, and myocardial infarction (2).

Thus the range of vascular pathology in hypertension makes it a key target for therapeutic interventions (2).

Regulation of vascular tone in hypertension

Maintenance of vascular tone is critical for the homeostatic function of blood vessels and blood supply to peripheral organs. In hypertension vascular tone is increased. This increase is primarily a result of an imbalance between vasoconstricting and vasorelaxant properties of vascular smooth muscle cells and endothelial cells (18, 19). In physiological conditions, maintenance of appropriate endothelial function provides vasorelaxant properties through release of vasoactive substances such as nitric oxide, prostacyclin, and/or endothelium-derived hyperpolarizing factor. In pathological conditions, such as hypertension, the endothelium may produce vasoconstrictor agents such as thromboxane A2, endothelin 1, or PGH2 (18, 19). An imbalance between production of vasoprotective and vasorelaxant factors and vasoconstrictor substances by the endothelium is a hallmark of endothelial dysfunction, which precedes numerous vascular pathologies and is a common feature in hypertension. Vasoactive substances exert their vasorelaxant or vasoconstrictive properties through effects on vascular smooth muscle cells, which are central for the regulation of vascular tone.

Vascular hypercontractility in hypertension is characterized by dysregulation of the contractile machinery of vascular smooth muscle—actin and myosin, mainly through changes in intracellular free Ca^{2+} concentration in response to various pro-constrictive agonists. Briefly, agonists activate phospholipase C (PLC)-coupled receptors, which trigger second messengers such as insitol trisphosphate (IP_3) and diacylglycerol (DAG). Increases in intracellular Ca^{2+} lead to activation of myosin light chain kinase (MLCK) that phosphorylates myosin light chain (MLC_{20}), promoting cycling of myosin cross-bridges with actin and consequent smooth muscle contraction (20). In contrast, vasorelaxation is induced by dephosphorylation of MLC_{20} by myosin light chain phosphatase (MLCP). Increased vascular contractility in hypertension is characterized by increased MLCK activity. Importantly, vascular smooth muscle myosin can also be phosphorylated in a Ca^{2+}-independent manner by additional proteins like Rho kinases (ROCK1 and ROCK2).

RhoA/Rho kinase activity is strongly activated by vasoconstrictors, such as angiotensin II and ET-1, characteristic of hypertension (21). This results in decreased myosin light chain phosphatase activation and consequent sustained vasoconstriction and blood pressure elevation. Ang II-induced hypertension in rodents exhibits increased vascular RhoA/Rho kinase activation (21). Human studies have suggested potential beneficial effects of ROCK inhibitors, although their effects are particularly prominent in preventing acute vasospasm, rather than more chronic vasoconstriction in hypertension (22).

Key vasoactive mediators

Nitric oxide

Nitric oxide (NO) bioavailability is reduced in hypertension contributing to increased vascular tone. Nitric oxide is produced in the vascular wall by nitric oxide synthases, which catalyse transfer of electrons from the prosthetic haem group of NOS to L-arginine, ultimately leading to NO and citrulline production (23, 24). There are three isoforms of NOS: (i) NOS I or (nNOS)—the neuronal form, (ii) NOS II—inducible nitric oxide synthase (iNOS), present in various cell types upon inflammatory stimulation (e.g. macrophages), and (iii) NOSIII (or eNOS)—constitutive enzyme expressed mainly in the endothelium (23). All three isoforms have a similar molecular structure and require multiple cofactors but eNOS is the most important in regulation of vascular tone. Nitric oxide produced by endothelium rapidly diffuses to interact with molecular targets in cells in the vascular wall and in the lumen (23). NO activates soluble guanylate cyclase in vascular smooth muscle cells (VSMC), leading to elevation of cGMP, activation of cGMP-dependent protein kinase (PKG), and vasorelaxation, the primary basis for blood flow and pressure regulation (23). NO also interacts with thiol groups and with metal centres in diverse protein targets, including membrane receptors, G proteins, ion channels, cytosolic enzymes, and transcription factors such as AP-1 and NFkB (25) further inhibiting Ca^{2+}-mediated vasoconstriction. eNOS knockout mice develop spontaneous hypertension (26).

Prostacyclin (PGI_2)

Prostacyclin is also released by the endothelium and acts synergistically with NO to control vascular tone. It is produced by endothelial cells and, to a lower extent, in the vascular media and adventitia during metabolism of arachidonic acid by phospholipase A_2, cyclo-oxygenase, and prostacyclin synthase. It can be released together with NO by numerous mediators and causes vasorelaxation of VSMC by activation of adenylate cyclase generating cAMP. However, in physiological conditions its contribution to vasorelaxation is less pronounced than that of NO (19). PGI_2 significantly inhibits platelet aggregation affecting other vasoprotective properties of endothelium.

Endothelium-derived hyperpolarizing factor (EDHF)

The nature of this factor remains unclear despite many years of research. Some initial studies suggested that it is identical to NO, but others showed that it can induce vasorelaxation independently of NO or PGI_2 (27). A role for K^+ has been suggested, as well as for hydrogen peroxide and metabolites of arachidonic acid metabolism (28).

Vasoconstricting mediators

The endothelium can also release factors inducing vasoconstriction. These include endoperoxides, tromboxane A2 (vasoconstrictor prostanoids), and endothelin-1. Importantly, the role of superoxide anion as an endothelium-derived contracting factor is also suggested through its reaction with NO, which will be discussed in detail in further sections of this chapter.

Angiotensin II (Ang II)

Ang II is an endocrine, autocrine/paracrine, and intracrine hormone produced from angiotensin I through the activity of angiotensin-converting enzyme in the lung, vascular endothelium, and in the kidney. Acting on AT1 receptors it directly leads to vasoconstriction, although various vascular beds vary in their sensitivity to Ang II. Certain proinflammatory stimuli, such as IFN-γ, increase vascular sensitivity to Ang II-induced vasoconstriction.

Endothelin 1

This is a potent vasoconstrictor, and is produced by endothelial cells from pre-proendothelin, and its production is increased in various pathologies, including hypertension. Endothelin 1, acting directly on VSMCs is a potent vasoconstrictor.

The autonomic nervous system in regulation of vascular tone

The central nervous system is essential for processing and integrating neurohumoral signals from the periphery, modulating autonomic nervous activity to maintain blood pressure, vascular tone, and fluid homeostasis. Autonomic control of blood pressure is largely regulated by distinct nuclei in the hypothalamus and brainstem. The circumventricular organs have an incompletely formed blood–brain barrier and can, therefore, sense circulating hormones, such as angiotensin II, resulting in increased sympathetic vasomotor activity. Importantly autonomic nervous system

endings are located in perivascular tissues (adventitia and perivascular adipose tissue) through which they regulate vascular function, and can affect vascular smooth muscle tone and endothelial function.

Endothelial dysfunction in hypertension

The relevance of endothelial cell function and NO production in vascular diseases is supported by observations that these processes are altered in patients with hypertension and in animal models of hypertension (29). The normal endothelium is anti-coagulant, anti-inflammatory, and promotes vasodilatation by production of nitric oxide (NO), prostacyclin, and other vasodilators (➔ Fig. 19.1). In the setting of pathological conditions, the endothelium can become dysfunctional and promotes thrombosis, inflammation, and vasoconstriction (30). The concept of endothelial cell dysfunction is defined as a loss of the normal vasoprotective characteristics (31). Endothelial dysfunction is an important predictor of cardiovascular outcomes and events in patients at risk of cardiovascular disease and, accordingly, there is increasing interest in measuring endothelial function in the clinical setting (38). This is linked to the fact that, in addition to regulating vascular tone, a substantial body of evidence suggests that NO has vasoprotective effects, including anti-inflammatory, anti-platelet, anti-proliferative, and antioxidant effects.

Alterations of NO bioavailability and production are key features reported in virtually all experimental models of hypertension and in clinical hypertension (32, 33). By contrast, endothelium-independent vasorelaxations to direct NO donors (nitro-glycerine, sodium nitroprusside) are most commonly unchanged in hypertension.

Endothelial dysfunction in human hypertension

Studies of vascular function in humans using numerous techniques, such as forearm plethysmography, coronary angiography, or *ex vivo* by means of organ bath studies, have shown significant impairment of endothelial function at high normal blood pressure levels (33, 34). Moreover, lowering blood pressure, use of vasoprotective drugs, or antioxidant therapy improves the functional deficit of vasorelaxations in hypertension (35, 36). However, this improvement is only partial, indicating that, whereas endothelial dysfunction is associated with the initial phase of blood pressure elevation, other factors play a role in the development of hypertension.

Regulation of nitric oxide bioavailability in hypertension

In vivo the activity of NO within the vessel wall depends on the balance between synthesis and breakdown. There are several mechanisms likely to be associated with impaired NO bioactivity in hypertension. Endothelial dysfunction could

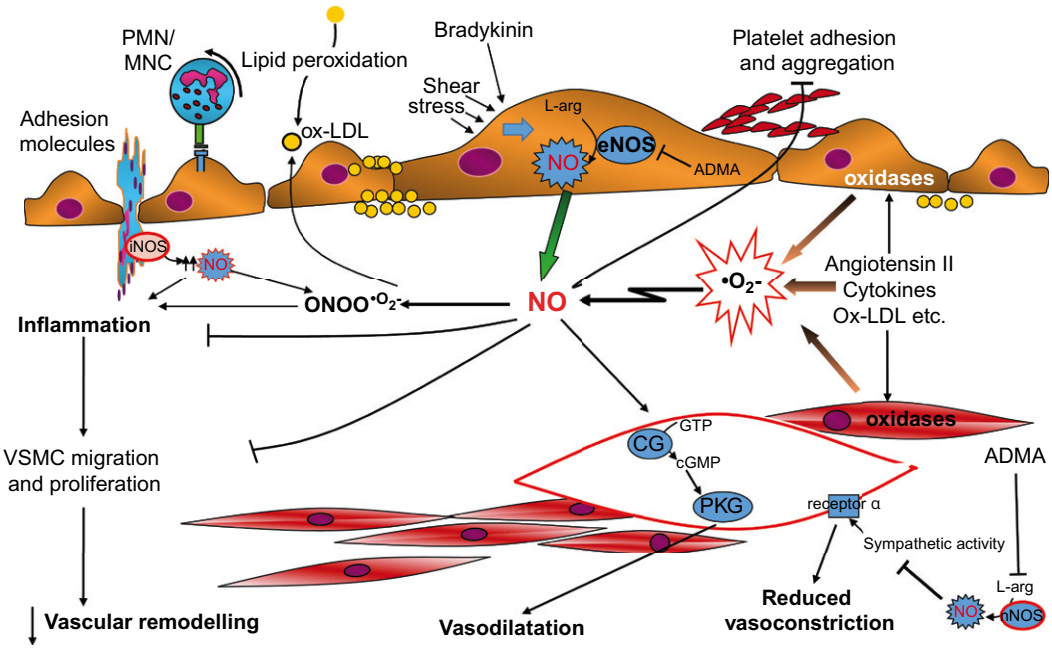

Fig. 19.1 Central role of endothelium in protecting vasculature from hypertension and vascular damage, and role of superoxide anion (O_{2^-}) in inducing endothelial dysfunction and its consequences.

(Reproduced from Guzik TJ and Harrison DG. Vascular NADPH oxidases as drug targets for novel antioxidant strategies. Drug Discov Today. 2006;11:524–33 with permission from Elsevier.)

be associated with variations in NOS protein levels or eNOS itself could be dysfunctional, resulting in reduced specific activity for NO production and reduced sensitivity to physiological activating stimuli. Although eNOS is constitutively expressed, eNOS gene expression may be regulated by a variety of different factors that are altered in hypertension, such as shear stress, cyclic strain, lyso-phosphatidylcholine, or ox-LDL. However, human studies have shown clearly that while eNOS levels are decreased advanced atherosclerotic lesions (37) in hypertension no clear deficiencies in eNOS expression, despite pronounced endothelial dysfunction (38, 39) have been found. In some situations eNOS protein may even be increased (39). Therefore, reductions in NO bioactivity in hypertension are not explained simply by loss of eNOS protein. As has been indicated earlier, the eNOS–caveolin interaction maintains eNOS in an inactive, membrane-bound state (40), and is reversed by calcium calmodulin activation (41).

In endothelial cells under hypertensive and atherosclerotic conditions, eNOS–caveolin 1 interaction is enhanced, therefore reducing eNOS activation and providing a possible explanation for reduced NO production in atherosclerosis (43, 44). Additionally, eNOS regulation Hsp90 and its phosphorylation by Akt (45–47) are altered in hypertension. Recent evidence points to availability of eNOS substrate and cofactors as important regulators. Initially thought to be important, L-arginine as a substrate has not proven to be effective in reducing blood pressure in hypertension. On the contrary, alterations of NO production from endothelial cells could derive from eNOS cofactor availability. In particular, tetrahydrobiopterin (BH_4), produced by vascular GTP cyclohydrolase I (GCH1), modulates NO production by NOS (48, 49). BH_4 is required to maintain eNOS dimerization (50). In hypertension vascular (and in particular endothelial) tetrahydrobiopterin levels are decreased, due to its oxidation, as well as active transport out of the cell (51, 52). NOS monomer transfers electrons to molecular oxygen rather than to arginine leading to formation of reactive oxygen species, instead of NO (53). Administration of exogenous BH_4 has been successfully used in pilot studies in patients with hypertension and was shown to improve endothelial function and to lower blood pressure (54), although sensitivity of BH_4 for oxidation is a problem in this approach (55). While oxidation of tetrahydrobiopterin is an important mechanism for endothelial dysfunction in hypertension, NO scavenging appears to be most prominent in human vasculature in the setting of numerous risk factors for atherosclerosis, including hypertension. The superoxide anion (O_2^-) rapidly reacts with NO leading to the production of the strong oxidant peroxynitrite ($ONOO^-$) (56). This reaction appears to be the most important mechanism of reduced NO bioavailability in hypertension and other vascular pathologies. Importantly, treatment with superoxide dismutase (SOD) or SOD mimetics (57) to reduce vascular O_2^- levels restores endothelial function in hypertension. In the setting of angiotensin II-mediated hypertension, vascular O_2^- production is increased and treatment with membrane-targeted forms of SOD also lowers blood pressure (58). In line with these findings, acute infusion of high concentrations of vitamin C improves endothelium-dependent vasodilatation in humans with atherosclerosis, likely via O_2^- scavenging (59), although antioxidant vitamins failed to provide sufficient clinical benefits in relation to cardiovascular risk or blood pressure lowering, pointing to the fact that the role of vascular oxidative stress in hypertension is more complex.

Vascular oxidative stress in hypertension

The critical role of vascular oxidative stress, as both a consequence and a cause of hypertension, has become clear during the past two decades (➔ Fig. 19.2).

Reactive oxygen species alone might not be capable of causing hypertension; they clearly augment elevations of blood pressure when other factors, such as elevated angiotensin II and salt loading, are present. The role of oxidative stress in vascular damage and hypertension was first demonstrated by Nakazono et al. who showed that a bolus administration of a heparin-binding form of superoxide dismutase acutely lowered blood pressure in hypertensive rats (60). Subsequently, Harrison demonstrated that membrane-targeted forms of SOD lower blood pressure in angiotensin II-induced hypertension (58, 61–63). The failure of antioxidant treatment to improve cardiovascular outcomes does not mean that reactive oxygen species have nothing to do with these conditions, but simply that the concept of oxidant stress is complex and new treatment strategies should be investigated. Understanding mechanisms of vascular oxidative stress in hypertension is critical for the development of specific interventions. All vascular cells are capable of producing ROS and major enzymatic sources of vascular superoxide include NADPH-dependent oxidases (64), xanthine oxidase (65), lipoxygenases, mitochondrial oxidases (66), and NO synthases (48).

Sources of vascular oxidant stress in hypertension

NADPH oxidases

NADPH oxidases are critical sources of reactive oxygen species in hypertension in both human disease and in

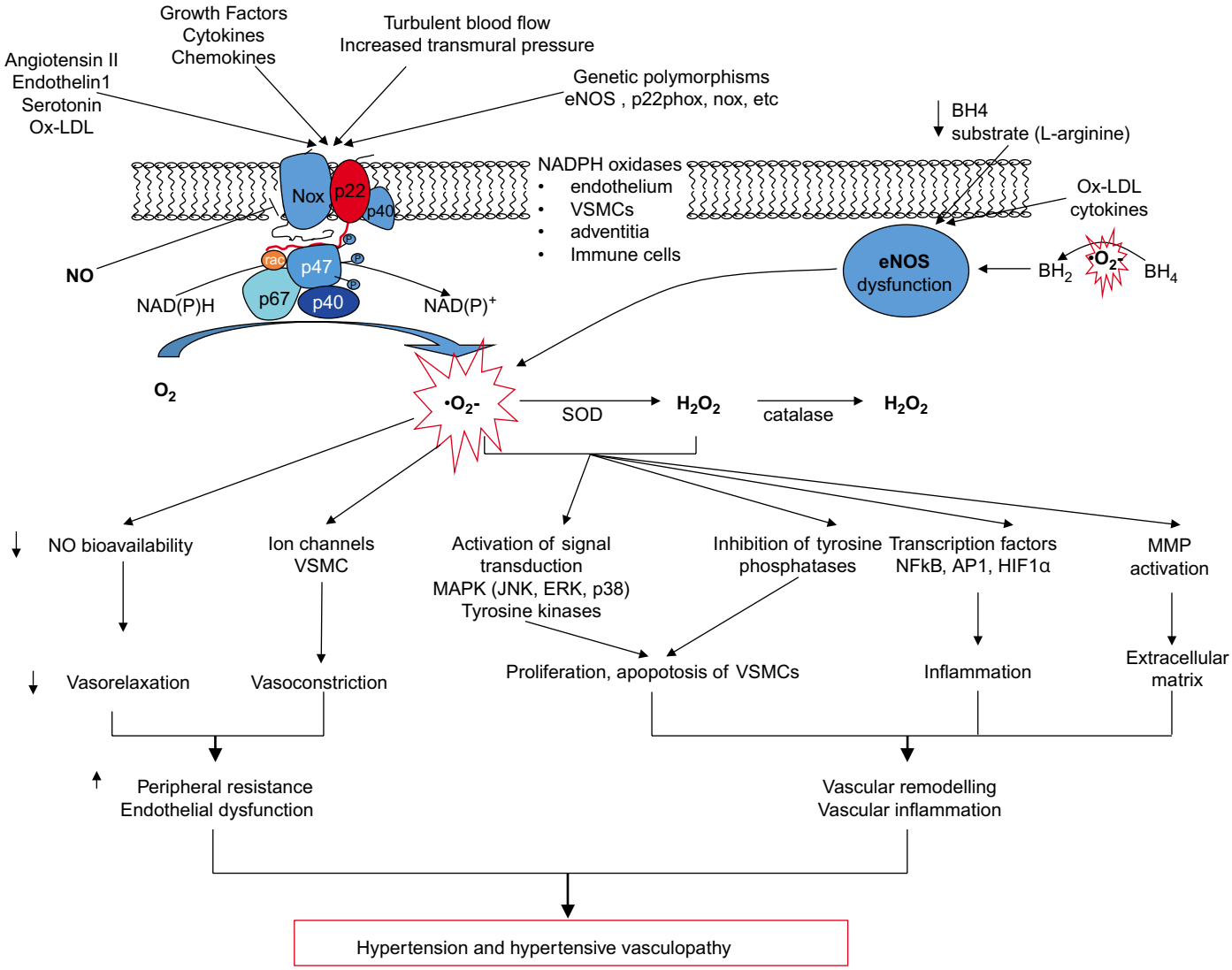

Fig. 19.2 Oxidants in the regulation of hypertensive vascular pathology.
(Reproduced from Guzik TJ and Harrison DG. Vascular NADPH oxidases as drug targets for novel antioxidant strategies. Drug Discov Today. 2006;11:524–33 with permission from Elsevier.)

animal models. These enzymes, also known as the Nox enzymes, not only directly produce ROS, but stimulate other ROS-generating enzymes (67). NADPH oxidases were first identified in phagocytic cells and were subsequently found in all vascular cells, including endothelial cells, VSMCs, and fibroblasts (68). They are expressed in human coronary and peripheral arteries (69, 70), and their expression and activity are closely associated with clinical (71) risk factors for atherosclerosis. These are complex enzymes composed of membrane and catalytic and cytoplasmic regulatory subunits (typically five protein components). There are at least seven variations of the NADPH oxidase, differentiated by their catalytic subunits known as the Nox proteins. These catalytic subunits possess flavin and haem-binding regions and generate O_2^- via one electron transfer from NADH or NADPH to oxygen. An exception to this is Nox4, which seems to predominantly produce H_2O_2. Of the various Nox isoforms, Nox1, Nox2, Nox4, and Nox5 are most important in hypertension. Nox1 is minimally expressed under basal conditions, but stimuli linked to hypertension, such as PDGF and angiotensin II, can increase its expression (72). Nox1 levels seem to be most highly upregulated *in vivo* in models of angiotensin II-dependent hypertension. Nox2, previously known as $gp91^{phox}$, is the large catalytic subunit of the phagocyte cytochrome b558. It is expressed in endothelial and adventitial cells of large vessels and in the vascular smooth muscle cells of smaller vessels, and may directly affect both NO bioavailability and contractile properties of the vasculature. Nox4 is constitutively expressed and active in vascular smooth muscle and endothelial cells, its expression appears

to exert protective effects, at least in respect to atherosclerosis, although its role in hypertension is less clearly defined. It is abundantly expressed in the kidney and as such was originally termed 'renox'. Nox4 may constitute an important compensatory mechanism protecting from development of endothelial dysfunction, as mice lacking Nox4 have exacerbated endothelial dysfunction in response to various stimuli. Nox5, which is expressed in testes, spleen, and in the uterus, is a calcium-dependent enzyme (73, 74) and is activated by angiotensin II and other pro-hypertensive factors. The role of human Nox5 in hypertension is unclear, but it has been implicated in human atherosclerosis (75) and renal injury in diabetic nephropathy (76). In atherosclerotic coronary arteries, Nox5 is expressed at various stages of pathology in either endothelial cells (early disease) or neointimal and inflammatory cells in the shoulder region of the plaque (75). All Nox enzymes, except Nox5, require $p22^{phox}$, which serves as a docking protein for other subunits and stabilizes the Nox proteins (77). The role of $p22^{phox}$ in human vasculature is confirmed by findings that genetic variation in CYBA gene encoding $p22^{phox}$ is associated with superoxide production in the vessels (78). Activation of Nox2 requires recruitment of cytosolic subunits, including $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and the small g protein rac-1 (72). A variety of pro-hypertensive stimuli, such as angiotensin II, stretch, endothelin-1, thrombin, and catecholamines, acutely activate the NADPH oxidases in both vascular smooth muscle and endothelial cells, probably by promoting phosphorylation of $p47^{phox}$ and by increasing expression of NADPH oxidase catalytic subunits. A putative causal role of NADPH oxidases has been demonstrated by numerous studies showing that lack of Nox1, Nox2, or Nox4 results in blunted hypertension, although the effects in more chronic hypertension models is less clear. The NADPH oxidase inhibitor apocynin reduces blood pressure in several models of hypertension (79–81). Recently, systemic administration of $p22^{phox}$ siRNA reduced angiotensin II-induced hypertension in rats (82).

In summary, the NADPH oxidases have been indicated as a main source of O_2^- in hypertension. In humans it has been confirmed that NADPH oxidase activity is inversely correlated with endothelial function. This relationship exists even when corrected for other major risk factors for atherosclerosis, including diabetes and hypercholesterolaemia (83).

Other enzymatic sources of vascular oxidant stress in hypertension

Other potential sources of vascular superoxide anion in hypertension include xanthine oxidase, nitric oxide synthases, enzymes involved in the metabolism of arachidonic acid (cyclo-oxygenases or lipo-oxygenases), and the mitochondrial electron transport chain, as well as cytochrome p450 Cyp2C9 subtype. Xanthine oxidoreductase activity is increased in heart failure and hypertension (67). The endothelial levels of xanthine oxidase are increased and correlate with the degree of impairment in endothelium-dependent vasodilation (62, 84). As discussed, in the absence of its cofactor tetrahydrobiopterin (HB_4) or its substrate L-arginine, the endothelial NO synthase is uncoupled and generates O_2^- instead of NO (48). This process was first identified in the context of hypertension. Importantly, in hypertension, NADPH oxidase is critical in the regulation of superoxide production from both eNOS (63) and xanthine oxidase (85).

Vascular oxidant injury as a mechanism of hypertension

Vascular oxidant stress is a hallmark of vascular injury (86). One of the key mechanisms through which ROS contribute to hypertension is their role in modulation of the loss of NO bioavailability and the development of endothelial dysfunction in the rapid reaction of NO oxidation by O_2^- to peroxynitrite and, subsequently, to nitrite and nitrate. This results in a loss of endothelium-mediated vasodilation, an increase in vasoconstriction, and, subsequently, an increase in systemic vascular resistance. Such an increase in systemic vascular resistance would elevate blood pressure, provided that cardiac output remained constant. This hypothesis has been supported by studies in which mice with targeted overexpression of the NADPH oxidase in vascular smooth muscle cells had augmented hypertension in response to angiotensin II infusion (87). Reactive oxygen species (ROS) can also modify endothelial NO production via oxidation of the zinc–thiolate centre of NOS by $ONOO^-$ (the product of O_2^-–NO interaction) and cause post-translational modification of calmodulin, and may interfere with actions of NO on the level of vascular smooth muscle. Irreversible oxidation of relevant cysteine thiols block S-glutathiolation and activation of SERCA, which increases cytoplasmic Ca^{2+} levels and impairs vascular relaxations and endothelial function (88). The effects of vascular oxidative stress in hypertension extend beyond those of NO bioavailability. ROS are important intracellular and intercellular second messengers that modulate many downstream signalling molecules, such as protein tyrosine phosphatases, protein tyrosine kinases, transcription factors, mitogen-activated protein kinases, and ion channels, which participate in generation of hypertension, as well as in subsequent vascular remodelling. ROS also increase intracellular free Ca^{2+} concentration, a major determinant of vascular reactivity. ROS influence signalling molecules

by altering the intracellular redox state and by oxidative modification of proteins.

When discussing vascular oxidative stress, it is important to emphasize that ROS are also very important in normal physiological processes. Produced at low concentrations, in a well-controlled manner, intracellular ROS regulate normal redox signalling involved in maintaining vascular function and integrity. Under pathological conditions, ROS contribute to vascular dysfunction and remodelling through oxidative damage, which is critical for both development of hypertension and vascular remodelling.

Vascular inflammation in hypertension

Vascular inflammation has been traditionally linked to end-organ damage in hypertension, as one of the key consequences of increased transmural pressure. Its role in atherosclerosis and vasculitis is discussed at length in other chapters (➔ Fig. 19.3; Chapters 4, 13, 14, and 17). Thus inflammation and immune activation, provide an important link between hypertension and atherosclerosis. Blood pressure increase is associated with an increase in the pro-inflammatory marker, C-reactive protein (CRP) (89), and there is a correlation between CRP levels and pulse pressure (90).

Direct evidence linking blood pressure regulation, inflammation, and the immune system came from basic animal studies. More than 30 years ago, Svendson showed that the delayed phase of DOCA salt hypertension was blunted in thymectomized animals (91). The T-cell modulating agent, mycophenolate mofetil, lowers blood pressure in Dahl salt-sensitive rats (92), and in rats with lead-induced hypertension (93). This agent also lowers blood pressure in humans with psoriasis and rheumatoid arthritis (94). In keeping with this, it has been estimated that 70% of patients with rheumatoid arthritis have hypertension (95). The incidence of hypertension is increased rather markedly in individuals with severe psoriasis, and to a modest extent in subjects with mild psoriasis (96). Pre-eclampsia is associated with an increase in lymphocyte markers and the cytokine profile of natural killer lymphocytes in the uterus (97, 98). T cells infiltrate the kidney in hypertensive models (99), and efforts to decrease this have proven effective in lowering blood pressure (100, 101). A recent analysis of almost 6,000 people with AIDS and reduced CD4 cells showed that the incidence of hypertension was significantly lower than in a population of matched non-infected individuals. Clinically, treatment with highly active anti-retroviral therapy for 2 years restored the incidence of hypertension to that of the control population (102). There are several explanations for this, including the fact that these individuals are generally very sick; another explanation is that T cells are required to develop hypertension.

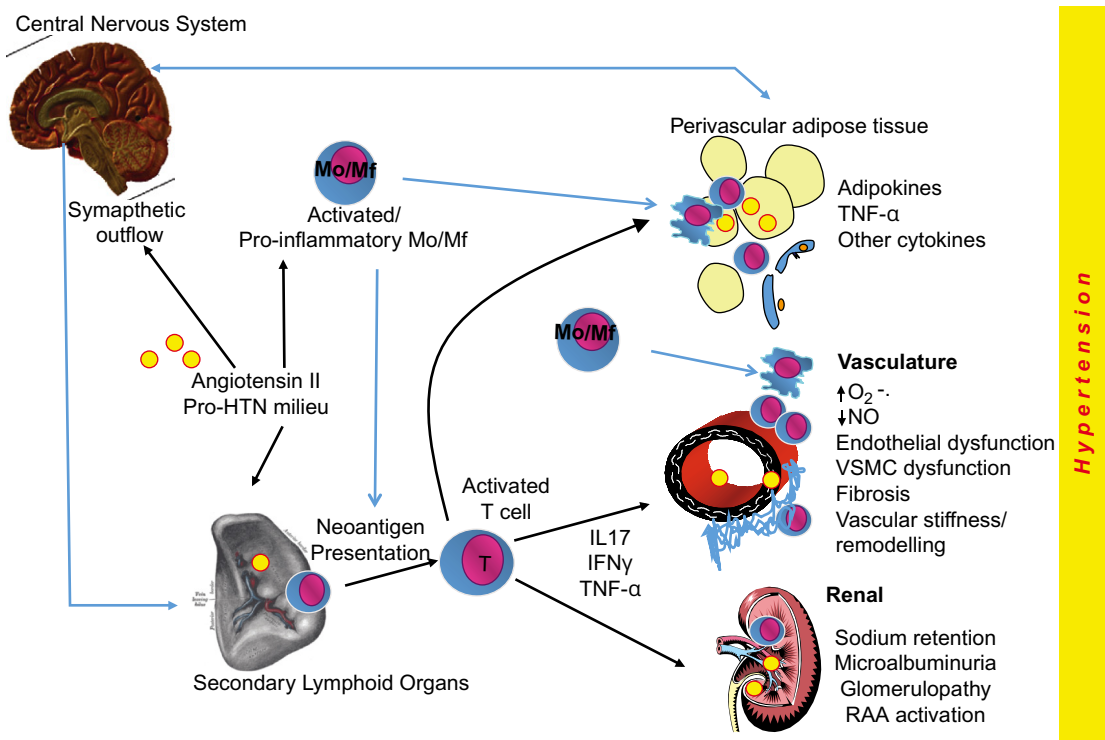

Fig. 19.3 Role of inflammation in hypertension.

The direct proof of involvement of T-lymphocyte activation in hypertension comes from findings that Rag1$^{-/-}$ mice as well as Rag1$^{-/-}$ rats, which lack both T and B cells, are resistant to the development of hypertension, and adoptive transfer of T cells, but not B cells, restores these responses (103). These findings have been confirmed in a number of models of hypertension in mice and rats (104–106). Similar decrease in the occurrence of hypertension has been observed in mice lacking functional monocytes (107). The mechanisms of these observations are unclear, but animal models indicate that there is a significant infiltration of T cells into the perivascular tissues, namely adventitia and perivascular adipose tissue, and in the kidneys in hypertension. Our recent data indicate that T cells infiltrating the perivascular space promote vascular ROS production, alter vascular function, and are essential for the development of hypertension (103). This is likely via release of cytokines: IFN-γ is critical in mediating endothelial dysfunction in hypertension and the TNF-α antagonist, etanercept, prevents both hypertension and vascular dysfunction caused by angiotensin II. Thus, modulation of T-cell function might be a new target for treatment or prevention of hypertension and associated vascular dysfunction.

Interestingly, angiotensin II infusion markedly increases the percentage of CCR5 positive cells in perivascular adipose tissue. Also, the level of vascular RANTES chemokine (regulated on activation normal T cells expressed and secreted) is elevated in the same conditions (103). The level of chemokine ligand 5 is increased in the kidney after acute infusion of sodium (108). This interaction of RANTES and its receptor CCR5 facilitates T-cell migration and accumulation in the perivascular adipose tissue and in the kidney. In these sites, activated T cells release proinflammatory cytokines such as TNF-α and INF-γ (103). Agents that interrupt the CCR5/RANTES axis might become a novel therapeutic strategy to effective treatment of hypertension and cardiovascular disease. Antagonist of CCR1/CCR5, met-RANTES has been used to inhibit atherosclerosis (109). Other CCR5 receptor antagonists are clinically available and include Maraviroc or TAK-779 (110–111).

In keeping with previous animal studies, T cell-derived inflammation also contributes to human hypertension (112). In humans this appears to be linked particularly to the immunosenescent, cytotoxic T cells characterized by the loss of CD28 and the acquisition of CD57 (112). Appearance of CD28^{null}CD57^{+} CD8^{+} T cells may be associated with repeated antigenic stimulation by neoantigens (113). The nature of these neoantigens that could stimulate immune responses in hypertension remains elusive. So far, isoketals, arachidonic acid metabolites, capable of modifying endogenous proteins and peptides to increase their immunogenicity, have been identified in the kidneys and vasculature in hypertension (114).

Activation of naïve T cells requires the concomitant signalling provided by T cells (TCR) and the co-stimulatory receptors (115). In this process, CD28 molecules interact with co-stimulatory receptors, such as CD80 and CD86 (B7-1 and B7-2, respectively), on antigen-presenting cells (APC), e.g. dendritic cells (115). Blocking this process using compounds such as CTLA4-Ig (cytotoxic T lymphocyte antigen-Ig), prevents experimental hypertension and inhibits vascular T-cell infiltration.

The major effect of immune cells in the vasculature in hypertension is probably achieved by release of effector cytokines, including IL-17, IFN-γ, and TNF-α, as well as TGF-β and IL-6. Lack of these cytokines results in reduced blood pressure in hypertension and/or in protection from development of endothelial dysfunction, in spite of hypertension. IL-17-producing T cells increase in peripheral organs, blood, and especially in the vasculature of hypertensive animals and humans (116). Chronic hypertension is also associated with increased IL-6 levels and an increase of arterial pressure and this effect may be blocked in IL-6 knockout mice in Ang II-induced pathology (117). Ang II induces IL-6 production through a mineralocorticoid receptor-dependent mechanism in humans (118). An interesting observation concerns patients with psoriasis, treated with mycophenolate mofetil (MMF), which targets B and T cells and was shown to reduce blood pressure and urinary excretion of TNF-α in essential hypertensive patients (94) and may exhibit vasoprotective properties.

IFN-γ produced by the T cells is also vital in regulating vascular oxidative stress and endothelial dysfunction in hypertension, linking immune activation and vascular inflammation to key effector mechanisms of the disease. Th1 and Th17 effector cells, via production of proinflammatory mediators, participate in the low-grade inflammation that leads to blood pressure elevation and end-organ damage. The suppressive arm of the immune system, and in particular FoxP3 expressing T regulatory lymphocytes, counteract hypertensive effects by suppressing innate and adaptive immune responses. Their decreases have been reported in hypertension and their adoptive transfer inhibits hypertension and protects from development of vascular inflammation, oxidative stress and hypertension.

Perivascular adipose tissue dysfunction in hypertension

Perivascular adipose tissue is a critical regulator of vascular function, which, until recently, has been greatly overlooked (119–121). Most arteries are surrounded by a significant

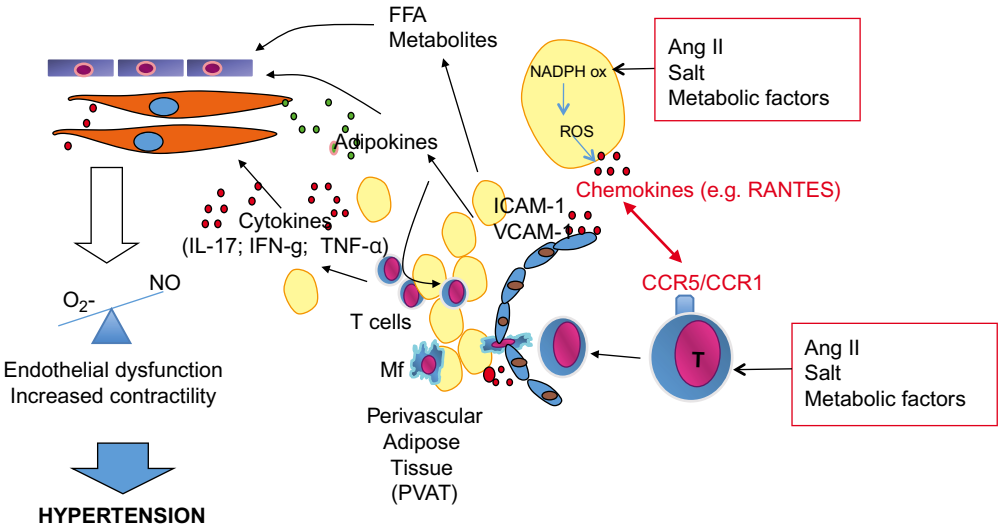

Fig. 19.4 Role of perivascular adipose tissue in the regulation of vascular function in hypertension.

amount of perivascular adipose tissue, which has long been considered to serve primarily a supportive, mechanical purpose. Recent studies, however, clearly show that adipose tissue is a very active endocrine and paracrine organ. While the majority of studies relate to typical visceral adipose tissue (VAT) the perivascular adipose tissue (pVAT) is also an important source of adipocytokines, as well as inflammatory cytokines (➲ Fig. 19.4) (121–123).

The role of perivascular adipose tissue in the regulation of vascular function, and thus in cardiovascular disease, is particularly evident in hypertension. Perivascular adipose tissue differs from VAT both morphologically and functionally, and especially with respect to the adipokines produced. Perivascular adipose tissue appears to play a very different role in physiological and pathological conditions. For example, in physiological conditions, some of the paracrine factors released from pVAT, such as adiponectin, induce vasorelaxation, whereas in pathological conditions, such as in hypertension, vasoconstrictor adipokines are produced. Lipoatrophic mice lacking adipose tissue depots, including pVAT, are hypertensive and show features of endothelial dysfunction, possibly due to the loss of adiponectin production (124). Perivascular AT may decrease contractile responses to vasoconstrictive agents, such as phenylephrine and norepinephrine, in physiological conditions (125). In contrast to this, in hypertension pVAT lacks these vasoprotective properties. Adiponectin may be an important regulator; however, many other adipocyte-derived vasoactive factors still await identification. The role of pVAT in the regulation of vascular function may also relate to the central nervous system (CNS). In particular, sympathetic nervous system endings are present in VAT but even more abundantly in pVAT (126, 127). At the same time it is known that the central nervous system can regulate vascular function by releasing neuromediators, in perivascular tissues, including the adventitia and perivascular fat. The role of the CNS in the latter has been demonstrated in numerous models of cardiovascular disease, in which disruption of CNS signalling leads to abrogation of hypertension or atherosclerosis and amelioration of vascular and endothelial dysfunction. This relationship between the brain and the vasculature has been termed the 'brain–vessel axis' (128).

ROS and perivascular Ang II

In the context of hypertension, perivascular adipose tissue can contribute to vascular oxidative stress, as adipocytes express NADPH oxidases and their activity is increased in hypertension (129). Furthermore, oxidative stress in adipocytes stimulates recruitment of inflammatory cells to pVAT and their transformation towards a proinflammatory phenotype in hypertension. Perivascular adipocytes also express angiotensinogen (130), although the regulation of its expression in cardiovascular pathology remains unknown. In hypertension, pVAT has been shown to produce and release Ang II and related peptides (Ang I, II, III, IV, Ang 1–9, Ang 1–7, Ang 1–5) (131).

Classical adipocytokines

Classical adipokines are also expressed and released by pVAT in hypertension, which may modify vascular function (132). Although leptin is one of the key pro-pathogenetic adipokines in obesity, and possibly in atherosclerosis, its expression appears to be reduced in primary hypertension in the absence of obesity. This may represent a phenotypic change of pVAT in hypertension from energy/lipid storage

towards proinflammatory secretory phenotype. However several human clinical trials supporting this strong correlation in myocardial infarction (133), coronary artery calcification (134), and stroke (135) suggest an important relationship between leptin and the development of clinically pertinent coronary lesions. Resistin is another typical adipocytokine, which has been implicated in cardiovascular pathology. Resistin levels are elevated in hypertension. Resistin increases vascular oxidative stress, acting directly on VSMCs and endothelial cells, and induces endothelial dysfunction. Resistin influences the protein expression of several vascular genes via PI3Kp85alpha. It can stimulate the release of PAI-1, vWF, and ET, and downregulates eNOS. The effect of resistin on PI3K signalling might contribute to endothelial dysfunction in hypertension (136). Recent studies also point towards high expression of visfatin in hypertension, which may regulate vascular function. Visfatin plasma levels are negatively associated with vascular endothelial function evaluated by flow medicated dilatation studies (FMD) and creatinine clearance, and positively associated with log urinary albumin excretion (137).

pVAT as a site of vascular inflammation

Perivascular adipose tissue in hypertension serves as a source for inflammatory cells, particularly T cells, B cells, and macrophages. Cells of the innate and adaptive immune system, such as macrophages and lymphocytes, accumulate in visceral adipose tissue but minimally in subcutaneous adipose tissue (138). This is possible as pVAT creates an ideal environment for the development of vascular inflammation, by releasing chemokines that attract effector and effector memory T cells and macrophages. RANTES is highly expressed within pVAT and its expression is exacerbated in hypertension. Adipocytokines attract inflammatory cells. Leptin and resistin have been shown to be a chemoattractant for inflammatory cells (139). Factors released by adipocytes can activate immune cells both resident and incoming recruited through chemotaxis. Both of these adipocytokines lead to activation of T cells and monocytes. Moreover, pVAT adipocytes release cytokines and alarmins, such as IL-6, IL-12, or IL-33, which modulate immune cell activation and differentiation.

Vascular dysfunction as a modulator of pVAT function

The relationship between pVAT and vascular function is bi-directional (120, 140, 141). Accumulating evidence demonstrates that vascular dysfunction and oxidative stress may precede dysfunction of adipose tissue and may even contribute to obesity. For example, mice with increased superoxide production selectively in vascular smooth muscle cells are characterized by increased propensity to diet-induced obesity and long-term hypertension associated with it. Moreover, soluble factors released by the vascular wall can alter expression of adipokines, such as adiponectin in the surrounding pVAT.

In summary, perivascular adipose tissue is an important regulator of vascular homeostasis in experimental hypertension. The clinical significance however awaits clarification.

Vascular remodelling in hypertension

Remodelling of large and small arteries contributes to the development and complications of hypertension. Growth, apoptosis, inflammation, and fibrosis contribute to vascular remodelling in hypertension (142). Although growth is the mechanism that is more classically associated with vascular remodelling, it has increasingly been appreciated that apoptosis, low-grade inflammation, and vascular fibrosis are dynamic processes that may also influence the degree of remodelling (142). In large arteries, remodelling is characterized by hypertrophy of intima and media, which is accompanied by intra- and perivascular fibrosis. Hypertrophy of media is associated with increased growth and proliferation of vascular smooth muscle cells, which is characteristic for hypertension and seems to be greatly dependent on initial blood pressure increase, as it is greatly prevented if blood pressure is lowered. The importance of this process in cardiovascular prognosis and risk is emphasized by the fact that intima–media thickness remains a key indicator of vascular damage in hypertension.

Vascular fibrosis and stiffening

Vascular stiffening commonly occurs in hypertension and further elevates systolic pressure. Arterial stiffness is primarily associated with fibrosis, which can take the form of perivascular fibrosis or fibrosis scattered throughout the media and neointimal of pathologically changed vessels. This process involves accumulation of collagen within the vessel wall along with changes in other extracellular matrix proteins, such as fibronectin, elastin, or proteoglycans. Collagen is the extracellular fibrillar component that may alter the passive pressure/diameter relation of arteries at higher pressures and induce a progressive stiffening of the vascular wall. Collagen accumulation within the medial layer is characteristic for remodelling of smaller, resistance arteries, and perivascular fibrosis, reducing their capacitance, is characteristic for larger vessels. While fibrosis is stimulated by increases of blood pressure and increased transmural pressure, recent evidence points to inflammation and oxidative stress as key pro-fibrotic triggering factors.

Numerous pro-hypertensive stimuli, such as aldosterone, angiotensin II, excessive salt, or endothelin 1, have been shown to very significantly promote vascular collagen synthesis and fibrosis independently from increases in blood pressure. Substantial research has focused on the role of TGF-β in tissue fibrosis and collagen deposition. Mechanisms involved in TGF-β-mediated vascular fibrosis are complex and include the Smad signalling pathway and activation of mitogen-activated protein (MAP) kinases. TGF-β blockade diminishes fibrosis in experimental models of hypertension. Moreover, the key role of matrix metalloproteinases has been described in the process of vascular remodelling leading to vascular stiffness. Hypertension is associated with very high increase in MMP-2, MMP-9, and thrombospondin-1, and reduced MMP-1 activity. These not only directly act on matrix remodelling, but may contribute to post-translational activation of TGF-β, stimulating fibrosis.

Clinical studies suggest that inflammation and arterial stiffness are related. Patients with inflammatory diseases, such as lupus erythematosus, rheumatoid arthritis, and psoriasis, have increased pulse wave velocity and animals lacking T and B cells are protected from development of fibrosis. Recent evidence clearly points to the fact that IL-17 is one of the key pro-fibrotic cytokines, as it stimulates collagen production from VSMCs and fibroblasts, linking vascular inflammation to fibrosis and remodelling.

In small arteries, which are critical for regulation of vascular resistance, remodelling may take two forms as defined by Mulvany et al.: inward eutrophic remodelling, and hypertrophic remodelling. These two distinct types of remodelling differ in the changes in the passive luminal diameter, which are increased in outward remodelling and decreased in inward remodelling. Media mass is linked to VSMC growth and ECM accumulation and may be increased (hypertrophy), unchanged (eutrophic), or reduced. Eutrophic vascular remodelling, characterized by reduced outer diameter and lumen with no change in media mass and cross-sectional area, is observed particularly at earlier stages of hypertension. Typical hypertrophic remodelling, in which the media thickens to encroach on the lumen, resulting in an increased media/lumen ratio, accompanies advanced disease. In summary, vascular remodelling, either in the larger vessels or small arteries, results in decreased distensibility, reduced elasticity, and increased arterial stiffness in hypertension leading to increased central systolic blood pressure. Microvascular remodelling is characterized by both fibrosis and narrowing of the lumen, resulting in an increase in vascular resistance. Thus vascular remodelling is a key contributor to the regulation of sustained hypertension and vascular damage.

Systemic biomarkers of vascular pathology in hypertension

Plasma levels of various markers of vascular injury have been studied in hypertension. Plasma markers of endothelial dysfunction, such as von Willeberand factor and ADMA, correlate with blood pressure and vascular damage. Similarly, inflammatory mediators and adipokines, such as resistin, have been linked to severity of vascular involvement in hypertension, although their independent prognostic value is being disputed. Numerous studies have shown links between levels of high-sensitivity C-reactive protein, soluble intercellular adhesion molecule 1 (ICAMs), soluble E-selectin (SelE), and angiotensin II, and vascular damage in hypertensive patients. More recently, biomarkers based on metabolomics, microparticles, and microRNA have been tested but validation of their utility is still required. Recent evidence indicates that levels of microparticles, as biomarkers of vascular damage, are increased in hypertension. Circulating microparticles (MP) are shed membrane vesicles resulting from apoptosis or activation of several cell types in response to various stimuli. While they may serve as markers of endothelial dysfunction or damage, dysfunctional circulating MP isolated from patients with acute coronary syndromes directly induce endothelial dysfunction *in vitro* and their levels are correlated with endothelial function in high-risk individuals. Microparticles released from endothelial cells and platelets are significantly increased in patients with severe hypertension and are correlated with the level of systolic and diastolic blood pressures. Thus, they can be used as circulating markers for endothelial injury in arterial hypertension. Increased levels of circulating endothelial microparticles have been found in patients with pre-eclampsia, a disease characterized by vascular inflammation, altered endothelial function, and arterial hypertension. Further research to identify ideal biomarkers of hypertension-associated vascular injury is required, so that risk can be predicted before vessels undergo irreversible damage.

Conclusions

Vascular pathology is a uniform feature of hypertension and is observed in various vascular beds and in vessels of various locations. Major vascular changes in the pathophysiology of hypertension are related to endothelial dysfunction, increased vascular contractility, vascular remodelling, and

inflammation. These processes contribute to increased vascular resistance leading to blood pressure elevation. Multiple factors, including activation of the renin angiotensin system, ET-1, salt, oxidative stress, immune cell activation, and perivascular tissue-derived adipokines, play a role in the vasculopathy of hypertension. A better understanding of molecular mechanisms that trigger and sustain vascular damage in hypertension will provide insights on new targets and novel approaches to promote vascular health and prevent development of hypertension.

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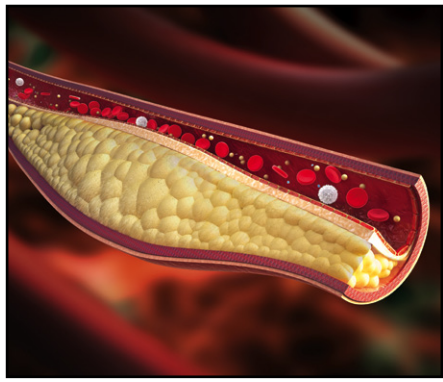

CHAPTER 20

Adventitia and perivascular adipose tissue—the integral unit in vascular disease

Zhihong Yang and Xiu-Fen Ming

Content

Introduction

Obesity is a major public concern and is associated with significant increase in cardiovascular morbidity and mortality because of unhealthy nutritional habits and sedentary lifestyle in our modern society (1, 2). According to World Health Organization (WHO)'s report published in January 2015, worldwide:

> obesity has more than doubled since 1980. In 2014, more than 1.9 billion adults, 18 years and older, were overweight. Of these over 600 million were obese. 39% of adults aged 18 years and over were overweight in 2014, and 13% were obese. Most of the world's population live in countries where overweight and obesity kills more people than underweight. 42 million children under the age of 5 were overweight or obese in 2013.
>
> <http://www.who.int/mediacentre/factsheets/fs311/en/>

Strictly speaking, obesity is not defined as an excess of body weight but as an increased adipose tissue accretion that adversely affects the healthy status of the human body. Obesity, particularly central obesity (genetically predisposed, or diet-induced, or associated with ageing), is often accompanied by a cluster of risk factors for cardiovascular diseases, which is called *metabolic syndrome*, and includes a group of the following risk factors in one person: (a) abdominal obesity, (b) atherogenic dyslipidaemia (high triglycerides, low HDL cholesterol, and high LDL cholesterol), (c) elevated blood pressure, (d) insulin resistance or glucose intolerance (the body can't properly use insulin or blood sugar), (e) pro-thrombotic state (e.g. high fibrinogen or plasminogen activator inhibitor-1 in the blood), and (f) proinflammatory state (e.g. elevated C-reactive protein in the blood). This cluster of risk factors explains the high incidence and prevalence of atherosclerotic coronary artery disease in this population (3). In 2009, a joint statement by the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity, suggested that three or more abnormal findings out of the above mentioned risk factors would qualify a person for a clinical diagnosis of metabolic syndrome with consideration of national or regional cut points for waist circumference (4). Highly associated with obesity is the prevalence of development of type 2 diabetes mellitus (T2DM), which represents a major contributor to cardiovascular diseases and death (5). About 90% of T2DM is attributable to

being overweight and obese, and the number of people with diabetes worldwide is projected to reach 366 million by 2030 (6). Because insulin resistance, i.e. decreased response or non-response of tissues or cells, in particular adipose tissue, skeletal muscle, and liver, to insulin, plays a central role in the development of obesity-associated metabolic syndrome, it was initially called insulin resistance syndrome (7).

In obesity-associated metabolic disorders, and also in coronary artery disease, fat mass, including ectopic fat mass and visceral fat, as well as perivascular fat mass, accumulates and is accompanied by cellular activation and inflammation in the perivascular tissues, including the adventitia and the adipose tissue, contributing to pathogenesis of vascular disease (8, 9). In line with this contention, atherosclerotic lesions develop primarily in coronary arteries encased by perivascular adipose tissue (pVAT), as shown in the atherosclerotic rabbit model (10). Also, studies with human post-mortem coronary arteries show that pVAT macrophage infiltration is highly correlated with atherosclerotic plaque size and macrophage infiltration in the adventitia and plaque (11), suggesting that pVAT plays an integral role in atherosclerotic lesion development. Indeed, in humans pVAT is anatomically co-localized with atherosclerotic lesions and correlates with plaque burden and calcifications (12–17).

Adventitia and pVAT as an integral unit of the vascular wall

The adventitia and pVAT are very dynamic in the regulation of vascular homeostasis and disease progression. Cells and signals originating from the adventitia and from pVAT are essentially involved in vascular remodelling, inflammation, and the vascular disease process (18–20). The adventitia is the interface between the vascular smooth muscle layer and pVAT, and contains fibroblasts, myofibroblasts, vaso vasorum, lymphatic vessels, nerves, and resident immune cells, including macrophages, dendritic cells, lymphocytes, and mast cells, as well as stem cells/progenitor cells (21–27). There is no separating fascia layer between pVAT and associated vascular wall (except for the cerebral arteries, which have no pVAT), so that pVAT directly abuts the adventitia of conduit arteries or constitutes an integral part of the vascular wall of small arteries or microvessels (28). pVAT works in concert with the cells within the adventitia to communicate with the smooth muscle and endothelial cells. Therefore, adventitia and pVAT shall be considered as an integral unit of vasculature. The traditional view on the control of vascular contractility and remodelling has focused on signals from the endothelial layer through production of endothelium-derived vasoactive factors (29). Recent research provides evidence suggesting that the vascular function is also regulated by signals originating from the cells in the adventitia and cells in the pVAT, including inflammatory cells and adipocytes (30). Adventitial progenitor cells are shown to be able to migrate into the developing neointima and significantly contribute to intimal cells in atherosclerotic lesions and transplanted arteriosclerosis in rodents models (25, 27, 31, 32), which is dependent on monocyte chemoattractant factor-1/chemokine (C–C motif) ligand 2. Interestingly, only few medial SMCs migrate into the intima in this transplantation vascular disease model (32). These findings indicate that the adventitia and peri-adventitial tissues/cells, including pVAT, function in concert to communicate with smooth muscle and endothelial cells to regulate vascular physiology, structural remodelling, and the development of vascular disease, e.g. associated with obesity and metabolic disorders.

Anatomical and functional features of pVAT

Three types of adipose tissue

Adipose tissue is composed of a complex set of cell types, including adipocytes, pre-adipocytes, immune cells, vascular cells (also called vascular stromal cells), and also collagen fibres (33). Adipose tissue is now classified into three types (see Fig. 20.1): (a) white adipose tissue (WAT), such as visceral and subcutaneous fat tissues, (b) brown adipose tissue (BAT) mainly found in the interscapular region in human infants and also inducible in adults under cold conditions, and (c) beige adipocytes (33). Morphologically, adipocytes in WAT contain a large, single fat droplet, whereas adipocytes in BAT contain multiple, smaller fat droplets and are rich in mitochondria and express high levels of UCP-1, a polypeptide residing in the inner mitochondrial membrane of these cells (33, 34). The main function of WAT is to store lipids and that of BAT is to generate heat, i.e. thermogenesis through UCP1, which dissipates the proton gradient generated by the mitochondrial electron transport chain and uncouples long-chain fatty acid oxidation from adenosine triphosphate (ATP) synthesis (34). Beige adipocytes are in between and show plasticity, i.e. with the ability to either store lipids or produce heat under different circumstances (35). The most well-known stimulus of BAT is cold, under which the BAT generates heat to maintain the body core temperature and clears lipids (33, 34). WAT can gain

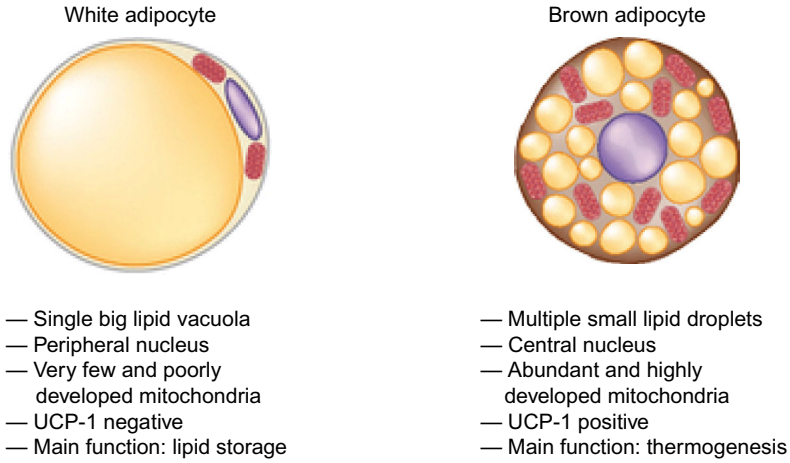

Fig. 20.1 Characteristics of three types of adipocytes. For details please see text.

the ability to upregulate thermogenesis function by cold exposure, physical activity, and under the stimulation of certain hormones, a process called 'browning' (33, 36). It is of note that the same type of adipose tissue at different locations may reveal variable functions, particularly in diseased conditions. For example, visceral adipose tissue and subcutaneous adipose tissue, although both are WAT type, the former produces a greater amount of proinflammatory cytokines and chemokines than the latter in type 2 diabetes (37), which may explain the closer relationship between central obesity and cardiovascular risk (38).

Heterogeneity of pVAT along the vascular bed

pVAT also contains a mixture of the various cell types (30). pVAT at different regions reveals different characteristics regarding morphology and functional responses (39). For example, in rodents, the peri-mesenteric, peri-carotic, and peri-femoral arterial adipose tissues are WAT, whereas the thoracic peri-aortic fat is BAT-like or a mixture of WAT and BAT (not known in humans) (30, 40–43). The abdominal peri-aortic adipose tissue has features of WAT in rodents and humans (42, 44). In response to diet-induced obesity, the expression and release of chemokines, as well as infiltration of macrophages, are more readily in abdominal peri-aortic adipose tissue than the thoracic periaortic adipose tissue (41, 42). These findings suggest that regional phenotypic differences in pVAT may be relevant to the vulnerability of different vascular beds to the development of vascular diseases, such as atherosclerosis and aneurysms. Interestingly, there is no adipose tissue surrounding the murine coronary artery, which does not develop atherosclerosis in this species. Moreover, human coronary arteries that are prone to atherosclerosis are surrounded by adipose tissue with characteristics of the 'beige' type (35, 45). Human epicardiac adipose tissue, a part of which belongs to peri-coronary adipose tissue, also reveals 'beige' features (46).

The origin of WAT, beige, and BAT adipocytes are from different lineage precursors, although they are all from mesenchymal/mesodermal stem cells (47) (➔ Fig. 20.2). Studies in rodents demonstrate that interscapular brown adipocytes are differentiated from *Myf5*$^+$ precursor cells, which are the same precursors for skeletal muscle cells (48). Subcutaneous and visceral white and beige adipocytes are derived from *Pdgfr*-α$^+$ progenitors (49) or from smooth muscle-like precursors (50). Cold exposure and β3-agonists have been shown to recruit beige adipocytes from differentiation of a *Pdgfr-α*$^+$ progenitor or from transdifferentiation of existing white adipocytes (51, 52). The transdifferentiation can also be induced by physical activity and by the skeletal muscle-derived hormone irisin, whereas over-nutrition switches beige back to white adipocytes (48, 52). There is evidence that aortic and mesenteric pVAT adipocytes are derived from *SM22α*$^+$ progenitor cells (53). Ectopic expression of PRD1-BF-1-RIZ1 homologous domain-containing protein-16 (PRDM16) transdifferentiates skeletal myocytes into brown adipocytes and also transdifferentiates VSMC into beige adipocytes (49, 50).

The physiopathology of pVAT in vascular disease

The adipose tissues, including pVAT, are organs with active secretory functions. Since the discovery of adipocyte-derived leptin in 1994 (54), adipose tissue is recognized as an important source of many mediators with profound biological functions. These mediators include factors released from adipocytes, the 'true adipokines', and factors released from non-adipocytes, e.g. inflammatory cells or stromal cells in

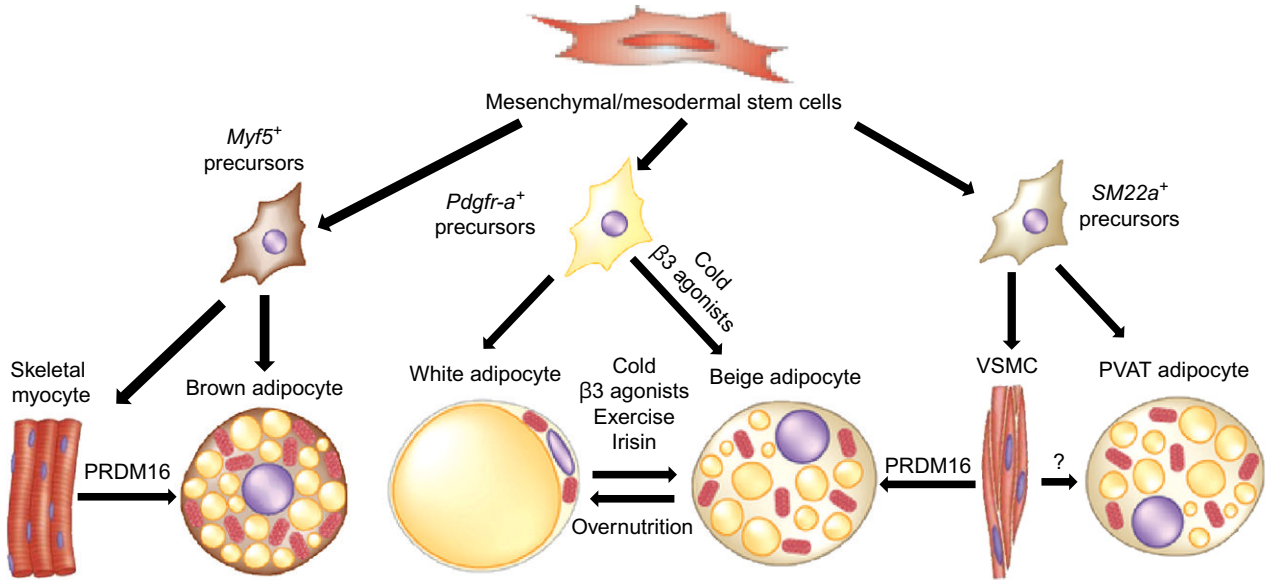

Fig. 20.2 Origin, differentiation, and transdifferentiation of adipocytes. For details please see text.
(Gesta S, Tseng YH, Kahn CR. Developmental origin of fat: tracking obesity to its source. Cell. 2007;131(2):242–56.)

the fat tissue—the 'pseudo-adipokines'. These factors are involved in the regulation of body weight, insulin sensitivity, inflammation, thrombosis, and vascular functions (55). pVAT releases mediators that include vascular relaxing and contracting factors, pro- and anti-proliferative factors, as well as pro- and anti-inflammatory cytokines. The pVAT-derived adipokines act as autocrine and/or paracrine hormones, and may be also released into the bloodstream where they function as endocrine hormones (56). Therefore, adipokines are considered as the link between obesity and the development of cardiovascular disease (57). The imbalanced production of the factors occurs in obesity and favours pathogenesis of cardiovascular diseases. For example, visceral adipose tissue exhibits a greater capacity to synthesize and release pro-atherogenic adipokines, as compared to the subcutaneous adipose tissue, which explains the increased risk of developing metabolic disorders and cardiovascular disease in patients with visceral adiposity (58, 59). This also appears true for pVAT, which participates in obesity-associated vascular disease through unfavourable production of adipokines and other mediators influencing the functions of vascular cells, i.e. adventitial cells, SMC, and endothelial cells (EC), leading to abnormal vascular contractility, structural remodelling, inflammation, and atherothrombosis.

pVAT and regulation of vascular tone in obesity

Adipose-derived relaxing factor(s) (ADRF)

Vascular tone is primarily determined by the contractile properties of medial SMC, which is regulated by neuronal, hormonal, and local mechanisms. Similar to the endothelium of arteries, evidence suggests that pVAT is able to modulate vascular tone. This aspect dates back to 1991 when Soltis and Cassis reported that pVAT influences vascular contraction (60). They demonstrated in isolated rat aortas that the contractile responses to norepinephrine were reduced in the artery with pVAT, as compared to that with pVAT removed. In 2002, Löhn and colleagues confirmed this observation and demonstrated that peri-aortic adipose tissue produces a relaxing factor(s), which is(are) named as adipose-derived relaxing factor(s) (ADRF) (61). ADRF inhibits vascular contractions evoked by many vasoconstrictor hormones, such as serotonin, angiotensin II, or phenylephrine (62, 63). The chemical features of ADRF remain obscure. However, it seems that it is not only a single factor, but has different natures depending on different vascular beds. So far, adiponectin, leptin, H_2S, NO, prostacyclin, PGE_2, angiotensin 1–7, hyperpolarizing factor(s), and H_2O_2, indoleamine 2,3-diooxygenase, etc., are proposed candidates of ADRF released from pVAT (64–70). Under pathophysiological conditions, e.g. in obese offspring of Wistar rats receiving nicotine during pregnancy and lactation, the functions of ADRF to inhibit vasoconstrictions induced by phenylephrine are decreased, which is associated with an increased amount of pVAT in the thoracic aorta and mesenteric arteries (71). In obesity induced by a high-fat diet, the function of pVAT to relax vascular smooth muscle is markedly reduced (72). Also, pVAT from obese subjects has markedly diminished vasodilatory capacity, as compared with lean controls (73). In an obesity mouse model, insulin-induced vasodilatory effects in intramuscular

resistance arteries are dependent on pVAT-derived adiponectin, production of which is decreased in obesity (66). The same phenomenon was observed in human samples (74). Conversely, reduction in body weight of obese subjects increases levels of adiponectin in pVAT and restores the anti-contractile effect of pVAT (75). Adiponectin is an adipocyte-derived 244 amino-acid peptide hormone and is the most well-characterized protective adipokine in type 2 diabetes and cardiovascular disease. Adiponectin is produced from pVAT and is a vasodilator that acts directly on its receptors, AdipoR1, on SMC. On the other hand, adiponectin also acts on endothelial cells to activate eNOS through PKB/Akt and to increase the bioavailability of the eNOS cofactor tetrahydrobiopterin (BH_4), causing vascular relaxation, as shown in human blood vessels (76).

Adipose-derived contracting factor(s) (ADCF)

As in the case of the endothelium, pVAT is also able to produce contracting factors tentatively called adipose-derived contracting factors (ADCF). All of the components of the renin–angiotensin system (RAS), except renin, are detected in pVAT in rats. This includes angiotensinogen, Ang-II, angiotensin-converting enzyme (ACE), renin receptor, and AT_1 and AT_2 receptors (77–81). The adipose tissue RAS may participate in obesity-associated development of hypertension (79), since local formation of angiotensinogen and Ang II is increased in rat adipocytes upon overfeeding (82, 83) or in obese hypertensive subjects (78, 84). Interestingly, electrical stimulation-induced contraction of vascular rings seems dependent on intact pVAT-derived angiotensin II (85). In addition, norepinephrine from adipose tissue sympathetic nerves is also found in pVAT (86). Moreover, pVAT produces a superoxide anion, which enhances arterial contraction, most likely by inactivating endothelial NO (87). Products derived from cyclo-oxygenase and chemerin are also suggested as ADCF in obesity (88, 89). Importantly, the production of ADCF or the pro-contractile effects of ADCF, are enhanced in obesity as reported in obesity animal models and humans (89, 90).

Vasocrine signalling mechanisms

Adipose tissue is an important source of proinflammatory cytokines in obesity. Production of cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β</βολδ), interleukin-6 (IL-6), and chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), etc., are increased in obesity (20, 91). The perivascular inflammation and oxidative stress in obesity and metabolic diseases function in concert to impair endothelial function or eNOS enzymatic activity, which is often related to eNOS-uncoupling, a situation under which eNOS enzyme produces superoxide anion instead of NO (92–94). This local paracrine function of pVAT in impairing endothelial function is called a 'vasocrine' signalling mechanism and is particularly important in small or resistant arterioles of the microcirculation system, where insulin exerts its vasodilatory effect, i.e. 'microvascular recruitment' effect of insulin (95). In the microcirculation, insulin-stimulated signalling pathways in endothelial cells are modulated by signals released from pVAT (➔ Fig. 20.3). One of the hallmarks of obesity is insulin resistance in peripheral tissues or organs, including the vasculature. In the endothelial cells of the vasculature, particularly the resistance arterioles, the physiological function of insulin is to phosphorylate eNOS at S1177 through activation of the insulin receptor/IRS/PI3K/Akt/eNOS cascade, resulting in enhanced eNOS activity and NO release, and, ultimately, vascular relaxation (96–98). On the other hand, insulin also stimulates production of the vasoconstrictor peptide endothelin-1 (ET-1) in endothelial cells through the mitogenic pathway Raf/MEK/ERK cascade (99) (➔ Fig. 20.3). In insulin-sensitive individuals, activation of the vasodilator pathway by insulin dominates, which causes postprandial increase in blood flow in the nutritive microcirculation, i.e. 'microvascular recruitment', favouring glucose

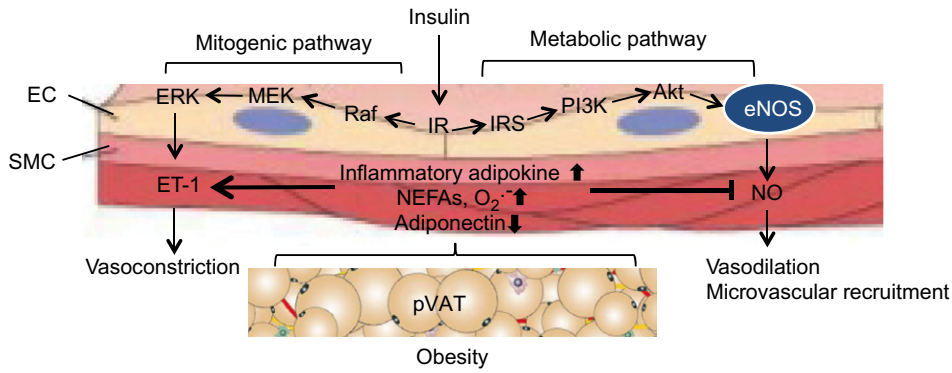

Fig. 20.3 Vasocrine signalling mechanisms of pVAT. For details please see text.

uptake in the insulin target organs (100). However, insulin-induced microvascular recruitment is blunted in obesity and insulin resistance (99). Importantly, vascular insulin resistance is selective for the PI3K/Akt/eNOS pathway (metabolic pathway), while the Raf/MEK/ERK pathway (mitogenic pathway) remains intact or even more active due to compensated hyperinsulinaemia caused by hyperglycaemia (99), which leads to reduced vasodilatation due to decreased endothelial NO production, or even vasoconstriction exerted by preserved or enhanced production of ET-1 (99) (➔ Fig. 20.3). In lean, heathy conditions, pVAT exerts paracrine effects (most likely through adiponectin production) and enhances insulin-induced vasodilation, which is abolished in obesity (66, 74). This can be explained by the fact that in obesity, the production of proinflammatory adipokines in pVAT, such as TNF-α, etc., is enhanced, which inhibits the insulin-mediated metabolic pathway but enhances the mitogenic pathway, leading to impaired NO production and enhanced ET-1 production (20, 72). Together with the sympathetic stimulating effects in the central nerve system and the water retention effect in the kidney by insulin, the vasocrine signalling mechanism of pVAT may participate in the development of vascular disease or enhanced vascular resistance and hypertension associated with obesity.

pVAT and regulation of vascular remodelling, inflammation, and atherosclerosis

Vascular remodelling

Besides the regulatory role in vascular tone, pVAT and/or adventitia are involved in regulation of vascular remodelling. The term 'vascular remodelling' is considered a process that mainly occurs in adventitia and media, where chronic inflammation plays a key role (101). It refers to a spatial reorganization of the vascular wall components resulting in geometric changes that can be either inward (constrictive), leading to lumen narrowing, which underlines the pathogenesis of major cardiovascular diseases such as atherosclerosis, restenosis after vascular intervention, hypertension, and also vascular aging (102–104), or outward (expansive), leading to vascular lumen enlargement and subsequently aneurysm formation (105, 106). Experimental evidence shows that adventitial myofibroblast proliferation/migration is primarily involved in constrictive vascular remodelling (31, 107–111). Moreover, SMC phenotype transformation, migration and proliferation, cell death, vascular inflammation, and changes in extracellular matrix composition, all act in concert to modify the pathological vascular remodelling process (101). Besides, vascular adventitia cells also send signals (not identified yet) to the endothelial cells, participating in vascular remodelling, since endothelium-specific inhibition of NFκB signalling in mice is able to attenuate intimal hyperplasia and aneurysm formation, when vascular injury was introduced from the adventitial side as shown in the femoral artery by placing a perivascular cuff surrounding the blood vessel (112). Conversely, endothelial cells also communicate to adventitia and pVAT to regulate vascular diseases. Indeed, endovascular injury upregulates proinflammatory adipokine production but inhibits the anti-inflammatory adiponectin production in pVAT in rodents (113, 114). Emerging evidence suggests that pVAT participates in vascular remodelling and disease process through release of growth factors and inflammatory cytokines, which stimulate SMC proliferation/migration and recruitment of adventitial myofibroblasts. Several growth factors released from adipose tissue cells are proposed, which include lysophosphatidic acid (115, 116), angiotensin II (117), TNF-α, leptin, fibroblast growth factor, insulin-like growth factor, and heparin-binding epidermal growth factor-like growth factor (118–122), whereas adiponectin is a SMC growth inhibitor (123). There is evidence that mature adipocytes, as compared to pre-adipocytes, release more growth factors for vascular SMC (124). The growth-promoting effect of peri-aortic adipose tissue for SMC is significantly enhanced with ageing and in diet-induced obesity (124).

Vascular inflammation—chronic low-grade inflammation

Chronic low-grade inflammation plays a critical role in obesity-associated insulin resistance (125, 126), facilitating atherogenesis under this condition (93, 127). Plasma concentrations of the proinflammatory mediators IL-6, TNF-α, plasminogen activator inhibitor-1, and C-reactive protein (CRP) are elevated in obese patients (120, 128–133). Moreover, intense clustering of these inflammatory cells is found in the interface between the adventitia and pVAT in atherosclerotic human biopsies (44, 134), and pVAT inflammation correlates with atherosclerotic size and vulnerability (11). In rodents, metabolic syndrome is associated with oxidative stress and inflammation in pVAT (135). An increased staining of $CD11c^+$ cells in epicardial fat tissue, a marker of proinflammatory M1 or 'classically activated' macrophages, is observed in patients with advanced coronary artery disease, whereas in patients without coronary artery disease, the anti-inflammatory macrophage (M2 or 'alternatively activated') is dominant (136). In parallel, adiponectin secretion from pVAT is suppressed in patients with obesity and/or CAD (76, 137). pVAT from patients with CAD was found to have higher mRNA and protein levels of inflammatory cytokines (IL-1β, IL-6, MCP-1, and TNF-α) than paired subcutaneous fat. Expression of these factors was associated with dense inflammatory infiltrates of macrophages, T cells,

and mast cells in epicardial adipose tissue (134, 138, 139), which may affect the pathological process of atherosclerosis. In atherosclerosis-prone apolipoprotein E–deficient mice, accumulation of inflammatory cells, such as T- and B-lymphocytes and macrophages in the adventitia, are much more pronounced than in the intima (21). In comparison with subcutaneous and visceral adipose tissue, pVAT exerts more inflammatory propensity, i.e. lower expression of adiponectin and higher interleukin-6, interleukin-8, and MCP-1 (28, 140). Conversely, the production of adiponectin, the anti-inflammatory adipokine, is much less in the pVAT as compared with the subcutaneous or peri-renal counterparts, even in healthy organ donors (28). The results suggest that pVAT is primed for inflammatory responses. This conclusion is further supported by the fact that in an obesity mouse model fed a high-fat diet, and in humans with atherosclerosis, the inflammatory gene TNF receptor superfamily member 11b (osteoprotegerin) secretion in perivascular adipocytes is elevated to a much higher level, as compared with the subcutaneous adipocytes (141). Also, in the restenosis animal models, chemokines (CK) and chemokine receptors (CKR), including MCP1 and its receptor CCR2 and other chemokines and receptors, start to be upregulated in pVAT at earlier time of post-injury and then progress toward the intima (142). Interestingly, maternal obesity induced by a high-fat diet accelerates atherosclerosis development in adult offspring specifically by augmenting inflammatory macrophage accumulation and subsequent increase in proinflammatory cytokine production in thoracic peri-aortic adipose tissue via initial early expression of macrophage colony-stimulating factor (M-CSF) (143). The inflammatory response is, however, not present in other adipose tissues such as epididymal fat in the offspring. Also, in the uninephrectomized ApoE-deficient ($ApoE^{-/-}$) mouse model and in the high-cholesterol diet mouse model, specific activation of the renin–angiotensin system (RAS) and macrophage infiltration occur in thoracic peri-aortic adipose tissue, which is shown to be partially responsible for the accelerated atherosclerotic development (144, 145). These peri-aortic-specific phenotypic alterations were absent when mice were treated with AT-1 receptor (AT1R) blocker and in AT1R-deficient $ApoE^{-/-}$ mice. Furthermore, transplantation of thoracic peri-aortic adipose tissue from AT1R blocker-treated $ApoE^{-/-}$ mice or from AT1R-deficient $ApoE^{-/-}$ mice markedly reduces atherosclerosis development, demonstrating the important role of pVAT–RAS system in contribution to atherogenesis. All the studies highlight the paradigm that vascular injury could come from the outside layer of vasculature including adventitia and pVAT.

Anti-atherosclerotic functions

On the other hand, pVAT also exerts anti-atherosclerotic components. Adiponectin is produced in pVAT (65), which is the best-known anti-atherosclerotic adipokine (140, 146, 147). Direct evidence showing that pVAT exerts anti-atherosclerotic properties is derived from the experiments demonstrating that removal of pVAT from the femoral artery enhances neointima formation after endovascular injury in a diet-induced obesity mouse model (148). This effect is attributable to adiponectin, which is released from adipose tissues, including pVAT (149, 150). Adiponectin exerts cardiovascular protective effects via multiple mechanisms, including anti-oxidative, anti-inflammatory, and anti-smooth muscle cell proliferative effects, and stimulation of eNOS activity through Akt and AMPK pathways (149, 150). Indeed, adiponectin deficiency accelerates atherosclerosis in $ApoE^{-/-}$ mice and, conversely, overexpression of adiponectin gene reduces atherosclerosis in the obesity mouse model (123, 151). In obesity, decreased adiponectin has been shown to play a role in vascular oxidative stress in patients with type 2 diabetes (150, 152). The anti-atherosclerotic components of pAVT are strengthened by the study with generation of the 'pVAT-less' mouse model on the $ApoE^{-/-}$ background (53). The control $ApoE^{-/-}$ mice have reduced atherosclerosis when housed under a mild cold temperature condition (16°C), as compared to the mice housed at thermoneutral conditions, which is associated with reduced plasma triglyceride levels, implicating that pAVT may result in lipid clearance (53). Interestingly, this effect is lost in the 'pVAT-less' mice on the $ApoE^{-/-}$ background.

Thus, the current concept is that pVAT exerts pro- and anti-inflammatory effects on vasculature to regulate

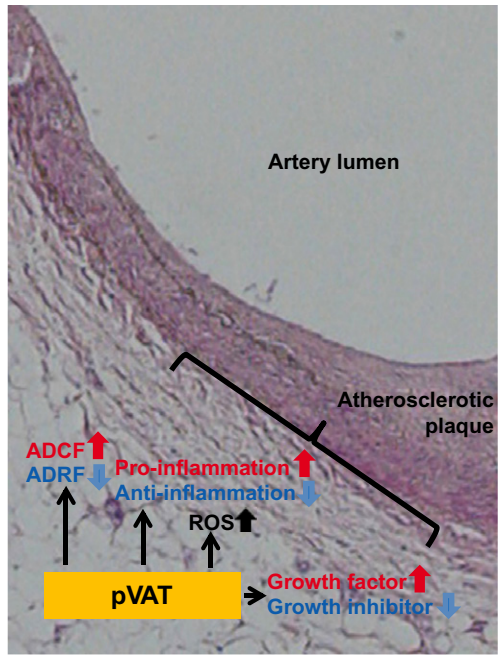

Fig. 20.4 pVAT dysfunction and development of atherosclerotic vascular disease. For details please see text.

vascular disease in obesity. A shift of this balance to a more proinflammatory state in pVAT in obesity may play a fundamental role in pathogenesis of cardiovascular disease under this condition.

Conclusions

Increasing evidence demonstrates that pVAT exerts profound effects on vascular structure and function under physiological conditions. Functional changes of the pVAT, in particular the imbalance between adipose-tissue-derived relaxing factors and contracting factors, and/or between growth-promoting and growth-inhibiting factors, and/or between proinflammatory and anti-inflammatory factors, occur under obesity and disease conditions (➔ Fig. 20.4). This imbalanced release of adipokines also impairs endothelial function. In concert with endothelial dysfunction, pVAT dysfunction favours vascular disease development in obesity and contributes to enhanced incidence of obesity-associated cardiovascular events.

Recommended reading

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