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Hervé Petite, Ph.D.
Laboratory of Orthopedic Research UMR CNRS 7052
Faculty of Laboratory Medicine Saint-Louis
Université Denis Diderot
Paris, France

Rodolfo Quarto, M.D.
Laboratorio Differenziamento Cellulare
Istituto Nazionale per la Ricerca sul Cancro
Centro Biotecnologie Avanzate
Genova, Italia

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Dedication

To my parents, Marcelle and Jean

CONTENTS

Part I
Building Blocks of Osteogenesis

Part II
Bone Healing

Part III
Tissue Engineering of Bone

Part IV
From the Bench to the Bedside

EDITORS

Hervé Petite
Laboratory of Orthopedic Research UMR CNRS 7052
Faculty of Laboratory Medicine Saint-Louis
Université Denis Diderot
Paris, France
E-mail: hpetite@infobiogen.fr
Chapter 11

Rodolfo Quarto
Laboratorio Differenziamento Cellulare
Istituto Nazionale per la Ricerca sul Cancro
Centro Biotecnologie Avanzate
Genova, Italia
E-mail: quarto@cba.unige.it
Chapter 11

CONTRIBUTORS

Elisabeth H. Burger
Department of Oral Cell Biology
Academic Center of Dentistry ACTA
Vrije Universiteit
Amsterdam, The Netherlands
E-mail: eh.burger.ocb.acta@med.vu.nl
Chapter 4

Ranieri Cancedda
SC Medicina Rigenerativa
Istituto Nazionale per la Ricerca sul Cancro
Centro Biotecnologie Avanzate
and
Dipartimento di Oncologia, Biologia e Genetica
Universita' di Genova
Genova, Italia
E-mail: cancedda@cba.unige.it
Chapter 15

Yang Yu Chien
Tsukazaki Hospital
Himeji, Japan
E-mail: yangyang1968@hotmail.com
Chapter 14

Guy Daculsi
Faculté de Chirurgie Dentaire
Centre de recherche sur les matériaux d'intérêt biologique
INSERM
Nantes, Cedex, France
E-mail: g.daculsi@sante.univ-nantes.fr
Chapter 6

Beatrice Dozin
Istituto Nazionale per la Ricerca sul Cancro
Centro Biotecnologie Avanzate
Genova, Italia
E-mail: dozin@cba.unige
Chapter 12

Paolo Giannoni
SC Medicina Rigenerativa
Istituto Nazionale per la Ricerca sul Cancro
Centro Biotecnologie Avanzate
Universita' di Genova
Genova, Italia
E-mail: giannoni@cba.unige.it
Chapter 15

Allen Goodship
The Institute of Orthopaedics
and Musculoskeletal Sciences
University College London
and
Royal Veterinary College
University of London
London, United Kingdom
E-mail: goodship@rvc.ac.uk
Chapter 7

Geneviève Guillemin
Laboratoire de Recherches
Orthopédiques
CNRS-UMR 7052
Université Denis Diderot
Paris, France
E-mail: nguillem@ext.jussieu.fr
Chapter 8

Didier Hannouche
Department of Orthopaedic Surgery
Hôpital Lariboisière
Paris, France
E-mail: didierh_2000@yahoo.fr
Chapter 14

Jeffrey O. Hollinger
Bone Tissue Engineering Center
Carnegie Mellon University
Pittsburgh, Pennsylvania, U.S.A.
E-mail: hollinger@cs.cmu.edu
Chapter 10

Julie Jadlowiec
Department of Biological Sciences
Bone Tissue Engineering Center
Carnegie Mellon University
Pittsburgh, Pennsylvania, U.S.A.
E-mail: juliej@andrew.cmu.edu
Chapter 10

Gerard Karsenty
Department of Molecular and Human
Genetics
Baylor College of Medicine
Houston, Texas, U.S.A.
E-mail: karsenty@bcm.tmc.edu
Chapter 1

Jenneke Klein-Nulend
Department of Oral Cell Biology
Academic Center of Dentistry ACTA
Vrije Universiteit
Amsterdam, The Netherlands
E-mail:
j.klein_nulend.ocb.acta@med.vu.nl
Chapter 4

Hannjörg Koch
Department of Orthopaedic Surgery
University of Pittsburgh
and
Bone Tissue Engineering Center
Carnegie Mellon University
Pittsburgh, Pennsylvania, U.S.A.
and
Department of Orthopaedic Surgery
University of Greifswald
Greifswald, Germany
E-mail: hkoch@uni-greifswald.de
Chapter 10

Sergei A. Kuznetsov
National Institute of Dental
and Craniofacial Research
National Institute of Health
Bethesda, Maryland, U.S.A.
E-mail: kuznetsov@yoda.nidcr.nih.gov
Chapter 2

Olivier Laboux
Faculté de Chirurgie Dentaire
Centre de recherche sur les matériaux
d'intérêt biologique
INSERM
Nantes, Cedex, France
E-mail:
olivier.laboux@sante.univ-nantes.fr
Chapter 6

Joseph M. Lane
Departments of Metabolic Bone Diseases
and Orthopaedic Surgery
Hospital for Special Surgery
New York, New York, U.S.A.
E-mail: LaneJ@hss.edu
Chapter 5

Delphine Logeart-Avramoglou
Laboratoire de Recherches
Orthopédiques
CNRS-UMR 7052
Faculté de Médecine Lariboisière
Saint-Louis
Université Denis Diderot
Paris, France
E-mail: logeart@ext.jussieu.fr
Chapter 9

Ivan Martin
Department of Surgery,
Research Division
University Hospital Basel
Basel, Switzerland
E-mail: IMartin@uhbs.ch
Chapter 12

Margriet Mullender
Deptartment of Oral Cell Biology
Academic Center of Dentistry ACTA
Vrije Universiteit
Amsterdam, The Netherlands
E-mail: m.mullender@fbw.vu.nl
Chapter 4

Ashley R. Poynton
Departments of Metabolic Bone Diseases
and Orthopaedic Surgery
Hospital for Special Surgery
New York, New York, U.S.A.
E-mail: ashpoynton@aol.com
Chapter 5

A. Hari Reddi
Center for Tissue Regeneration
and Repair
Department of Orthopaedic Surgery
School of Medicine
University of California, Davis
Sacramento, California, U.S.A.
E-mail: ahreddi@ucdavis.edu
Chapter 3

Paul Robbins
Department of Molecular Genetics
and Biochemistry
University of Pittsburgh
Pittsburgh, Pennsylvania, U.S.A.
E-mail: probb@pitt.edu
Chapter 10

Pamela Gehron Robey
National Institute of Dental
and Craniofacial Research
National Institute of Health
Bethesda, Maryland, U.S.A.
E-mail: pamela.robey@nih.gov
Chapter 2

Dirk Schaefer
Department of Surgery,
Research Division
University Hospital Basel
Basel, Switzerland
E-mail: dschaefer@uhbs.ch
Chapter 12

Charles Sfeir
Department of Oral Medicine
and Pathology
McGowan Institute of Regenerative
Medicine
University of Pittsburgh
Pittsburgh, Pennsylvania, U.S.A.
E-mail: csfeir@pitt.edu
Chapter 10

Minoru Ueda
Department of Oral and Maxillofacial Surgery
Graduate School of Medicine
Nagoya University
Nagoya, Japan
E-mail: mueda@tsuru.med.nagoya-u.ac.jp
Chapter 13

Véronique Viateau
Laboratoire de Receherches Orthopédiques
CNRS-UMR 7052
Université Denis Diderot
Paris, France
and
Ecole Nationale Vétérinaire d'Alfort
Maisons Alfort, France
E-mail: veronique.viateau@wanadoo.fr
Chapter 8

Pierre Weiss
UFR Odontologie
Centre de recherche sur les matériaux d'intérêt biologique
INSERM
Nantes, Cedex, France
E-mail: pweiss@sante.univ-nantes.fr
Chapter 6

Xiangli Yang
Department of Molecular and Human Genetics
Baylor College of Medicine
Houston, Texas, U.S.A.
Chapter 1

PREFACE

The purpose of this book is to provide students, young investigators as well as established researchers and clinicians with comprehensive reviews on fundamental and applied aspects of regeneration of bone and to offer insights into future developments. The book is divided in four different parts which are entitled "Building Blocks of Osteogenesis", "Bone Healing", "Tissue Engineering of Bone" and "From the Bench to the Bedside".

The first section gives an overview of the building blocks of osteogenesis. The opening chapter by Yang et al describes transcription factors that are essential in bone development. It followed by chapters by Kutzenov et al and Reddi which give respectively comprehensive overviews on skeletal stem cells and bone morphogenetic proteins. Finally, Burger et al stress the importance of mechanical forces such as fluid shear stress and cell stretching on bone formation in the last chapter of this section.

The second section deals with bone healing. Fracture repair and the factors leading to its failure are the central point of the chapter by Poynton et al whereas the interest of calcium phosphate bioceramics and their potential role in bone repair are the focus of the chapter by Daculsi. Mechanical forces are key factors in bone healing. Their roles and potential implications in skeletal tissue engineering are described in the chapter by Goodship. Development of new strategies for bone repair requires appropriate animal model systems to assess their efficacy. This issue is critically analyses by Viateau et al.

The third section relates the recent advances that have been made in the fast advancing field of tissue engineering of bone. First, growth factors needs to be delivered in a timely fashion in the lesion to achieve maximum efficacy. Logeart-Avramoglou gives us the fundamentals behind growth factor delivery with a special emphasis on BMPs. Alternatively, growth factors can be delivered at the gene level. Sfeir et al provide a current review on the gene delivery technology currently available and their applications to bone. Tissue engineered bone repair have also been attempted by the transplantion of skeletal stem cells. An overview of this therapeutical approach is given by Petite and Quarto. Repair of osteochondral lesions represents a major challenge for the orthopaedic surgeon. Attempts using tissue engineering strategies have been made to address this issue and are critically presented by Martin et al. Similarly, Ueda introduces us to strategies for maxillo-facial bone regeneration using tissue engineering concept.

The fourth and last section of this book is entitled "from the bench to the patient". Giannoni and Cancedda address relevant issues that tissue-engineering researchers must consider when planning new strategies, especially in the bone and cartilage field. Finally, Hannouche will give us the view of the surgeon and will provide an update on the clinical application of these new modalities.

Hervé Petite and Rodolfo Quarto

Part I
Building Blocks of Osteogenesis

CHAPTER 1

Transcription Factors in Bone:
Developmental and Pathological Aspects

Xiangli Yang and Gerard Karsenty

Many transcription factors involved in bone development have been identified through studies of human patients with genetic diseases and of genetically modified mice. Some of them are specific for a particular cell lineage and act as developmental regulators of cell differentiation. Others are not bone cell-specific but are predominantly expressed in bone tissues during development and more importantly have a function restricted to controlling bone cell proliferation or differentiation. The molecular elucidation of mechanisms governing bone cell differentiation is of particular importance considering the incidence and severity of the diseases that affect skeletal development and function. In this review, we focus on the transcription factors that are essential for regulation of cell differentiation during skeletal development. In the later part of this review, we will provide examples of transcription factors affecting patterning of the skeleton and whose functions were uncovered by human genetic studies.

Bone Development

Bone and cartilage are the two tissues forming skeleton in vertebrates. These two tissues contain three specific cell types scattered within an extracellular matrix. Osteoblasts or bone-forming cells and osteoclasts or bone-resorbing cells reside in bone and chondrocytes in cartilage. The osteoblasts are derived from mesenchymal stem cells and are located on the bony surfaces. They are responsible for synthesizing matrix proteins that subsequently become mineralized, a process called bone formation. Osteoclasts are cells of hematopoietic origin that are responsible for resorbing extracellular matrix (ECM), a process called bone resorption. Chondrocytes in cartilage shares the same origin with osteoblasts; they also play important roles in endochondral ossification (see below).

The earliest forms of skeleton are the primitive mesenchymal condensations that formed when undifferentiated mesenchymal cells migrate into areas destined to become bone. In mouse, mesenchymal condensation begins to form at 9.5 days post coitum (dpc). These mesenchymal condensations can eventually become bone through two distinct processes. During intramembranous ossification, cells of mesenchymal condensations differentiate directly into osteoblasts without any intermediate step. Bones formed through this process include frontal, parietal, and parts of the temporal and occipital bones, the majority of the facial bones, and the clavicles.[1] All other bones are formed through endochondral ossification, a two-step process. At 11.5 dpc of mouse embryonic development, cells in the mesenchymal condensation differentiate first into chondrocytes to create a cartilaginous anlage of the future bone. This process, also called chontrogenesis, will give rise to essentially an entire skeleton consisting of cartilaginous elements. In the center of each cartilaginous anlage, cells stop dividing and become hypertrophic chondrocytes, a subpopulation of chondrocytes surrounded by a calcified ECM. Vascular invasion from this ECM will bring in osteoblast progenitors that will form ossification centers. The

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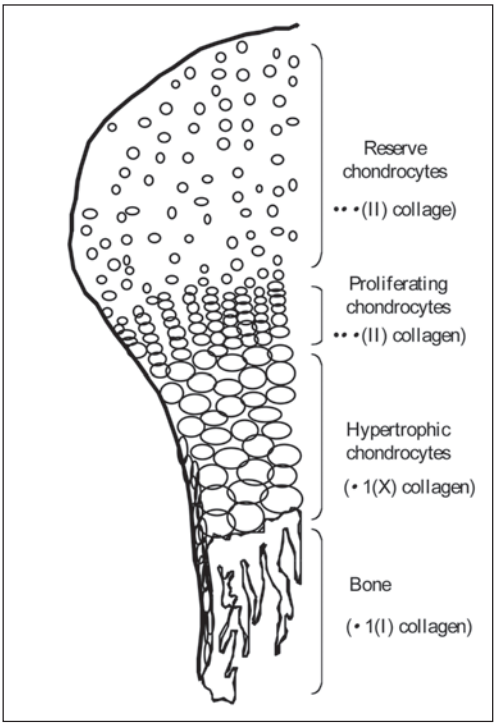

Figure 1. Diagram of growth plate.

osteoblast progenitors will differentiate into mature osteoblasts that secrete a specific bone ECM that will replace the cartilaginous ECM. At both ends of a bone, chondrocytes become restricted to a narrow zone forming the growth plate, which is responsible for longitudinal bone growth.[2] A typical growth plate contains resting, proliferating, prehypertrophic, and hypertrophic chondrocytes (Fig. 1).

Beyond embryonic development and throughout adult life, bone undergoes continual resorption and formation through a dynamic process called bone remodeling. Bone remodeling starts when osteoclasts resorb mineralized ECM that is then replaced by a new bone ECM secreted by osteoblasts.

Transcription Control of Chondrocyte Differentiation

Beyond their morphological differences, various chondrocyte subpopulations express specific molecular markers.[3] These various molecular markers help also to define each specific subpopulation. In the reserve zone, cells are small and resting. In the adjacent proliferative zone, however, cells are rapidly dividing to increase their numbers which contributes to the ongoing growth of bone. Both resting and proliferating chondrocytes express α1(II) collagen. Cells in the prehypertrophic zone express α1(II) collagen albeit at a lower level, Indian hedgehog, and PPR, a gene encoding the receptor for both parathyroid hormone (PTH) and PTH-related peptide (PTHrP). Finally, chondrocytes of the hypertrophic zone express α1(X) collagen but not anymore α1(II) collagen.

The earliest molecule required for specifying a cell fate during skeletogenesis identified to date is Sox9, a high-mobility-group (HMG) domain containing transcription factor. It was shown that Sox9 is the gene inactivated in campomelic dysplasia patients, a disease characterized by skeletal malformation and XY sex reversal.[4] In mouse embryo, Sox9 is expressed

predominantly in mesenchymal condensations[5] and prehypertrophic condrocytes[6] where α1(II) collagen is also expressed. The coexpression of Sox9 and α1(II) collagen, an early and abundant marker of chondrocyte differentiation, implicated that Sox9 may play a role in specifying chondrocytic lineage. Subsequently, Sox9 was found to bind directly to chondrocytic specific cis-acting elements in the α1(II) and α2(XI) collagens and to control the expression of these genes in cells of the chondrocytic lineage,[7,8] thus providing a direct evidence that Sox9 may be a regulator of chondrogenesis. The most convincing evidence that Sox9 is required for the formation of mesenchymal condensation came from mouse genetic studies. Indeed, in chimaeric mouse embryos, Sox9 -/- embryonic stem (ES) cells but not wild type (wt) ES cells are excluded from the mesenchymal condensations indicating that Sox9 is required for their formation. Moreoever, teratomas derived from Sox9 -/- ES cells, but not from wt ES, failed to develop cartilage in mouse chimaeras.[8,9] Interestingly, although Sox9 heterozygous deficient mice are phenotypically similar to human patients, the severity of the phenotype is more pronounced in mice than in humans. Sox9 +/- mice die perinatally with cleft palate, hypoplasia and bending of many long bones. Other abnormalities, such as expanded hypertrophic chondrocyte zone and premature mineralization of many bones also exist in these mutant animals.[10] These data suggest that Sox9, in addition to controlling the mesenchymal condensation of cartilage primordia, may also prevent chondrocytic hypertrophy. The latter action of Sox9 may be mediated by a PTHrP signaling pathway.[11]

Two other HMG-containing proteins, L-Sox5 and Sox6, are found to be coexpressed with Sox9 in all precartilaginous condensations and continue to be expressed in hypertrophic chondrocytes of mouse embryos.[12] In vitro, they bind the chondrocyte-specific enhancer of a1(II) collagen and induce its expression in non-chondrocytic cells.[12,13] Together with Sox9, they induce endogenous expression of chondrocyte differentiation marker genes, such as α1(II) collagen, and aggrecan in vivo. L-Sox5 and Sox6 that have no activation domain, have a high degree of sequence identity to each other but have no similarity to Sox9 except for the HMG-box. Consistent with their identical pattern of expression, Sox5 and Sox6 have redundant function in vivo, since single null mutant mice are virtually normal. However, targeted deletion of both Sox5 and Sox6 leads to embryonic lethality due to a generalized chondrodysplasia,[14] demonstrating their essential role in control of chondrocyte differentiation. Sox5 and Sox6 are possibly genetically downstream of Sox9 since the chondrogenesis is not completely blocked in the double mutant animals.[14] The intramembranous bone collars develop normally in double mutant animals[14] ruling out the involvement of these Sox proteins in the control of intramembranous ossification.

A group of nuclear factor of activated T cells (NFATs) are transcription factors that regulate the expression of many cytokines in lymphocytes[15] and also cardiac valve formation.[16,17] One of these proteins, NFAT1, whose activity is essential for cardiac valve morphogenesis also modulates chondrogenesis in adult animals. Indeed NFAT1 acts as a repressor of chondrocyte differentiation since its overexpression in chondrocytic cell lines suppresses the expression of molecular markers of chondrocyte and NFAT1-deficient mice develop fixed joints due to spontaneous ectopic formation of cartilage after 6 months. The newly formed cartilage contains ordered and columnar chondrocytes with distinct morphologies and is eventually replaced by bone, recapitulating the process of endochondral ossification.[18] Curiously NFAT1 mutation affects female more severe than males. These NFAT1-deficient mice provide a good animal model for studies of age-related skeletal diseases.

Chondrocytic hypertrophy is a mandatory step for endochondral bone development to occur and core binding factor α 1 (Cbfa1), or Runx2, is the only transcription factor that has been demonstrated so far to induce chondrocyte hypertrophy in vivo.[19,20] In cartilage Cbfa1 expression is restricted to prehypertrophic chondrocytes.[20,21] Cbfa1-deficient mice have a delayed chondrocyte hypertrophy in many long bones.[22,23] Continuous expression of Cbfa1 in hypertrophic chondrocytes induced α1(X) collagen expression and chondrocytic hypertrophy in transgenic mice. Moreoever, expression of Cbfa1 in non-hypertrophic chondrocytes in Cbfa1-deficient genetic background corrected the lack of hypertrophic chondrocytes of these

mutant mice.[19,20] This observation suggests Cbfa1 is necessary for chondrocyte hypertrophy and that this function is independent of its osteoblast differentiation ability (see below).

Transcriptional Control of Osteoblast Differentiation

In addition to being a hypertrophic chondrocyte differentiation factor, Cbfa1/Runx2, a mouse homolog of the drosophila Runt protein,[24] is the only transcription factor reported to date to act as an inducer of osteoblast differentiation. Runt proteins are a group of transcription factors that are conserved from *C. elegans* to human.[25] They all share a typical DNA binding domain called Runt domain of 128 amino acids long.[26] Cbfa1 was initially identified as one of the two key regulators for the osteoblast-specific expression of osteocalcin gene.[27,28] Cbfa1 has all characteristics of a differentiation regulator in the osteoblast lineage. First, its expression correlates with osteogenesis during development. The onset of its expression is as early as 9.5 dpc in notochord[22] and starting at 10.5 dpc Cbfa1 is expressed in all mesenchymal condensations.[28] Later during development Cbfa1 is expressed at high levels in osteoblasts an at lower levels in prehypertrophic chondrocytes but not in any other cell types.[20] Second, Cbfa1 is necessary for osteoblast differentiation. Targeted inactivation of Cbfa1 in mice generated mice that have no osteoblasts.[29] Inactivation of only one Cbfa1 allele causes cleidocranial dysplasia syndrome, a disease characterized by a delay in osteoblast differentiation in bones forming through intramembraneous ossification in mice and human.[30] Third, Cbfa1 is also sufficient to induce osteoblast differentiation in vivo and in vitro. In vivo, ectopic expression of Cbfa1 in transgenic animals leads to ectopic endochondral ossification.[20] In vitro, forced expression of Cbfa1 in non-osteoblast cells induces osteoblast-specific expression of all bone marker genes, including osteocalcin and bone sialoproteins.[28]

Besides Cbfa1, two broadly expressed AP1 family members, ΔFosB and Fra-1, have been shown to act as positive regulators for osteoblast differentiation by gain-of-function studies.[31,32] ΔFosB is an alternative spliced product of FosB, an oncogene and a transcription repressor of c-Fos.[33] ΔFosB lacks the last 101 amino acids of FosB and functionally it differs from FosB because it lacks oncogenic ability and fails to suppress c-Fos transcription. FosB is expressed at the highest levels in bones and cartilages.[33] Mice lacking FosB develop normally.[34,35] However in agreement with its distribution, generalized overexpression of ΔFosB in transgenic mice leads to a postnatal osteosclerosis secondary to an increase in osteoblast differentiation.[32] Mirroring that of ΔFosB, forced ubiquitously expression of Fra-1 caused a similar bone phenotype.[31] Targeted deletion of Fra-1 in mice led to embryonic lethality around 10 dpc and mice rescued to birth through blastocyst injection contained mature osteoblasts,[36] indicating that Fra-1 is not essential for osteoblast differentiation.

Several other transcription factors are being characterized in several laboratories. They may control osteoblast differentiation in Cbfa1-dependent or independent pathways. A zinc-finger protein AJ18 has been recently identified to modulate Cbfa1's activity by competing with the same consensus sequence.[37] Another example is Osf1, the protein that binds and activates one of the two osteoblast-specific cis-elements (OSE1) in osteocalcin gene.[27] Unlike Cbfa1, Osf1 is only present in non-mineralized osteoblasts.[38] These factors are not the only ones are being studied. The description of the function of each of these factors in vivo will greatly increase our understanding of osteoblast differentiation.

Transcription Control of Osteoclast Differentiation

Unlike the two other cell types of the skeleton, the osteoclasts are derived from myeloid origin which gives rise also to macrophages and monocytes. Hence, it is not surprising that many growth factors such as macrophage colony-stimulating factor and receptor activator of NFκB ligand, and transcription factors such as Pu.1 and NFκB that are involved in haematopoietic cell differentiation also affect osteoclast differentiation. Pu.1 is an ETS (a winged helix-turn-helix wing motif) domain-containing protein whose absence causse multilineage defects in the generation of B lymphocytes, monocytes, and granulocytes.[39] Accordingly, its expression pattern is lineage- and stage-restricted in the myeloid, B-lymphoid origin and early

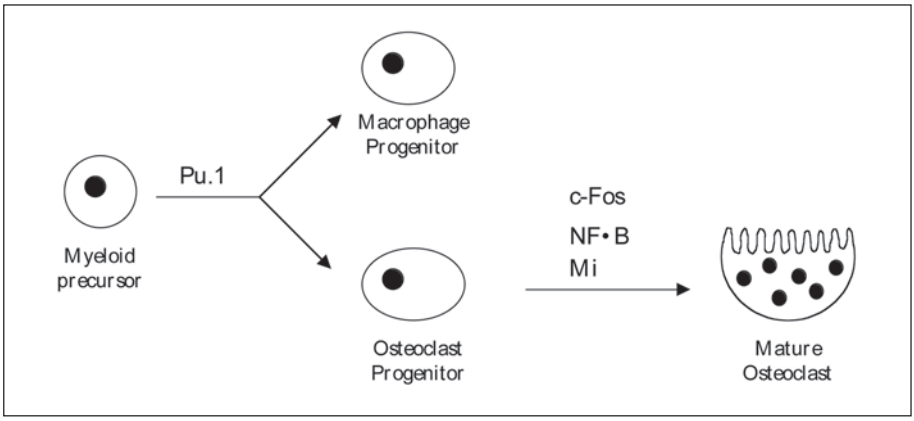

Figure 2. Transcription factors in osteoclast differentiation.

granulocytic cells.[40] Pu.1-deficient mice lack both osteoclasts and macrophages,[41] demonstrating that Pu.1 controls the differentiation of these two types of cells. The action of Pu.1 is cell-autonomous since the defect was completely rescued by bone marrow transplantation[41] or forced expression of Pu.1 in Pu.1-deficient monocyte progenitors.[42] Pu.1 -/- progenitors could commit to the monocytic lineage but these cells could not mature to macrophages,[43] suggesting that Pu.1 is only required at later stages of macrophage differentiation. Pu.1 is genetically upstream of M-CSF in control of osteoclast differentiation (Fig. 2) as Pu.1 directs cell-specific expression of M-CSF.[44]

Early observation that FBJ murine osteosarcoma virus induces osteosarcomas in mice[45] had drawn attention to v-Fos, the gene responsible for this malignant transformation. In situ hybridization shows that c-Fos, the cellular counterpart of v-Fos, is expressed in cartilage, bone, and tooth during mouse development[46] while overexpression of c-Fos in mice causes osteosarcoma.[47] These results made c-Fos a good candidate to control osteoblast differentiation in vivo. A major surprise came from later loss-of-function studies. Indeed, homozygous c-Fos -/- mice develop osteopetrosis, a disease characterized by increased bone mass, due to the absence of osteoclast,[48] demonstrating that c-Fos is required for the differentiation of monococyte precursors into osteoclasts. The action of c-Fos is also cell-autonomous because the arrest of osteoclast differentiation can be released by bone marrow transplantation or ectopic expression of c-Fos.[49] The fact that c-Fos-deficient mice also hav ane increased number of macrophages implicates a reciprocal regulatory mechanism in which c-Fos may act as a repressor for macrophage maturation. The absence of macrophages in c-Fos-deficient mice argues that c-Fos lies downstream of Pu.1 in controlling osteoclast differentiation.

c-Fos shares high homology with Fra-1 at the DNA-binding (basic) and leucine zipper domains but Fra-1 lacks a transcription activation domain.[50] Recently, mice carrying a Fra-1 allele inserted in the c-Fos locus were generated, these mice did not show any bone developmental abnormality[51] suggesting the c-Fos-dependent osteoclast differentiation does not require the transactivation ability of c-Fos. This result is consistent with the observation that Fra-1 overexpresion in a c-Fos-deficient mice rescues osteopetrosis phenotype.[52] However, Fra-1 is not required for osteoclast differentiation since Fra-1-deficient mice that have been rescued by blastocyst injection contained functional osteoclasts.[36]

Two forms of NF-κB, P50 and P52 are closely related transcription factors regulating immune and inflammatory responses. The transcription activity is tightly regulated post-translationally.[53] Defects in animals caused by targeted deletion of either one of them are restricted in immune systems. Knockout of the gene encoding for P50 leads to an impaired ionizing radiation-induced NF-κB activation,[54] whereas targeted mutation of P52 have

reduced peripheral B cells and defects in humoral responses in mice.[55] However, when both P50 and P52 were deleted simultaneously, the mutant mice developed osteopetrosis as a result of an arrest in osteoclast differentiation. Similar to Pu.1-deficient mice, the osteopetrotic phenotype can be rescued by bone marrow transplantation.[56] Lack of NFκB impairs the expression of cytokines including IL-1, TNFα, and IL-6 that are also required for osteoclast maturation.

Two mutant alleles at the microphthalmia (mi) locus have been isolated from mice showing sclerosing bone dysplasias in skeleton.[57] Mi locus encodes a basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor called microphthalmia transcription factor (MITF). Heterodimerization of MITF with its closely related family members, TFEB, TFE3, and TFEc enable them to bind and activate target genes. Both Mitf and Tfe3 are expressed at high levels in osteoclasts.[58,59] Mutations in Mi cause mild osteopetrosis in mice due to defects in osteoclastogenesis, providing a direct evidence that MITF is required for osteoclast differentiation. MITF and TFE3 are also found to regulate osteoclast activity through controlling cathepsin K expression.[60] However, mutations in human homologue of the mouse Mi gene do not lead to skeletal abnormality (see below).

Human Muations in Transcription Factors Affecting Skeletal Development

Mutations affecting the DNA binding or the transactivation ability of transcription factors in humans can affect many aspects of skeletogenesis. As a matter of fact, molecular elucidation of many human skeletal dysplasias have been a major determinant in our improved understanding of skeletal development. There mutations can affect transcription factors regulating skeletal patterning or transcription factors regulating cell differentiation. Mutation of genes affecting patterning skeleton is best illustrated by the study of synpolydactyly, an autosomal dominant disease caused by mutation in HoxD13. Synpolydactyly patients have a characteristic manifestation in both hands and feet, typically have 3/4 finger and 4/5 toe syndactyly with a duplicated digit in the syndactylous web. This condition is caused by expansions of a polyalanine stretch in the amino-terminal region of HoxD13,[61,62] which impair the function of HoxD13 protein. Hox proteins are a family of transcription factors highly conserved from fruit fly to man. They all contain a DNA-binding motif called homeodomain which is 60 amino acid long. Hox genes are distributed in four different clusters and each of them contains 9 to 13 individual genes whose loci arranged in an order corresponding to their expression pattern.[63,64] HoxD13 is the 13th gene of the 4th cluster.

Msx1 and Msx2 genes belong to a unique homoebox gene subfamily since their loci are outside of the 4 Hox clusters. Msx1 and Msx2 expression is restricted in the developing craniofacial complex. Consistently, haploinsufficiency of Msx1 in humans shows orofacial clefting[65] and tooth agenesis[66,67] and deletion of Msx1 in mice caused cleft palate and abnormalities of craniofacial and tooth development.[68] Inactivation of Msx2 in humans gives rise to craniosynostosis,[69] an autosomal dominant disease characterized by craniofacial malformations or enlarged parietal foramina (PFM) caused by deficient ossification around the parietal bones.[70,71] Mutations in human Msx2 can either affect its DNA-binding affinity leading to loss-of-function mutation as in the case of PFM[70] or stabilize DNA binding of Msx2 to its target DNA leading to gain-of-function mutation as in Boston type craniosynostosis.[72] Interestingly, the defects caused by Msx2 mutation in mice are milder than those in humans. Cranial ossification defect is limited to the central frontal bone in Msx2-null mutant mice but heterozygous mutant mice are normal.[73]

Another autosomal dominant disorders of craniosynostosis in humans is Saethre-Chotzen syndrome (SCS), which is characterized by craniofacial and limb anomalies. Haplo-insufficiency of Twist, a transcription factor containing a basic helix-loop-helix motif, has been identified as the cause for SCS.[74,75] In veterbrates, Twist is expressed starting at 8 dpc.[76] Null-mutation of this gene in mouse is embryonic lethal, the mutant embryos die around 11.5 dpc.[77]

Heterozygous and transgenic mice show facial malformation including premature fusion of the cranial suture in twist +/- mice and a short snout and a twisted upper jaw caused by developmental defect in the first branchial arch.[77,78] Mutations affecting DNA binding, nuclear localization, or protein stability are all involved in the haplo-insufficiency of Twist.[79-82]

Unlike gene mutations disturbing skeletal patterning and certain cranial biogenesis discussed above, mutations affecting osteoblast or osteoclast differentiation lead to more generalized bone defects. Diseases arising from mutations disrupting osteoblast differentiation are best characterized in cleidocranial dysplasia (CCD), a heritable skeletal disorder characterized by abnormal clavicles, patent sutures and fontanelles, supernumerary teeth, short stature, and a variety of other skeletal changes. In both humans and mice, haplo-insufficiency of Cbfa1 is the cause of CCD.[30,83] Numerous studies identified the majority of mutations are missense mutations in conserved residues within Runt or PST domains, the two domains responsible for DNA binding or transactivation ability, respectively.[83-88]

In humans, MITF mutations cause Waardenburg syndrome type 2A and Tietz syndrome, autosomal dominant disorders resulting in deafness and hypopigmentation. Mice deficient in mi locus also show similar symptoms to humans, but these animals also develop osteopetrosis.[57] Mutations leading to impairment of its DNA-binding, dimerization, or transactivation of MITF at the mi locus affect the development of several different cell types, including osteoclasts, melanocytes, and mast cells.[89]

Conclusions

In the past 5 years, tremendous progress has been made in identifying transcription factors involved in the specification of cell lineage of the skeletal system. Mice and humans harboring mutations affecting skeletal development have provided excellent models to understand the function of these factors. Knowledge gained from the analysis of these genes has allowed us to establish a genetic cascade of transcription factors involved in controlling the differentiation and function of chondrocyte, osteoblast, and osteoclast. However, many questions remain to be answered. We know little about the genes whose expression is controlled by Sox9, Cbfa1, Pu.1 and c-Fos that allow them to cause progenitor cells to differentiate into chondrocytes, osteoblasts, and osteoclast, respectively. It also remains to identify the factors that act in concert with Cbfa1 to regulate osteoblast differentiation. Since strict temporal and tissue-specific control of gene expression in eukaryotes is often achieved by coordinate assembly of multiple transcription factors, we can expect that many more key regulators will be identified in the next 5 years.

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Chapter 2

Skeletal Stem Cells

Sergei A. Kuznetsov and Pamela Gehron Robey

Evolution of Skeletal Stem Cell Biology

More than 30 years ago, Friedenstein demonstrated that in low-density marrow cell cultures, discrete colonies of adherent fibroblast-like cells are formed.[20,22] Each colony was generated by proliferation of a single precursor cell[12,20,47] referred to as a colony forming unit-fibroblast (CFU-F).[19] The colonies could be easily detached with trypsin and expanded many fold by consecutive passages. Strains thus generated consisted of virtually homogenous cells (with the exception of mouse cultures, which also contain hematopoietic cells) displaying fibroblastic morphology, called marrow stromal fibroblasts (MSFs), by Friedenstein and coworkers.[21,24] MSFs lacked basic characteristics of macrophages, shared some properties of fibroblastic cells of other tissues[11,17,57,65] and, additionally, demonstrated some characteristics of smooth muscle and endothelial cells (Table 1). Consequently, the more general term "bone marrow stromal cell" (BMSC) has evolved in view of their properties that distinguish them from fibroblasts of other connective tissues.

The identity of BMSCs in situ has long remained elusive. Morphological data point to cells with ill-defined morphology and multiple names: adventitial cells, reticular cells, stromal fibroblasts, preadipocytes, undifferentiated mesenchymal cells, Westen-Bainton cells. In the post-natal marrow, these cells form both an adventitial (outer) coating of the sinusoid wall and branching extravascular meshwork that provides the physical substrate for myelopoiesis. Westen-Bainton cells are characterized by extensive, elongated and attenuated cell processes with membrane-bound alkaline phosphatase activity.[61] They express low levels of collagen types I and III and osteonectin, indicative of their partial fibroblastic character.[8,51] In human post-natal marrow, Weston-Bainton cells also demonstrate characteristics of preadipocytes.[5] In different animal species, varying proportions of these cells survive mechanical dissociation and plating in vitro. Of those that survive and attach to the culture vessels, the great majority begin to proliferate to form BMSC colonies, and are likely CFU-Fs, at least in part.

It is now well known that when animal and human BMSCs are transplanted in vivo, bone, cartilage, and fibrous tissue,[1,2,13,33,37,50] or bone accompanied by hematopoiesis-supporting stroma, including adipocytes[24,28,35,38,43-45,49] are formed, depending on the technique that is utilized. The ability of BMSCs to proliferate extensively and to differentiate into diverse tissues led to the hypothesis that the BMSC population is composed of multi-potential progenitors, akin to the hematopoietic stem cells.[51] The term "skeletal stem cells" (SSCs) was introduced based on the fact that they could differentiate into all major tissues of the skeleton.

In recent years, SSCs have been found to have unexpected and "nonconventional" differentiation capacities. Recently, myogenic cells,[59] to skeletal muscle,[18] and tendocytes[3,64] were added to the list of phenotypes that can be generated by SSCs, which represents differentiation into other tissues that arise from mesoderm. It has also been suggested that SSCs are able to differentiate into neurons,[62] astrocytes,[42] and cardiomyocytes,[30,60] and finally, to endothelium[53] which derive from different embryonic tissues. However, some of these conclusions of trans-germal differentiation were made based solely on in vitro experiments and the use of

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Table 1. Phenotypic characterization of multi-colony derived strains of human marrow SSCs and guinea pig blood-borne adherent cells, and of single-colony derived strains of human and rabbit blood-borne adherent cells

Marker*	Rabbit Polygonal Nonosteogenic	Rabbit Fibroblast-Like Osteogenic	Guinea Pig Osteogenic	Human Fibroblast-Like Nonosteogenic	Human Fibroblast-Like Osteogenic	Human Marrow SSCs
FIBROBLASTIC						
Col I	++	+	++	++	++	++
Col III	-/+	-/+	+	+	+	++
Fibronectin	++	++	++			
CD-44 (H9H11)				++	++	++
β1 integrin subunit				++	+/++	++
OSTEOGENIC						
ON	++	+/++	++	-/+	++	++
OPN	+/++	+	++	-/+	-/+	-/+
BSP	+/++	+	+	-	-	-/+
OC				-/+	-/+	-/+
ADIPOCYTIC						
CEBPα				-/+	-/+	-/+
PPARγ2	-	-		-	-	-
ENDOTHELIAL						
CD34				-	-	-
Muc-18				-	-	+
Factor VIII				-	-	-
Endoglin				-	-	+
PAL-E	-	-				
EN4			-			
MARROW STROMAL CELLS						
Stro-1				-	-	-/+
VCAM-1				++	++	+
SMOOTH MUSCLE						
α-smooth muscle actin	+	++	++	++	++	-/+/++
Smooth muscle ACL-10002	-/+	+	+/++			
HISTOCHEMICAL						
Alkaline Phosphatase	-	-	-	-	-	-/+/++
Acid Phosphatase	+	+	+	+	+	+
α-Naphthyl acetate esterase	-	-		+/++	+/++	+
Oil Red O (Lipid Accumulation)						
20% Fetal Bovine Serum	-/+	-	-/+	-/+	+	-/+
20% Rabbit Serum	+/++	-/+/++	++	++	++	++

*Markers for leukocytes, monocytes/macrophages, skeletal muscle, and neuronal cells were negative in all cell types studied. ++ intensive staining; + weak staining; - negative staining

artificial inducing stimuli,[30,53,59,62] and these data need to be corroborated by physiologically relevant in vivo observations. In other studies, analogous assertions were based exclusively on improved clinical outcome, with no specific markers proving SSC differentiation towards

tendocytes.[3,64] Yet another study used whole bone marrow, rather than purified SSCs, to initiate a skeletal muscle differentiation.[18] Contribution of hematopoietic stem cells or other potential stem cells present in marrow to these lineages should be excluded before giving all credit to SSCs.

SSC in Vivo Transplantation as the Assay of Their Multi-Potentiality

In vivo transplantation has been a major tool for studying differentiation potential of stem cells in general, and remains the "gold standard" for studying SSC multi-potentiality. Being highly adhesive in nature, SSCs are very sensitive to the microenvironment in which they are transplanted. Usually, no hard tissue is formed when an SSC suspension is injected subcutaneously or intramuscularly, when SSCs are implanted as a cell pellet without vehicle[33,63] or within rapidly resorbed vehicles.[25,25] For chondrogenic differentiation, high cell density and relatively anaerobic conditions are required, such as in micromass or pellet cultures. To initiate osteogenic differentiation, SSCs require the presence of a scaffold of a particular composition and architecture, depending on the animal species, that can support their growth and differentiation. Based on vehicle design, SSC transplantation techniques can be divided into two groups: closed and open.

In a closed system, cells are transplanted within diffusion chambers constructed of Millipore filters (0.22 μm or 0.45 μm porosity), such that the transplants receive nutrients but have no direct contact with the host cells. SSCs transplanted in closed systems form bone, cartilage, fibrous tissue, and sometimes fat. Bone is developed adjacent to the filters, while cartilage is found towards the center of the chamber[1,2] suggesting that SSC differentiation may be affected by the nutritional environment, including oxygen gradients. This conclusion is supported by the observation that in chambers with a narrow gap between the filters (0.1 mm), bone without cartilage is formed, while in chambers with a wider gap (2 mm), both bone and cartilage are developed.[23]

Transplantation into an open system (usually subcutaneously or under the kidney capsule) allows contact with recipient cells and is followed by rapid vascularization of the graft. In these conditions, SSCs rarely differentiate into cartilage, but in addition to bone and fibrous tissue, they form hematopoiesis-supporting reticular stroma and associated adipocytes (Fig. 1A). Vast fields of hematopoiesis are developed in close proximity to the new bone. In the absence of bone formation, hematopoiesis is never observed. In open transplants, SSCs demonstrate species-specific sensitivity to the nature of transplantation vehicle. Mouse SSCs form bone when transplanted within collagen sponges.[25,44] Human SSCs, however, form very little bone or no bone at all in vehicles such as collagen sponges, human demineralized bone matrix, polyvinyl sponges, and poly(L-lactic acid).[45,58] Human SSCs consistently formed extensive bone only in vehicles containing hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic in the form of blocks, powder, or Collagraft™ (mixture of ceramic powder with bovine collagen type I).[43,43] Furthermore, the development of the new bone is strongly influenced by size and shape of vehicle particles[46] emphasizing, once again, the importance of vehicle structure for SSC osteogenic differentiation.

In addition to studying SSC populations en masse, in vivo transplantation assay allows analysis of the heterogeneity of SSC populations. For this purpose, progeny of individual CFU-Fs can be expanded and transplanted separately. This type of assay provided similar results for SSCs derived from several species. Mouse and guinea pig SSC colonies were transplanted in vivo and in both cases, approximately 20% of the colonies formed bone with or without hematopoiesis, while the remaining colonies formed just fibrous tissue or no donor tissue at all.[12] Rabbit single-colony derived SSC strains were transplanted in diffusion chambers, either autologously or into immunodeficient mice, and bone, either with or without cartilage, was formed by 48.3%[23,31] and by 36.8% of the strains.[4] Human single-colony derived SSC strains were transplanted into immunodeficient mice in HA/TCP powder and Collagraft. Out of 34 strains, 20 (58.8%) formed bone while the remaining strains formed fibrous tissue. Among the 20 osteogenic strains, 8 (23.5%) developed extensive bone accompanied by hematopoiesis,

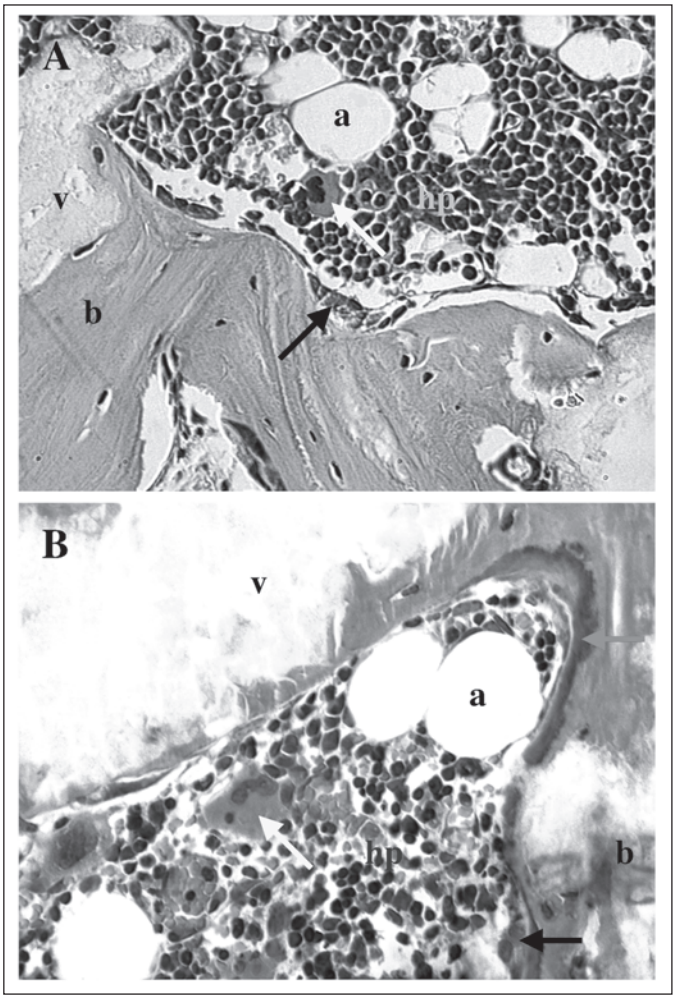

Figure 1. Bone formation in 8 week old transplants of SSCs from human bone marrow (A) and guinea pig blood (B). Human transplant (A) was demineralized, embedded in paraffin and sections were stained with hematoxylin and eosin; guinea pig transplant (B) was undemineralized, embedded in glycol methacrylate and sections were stained with Goldner's modified trichrome. Bone (b) is deposited on the surfaces of hydrohyapatite/tricalcium phosphate ceramic vehicle particles (v) by mature osteoblasts (black arrows). In undemineralized sections (B), bone (green) can be distinguished from osteoid (red, red arrow). A fully functional hematopoietic marrow (hp) is formed adjacent to the new bone; it is complete with megakaryocytes (yellow arrows) and adipocytes (a). To view color figure, please go to http://www.eurekah.com/eurekahlogin.php?chapid=1916&bookid=132&catid=58.

while 12 (35.3%) formed less abundant bone without hematopoiesis.[43] These data demonstrate that while SSCs are clonogenic, and proliferate extensively, only a subset of CFU-Fs giving rise to SSCs are multi-potential and have the ability to differentiate along several directions, including bone and hematopoiesis-supportive stroma with associated adipocytes.

In vivo transplantation assays not only reveal general directions into which a SSC population can or cannot differentiate but are a far more sensitive tool able to uncover subtle differences between differentiation abilities of skeletogenic precursor populations. Upon

transplantation, rabbit SSCs derived from red or yellow marrow form heterotopic ossicles containing stroma with abundant hematopoiesis, or hypocellular stroma with mainly fat cells, respectively, thus mirroring the composition of the original tissues.[52] SSCs derived from human cementum and dental pulp form unique hard tissues reminiscent of cementum and dentin, respectively, and yet distinct from typical bone.[34,36] SSCs from transgenic mice deficient for membrane-bound matrix metalloproteinase reproduce impairment of both osteogenic capacity and collagenolytic activity.[39] SSCs from patients with fibrous dysplasia of bone and the McCune-Albright syndrome recapitulate abnormal features of the fibrous dysplastic lesion.[6] SSCs from a patient with a rare, newly described syndrome, gnathodiaphyseal dysplasia, closely imitate pathological characteristics of the native lesion.[7] The two latter findings open a novel direction in developing small animal models of human skeletal disorders. Taken together, these data demonstrate that skeletal stem cells "remember" the exact features of the part of skeleton they originated from, preserve this "memory" during prolonged ex vivo expansion, and reveal it upon in vivo transplantation.

Use of in Vivo Transplantation Assay for Identification of SSCs from Nonskeletal Tissues

As early as the 1960s, Friedenstein developed a concept of determined and inducible osteogenic precursor cells. The former, DOPC, included BMSCs and other spontaneously osteogenic cell populations of skeletal origin. The latter, IOPC, comprised a number of extraskeletal populations requiring inductive stimuli to initiate osteogenic differentiation. The analysis of osteogenic potential of these populations was performed entirely by means of in vivo transplantation. When cell suspensions of thymic, splenic, and peritoneal origin, as well as blood leukocytes, were transplanted in diffusion chambers together with an "inducer" (epithelium of the urinary bladder, or decalcified bone matrix), bone was formed inside the chambers.[22,27,29] These findings directly proved nonbone related tissues include precursor cells capable of bone formation under the influence of inductive stimuli.[19-21] Transplantation of these cells into an open system shed some light on the potential origin of IOPCs. Epithelium of the urinary bladder was implanted autologously into a guinea pig's tibia locally irradiated with 1000 to 5000 rad. Despite pronounced radiation injury, the course of bone induction was unaffected suggesting that IOPC were not local but migrated from nonirradiated areas,[19] possibly via the blood stream.

Evidence that spontaneously osteogenic SSCs can also be found outside the skeleton came from studies of pericytes, cells embedded within the basement membrane of microvessels (venules, arterioles, and capillaries). Pericytes grown from retinal and brain microvessels formed mineralized nodules in vitro and express multiple osteogenic markers, such as alkaline phosphatase, bone sialoprotein, osteonectin, osteopontin, and osteocalcin.[9,10,15,55,56] Pericytes inoculated into diffusion chambers and implanted into athymic mice, and recovered 4 to 8 weeks later, the chambers reproducibly contained bone, cartilage, soft fibrous tissue, and adipocytes.[15] These findings demonstrate that pericytes represent precursor cells able to differentiate along osteogenic, chondrogenic, and adipogenic pathways and are true SSCs. Moreover, based on morphological and histochemical evidence, it can be speculated that bone marrow Weston-Bainton (reticular, adventitial) cells, the very cells that give rise to BMSC colonies upon in vitro explantation, represent a subtype of pericytes. The existence of ubiquitous, microvessel-associated system of primitive multi-potential SSCs has been postulated.[7]

Recent studies have also identified cells in adipose tissue that have osteogenic properties. Preadipocytes, or stromal cells, were isolated from human subcutaneous adipose tissue by collagenase digestion, centrifugation, and plastic adherence. In vitro, these cells were able to support hematopoiesis.[32] In medium supplemented with calcitriol, the adipose tissue-derived cells demonstrated synthesis of osteoblastic mRNAs (Cbfa1, alkaline phosphatase, osteocalcin, and osteopontin) and they also developed a mineralized matrix after ascorbic acid had been added.[41] When these cells were grown in three-dimensional alginate cultures in the presence of chondrogenic supplements (ITS+, pyruvate, ascorbate 2-phosphate, dexamethasone, and

Table 2. Colony forming efficiency, morphology and osteogenic potential of circulating skeletal stem cells

Animal Species	CFE x 10^6 Cells (Range between Donors)	# Polygonal/ Fibroblastic Colonies (% Polygonal)	# of Osteogenic Clones/Clones Transplanted
Mouse	0.93 (0-3.8)	5/33 (13%)	1/8
Rabbit	0.18 (0-0.58)	6/41 (13%)	3/19
Guinea Pig	2.7 (1.1-3.9)	15/319 (5%)	2/4*
Human	Rare (0-0.025)	0/2 (0%)	1/2

*Guinea pig cultures were derived as polyclonal rather than single colony derives strains

TGF-β1), they synthesized cartilage matrix molecules, including collagen type II, VI, and chondroitin 4-sulfate.[16] These cells were assayed by in vivo transplantation in three-dimensional alginate gels. Four and 12 weeks post-transplantation, the cells demonstrated significant production of cartilage matrix molecules,[16] though no morphologically recognizable cartilage tissue was formed. Finally, using a different transplantation technique, bone tissue was formed in vivo by transplanted adipose tissue-derived cells (J.H.F. Justesen, personal communication). These combined data strongly suggest that subcutaneous adipose tissue contains SSCs with at least quadruple differentiation potential: osteogenic, chondrogenic, adipogenic, and stromal. It remains to be found, however, whether these putative subcutaneous SSCs represent a distinct stem cell type or are derived from pericytes from subcutaneous microvessels.

Recently, adherent, clonogenic cells have been isolated from the blood of four mammalian species: mouse, rabbit, guinea pig and human.[46] Colony forming efficiency (CFE), or the number of colonies per 10^6 nucleated cells, varied significantly between species, as well as from one individual donor to another (Table 2). In all species except human, colonies of two distinct types were observed. The majority of the colonies were composed of cells with fibroblastic morphology, but a variable, small proportion of colonies (Table 2) consisted of cells exhibiting a distinctive polygonal shape. Cloned strains of both types, however, demonstrated virtually identical immunophenotype, staining positively for some, but not all markers of fibroblastic, osteogenic, and smooth muscle cells but negatively for markers of endothelial cells, hematopoietic cells, and monocytes/macrophages (Table 1). The blood-derived adherent cells, therefore, phenotypically resembled but were distinguishable from BMSCs. Notably, the human blood-derived adherent cells were negative for the marrow stromal marker, Stro-1, as well as for endoglin and Muc-18, all of which were expressed in human BMSCs. Both blood-derived adherent cell and BMSC populations were negative for PPARγ2 but contained cells positive for another adipogenic marker, CEBPα. Upon culture with medium containing rabbit serum, a known inducer of adipogenesis,[14] blood-derived adherent cells from all species underwent adipogenic differentiation.[46] In micromass cultures in the presence of chondrogenic supplements,[40] a proportion of guinea pig clonal and polyclonal strains of blood-derived adherent cells demonstrated vigorous cartilage formation (Kuznetsov and Gehron Robey, unpublished observation).

Clonal strains of blood-derived adherent cells from mouse, rabbit and human cultures and polyclonal strains from guinea pig cultures were transplanted within HA/TCP powder vehicles into the subcutis of immunocompromised mice to determine the capability of forming bone. Histology-proven bone was formed by 12-50% of the strains, depending on the animal species

(Table 2). Only the strains that displayed a fibroblastic morphology formed bone. In situ hybridization using species-specific DNA repetitive sequences as probes demonstrated that bone tissue formed in transplants of human and guinea pig blood-derived adherent cells had donor origin.[46] In some transplants, a complete hematopoietic marrow, including adipocytes, accompanied the new bone (Fig. 1B). These findings clearly demonstrate that SSCs with multiple differentiation potential similar to that of BMSCs (osteo-chondro-adipo-stromo-fibrogenic) are present in peripheral blood cells, albeit with highly variable frequency. Obviously, many intriguing questions related to the circulating SSCs remain to be answered, such as their origin, destination, and participation in physiological and/or pathological skeletogenic events.

Conclusions

Recently, several discoveries have had high impact on the field of skeletal stem cell biology. First, it was shown that the differentiation potential of SSCs is not necessarily limited to skeletal tissues, such as bone, cartilage, adipocytes, hematopoiesis-supporting stroma, and fibrous tissue. Rather, SSCs may differentiate into a much broader spectrum of tissues, including muscle, endothelial, and even neural cells, thus displaying a phenotypic plasticity. Secondly, it now appears that SSCs are located outside of bone marrow, having a much wider distribution throughout the body, including the dental pulp, the microvasculature, circulating blood cells, and subcutaneous fat. These exciting data have considerably modified our views on tissue regeneration in adult mammals, and they have also opened novel, potentially very promising therapeutic approaches. In vivo transplantation assays has provided convincing evidence of the varied differentiation capacities of SSCs. However, either orthotopic transplantation or systemic injection, followed by careful examination of the fate of transplanted cells, will be required to answer the question whether, in the context of a real organ physiology, SSCs can integrate into the organ, differentiate along a physiologically relevant pathway, and play a tangible role in the organ's function.

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CHAPTER 3

Bone Morphogenetic Proteins and Tissue Engineering of Bone

A. Hari Reddi

Abstract

Tissue engineering is the emerging science of design and manufacture of tissues including bones and joints. The three key ingredients for both tissue engineering and morphogenesis are inductive morphogenetic signals responding stem cells and the assembly of extracellular matrix. The long-term goal of our research is to produce spare parts for the human musculoskeletal tissues. Regeneration of musculoskeletal tissues recapitulates embryonic development and morphogenesis. Morphogenesis is the developmental cascade of pattern formation, body plan establishment leading to adult form and function. Therefore, signals involved in morphogenesis will be of utility in tissue engineering of bones and joints. Although bone and articular cartilage are adjacent tissues, there is marked difference in their regenerative potential. Bones has maximal regenerative potential; on the other hand cartilage is feeble. BMPs have pleiotropic roles in initial pattern formation, cell differentiation and maintenance of bone and articular cartilage. The regenerative potency of bone is due to bone morphogenetic proteins (BMPs) in the bone matrix. BMPs act via BMP receptors and Smads 1, 5 and 8 to initiate lineage of cartilage and bone. The homeostasis of tissue engineered bone and cartilage is dependent on the interface of tissue extracellular matrix and biomechanics. The use of BMPs in gene therapy and isolation of stem cells and their microenvironmental niche of extracellular matrix results in functional bone and cartilage. In conclusion, these are exciting times in functional tissue engineering of bone.

Introduction

One of the challenges confronted by an orthopaedic surgeon is the repair and restoration of large segment skeletal defects resulting from resection of a malignant bone neoplasms. Although large segment bone allografts have gained increasing acceptance it has the drawbacks of potential fractures.[3,38] The problem of bone fractures in patients with postmenopausal osteoporosis, metastases due to breast and prostate cancer and metabolic diseases such as diabetes requires the application of principles of tissue engineering to bone.[8,13,16,27,28]

Tissue engineering is the science of design and fabrication of new tissues for functional restoration of impaired organs and replacement of lost parts due to cancer, disease and trauma.[26,27] Among the many tissues in the body, bone has the highest potential for regeneration and therefore is a prototype paradigm for the enunciation of principles of tissue engineering in general. The accumulating knowledge in tissue engineering will lead to the design of bone with predetermined shapes for orthopaedic surgery applications.

The three key ingredients for tissue engineering and tissue regeneration are signals, stem cells and scaffolding. The specificity of signals is dependent on tissue morphogenesis and inductive cues in the embryo and they are generally recapitulated during regeneration.[30] Bone

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grafts have been utilized by orthopaedic surgeons for over a century. Urist (1965)[40] made the key discovery that intermolecular implantation of demineralized, lyophilized segments of allogeneic rabbit bone induced new bone formation. Bone induction is a sequential multistep cascade and the three key steps are chemotaxis, mitosis and differentiation.[25,29,30] Chemotaxis is the directed migration of cells in response to a chemical gradient of signals released from the demineralized bone matrix.[4] The migration and attachment of progenitor cells to the collagenous matrix is mediated by fibronectin. On day 3 there is a peak in proliferation of cells in response to growth factors released from the insoluble demineralized matrix.[24] Chondrogenesis is maximal on days 7-8 and is followed by vascular invasion and osteogenesis on day 9. Bone formation is maximal on days 10-12 as indicated by alkaline phosphatase activity and is followed by increases in osteocalcin, the bone γ-carboxyglutamic acid containing protein (BGP). The newly formed ossicle is filled with hematopoietic marrow on day 21.[25] The demineralized bone matrix-induced bone morphogenesis system led to the isolation of bone morphogenetic proteins (BMPs) the primordial signals for morphogenesis of bone and a variety of organ systems beyond bone such as brain, heart, kidney, lungs, liver, skin and teeth. Hence, one can refer to BMPs as body morphogenetic proteins.

Bone Morphogenetic Proteins

The demineralized bone matrix was extracted by dissociative agents such as 4 M guanidine HCL, 8 M urea or 1% sodium dodecyl sulfate at pH 7.4.[35,36] Approximately three percent of the proteins were solubilized and the residue was predominantly type I insoluble bone collagen. Although the soluble extract or insoluble collagen were not osteoinductive singly when recombined and reconstituted together it restored bone induction. Thus, there is a collaboration between a soluble signal and an insoluble substratum of collagen to initiate new bone formation. The soluble signal was purified by heparin affinity chromatography, hydroxyapatite columns, and molecular sieve chromatography. The final purification was accomplished by preparative gel electrophoresis and novel BMPs were isolated, cloned and expressed.[15,21,27,45]

Table 1 summarizes the 15 BMPs in the mammals and are related to members of the TGFβ superfamily. BMPs are dimeric due to a critical intermolecular disulfate linkage. The dimeric conformation is critical for bone induction and morphogenesis. Each of the two monomers is biosynthesized as a molecule of over 400 amino acids. However, the mature BMP monomer derived by proteolytic processing is about 120 amino acid polypeptide. BMPs are pleiotropic signals. Pleiotropy is the property of a gene or protein to act in a multiplicity of steps. BMPs act on the three key steps in the sequential cascade of bone morphogenesis such as chemotaxis, mitosis and differentiation of transient stage of cartilage and the permanent induction of bone. Thus, all BMPs are chondrogenic, therefore can be considered as cartilage morphogenetic proteins.

Cartilage morhogenesis is a key rate-limiting step in the bone morphogenetic cascade, and is critical for bone and joint development. In this reference it is noteworthy that articular cartilage served as a starting point for the isolation of novel cartilage-derived morphogenetic proteins (CDMPs) using a chondrogenesis bioassay based on dissociative extraction and reconstitution of chondrogenic proteins.[27,28]

Although BMPs were first isolated, cloned and expressed from bone, they have actions beyond bone. Genetic evidence based on gene knockouts have implicated BMPs in development and morphogenesis of brain, eye, heart, kidney, liver, lung, ovary, skin, teeth, testis and in a variety of tissues during various steps of epithelial-mesenchymal interactions during embryogenesis. It is indeed gratifying to note that BMPs is the basis of key developments in morphogenesis of many tissues.

BMPs elicit their biological actions by their interaction with types I and II BMP receptors. There are two kinds of type I BMP Receptors, types IA and IB.[9,37] BMPs receptors are protein kinases that phosphorylate cytoplasmic substrates called Smads 1, 5 and 8. The phosphorylated Smads 1, 5 and 8 partner with a coSmad called Smad 4 and enter the nucleus to turn on

Table 1. The superfamily of BMPs

BMP Subfamily	BMP*
BMP 2/4	BMP 2
	BMP 4
BMP 3	BMP 3
	BMP 3B
DP-1/BMP 7	BMP 5
	BMP 6
	BMP 7
	BMP -8
	BMP 8B
OTHERS	BMP 9
	BMP 10
	BMP 11
	BMP 15
	BMP 16
CARTILAGE-DERIVED MORPHOGENETIC	BMP 14/CDMP1/GDF5
PROTEINS (CDMPs), GROWTH/DIFFERENTIATION	BMP 13/CDMP2/GDF6
FACTORS (GDF)	BMP 12/CDMP3/GDF7

* BMP 1 is procollagen C-proteinase related to Drosophila Tolloid and does not contain the canonical seven cysteines of classical BMPs listed in this table.

BMP-response genes. The phosphorylation of Smads 1, 5 and 8 by BMP receptors is inhibited by inhibitory Smad 6. Thus, the BMP signaling system is an intrically regulated homeostatic machine such as a thermostat in an airconditioner.[27] BMP-BMP receptor signaling system in the mesenchymal stem cells results in bone induction and morphogenesis.

Stem Cells

Mesenchymal stem cells derived from mesoderm are the common progenitors for the various lineages of the musculoskeletal system such as bone, cartilage, ligaments, muscle and tendon. The exciting advances in stem cell biology is opportune for the introduction of BMP genes by gene therapy into responding stem cells. The fundamental work of Friedenstein and Owen[5,20] laid the foundations for recent excitement in bone marrow-derived mesenchymal stem cells for bone tissue engineering.[2,23]

The characterization of stem cells including unique markers will permit isolation by flurescent-activated cell sorters (FACs). These isolated stem cells can be transduced by gene therapy.[18,19] Thus stem cell and BMP gene therapy in combination is a platform which can be applied to other tissues beyond bone in tissue engineering. Refinement of viral and nonviral vectors and novel physical techniques including electroporation, sonoporation of plasmid DNA into cells may enhance the efficiency and efficacy of gene therapy for bone tissue engineering.

Scaffolding: Biomimetic Biomaterials

The isolation, cloning and expression of BMPs and the advances in stem cells will permit the rational design of the bones of predetermined shapes using scaffolds for tissue engineering of bone. A scaffold in the context of bone tissue engineering is the extracellular matrix of bone, the unique microenvironmental niche for bone morphogenesis. What are biomimetic biomaterials in the context of the extracellular matrix scaffolding? Biomaterials that mimic native extracellular matrix scaffolding are biomimetic as they imitate nature. The biomimetic biomaterials in the musculoskeletal tissues include collagens, proteoglycans, component glycosamigolycans and

hyaluronan. Adhesive proteins fibronectin and laminin are critical in attachment of cells to ECM. Hydroxyapatite in the mineral phase of bone is a natural biomimetic biomaterial. BMPs bind to collagens I and IV, heparan sulfate, heparin and hydroxyapatite.[22,27] The geometry of the hydroxyapatite is critical for delivery of BMPs for bone induction. Consistently, optimal bone morphogenesis was observed by hydroxyapatic discs compared to beads. This profound difference is independent of the pore size in the range from 200 to 500 μm. The chemical composition of the two hydroxyapatites were identical illustrating the key role of three-dimensional architecture of the substratum the geometry for tissue engineering.[12,27,32,33] The role of Bioceramics in medical applciations is well known.[10] In subhuman primates hydroxyapatite appears to be "osteoinductive".[31] It is likely that BMPs in circulation in the vascular system may bind to hydroxyapatite and secondarily induce bone formation. Thus, an osteoconductive biomaterial such as hydroxyapatitte progressively becomes an osteoinductive substratum.

The controlled release of morphogenes and growth factors from biodegradable polymers of poly (DL-lactic-co glycolyic acid, PLGA) and polyethylene glycol (PEG) is a critical area for tissue engineering.[14] Biodegradable block copolymers of PLGA and PEG are optimal delivery systems for BMP2.[34] Recombinant BMP4 and purified BMP3 bind to types I and IV collagen and heparin.[22] A comparison of several delivery systems indicated collagen is the most optimal delivery system for bone induction.[17] It is likely in the native demineralized bone matrix BMPs are bound to collagenous extracellular matrix scaffolding. The role of the biomimetic biomaterial in the delivery of recombinant BMPs for bone tissue engineering is critically dependent on the pharmacokinetic of release of BMPs.[39] The local retention of BMPs by the biomimetic biomaterial such as collagen sponge as hydroxyapatite as composites of collagen and hydroxyapatite may have profound influence on the osteoinduction by a tissue engineering device. Cells may be transplanted in various matrices.[41]

Clinical Applications of BMPs

The proof of concept that an osteoinductive composite of BMPs and scaffolding can be used to fabricate a tissue engineered bone was demonstrated.[11] In this experiment a vascularized muscle flap was placed in a mold mimicking the head of the femur of rat and was injected with BMPs and collagenous matrix. It is noteworthy that a true transformation of muscle into bone mirroring the shape of the femur was accomplished demonstrating the proof of principle for tissue engineering of bone.[11] The outstanding regenerative potential of bone is common knowledge. However, in the repair of massive segmental bone loss due to tumors, trauma or fractures due to metabolic diseases such as diabetes and osteoporosis, it is common orthopaedic practice to aid and abet the healing site with autogenous bone graft. The limited supply of autograft bone, the associated donor site morbidity[46] including infections and pain is a major challenge. The availability of recombinant BMPs and biomimetic biomaterials and stem cells has set the optimal stage for tissue engineering to enter the operating suites in orthopaedic surgery.

An auspicious beginning was made by the use of BMP 7 in treatment of tibial nonunions.[6,7] In addition to orthopaedics BMPs have been used in clinical dentistry in the realms of maxillofacial, surgery bone augmentation and integration of dental implants.[1,44] Despite these positive advances, many clinical challenges remain. In addition to optimization of the dose of BMPs, pharmacokinetics of release, the optimal delivery from biomimetic biomaterials and the optimal sterilization including irradiation.[42,43] The recent approval of recombinant BMP2 for spine fusion appears to be the first use of a recombinant morphogen in orthopaedic surgery and tissue engineering of bone.

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Chapter 4

Mechanobiology of Bone

Elisabeth H. Burger, Jenneke Klein-Nulend and Margriet Mullender

Abstract

Mechanical force is an important regulator of bone formation and resorption. Bone tissue remains adapted to the magnitude and direction of its daily loadings throughout life, as a result of continuous adaptive remodeling. In culture, bone cells demonstrate a high responsiveness to mechanical strain, both resulting from fluid shear stress and from cell stretching, but the type of response seems to differ: fluid shear stress causes the rapid production of nitric oxide and prostaglandins, while cell stretching leads to cell alignment and cell proliferation. Fluid shear stress occurs in the osteocyte canaliculi during dynamic loading of intact bone, while cell stretching occurs a.o. in osteogenic soft tissue during distraction osteogenesis. Here we discuss the concept that the response to fluid shear stress in vitro reflects mechanotransduction by osteocytes in intact remodeling bone. The response to cell stretching however may reflect the osteogenic response to stretching of soft tissue as occurs in distraction osteogenesis. In tissue engineering, both stimuli offer possibilities for enhancing bone cell growth in vitro.

Introduction

An important issue in Bone Tissue Engineering is the phenomenon of mechanical adaptation, also called Wolff's Law.[1,2] Mechanical adaptation of bone tissue means that not only the form of a whole bone but also it's internal structure is adapted to the magnitude and direction of the forces acting upon it during daily movement of the body. At the tissue level, mechanical adaptation is the cellular process whereby living bone adapts it's mass and structure to prevailing loads to obtain a higher efficiency of load bearing. Adaptation will improve an individual's survival chance, because bone is not only hard, but also heavy. Too much of it is probably as bad as too little, as it leads either to uneconomic energy consumption during movement (when bone mass is too high) or to an enhanced fracture risk (when bone mass is too low). This easily explains the usefulness of mechanical adaptation, even if we do not know the cellular mechanism by which it is obtained. Adaptation continues throughout life, also when bone tissue is renewed, as is necessary for most tissues in the body. Renewal occurs by resorption of old bone and formation of new bone in a process called remodeling.[3] Changes in the magnitude and direction of loads are followed by changes in both mass and orientation of the bony tissue, as originally shown by Wolff,[1] and in modern medicine by the loss of alveolar bone from the edentulous jaw and the growth of bone around hip implants.[4] Maintenance of an adapted structure during remodeling means that the daily loads to which the bone is subjected somehow regulate the activities of the cells involved in remodeling. The question is how this regulation occurs.

A completely different case of mechanical regulation of bone tissue formation is distraction osteogenesis (DOG), a method applied in orthopedic and oral surgery to lengthen bones. In DOG, force is applied by mechanically separating the two bony walls of an osteotomy, after the gap has filled with osteogenic connective tissue.[5] This collagenous connective tissue that

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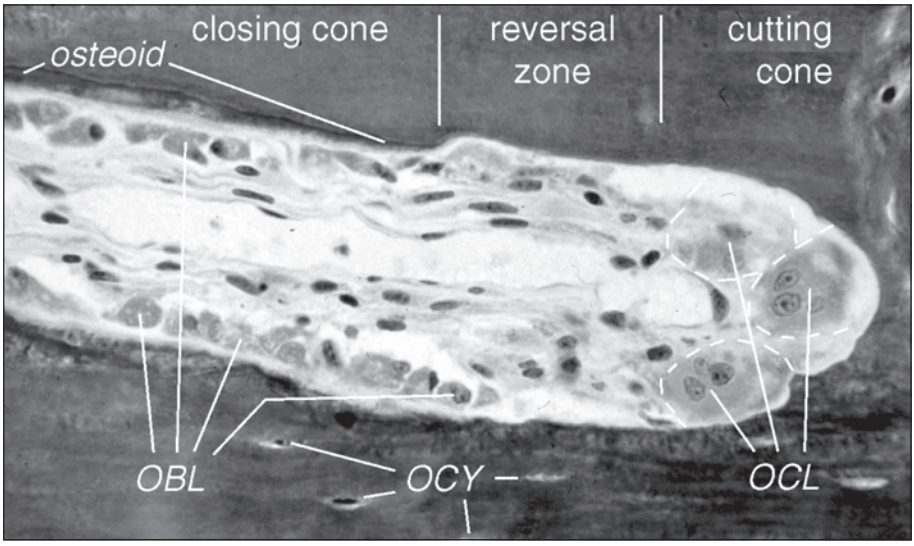

Figure 1. The cellular activity during bone remodeling. At the tip (cutting cone) multi-nucleated osteoclasts (OCLs) excavate the mineralized bone tissue. At some distance, after the resting zone, osteoblasts (OBLs) appear at the surface to refill the tunnel with osteoid that is subsequently mineralized. Osteocytes (OCYs) are former osteoblasts that were entombed within the bone matrix, but remained connected to the bone surface by numerous long slender protrusions (not visible). Typical outer diameter of an osteon in human cortical bone is about 200 μm. Picture adapted from Schenk and Willenegger, 1964, courtesy R. Schenk.

connects the two bone surfaces is stretched, and as a result bone grows rapidly from the two surfaces towards the distraction gap.[6] In this case the applied force causes strain not in the bone itself, but in the soft osteogenic tissue that has filled the gap.

In the following chapter we will discuss currents concepts of the cell biology of these two processes. We will argue that mechanotransduction in these cases is different, and that different cell types are involved. We will then argue that the two most widely used models for applying force on bone cells in culture, cell stretching and fluid shear stress, may represent each of these two processes. In tissue engineering of bone, application of these force models during cell growth in culture may each have their particular beneficial effect.

Mechanotransduction during Adaptive Bone Remodeling

In adult bone, osteoblastic and osteoclastic activity is largely confined to bone remodeling.[7] osteoclasts, large multinucleated cells related to macrophages, dig a tunnel in compact, cortical bone or a trench along the surface of trabecular bone.[8] They are followed by osteoblasts, smaller mononuclear cells that are related to fibroblasts and chondrocytes. The osteoblasts form bone-specific extracellular matrix that calcifies, thereby replacing the old resorbed bone. During bone formation a number of osteoblasts differentiate into osteocytes and become entombed in the new matrix.[9,10] The group of osteoclasts plus ensuing osteoblasts is called a Basic Multicellular Unit or BMU.[3,11] The moving resorption front where the osteoclasts degrade existing bone is called the cutting cone, and the tunnel or trench which the osteoblasts gradually fill with new bone the closing cone (see Fig. 1). The new bone, organized as osteons in cortical bone and hemi-osteons in trabecular bone, is aligned along the dominant local loading direction, suggesting local strain gradients as regulating principle.[12-14] This leads to the question how strains are sensed in bone tissue, and by which cells. Many recent studies show that mechanosensing in intact bone is primarily a task for the *osteocytes*, the mature, long-lived, terminal differentiation stage of osteoblasts that lie buried in the mineralized bone matrix (see

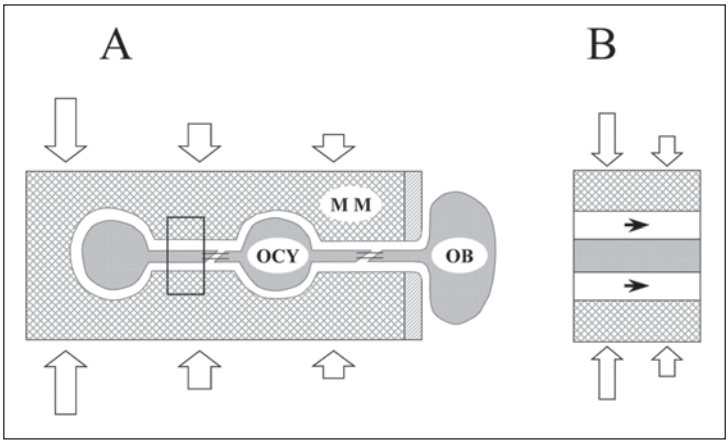

Figure 2. The concept of strain-derived canalicular flow. Deformation of the mineralized matrix (MM) causes fluid pressure gradients within the canalicular system, resulting in fluid flow over the octeocyte (OCY) processes. In response to the fluid shear stress osteocytes may signal the osteoblasts (OB) at the bone surface.

Burger and Klein-Nulend[10] for a review). Comparison of the responsiveness of osteocytes, osteoblasts and preosteoblasts to mechanical strain in vitro, showed that the osteocytes were most responsive, more than osteoblasts and these more than preosteoblasts.[15-19] Thus, in the course of differentiation from immature preosteoblast via osteoblast to osteocyte, bone cells increase their sensitivity to mechanical strain. The manner whereby osteocytes sense the strains of the mineralized matrix must be considered in light of the very small strains in bone during daily loading, as compared to muscle tissue, for example.[20,21] This has led to the concept of strain-derived canalicular fluid flow, or it's derivative fluid drag force, as the physical mediator of mechano-sensing by osteocytes in bone tissue[20,22,23] (Fig. 2). Several experimental studies support this concept. Animal studies show that bone formation in response to loading is sensitive to strain rate, and that large strains alone are not sufficient to activate bone cells, suggesting interstitial fluid flow as the physical stimulus involved.[24,25] In cell culture studies, fluid flow is the most efficient cell activator,[26] and osteocytes show strong responses to fluid flow, including rapid release of nitric oxide (NO) and prostaglandins.[15-19] Together these findings suggest that the osteocyte network with it's accompanying lacuno-canalicular porosity, is the site of mechanosensing in bone tissue. Mechanotransduction thus includes the translation, by osteocytes, of canalicular flow into cell signals that can recruit osteoclasts and osteoblasts.[10]

Mechanotransduction during Distraction Osteogenesis

Distraction osteogenesis (DOG) can be regarded as a form of bone engineering in vivo. It is a well-established technique, initially used for lengthening of long bones,[5] but it has also found other applications such as the treatment of limb deformities, bone defects and the creation of craniofacial bone. The technique involves separation of a bone by osteotomy and fixation by an external fixator. During a short latency period of about a week a collagenous connective tissue, or callus, is formed. This newly formed soft tissue is then subjected to controlled distraction. For appropriate distraction rates the tissue in the gap expands. Gradually ossification of the soft tissue occurs and as a result the gap is bridged by new bone (Fig. 3). After consolidation, the newly formed bone is remodeled as in existing bone.

Similar to fracture healing, soft tissue and bone formation in DOG is thought to be controlled to a large part by growth factors, low-molecular weight glycoproteins. These factors are released by the blood clot, the inflammatory cells that invade the fracture site and the bone

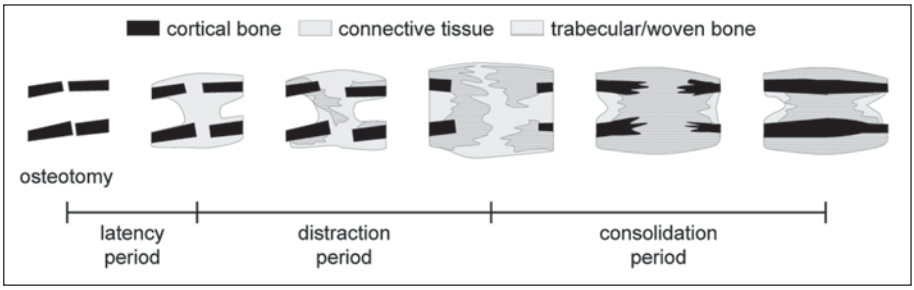

Figure 3. Schematic representation of tissue development in distraction osteogenesis (DOG). After osteotomy the bone is stabilized. In this latency period, a fracture callus forms. During distraction, the callus amplifies, while ossification starts around the cortical edges and progresses in the direction of the applied strain. At the end of distraction, the bone is stabilized again and the osteotomy gap is bridged by bone. Hereafter, full consolidation of the gap takes place. The composition of the callus tissue varies between fibrous connective tissue and fibrocartilage.

itself. During clotting, various factors are released, such as platelet derived growth factor (PDGF) and transforming growth factor-beta (TGF-β). Thereafter, inflammatory cells invade the wound rapidly. They also secrete factors e.g., fibroblast growth factor (FGF) as well as PDGF and TGF-β.[27] In addition, they are thought to produce cytokines including interleukin-1 (IL-1) and interleukin-6 (IL-6).[28] Growth factors released by bone include bone morphogenetic proteins (BMPs), TGF-β, PDGF, insulin-like growth factors I and II (IGF-I, IGF-II) and FGFs.[27] Hence, within the first couple of hours after osteotomy or fracture a multi-factorial environment is created. This complex biochemical milieu is thought to be responsible for the invasion, proliferation and differentiation of cells in the wound. Interestingly, the majority of these factors play distinct roles in the ontogenesis of the embryonic skeleton.[28] The coordinated regulation of successive events by these factors is still poorly understood. It is clear, however, that once connective tissue is formed, its further development depends to a large extent on the mechanical context.

Bone repair can follow various pathways with various combinations of bone formation via direct, endochondral or intramembranous ossification. In fracture healing these pathways are modulated by the stiffness of the fixation.[29] When the fracture is fixed rigidly, direct bone formation, without the formation of a callus can occur. Apparently, a stable environment with relatively low strains stimulates the direct differentiation towards bone forming osteoblasts. Under less rigid fixation bone healing occurs by endochondral ossification. Interfragmentary movement stimulates the growth of callus tissue, but it inhibits the conversion of soft connective tissue into bone.[29-32] In contrast, pure axial loading of the callus and load bearing seem to stimulate the formation of bone.[33,34]

In DOG the applied strain and frequency affect the pathway and the success of bone development.[35,36] If the distraction is too slow, bone formation will catch up with the formation of new connective tissue. Consolidation will happen too soon and further distraction is impossible. Too large distraction rates (i.e., large strain values) lead to disorganized fibrous tissue formation without the formation of bone, resulting in nonunion.[35,37] Within this window, distraction is usually successful. The course of tissue development is modulated by the distraction magnitude and rate.[37-39] For instance, Meyer et al[40] showed that low strains of 0.2% lead to intramembranous bone formation, with active osteoblasts in the whole distraction gap and no clear anisotropy. Strains of 2% gave highly oriented collagen fibrous tissue, with bone formation at the borders of the gap in the form of trabeculae growing in the direction of tension. Furthermore, high strains of 20% or 30% result in loosely arranged collagen, tissue failure, and lack of mineralization.

In fracture healing and DOG, cells within the callus tissue are loaded quite differently compared to bone cells in normal healthy bone. The strain values during distraction are much higher than in adult bone. These higher strains are found to stimulate cell proliferation, matrix production and cell and matrix alignment in vitro.[41-45]

Mechanical Stimulation of Bone Cells in Vitro

Fluid Shear Stress: Technique, Cell Responses

In healthy, adequately adapted bone, small matrix strains produce flow of extracellular tissue fluid through the lacuno-canalicular network.[46,47] This strain-derived extracellular fluid flow may help to keep osteocytes healthy, particularly the deeper ones, by facilitating exchange of nutrients and waste products between the Haversian channel and the osteocyte network of an osteon.[48] However a second function of this strain-derived interstitial fluid flow is thought to be the transmission of "mechanical information". The magnitude of interstitial fluid flow through the lacuno-canalicular network is directly related to the amount of strain of the bone organ.[49] Because of the narrow diameter of the canaliculi, bulk bone strains of about 0.1% will produce a fluid shear stress in the canaliculi of roughly 1 Pa,[20] enough to produce a rapid response in e.g., endothelial cells.[50,51] Experimental studies in vitro have demonstrated that osteocytes are also quite sensitive to fluid shear stress of such magnitude.[15-17,19] These results suggest that the combination of the cellular 3-dimensional network of osteocytes and the accompanying porous network of lacunae and canaliculi, acts as the mechano-sensory organ of bone. The flow of interstitial fluid through the bone canaliculi will have two effects, a mechanical one derived from the fluid shear stress, and an electrokinetic one derived from streaming potentials.[52,53] Either of the two, or both in combination, might activate the osteocyte. For instance, streaming potentials might modulate the movement of ions such as calcium across the cell membrane,[54,55] while shear stress will pull at the macromolecular attachments between the cell and it's surrounding matrix.[56] Both ion fluxes and cellular attachment are powerful modulators of cell behavior, and therefore good conveyors of physical information.[57,58]

The discovery of an antigen against chicken osteocytes made it possible to specifically study the mechanosensitivity of osteocytes.[59] With the immunodissection technique three separate cell populations with a high (≈ 90%) degree of homogeneity can be prepared, representing (1) osteocytes with the typical "spider-like" osteocyte morphology and little matrix synthesis, (2) osteoblasts with high synthetic activity of bone matrix-specific proteins, and (3) osteoprogenitor cells (from the periosteum) with a fibroblast-like morphology and very high proliferative capacity.[59] As the cells are used within 2 days after isolation from the bone tissue, they may well represent the three differentiation steps of osteoprogenitor cell, osteoblast and osteocyte. By contrast, the mixed cell cultures derived from bone that are generally used to represent "osteoblastic" cells, likely contain cells in various stages of differentiation. Therefore, changes in mechanosensitivity related to progressive cell differentiation cannot be studied in such cultures.

Using these immuno-separated cell populations, osteocytes were found to respond far stronger to fluid flow than osteoblasts, and these stronger than osteoprogenitor cells.[15-17,19] Pulsating fluid flow (PFF) with a mean shear stress of 0.5 Pa (5 dynes/cm^2) with cyclic variation of plus or minus 0.2 Pa at 5 Hz, rapidly stimulated the release of nitric oxide (NO) and prostaglandin E_2 and I_2 (PGE_2 and PGI_2) from osteocytes within minutes.[15,17] Osteoblasts showed less response, and osteoprogenitor cells (periosteal fibroblasts) still less. Intermittent hydrostatic compression (IHC) of 13,000 Pa peak pressure at 0.3 Hz (1 sec compression followed by 2 sec relaxation) needed more than 1 h application before prostaglandin production was increased, again more in osteocytes than osteoblasts, suggesting that mechanical stimulation via fluid flow is more effective than hydrostatic compression.[16] One hour treatment with PFF also induced a sustained release of PGE_2 from the osteocytes in the hour following PFF treatment.[17] This sustained PGE_2 release, continuing after PFF treatment had been stopped, could be ascribed to

the induction of prostaglandin G/H synthase-2 (or cyclo-oxygenase 2, COX-2) expression.[19] Again osteocytes were much more responsive than osteoblasts and osteoprogenitor cells, as only 15 min treatment with PFF increased COX-2 mRNA expression by 3-fold in osteocytes, but not in the other two cell populations.[19] Upregulation of COX-2, but not COX-1 by PFF had been shown earlier in a mixed population of mouse calvarial cells[60] and was also demonstrated in primary bone cells from elderly women,[61] while the expression of COX-1 and 2 in osteocytes and osteoblasts in intact rat bone has been documented.[62] The in vitro experiments on immuno-separated cells suggest that as bone cells mature, they increase their capacity to produce prostaglandins in response to fluid flow.[10] First, their immediate production of PGE_2, PGI_2 and probably $PGF_{2\alpha}$[60] in response to flow increases as they develop from osteoprogenitor cell via the osteoblastic stage into osteocytes. Second their capacity to increase expression of COX-2 in response to flow, and thereby to continue to produce PGE_2 even after the shear stress has stopped,[19] increases as they reach terminal differentiation. As induction of COX-2 is a crucial step in the induction of bone formation by mechanical loading in vivo[63] these results provide direct experimental support for the concept that osteocytes, the long-living terminal differentiation stage of osteoblasts, function as the "professional" mechanosensors in bone tissue.

Pulsating fluid flow also rapidly induced the release of NO in osteocytes, but not osteoprogenitor cells.[17] Rapid release of NO was also found when whole rat bone rudiments were mechanically strained in organ culture[18] and in human bone cells submitted to fluid flow.[64] In line with these in vitro observations, inhibition of NO production inhibited mechanically induced bone formation in animal studies.[65,66] NO is a ubiquitous messenger molecule for intercellular communication, involved in many tissue reactions where cells must collaborate and communicate with each other.[67] An interesting example is the adaptation of blood vessels to changes in blood flow. In blood vessels, enhanced blood flow as for instance during exercise, leads to widening of the vessel, to ensure a constant blood pressure. This response depends on the endothelial cells, which sense the increased blood flow, and produce intercellular messengers such as NO and prostaglandins. In response to these messengers, the smooth muscle cells around the vessel relax, to allow the vessel to increase in diameter.[51] The capacity of endothelial cells to produce NO in response to fluid flow is related to a specific enzyme, endothelial NO synthase or ecNOS. Interestingly, this enzyme was found in rat bone lining cells and osteocytes[68,69] and in cultured bone cells derived from human bone.[70] Treatment with pulsatile fluid flow increased the level of ecNOS RNA transcripts in the bone cell cultures,[70] a response also described in endothelial cells.[71,72] Enhanced production of prostaglandins is also a well described response of endothelial cells to fluid flow.[51,71] It seems therefore that endothelial cells and osteocytes posses a similar sensor system for fluid flow, and that both cell types are "professional" sensors of fluid flow. *Mutatis mutandis*, this is an indication that osteocytes sense bone strains via the (canalicular) fluid flow resulting from bone strains.

Mechanotransduction starts by the conversion of physical loading-derived stimuli into cellular signals. Several studies suggest that the attachment complex between intracellular actin cytoskeleton and extracellular matrix macromolecules, via integrins and CD44 receptors in the cell membrane, provides the site of mechanotransduction.[15,73-76] An important early response is influx of calcium ions, through mechanosensitive ion channels in the plasma membrane and release of calcium from internal stores.[54,55,75,77,78] The signal transduction pathway then involves protein kinase C and phospholipase A_2 to activate arachidonic acid production and PGE_2 release.[75] However, many other steps in the mechanosignaling cascade are still unknown, in osteocytes as well as other mechanosensory cells.

Cell Stretching: Technique, Cell Responses

To mimic the effect of physiological bone loading in monolayer cell cultures, several authors have used cell stretching, via deformation of the cell culture substratum[18,42,69,79-82] (see Burger and Veldhuijzen[83] for a review of the older literature). Stretch-loading by hypo-osmotic cell swelling was also used.[84] The results are generally in agreement with the studies using fluid

flow, including the high sensitivity of osteocytes to strain. Several different stretch devices have been used. Most devices apply stretch to a membrane or flexible culture dish on which the cells are cultured. The applied stretch can be uniaxial (the membrane is stretched in one direction) or biaxial (where the membrane is stretched in all directions simultaneously). In the latter case a circular membrane is usually used that is stretched in all directions. The disadvantage of such a system is that the applied strain is not homogeneous in size and direction. Other systems use four-point bending of plastic or glass coverslips on which cells are cultured.[26,85-87] See Brown[88] and Basso and Heersche[89] for reviews of the mechanical loading devices used to apply mechanical loads to cells in vitro. In addition, 3-dimensional collagen culture models were used to evaluate the response of fibroblasts or osteoblasts to stretch.[90-92] It was found that collagen fiber and cell alignment depended on the applied force. Many different responses to mechanical stretch have been reported. These include synthesis of active regulatory molecules (growth factors, hormones, etc.),[93,94] changes in matrix synthesis,[41,95] cell alignment,[44,45,96] and enzyme release.[42] However, the mechanisms involved in mechano-sensing are still ambiguous. Modulation of cytosolic free calcium,[97] stretch-sensitive ion channels,[87,98] or integrin-cytoskeletal interactions[73,99-101] have all been suggested to play a role.

The advantage of cell straining via the cell culture substratum is, that this technique allows to precisely determine the amount of cell strain that is applied, provided that the cell does not modulate it's attachment to the substratum during stretching. Cell strain in vitro can therefore be matched to bone tissue strain in vivo during exercise. Compared with physiological strains in bone, relatively high strains are necessary to evoke cell responses.[78,85] It should be noted that the application of mechanical strain in vitro is always accompanied by another mechanical stimulus; fluid flow. Stretch of the substrate will inevitably cause movement of the medium with respect to the substrate and thus fluid shear. Two independent studies concluded that the actual physical cell stimulus during substratum-mediated cell stretching is the flow of culture medium over the cell surface.[26,78] When this medium fluid flow was prevented, 10% cell stretch was needed to induce increase of cell calcium levels.[78] You et al[78] suggest that bone cell mechanotransduction may involve two distinct pathways relating to two different events. One is the mechanical adaptation of intact bone that occurs throughout life mediated by canalicular loading-induced fluid flow derived from small bone strains of the order of 0.1%. The other relates to fracture healing, when large cell deformations of the order of 10% can be expected. Such large cell deformations are also applied during DOG and in orthodontic tooth displacement, where rapid activation of osteoprogenitor cells occurs.[102] It seems therefore that osteoprogenitor cells are activated to express their osteoblastic phenotype when they experience large strains in biological processes related to rapid bone healing. However the more subtle effects of physiological bone loading seem to be mediated by fluid flow in the osteocyte network in intact bone.

Relation of Cellular Responses in Vitro with Mechanobiology of Bone in Vivo

After evaluating the effects of fluid shear stress and cell strectching in vitro, we will now return to the intact bone tissue. Can we link the cellular responses as observed in bone cell cultures to the behavior of bone tissue during adaptive remodeling or DOG? The former paragraphs have shown the intrinsic differences between these two processes. Here we will discuss the concept that fluid shear stress applied to bone cells in vitro mimics the canalicular fluid shear stress of osteocytes in remodeling bone. Stretching of bone cells in vitro however likely mimics the osteogenic stimulus in the fibrous central zone during DOG.

Fluid Shear Stress and Adaptive Bone Remodeling

We recently described a link between local strains around a remodeling (hemi-)osteon and the activation of osteoclasts and osteoblasts.[103] A finite element analysis of the deformation of the bone tissue around a progressing BMU showed opposite strain fields around the cutting and closing cone. These opposite strains appeared both under compressive and tensile axial

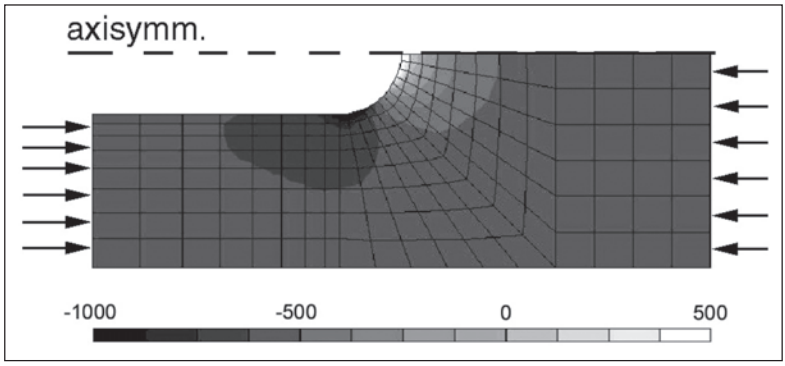

Figure 4. Volumetric strain within the bone matrix around the progressing end of an osteonic BMU at maximum loading during during the walking cycle. The direction of loading is indicated by arrows. A superficial area of volumetric expansion appears at the tip of the cutting cone. At the base of the (hemi-) cone an area of high volumetric compression appears. Values are microstrains. Figure taken from Burger et al, 2002, with permission.

loading of the remodeling piece of bone. Decreased strain was found in front of the cutting cone, just where resorption continues to proceed. Elevated strain however was found behind the cutting cone, where osteoblasts are active.[103] This observation suggested that the subsequent activation of osteoclasts and osteoblasts in a BMU could be regulated by the local strains in the surrounding bone. As these strains derive directly from the direction and magnitude of the daily loads, we have an explanation how a BMU can produce a (hemi-) osteon that is aligned to the prevailing mechanical loads of the piece of bone. In recent papers,[104,105] we subsequently calculated the canalicular fluid flow in the bone around cutting- and closing cone of a progressing BMU, using parameters for the poro-elasticity of cortical bone developed earlier.[106] These studies show that physiological loading produces stasis of extra-cellular fluid in front of the cutting cone of a progressing BMU, while enhanced extra-cellular fluid flow occurs around the closing cone[105] (Figs. 4 and 5). Thus, osteoclastic resorption occurs in the direction of nonstressed osteocytes, while osteoblastic bone formation occurs where osteocytes receive enhanced fluid shear stress. Together these observations explain the alignment of the new (hemi-)osteon along the direction of loading, as well as the refilling of the resorption tunnel or trench by osteoblasts.

In vitro, fluid shear stress rapidly induces production of NO and prostaglandins (PG) by osteocytes.[15,17] Both molecules are involved in maintaining the viability of the producing cell, a form of autocrine regulation.[107] Lack of NO and PG production by cells has been related to their programmed cell death or apoptosis.[107,108] We have therefore proposed that the production of NO and PG by shear stressed osteocytes serves to keep them vital.[104] Absence of fluid shear stress in front of the cutting cone will therefore lead to osteocyte apoptosis, as a result of insufficient production of NO and PG. Apoptotic osteocytes have been shown to attract osteoclasts, which act as the macrophages of bone by phagocytosing the apoptotic osteocytes while resorbing their extracellular matrix.[109-113] The progression of a BMU along the direction of loading, and therefore the orientation of the new (hemi-)osteon that is formed, can thus be explained. The magnitude of the daily loads however will determine the magnitude of the fluid shear stress around the closing cone, which will determine the amount of activation of osteoblasts to refill the closing cone and thereby the amount of bone mass.

Mechanical Strain and DOG

Interfragmentary movement after bone fracture and distraction in DOG cause much larger tissue strains (about 2% to 10%) then normal loading in bone does. These strains are largest in

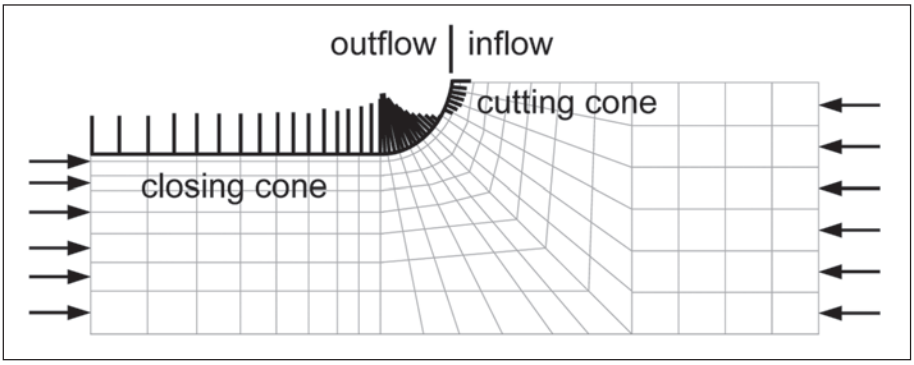

Figure 5. Fluid flow pattern in a remodeling osteon at maximum load during a walking cycle. Volumetric expansion leads to influx of canalicular fluid at the tip of the cone. At the base of the cone, high volumetric compression produces high efflux of canalicular fluid. Figure taken from Burger et al, 2002, with permission.

the soft callus tissue. In the callus a mix of cell types is present, fibroblast-like cells, osteoblasts and chondrocytes.[36] Fibroblasts as well as bone cells and chondrocytes have been shown to be mechano-sensitive, although the responses depend on cell-type. Responses to strain include synthesis of growth factors, cytokines and hormones,[93,114,115] changes in matrix synthesis,[41,42,45,95] and cell alignment.[44,45,90] Tensile forces have been shown to stimulate differentiation of mesenchymal cells towards a fibroblastic phenotype.[45] Hence, mechanical stimuli affect the preferential differentiation towards certain tissue types and may play an important role in the control of tissue development. Pauwels[116] suggested preferential differentiation of tissue to occur on the basis of the amount and type of mechanical deformation. This idea has been refined further and translated into quantitative models.[117-119] Based on the assumption that cells differentiate into various phenotypes in response to biophysical stimuli, the models successfully predict patterns of fracture healing.[118,119] These theoretical models confirmed the feasibility of mechanical controlled tissue differentiation. Several experimental findings also support this hypothesis. For instance, a relationship between mechanical loading and BMP expression was found in vivo.[34,120,121] BMPs are thought to be involved in controlling the differentiation of cartilage and bone. In addition, Sato et al[122] found that the expression of the mRNA for various bone matrix proteins was stimulated by distraction of the callus and that mechanical tension-stress modulated cell shape and phenotype.

The mechanical context not only affects tissue growth and differentiation, but also forms the boundary conditions in which various tissue types can thrive. Too large strains, for instance, inhibit or even prevent ossification[35,37,123] and even larger strains are also deleterious for fibrous tissue.[40] High strains applied to osteoblasts or fibroblasts in culture cause them to align away from the principal strain direction,[44,124] indicating that they try to avoid these large deformations. The exact load on the cells is not known because of the complex viscoelastic nature of the tissue. The magnitude and distribution of strain over the distraction area depend on several factors, which change over time. These factors include the relative stiffness of the local tissue, the distraction rate, the stiffness of the fixator and the amount of loading. If areas within the soft tissue are sufficiently stable, whether by modulated tissue stiffness or due to external conditions, ossification occurs.

Mechanical Stimuli and Bone Engineering

We have argued that adaptive bone remodeling and osteogenesis are two distinct processes. Osteocytes are believed to play a role as mechanosensors in adult bone. In fracture healing and DOG new bone is formed via a cascade of events, akin to embryonic bone development, which

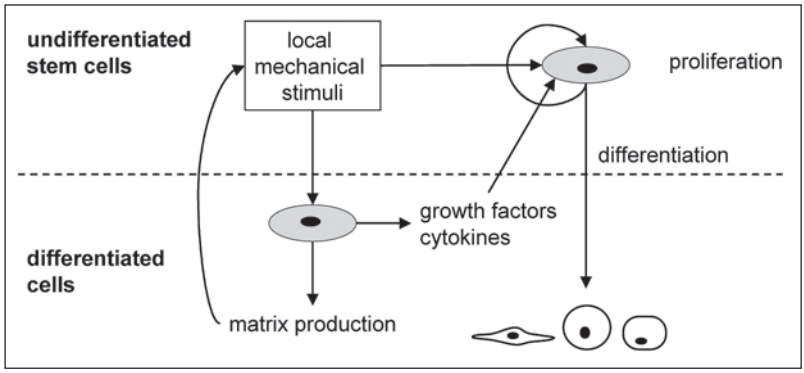

Figure 6. Tissue differentiation in fracture healing and DOG is affected by many factors. Here is depicted a simplified scheme of how mechanical factors can play a role in this process. Mechanical load stimulates cells to produce cytokines and growth factors, which affect cell differentiation and proliferation. It may also directly stimulate the differentiation of a cell towards a certain phenotype. Mechanical load also stimulates matrix production, which in turn affects the tissue properties and thereby the mechanical loads on the cells.

is influenced by mechanical loading. We are a long way from understanding the complex multifactorial process of fracture healing and DOG. Also the precise role of mechanical factors is still poorly understood. On the one hand, the gradual stiffening and stabilization of the tissue is required for ossification. On the other hand, tissue deformation plays a role in the stimulation of cell proliferation, matrix production and the release of various growth factors. The interaction of these factors result in the progressive development of the tissue (Fig. 6). Presently, in vitro experiments are only able to mimic aspects of this process. In vivo bone engineering is, however, a very promising practice. Several factors determining the success of bone formation can be identified. These factors include the release of growth factors and cytokines, the availability of a population of mesenchymal stem cells, good blood supply and appropriate mechanical conditions. The future of bone engineering lies in the manipulation of these factors. The possibility of treatments using growth factors, such as BMP-2, has already been studied extensively.[125] In DOG, the appropriate cellular and chemical environment is created by the callus tissue and the existing bone. Bone development is further stimulated by manipulation of mechanical factors.

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Part II
Bone Healing

Chapter 5

Bone Healing and Failure

Ashley R. Poynton and Joseph M. Lane

Introduction

Fracture healing is a complex process resulting from the interaction of cellular elements that are activated and controlled by an array of proinflammatory cytokines and signaling proteins. This process is both temporal and spatial in nature and results in the formation of new bone that has properties very similar to the original prefracture structure. In children, the repair process simulates regeneration and results in bone that is both structurally and mechanically identical to native bone. This is a unique healing process compared to elsewhere in the body where healing results in scar formation. The ability of the skeleton to repair itself in this manner is a vital survival mechanism. Despite this ability, the repair process may fail. There are many reasons why this may occur, some are obvious and well understood while little is known about others, particularly at a cellular level.

To understand the mechanisms of failure of fracture healing one must have a comprehensive knowledge of the fracture repair process. To achieve this, animal models have been developed that allow the repair process to be studied in a detailed and reproducible manner.[1,2] The series of biological events, and their physical and chemical controlling factors, that lead to successful fracture healing have become more defined. However, many questions remain concerning the precise mechanisms of intercellular signaling and related issues.

The purpose of this chapter is to outline the current available knowledge on fracture repair and discuss the factors leading to its failure.

Overview of the Bone Repair Process

Prior to any discussion of fracture healing it is important to make the distinction between primary and secondary healing as most of the data on fracture healing pertains to the latter. Primary fracture healing can be defined as primary cortical healing which involves a direct attempt by the cortex to reestablish itself. This type of healing occurs when there is precise anatomical reduction of the fracture followed by rigid internal fixation. The key component here is very low inter-fragmentary strain.[3-5] When these conditions exist the formation of discrete remodeling units known as cutting cones occurs.[3,4] Osteoclasts on one side of the fracture begin to resorb bone and form tunnels that cut across the fracture line. This establishes a new Haversian system and allows blood vessel penetration. Endothelial and perivascular mesenchymal cells follow and become the osteoprogenitor cells for osteoblasts. In this method of bone repair endochondral ossification is absent and there is little or no callus formation callus. It is seen only with open reduction and rigid internal plate and screw fixation, and naturally for stress fractures.[3,4]

Secondary fracture healing is a far more common process of bone repair and has been studied in great detail in animal fracture models.[1-3]

The key tissue in secondary bone repair is the periosteum which contains committed osteoprogenitor cells and uncommitted undifferentiated mesenchymal cells both of which contribute to the process.[3] The response of the periosteum to injury is a fundamental one that

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occurs beneath the periosteum but its ability to cross the fracture gap is limited.[4] This response is enhanced by motion and increases in interfragmentary strain.[4,5] The response is retarded by rigid internal fixation.[4,5] McKibbin, in his classical article on fracture healing describes four types of fracture repair.[4] The primary callus response is: periosteal, rapid, very tolerant to both motion and rigidity, with limited ability to bridge gaps. External bridging callus forms relatively rapidly, has considerable ability to bridge gaps, is tolerant to movement, but inhibited by rigidity. Late medullary, or internal callus, forms at a slower rate, is best at bridging gaps, and is moderately tolerant to both motion and rigidity. Primary cortical repair, as outlined above, is the slowest form of fracture healing, and is unable to bridge gaps, completely intolerant to motion and will only occur in the presence of total rigidity.[4]

Two types of bone formation occur during fracture healing and will be discussed in greater detail below: intramembraneous ossification forms bone directly without first forming cartilage, resulting in "hard callus" formation, while endochondral ossification involves bone formation via a cartilage anlage.

The bone marrow response to fracture is rapid, within a few hours there is loss of normal architecture of the bone marrow elements, disappearance of blood vessels in the region adjacent to the fracture clot and a reorganization of the cellular complement of the bone marrow into regions of high and low cellular density. Within 24 hours of fracture, polymorphic cells that have transformed form endothelial cells in the cellular areas of the bone marrow express osteoblastic phenotype and begin to form bone. It would appear from this that the bone marrow has an important function in the early phase of fracture healing.[5]

The external soft tissue is the final vital component for successful fracture healing. Rapid cellular activity occurs early in the repair process and results in the development of an early bridging external callus. This response is stimulated by motion and its prime objective is to achieve early fracture stability to enable the repair process to continue unimpeded.[5] This process may be negatively influenced by significant soft tissue trauma, excessive soft tissue stripping during surgery, and rigid internal fixation. The external "soft callus" formed subsequently becomes bone through the process of endochondral ossification.[3]

The Sequence of Fracture Healing

Secondary fracture healing thus involves a combination of intramembraneous and endochondral ossification. However the formation of bone via these pathways is both spatially and temporally different. The overall sequence of events in fracture healing includes, the immediate response to injury, intramembraneous bone formation, chondrogenesis, endochondral bone formation, and bone remodeling.

Endochondral ossification occurs through a series of steps as follows: Initial or inflammatory stage, angiogenesis and initial cartilage formation, cartilage calcification, cartilage removal and bone formation, and finally, bone remodeling.

Bone formation through the intramembraneous ossification pathway occurs earlier in the fracture repair process from committed osteoprogenitor cells.

The various stages of fracture healing result from a complex interplay of cellular components with an array of pro-inflammatory cytokines and growth factors. The earliest event in the healing process is the formation of a fracture hematoma and it would appear that this is an important source of signaling molecules that have the capacity to initiate the cascades of cellular events that are critical to fracture healing.[3,7] The first detectable inflammatory factors following fracture include platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β) released by degranulating platelets.[7,8] This is closely followed by infiltrating macrophages and other inflammatory cells that secrete fibroblast growth factor (FGF) and additional PDGF and TGF-β.[7,8] The pro-imflammatory cytokines interleukin-1 and -6 (IL-1 and IL-6) are thought to play an important role also and are presumably secreted at this time.[8,9] Their exact role remains to be elucidated. Peak expression of both IL-2 and IL-6 occurs on day 1 post fracture and declines rapidly until day 3 when levels are barely detectable.[8,10,11] Therefore,

in the acute phase following a fracture, a series of inflammatory mediators are present and initiate the repair process at the various sites, namely, periosteum, bone marrow, and surrounding soft tissue. These factors are important in regulating cell proliferation, differentiation of committed mesenchymal stem cells, chemotaxis and angiogenesis.[3,10,11]

Angiogenesis is an essential component of successful bone repair and numerous angiogenic factors have been identified that have roles in both normal and pathological processes.[12] Wound angiogenesis is initiated by the early rapid release of stored growth factors such as FGF-2 (basic FGF) and several studies have demonstrated its role in bone healing.[13-16] Endothelial cell growth factor (ECGF) has been shown to enhance angiogenesis and osteogenesis in a rat model.[17]

Vascular endothelial growth factor (VEGF) has been widely studied and has been shown to be an important stimulator of angiogenesis. VEGF is produced by osteoblastic cell lines in vitro,[18] a process that is stimulated by insulin-like growth factor I (IGF-I)[19] and bone morphogenetic proteins (BMPs).[20] Sustained delivery of recombinant VEGF in rabbits stimulated neovascularization and DNA synthesis in endothelial cells.[21] Gene therapy techniques leading to VEGF expression has resulted in local neoangiogenesis in mice[22] and revascularization of avascular muscle in a rabbit model.[23]

Vascular invasion brings pericytes that function as mesenchymal stem cells that also produce various growth factors. There is evidence to show that following fracture, periosteal microvessel endothelial cells and pericytes increase in number and are transformed to mesenchymal stem cells that subsequently develop into chondroblasts.[6]

As outlined above the bone marrow is the first local tissue to react to a fracture and it has been shown that within 24 hours of injury that cells in the areas of high cellular density undergo differentiation and take on an osteoblastic phenotype. In addition to changes in the bone marrow, osteoblasts lining the cortical bone surface are activated, and adjacent periosteal cambium derived preosteoblasts divide and begin differentiating. These resident and differentiating osteoblasts lay down new bone via an intramembraneous pathway and form woven bone (hard callus) adjacent to the fracture site. This process of early intramembraneous ossification can be detected in experimental models of fracture healing by studying the expression of osteoclacin and type I collagen which indicate osteoblastic activity.[11] Expression occurs as early as 24 hours post injury and continues for the first 7 to 10 days of fracture healing, as evident with the formation of woven bone opposed to the cortex within a few millimeters from the site of the fracture on either side.[3,11] Proliferation in this region disappears by day 14 however osteoblastic activity continues beyond this time point

In secondary fracture healing, however, endochondral ossification provides the most important source of new bone formation. This process occurs in various stages, the acute inflammatory stage, as outlined, is the important first step. Mesenchymal cell proliferation can be detected as early as day 3 post fracture and remains high for several days.[11] The subsequent chondrogenesis by these mesenchymal cells and the proliferation of new chondrocytes continues from day 7 to day 21, leading to the formation of a cartilaginous callus (soft callus) that bridges the fracture site and provides initial stabilization. This chondrogenic phase is hallmarked by the expression of collagen types II and X which peaks at day 7.[11] As endochondral ossification progresses type II collagen expression subsides more rapidly than type X collagen expression. The sequence of events that culminates in endochondral ossification is very similar to the events in the proliferative zone of the growth plate. By day 9 post fracture the chondrocytes of the soft callus adjacent to the woven bone of the hard callus begin to elongate, form elaborate vesicular structures and turn off type II collagen and aggrecan expression characteristic of maturing chondrocytes.[11] These chondrocytes undergo hypertrophy and display the characteristic expression of type X collagen typical of hypertrophic chondrocytes of the growth plate. In addition, these cells produce and release neutral proteases that begin breaking down the cartilaginous matrix, including proteoglycans, in preparation for calcification.[11] The mineralization of soft callus proceeds in a spatially organized manner with hypertrophy of chondrocytes and calcification beginning at the interface between the maturing cartilage and newly formed woven

bone. Following closely after hypertrophic chondrocyte mineralization of the matrix is angiogenesis, and the formation of new vascular structures which infiltrate along with accompanying osteoblasts. The resident hypertrophic chondrocytes begin to undergo programmed cell death, or apoptosis, and the mineralized matrix is replaced by woven bone laid down by the incoming osteoblasts.[3] This new bone will subsequently be remodeled by coordinated osteoblastic/osteoclastic activity in response to the mechanical stresses peculiar for that bone.

As outlined previously, the mechanical environment that a healing fracture is exposed to significantly influences the differentiation of mesenchymal tissue into bone, cartilage, or fibrous tissue.[5] Cyclic motion resulting in shear stresses cause cell proliferation and the production of a large callus in the early phases of fracture healing. Low stress and strain at the fracture site is associated with direct intramembraneous bone formation. Low to moderate tensile strain and hydrostatic tensile stress may stimulate intramembraneous ossification. This is dependent on vascularity, with low vascularity promoting chondrogenesis. Hydrostatic compressive forces stimulate chondrogenesis whereas high tensile strain results in a net production of fibrous tissue. Tensile strain with a superimposed hydrostatic compressive stress will stimulate the development of fibrocartilage.[5]

The process of fracture healing is well described and universally accepted. The precise mechanisms whereby the different cellular events are initiated and controlled have been investigated in detail. While not fully understood, these processes involve a complex series of inflammatory mediators, growth factors, and signaling proteins, many of which have been isolated. The temporal expression of these factors in fracture healing has become clearer.

The Role of Pro-Inflammatory Cytokines in Fracture Healing

The initial inflammatory phase of fracture healing is a vital stage in the repair process. Influx of macrophages and other inflammatory cells leads to a secretion of an array of pro-inflammatory cytokines. IL-1 and IL-6 are accepted as being ubiquitous to tissue injury, including fracture healing, other cytokines such as the TNF family are also important. Both IL-1 and TNF-α have been shown to have important regulatory roles in bone remodeling and homeostasis.[24-29] Through a variety of mechanisms these factors regulate osteoclastic activity.[30,31] TNF-α participates in the cellular response to trauma by inducing acute phase proteins and increasing adhesiveness of leukocytes on vascular endothelial surfaces. It is a primary mediator of immune responses. A novel member of the TNF receptor family, osteoprotegrin (OPG), and its specific ligand receptor activator of NK-κB ligand (RANKL) in conjunction with macrophage colony stimulating factor (M-CSF) have been shown to be key regulators in the control of bone mass through their modulation of the bone resorptive cycle.[32-34] OPG is a secreted soluble TNF receptor family member that binds to RANKL and prevents it from stimulating osteoclastogenesis. RANKL up-regulation is an important component of the bone repair process. It is responsible for osteoclast mediated activities including; removal of devascularized bone, any remodeling that may occur during fracture repair, and ultimately for callus remodeling along the lines of stress, once union is achieved.

The role of these factors in the response to skeletal injury has become somewhat clearer in recent times.

Kon et al[11] have studied the expression of IL-1 (α and β), TNF-α, and their receptors, and, OPG, RANKL, and M-CSF. OPG was expressed in unfractured bones but had two distinct phases of increased expression following fracture. The first phase was within 24 hours of fracture and the second occurred at day 7 which is the time of peak cartilage production. RANKL was almost undetectable in unfractured bones but was strongly induced throughout the period of fracture healing with maximum expression on days 3 and 14. M-CSF followed the temporal profile of RANKL but had higher expression in unfractured bones.

TNF-α, IL-1α and IL-1β all showed peaks in expression within the first 24 hours following fracture, depressed levels during cartilage formation, and, a second increase in expression on days 21 and 28 when bone remodeling was initiated. TNF-α and IL-1α expression was seen in

macrophages and inflammatory cells in the early phases however both were also expressed by mesenchymal and osteoblastic cells later during the healing process. TNF-α expression was also detected at very high levels in hypertrophic chondrocytes. TNF-α and IL-1RII receptors showed identical expression as their ligands. IL-1-RI receptors were expressed only during initial inflammatory phase between days 1 and 3.

This important work has gone a long way to clarify the temporal roles of the various cytokines and their receptors in fracture healing. It would appear therefore that strong induction of TNF-α and IL-1 occurs within 24 hours following injury and is accompanied by a large influx of immune cells to the fracture site. RANKL level peaks, and is maintained, slightly later than the immediate inflammatory burst. This may mean that both TNF-α and IL-1 are involved in its induction. Its production may also be stimulated by local osteoblasts and stromal cells at the fracture site. The finding that TNF-α is not only synthesized by inflammatory cells but also by mesenchymal cells, osteoblasts and hypertrophic chondrocytes would suggest that it has different roles at different times during fracture healing. TNF-α may potentially regulate the initiation of fracture healing including mesenchymal cell proliferation and differentiation in the periosteum. The increasing expression of the ligands and receptors for both TNF-α and IL-1 on day 21 and day 28 following fracture suggests that these cytokines are most likely involved in promoting bone remodeling at later times during bone healing. TNF-α may have a potential autocrine regulator of chondrocyte maturation or apoptosis.[11]

This study[11] has also shown for the first time that OPG, RANKL and M-CSF function during endochondral resorption in fracture healing in a similar manner to that which is observed during bone remodeling.[35,36] Given the expression of OPG at moderately high levels in unfractured bones and the sharp peak in expression during the phase of maximal cartilage formation and then decline as endochondral resorption progresses it is reasonable to assume that it has a role as a negative regulator of cells involved in calcified tissue resorption. RANKL is expressed at very low levels in intact bones but shows a dramatic up-regulation immediately after fracture in conjunction with M-CSF. Both remain elevated throughout the entire fracture healing process. This would suggest that the expression of these cytokines is tightly coordinated in their regulation of endochondral resorption. It is also apparent that both TNF-α and IL-l play important roles in bone remodeling. However, given that low levels are expressed during the period of endochondral resorption these factors may function differently here as compared to their vastly increased expression from day 21 to 28 after fracture. This would suggest an important role in the remodeling of woven bone.

Growth Factor Regulation of Fracture Repair

The majority of work carried out to date on the role of growth factors in bone repair has concentrated on the following: acidic and basic fibroblast growth factor (FGF-1 and FGF-2), PDGF and the TGF-β superfamily that includes; multiple isoforms of TGF-βs, BMPs, specifically BMP-2, -3, -4, and -7, and growth and differentiation factors (GDFs).[3,6,7,10,36-38] Research has also centered on the expression of receptors to these growth factors during fracture healing, growth factor signal effectors (particularly related to the TGF-β superfamily) and growth factor inhibitors.[11, 40]

Fibroblast Growth Factors

The FGFs are a family of structurally related polypeptides characterized by a high affinity to heparin. The most common in normal adult tissue are acidic FGF and basic FGF (FGF-1 and FGF-2). FGFs are abundant in bone extracellular matrix and transduce signals to the cytoplasm of cells via a family of transmembrane receptors with tyrosine kinase activity. Both FGF-1 and -2 can be detected in the early stages of fracture repair in the granulation tissue at the fracture site, macrophages and other inflammatory cells being the most likely source.[7,41] Later FGFs are expressed by mesenchymal cells, maturing chondrocytes and osteoblasts and have been shown to enhance TGF-β expression in osteoblastic cells.[7]

The FGFs have a mitogenic effect on fibroblasts, chondrocytes and osteoblasts and this is thought to be their primary role.[42,43] FGF-1 appears to primarily affect chondrocytes as its peak expression correlates with peak chondrogenesis. FGF-1 has been shown to increase callus volume in a rat femur segmental defect model.[41] FGF-2 has greater mitogenic and angiogenic potency than FGF-1. It is thought to have a role in chondrocyte maturation and endochondral bone formation. It may also have a role in bone resorption. FGF-2 treatment has been shown to increase callus size in canine and diabetic rat models.[44,45] Other studies have failed to show an effect, however the timing of FGF treatment would appear to be critical. If FGFs are administered within the first 24 hours following fracture then an increase in callus size occurs.[44-46] When FGF-2 was applied to fresh rabbit fibular osteotomies it resulted in the stimulation of callus formation, increased bone formation, and earlier restoration of mechanical strength at the fracture site.[46]

Insulin-Like Growth Factor

The IGF family consists of two polypeptides known as IGF-I and IGF-II both of which have been shown to promote cellular proliferation and matrix synthesis by osteoblasts and chrondroblasts. IGF-II is 50-100 times more abundant in human bone compared to IGF-I however IGF-I is 4 to 7 times more potent and is more dependent on growth hormone. IGF-I may participate in endochondral fracture repair. Studies suggest that it accelerates the repair of intramembraneous bone defects.[47] IGF-II may also play a similar role in fracture healing, however, further studies are required to determine the exact role of these factors.

Platelet-Derived Growth Factor

PDGF is a dimeric molecule consisting of disulfide bonded A- and B- polypeptide chains. It can exist either as a homodimeric (PDGF-AA or -BB) or a heterodimeric form (PDGF-AB). The different PDGF isoforms exert their effect on target cells by binding with different specificity to two structurally related protein tyrosine kinase receptors denoted as the α and β receptors.[48] PDGF is initially released by degranulating platelets in the fracture hematoma and may be important in promoting chemotaxis.[49,50] Homodimeric PDGF-AA predominates in the early phases and is expressed by macrophages in addition to that released by platelets. This is also detectable in both early and mature chondrocytes.[7] PDGF-BB is the primary isotype seen in osteoblasts.[7] PDGF has been shown to increase callus density and mechanical strength in a rabbit tibial osteotomy model.[51] Histologically PDGF treated osteotomies revealed a more advanced stage of osteogenic differentiation as compared to controls. PDGF may also have a role in bone resorption. Zhang et al[52] have shown the PDGF promotes adult osteoclast bone resorption directly through the PDGF-β receptor. A dose dependent relationship between volume of Howship's lacuna and quantity of resorption pits was established. PDGF influences expression of IL-6, a key cytokine that induces osteoclast recruitment. This role is mediated through the activation of transcription factors through interaction between PDGF and PDGF-β receptor located on osteoblasts.

Transforming Growth Factor β Superfamily

The TGF-β superfamily is a large group of structurally related polypeptides that includes multiple isoforms of TGF-βs, BMPs and GDFs. Several members of the subfamilies have been shown to promote the differentiation of mesenchymal stem cells into the osteogenic and chondrogenic lineages. A number of BMPs have been shown to promote bone repair both in controlled animal models of fracture healing, segmental defects and spinal fusion, and in clinical trials.[53-76] The prerequisite for their successful action is the presence of responding cells. The role of these factors in fracture healing is becoming increasingly understood. Bostrom et al[77] were one of the first investigators to describe the temporal expression of BMP-2 and -4 during fracture healing.

Recently Cho et al[10] have characterized the differential temporal expression of members of the TGF-β superfamily during murine fracture healing. This study also looked at various cytokines (IL-6, IL-1β), cartilaginous matrix proteins, including type II collagen and osseous matrix proteins, including osteoclacin. The members of the TGF-β superfamily analyzed included: BMP-2, -3, -4, -5, -6, -7, -8, GDF-1, -3, -5, -6, -8, -9, -10 and TGF-β1, -β2, -β3. The findings of this study were as follows; Peak expression of IL-1β and IL-6 was seen 1 day following fracture after which these cytokines showed a rapid decline until day 3 to near undetectable levels. Peak expression of collagens type II and X occurred on day 7 and characterized the chondrogenic phase. As endochondral ossification progressed type II collagen expression subsided. Expression of type X collagen was slower to regress resulting in a 4.7 fold difference between these at day 14. Osteogenesis was observed over the whole period of fracture healing, reflected by increasing expression of type I collagen and osteoclacin (OC) beginning within 24 hours of injury. Maximal osteogenic differentiation, using these criteria, was seen between days 14 and 21.

The temporal expression of members of the TGF-β superfamily in relation to the phases of fracture repair were as outline below; BMP-2 was the earliest to be expressed and also showed a second elevation late during the period of peak osteogenesis. In contrast, BMP-4, -7, -8, and -3 showed a clear restriction in expression to the osteogenic period between days 14 and 21. BMP-5 and -6 were induced at the onset of injury and were elevated throughout the healing period.

The GDFs showed a more restrictive expression pattern with GDF-6 specifically induced on day 1 with little expression beyond this time point. GDF-5 was restricted to day 7 and day 14 when the chondrogenic phase was most pronounced. GDF-1 was also restricted to the chondrogenic phase though levels of expression were very low. GDF-3, -6, and -9 were not detected at any time.

Analysis of TGFs showed that TGF-β1 was expressed at very high levels in unfractured bones and showed a sharp rise on day 1 following fracture returning rapidly to baseline levels. TGF-β2 and TGF-β3 were only highly expressed following fracture and were not detected to any extent in unfractured bones. Both showed peak expression within the chondrogenic period however TGF-β3 remained elevated approximately 1 week more than TGF-β2 (until 21 days).

A summary of the temporal expression of the TGF-β superfamily found in this study is as follows: TGF-β1, BMP-2 and GDF-8 during the inflammatory phase, GDF-5, TGF-β2 and TGF-β3 during the chondrogenic phase, and BMP-3a, -4, -7, -8 during the osteogenic phase.

The authors[10] went on to determine the relative importance of each of these growth factors in terms of total expression over the time course of fracture healing, and the relative basal to peak levels of expression for the time point having the highest fold induction. In general it was found that those factors showing the highest relative peak levels of expression also had the greatest levels of overall expression. Among these, TGF-β1 was the highest followed by BMP-3, -5, TGF-β3 and GDF-10. Both GDF-5 and -8 had undetectable basal levels of expression and showed the lowest levels of induced expression but because of their near absence of basal expression in intact bone these factors showed the greatest fold up-regulation.

It is interesting that molecules that are structurally very similar have very different roles in fracture healing. An example of this is BMP-2 and BMP-4 which share high sequence similarity but are expressed differently during the bone repair process. Early induction of BMP-2 occurs on day 1 following fracture and is sustained until day 21. BMP-4 however reaches its peak expression during osteogenesis. Given this, it is possible that BMP-2 may serve as a trigger in the fracture healing cascade and regulates the subsequent expression of other BMPs. It has been shown that BMP-2 up-regulates the expression of BMP-3 and -4 in fetal rat calvarial osteoblasts and places the expression of these genes downstream of BMP-2 during osteoblastic differentiation.[78] It would appear that the coordinated expression of multiple BMPs during fracture healing is an important concept in bone repair, with BMP-2 being a possible trigger to the cascade.

The specific roles of each of the other members of the TGF-β superfamily are becoming clearer. It has been shown that BMP-3 can induce ectopic endochondral ossification in rats.[79] It may also have a role in chondrogenesis gives its ability to induce chondrogenic phenotypic expression in various cells.[80-82] Recent data suggests that BMP-3 may be an antagonistic regulator of osteogenic BMPs.[83,84] Cho et al[10] found BMP-3 to be maximally expressed during the period of peak osteogenesis and suggested that it may have a regulatory function here.

GDF10 (BMP-3b) is closely related to BMP-3 and is up-regulated on day 7 and 21 following fracture, it may have a role in both intramembraneous ossification and chondrogenesis.[10] BMP-5 and -6 show a strong and persistent expression during the first 3 weeks of fracture healing.[10] Developmental studies have shown roles for these factors in the regulation of mesenchymal condensation and endochondral progression and in the stimulation of chondrocyte maturation.[85-90] BMP-6 appears to be an autocrine factor initiating chondrocyte maturation, a role it may share with BMP-2.[91] BMP-7 and -8 (OP-I and OP-2) are similar in structure. Both of these factors show a high restricted expression pattern during fracture healing that parallels the osteogenic phase of fracture healing at the time when bone formation is maximal and resorption of calcified cartilage is taking place.[10]

GDF expression during fracture healing has been outlined above. It would appear that GDF-5 is important in chrondrogenesis during fracture repair as its expression is restricted to this phase.[10] This correlates with developmental studies,[91] and studies that have shown potent chondrogenesis with ectopic implantation of GDF-5.[92] Restricted GDF-8 expression during day 1 of fracture healing implies its presence in primitive mesenchymal stem cells and its potential role as an autocrine inhibitor of myogenic lineage differentiation.[10]

The expression of TGF-β in fracture healing is well documented.[10,38,94-96] These factors are produced by a variety of cells including osteocytes, osteoblasts and chondrocytes. Cho et al[10] using multiprobe ribonuclease protection assay (RPA) were able to compare the relative expressions of several of the TGF-β isoforms. It would appear the TGF-β2 and -β3 function during chondrogenesis and play more important roles than TGF-β1.

The actions of the BMPs are mediated through a heteromeric receptor complex of type I (BMPR-IA, or -IB or ALK-2/ActR-I), and type II (BMPR-II, ActR-II or ActR-IIB) receptors.[97,98] Once activated, the receptor complex transduces signals intracellularly through activation of specific Smad proteins[97-99] which form heteromeric complexes that accumulate in the nucleus where they participate in regulation of gene expression.[97] The Smad proteins can be subdivided in three groups: the signal transducing receptor regulated Smads (R-Smads, e.g., Smad1, Smad2, Smad3, Smad5 and Smad8), the common mediator Smads (coSmads such as Smad4) and the inhibitory Smads (I-Smads, e.g., Smad6 and Smad7). Whereas Smad2 and Smad3 function in the signaling by TGF-β and activin, Smad1, Smad5 and Smad8 are involved in BMP-signaling.[97-99]

The temporal and spatial activation of specific Smad proteins during fracture healing requires further investigation. This may give an even more accurate picture of the sequential importance of the various growth factors as detection of activated Smad indicates a definite effect of a growth factor in terms of altering gene expression.

The role of growth factor inhibitors in fracture repair remains unclear. However, inhibitors such as noggin appear to have a role in the regulation of callus formation.[40] The importance of growth factor inhibitors in the pathogenesis of fracture nonunion is unknown at present.

Failure of the Bone Repair Process

Despite considerable advances in our knowledge of the biological mechanisms of fracture healing, delayed union and nonunion remain a significant clinical problem in certain patient groups. While the precise mechanisms of bone repair failure at a cellular level remain largely undetermined many of the factors that contribute to this failure are well recognized and understood. These can be broadly categorized into general systemic factors and local factors pertaining to the fracture site itself.

The general factors that influence fracture healing are related to the systemic status of the patient and exogenous systemic influences. These include age, nutritional status, anemia, diabetes, hormonal deficiency, drugs (specifically corticosteroids and nonsteroidal anti-inflammatory drugs), smoking and alcohol abuse.

The influence of age on fracture healing is a well recognized phenomenon. The rate of fracture healing in children is vastly superior to that of adults and may be related to periosteal cellularity and vascularity[100,101] and a decline in cellular response to growth factors with age.[4]

For successful healing, particularly of long bone fractures, adequate nutrition is required including adequate intake of calcium and phosphorous. This is particularly relevant it the elderly population. Delays in callus formation occur with calcium and phosphorous deficiencies due to defective mineralization of the callus matrix, an extreme example of this is a Looser's zone in osteomalacia which is unmineralized osteoid. Protein deficiency has also been shown to affect the mechanical properties of fracture callus.[102]

Anemia has a significant effect on fracture healing with a 33% nonunion rate demonstrated in anemic rats.[103] Deficient mineralization occurs probably secondary to diminished oxygen tension and results in decreases callus formation and reduced callus strength.

The effects of diabetes mellitus on fracture healing are profound and are related to a multitude of factors related to nutritional, neurogenic, vascular and cellular causes.[100]

The most common hormone deficiency to affect fracture healing is estrogen. Post menopausal women have a higher nonunion rate than men.[100,104] The effects of osteoporosis on fracture healing have been demonstrated in an ovariectomized calcium deficient rat model. A 40% reduction in fracture callus cross-sectional area and a 23% reduction in bone mineral density in healing femurs were observed. Callus biomechanical parameters were also significantly reduced. Histomorphological analysis revealed a delay in fracture callus healing with poor development of mature bone.[104] However, others have found that although fracture healing is delayed in oophorectomized rats, it returns to near normal with the introduction of estrogen replacement, despite the presence of established osteoporosis.[106] This may suggest that the delay in fracture healing observed is related to the oophorectomy model rather that to osteoporosis per se.

The effect of systemic corticosteroids on fracture healing may be significant[107] and is primarily related to the fact that they inhibit differentiation of osteoblasts from mesenchymal cells[108] and may exert effects on callus tissue by altering the expression of local growth factors.[109]

Nonsteroidal anti-inflammatory drugs have been shown to delay fracture healing in both clinical series[110] and animal models.[111,112] The mechanism of action is thought to be related to inhibition of prostaglandins resulting in delayed ossification by diminishing regional blood flow or hindering primitive osteoblasts. More recently, selective COX-2 nonsteroidal anti-inflammatory drugs have been shown to impair fracture healing.[113]

The detrimental effect of smoking on fracture healing is well documented.[114,115] Smoking inhibits cellular proliferation during wound healing and promotes vasoconstriction.[116,117] Nicotine is thought to be the most potent mediator on the healing process via its vasoconstrictive effects, carbon monoxide may also have a detrimental effect.[117,118] Significant reduction in callus formation and callus mechanical properties secondary to nicotine exposure have been demonstrated in animal fracture models.[119] Chronic alcohol consumption has a detrimental effect on the bone repair process. There is evidence that ethanol inhibits osteoblast function and that chronic exposure induces systemic bone loss, particularly at high doses.[100] Animal studies have shown that ethanol exposure inhibits bone repair in nutritionally deprived rats[120] but not in rats with normal caloric intake.[121]

Factors that affect the local fracture milieu are equally important and include; prefracture condition of the limb, degree of energy imparted to the limb, location of the fracture, condition of the soft tissue, contamination and infection, surgical insult, fixation method, fracture gap, and degree of post-stabilization interfragmentary motion.

The condition of the limb prior to fracture is particularly relevant, preexisting vascular disease, ulceration, previous trauma, edema, radiation or neurological impairment pose significant impairment to successful fracture healing. Those that affect the vascular bed and consequent availability of progenitor cells are especially important.

The degree of energy imparted to the limb at the time of injury has significant consequences. High energy fractures result in comminution of the fracture and disruption of the soft tissue envelope. The association between high energy and retarded bony healing has been long recognized this is exacerbated if the fracture is open,[122,123] or if there is an associated neurovascular injury[124-126] or compartment syndrome.[127-129] Fracture location is also important. Metaphyseal fractures heal more readily than those involving the diaphysis.[130] Fractures with a surrounding bulky soft tissue envelop are more likely to heal than those covered by only skin and subcutaneous tissue e.g., middle third tibial diaphyseal fractures.

Surgical insult may, in some cases, be the final sequence in a series of events that contribute to failure of bone repair. Additional soft tissue injury and periosteal stripping may disrupt blood supply even further. Surgical strategies must be cognizant of this and aim to avoid exacerbating soft tissue injury. Fracture fixation has important consequences that are not always beneficial. Fracture gap and segmental defects following fixation is an important concept. It has been stated that a gap of greater than 2 mm may adversely affect healing.[131] Fixation method becomes important in these situations as this determines the degree of interfragmentary motion, shear and stress. As previously outlined, complete elimination of interfragmentary motion results in primary bone healing via intramembraneous ossification. Cutting cones are important in this process. For these to function there must be interfragmentary contact.[3] If a fracture gap exists in the presence of rigid internal fixation then failure of bone healing is likely to occur. Interfragmentary motion is beneficial to fracture healing up to a point.[3-5] The degree of motion at the fracture site affects the differentiation of mesenchymal cells either towards osteogenic or chondrogenic pathways. Creating a stable mechanical environment decreases the overall amount of cartilage that forms at the fracture site. So called micromotion at the fracture site stimulates chondrogenesis and subsequent endochondral ossification. However excessive motion disrupts this process and results in fibrous nonunion. The exact mechanism whereby this occurs is not fully understood. Le et al[132] have demonstrated that mesenchymal cells in the callus commit to a chondrogenic or osteogenic fate during the initial stages of healing and this is influenced by the mechanical environment of the fracture site. For example, by day 4.5 of nonstabilized fracture repair mesenchymal cells in the callus had begun to express indian hedgehog, (ihh, a regulator of chondrocyte maturation during skeletogenesis) indicating their differentiation along a chondrogenic lineage. However, in stabilized fractures this was not detected until day 7. This would suggest that mechanical environment influences cell differentiation at an early stage of fracture healing. It has been postulated the mechanical forces may influence mesenchymal differentiation by either preventing or facilitating vascular ingrowth into the fracture sire. Hypovascularity and alterations in pH may stimulate cartilage formation.[6,132] Aspenberg et al[133] demonstrated reduced expression of BMP-3 due to mechanical loading and suggested a link between mechanical stimuli and tissue differentiation. It would therefore appear that temporal or spatial presence or absence of certain signaling proteins may be affected by the mechanical environment of the fracture and this in turn may influence the conversion of cell from osteogenic to chondrogenic or fibroblastic phenotypes. However other authors have failed to demonstrate altered expression of growth factors or their receptors in either animal[134] or human nonunions.[135]

The exact cellular pathophysiology of bone repair failure remains undiscovered. However, a complete understanding of this process is now closer that ever before. Modulation of the repair process using exogenously applied growth factors is a clinical reality. The most efficient way to use this technology in the setting of fracture nonunion remains unresolved. However, it is conceivable that during the present decade nonunion will no longer exist as a clinical problem.

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Chapter 6

Calcium Phosphate Bioceramics:
An Alternative to Autograft and Allograft?

Guy Daculsi, Olivier Laboux and Pierre Weiss

Although bone tissue possesses the capacity for regenerative growth, the bone repair process is impaired in many clinical and pathological situations. For example massive bone loss caused by trauma and tumor resection as well as deformities require reconstructive surgery. In this context, there was a critical need to develop implant technologies to promote bone healing. Cortical and cancellous bone grafts are the materials of choice for bone filling or reconstruction, but their clinical use involves some difficulties. Septic complications, viral transmission and unavailability of native bone have therefore led to the development of synthetic bone substitutes. Allograft bone, or tissue harvested from a cadaver, while more readily available, may carry with it the risk of disease transmission and is also difficult to shape.[1-3] A significant additional limitation of allograft bone is the delayed remodeling by the host. In the case of very large defects, the allograft may remain in the implant site throughout the patient's life, creating an area more prone to fracture or infection.

The development of calcium phosphate ceramics and other related biomaterials for bone graft involved a better control of the process of biomaterials resorption and bone substitution. Synthetic bone graft materials available as alternatives to autogeneous bone for repair, substitution or augmentation, in particular synthetic biomaterials include, special glass ceramics described as bioactive glasses; calcium phosphates (calcium hydroxyapatite, HA; tricalcium phosphate, TCP; and biphasic calcium phosphate, BCP). These materials differ in composition and physical properties from each other and from bone;[4-7] and must be take in consideration for more efficient bone ingrowth at the expense of the biomaterials and to adapt to new development of dedicated biomaterials. In the last decade synthetic calcium phosphate materials, principally calcium hydroxyapatite (HA) ceramics, was commercially used. However the concept of bioactivity (release of ions of biological interest) well described for glass ceramic[8] was not particularly take into account for HA and other related biomaterials (ACP amorphous calcium phosphate, CdA calcium phosphate deficient apatite). HA until recently was considered to be non able to be resorbed. Calcium phosphate biomaterials differ in their solubility or extent of dissolution: ACP >> α-TCP >> β-TCP > CdA >>

These ceramics are osteoconductive (act as a support for new bone formation requiring the presence of porosity) and able to be resorbed(degradable through chemical and cellular processes). They are also biocompatible (do not induce adverse local tissue reaction, immunogenicity or systematically toxicity). Past decade, these biomaterials have been marketed and approved for use in humans as bone substitutes. Various presentations are currently used in orthopaedic and maxillo-facial surgery such as wedges, blocks or granules (Fig. 1). Owing to their bone substitution properties, CaP ceramics have naturally been considered as a potential matrix for tissue engineering and the development of a bioactive drug delivery system (DDS) in bone sites.[9]

The paper presents the current knowledge on calcium phosphate bioceramics, bone tissue engineering and calcium phosphate drug delivery.

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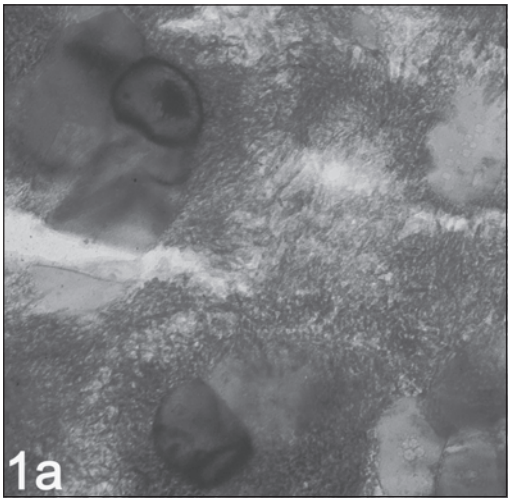

Figure 1a. Formation of microcrystals similar to those of bone apatite crystals.

Calcium Phosphate, the Artificial Bone

This paper is focused on calcium phosphate ceramics (CaP) and illustrated more particularly by biphasic calcium phosphate (BCP), an artificial bone concept developed 15 years ago, and currently the larger family of products elaborated and distributed in the EC market. The concept is determined by an optimum balance of the more stable phase of HA and more soluble TCP. The material is soluble and gradually dissolves in the body, seeding new bone formation as it releases calcium and phosphate ions into the biological medium.[10-12]

The interest of BCP concept is the dissolution control and due to the structure, the bone ingrowth at the expense of the ceramic. The extent of dissolution depends on the β-TCP/HA ratio, the higher the ratio, the higher the extent of dissolution.[11,13,14] Few reports between 1920 and 1975 described calcium phosphate materials (named at that time "tricalcium phosphate") as a promoter for bone formation[15,16] or periodontal restoration.[17] Between 1920 and 1975, a very limited number of scientific articles reported that the use of calcium phosphate materials, described as 'tricalcium phosphate', to repair bone defects successfully promoted bone formation;[15,16] or periodontal defects.[17] The 'tricalcium phosphate' material used by Nery was subsequently identified by LeGeros in 1988 as consisting of a mixture of 20% β-TCP and 80% HA.[18,19] This material and other mixtures of β-TCP and HA were later described as a biphasic calcium phosphate, BCP.

The main attractive feature of bioactive bone graft materials such as CaP ceramic is their ability to form a strong direct bond with the host bone resulting in a strong interface compared to bioinert or biotolerant materials which form a fibrous interface.[20,21]

The formation of this dynamic interface is believed to result from a sequence of events involving interaction with cells; formation of carbonate hydroxyapatite CHA (similar to bone mineral) by dissolution/precipitation processes.

Physicochemical Events

The BCP materials elicit responses from bone cells and related cells in vitro and in vivo that are similar to those elicited by bone. These materials allow cell attachment, proliferation and expression. The first biological events after BCP ceramics implantation are biological fluid diffusion, followed by cells colonization.

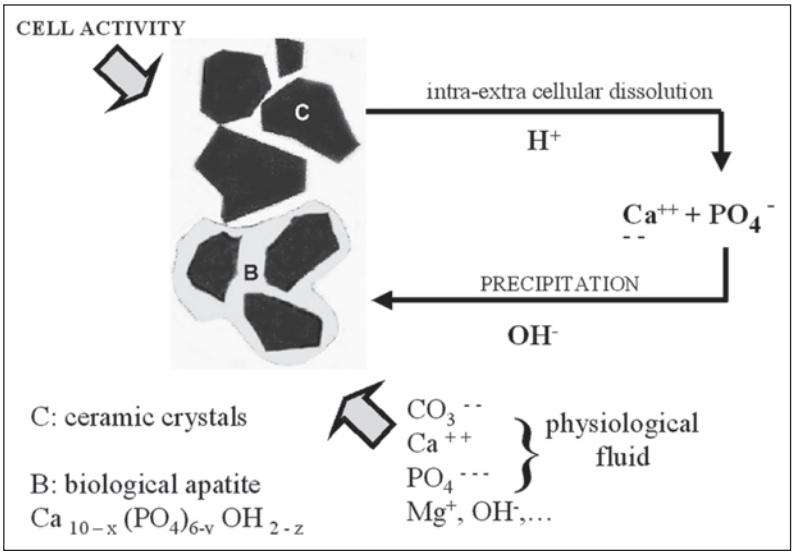

Figure 1b. Physicochemical process of biological precipitation

Biodegradation and Biodissolution

The biodegradation of BCP included the dissolution of the individual HA or β-TCP crystals.[11,22] The proportion of HA to β-TCP crystals in BCP appeared greater after implantation because of the higher reactivity or solubility of β-TCP compared to HA. The propensity to the resorption (reflecting in vivo dissolution) of BCP ceramics depends on their β-TCP/HA ratios the higher the ratio, the greater the resorption. Formation of microcrystals (which are able to diffract X-rays) with Ca/P ratios similar to those of bone apatite crystals were also observed after implantation (Fig. 1a and 1b). The abundance of these crystals were directly related to the initial β-TCP/HA ratio in the BCP: the higher the ratio is, the greater the abundance of the microcrystals associated with the BCP crystals are. According to these data it is possible to control the kinetic of dissolution and precipitation, and subsequently the bioactivity.

Physicochemical Events

The Bonding Zone

Using high resolution TEM Daculsi et al[12] demonstrated for the first time that the formation of these microcrystals after implantation were non-specific, i.e., not related to implantation site, subjects of implantation, and types of CaP ceramics. The coalescing interfacial zone of biological apatite and residual crystals provides a scaffold for bone-cell adhesion and further bone ingrowth.[21] The restoring process involves a dissolution of calcium phosphate crystals and then a precipitation of CHA needle-like crystallites in micropores close to the dissolving crystals. The coalescing zone constitute the new biomaterial/bone interface which includes the participation of proteins and CHA crystals originating from the CaP materials but does not include the biomaterial surface. The following events of bone ingrowth and the newly formed bone progressively replaces the initially formed CHA from the CaP biomaterials.

The process of cell colonization, adhesion, phagocytosis and osteoclastic resorption, ECM elaboration and mineralization, bone in growth and bone remodelling associated with the biological apatite precipitation during CaP ceramics dissolution, are continuously in progress. Consequently the interface is not static but dynamic, in constant evolution, taking into account bone physiopathology, biomechanical factors and bone maturation (Fig. 2).

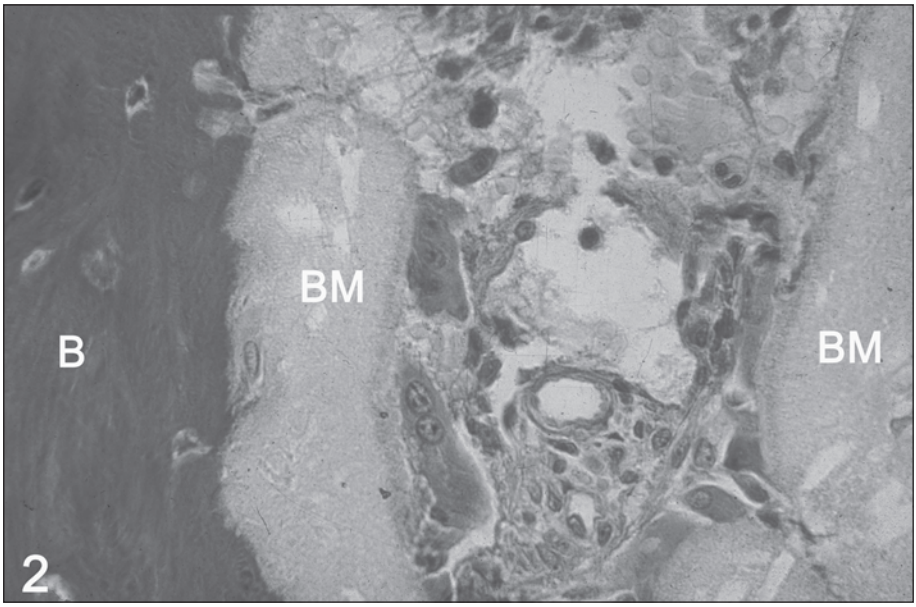

Figure 2. Bone ingrowth into Triosite® macropores (BM) in human spine arthrodesis after one year of implantation showing vascular vessels, osteoclasts, osteoblasts and newly formed bone (B).

The review of twelve years of human and animals use of micro and macroporous biphasic calcium phosphate materials (Triosite®, MBCP®) demonstrated that the bone ingrowth (Fig. 3), during the first year in both human or animal is limited to 2.5 mm from the surface of parallepipedic blocks. The bone ingrowth over 2.5 mm (on larger blocks than 5mm in diameter) depends of the mechanical strain on the implantation site. The mechanical strain acting on bone remodelling and on the first bone ingrowth filling the macroporores. The filling of the outer macropores forbid the osteogenic cell invasion of the inner macropores without a bone/ceramic remodelling. The kinetic of ceramic resorption/bone ingrowth indicates that in cortical site after one year (both in human and animals) 50% of the ceramic was replaced by cortical lamellar bone. In spongious bone large differences appeared from animal species and in human. The filling of bone cavities realized by granules (from 1 to 3 mm) show others kinetics of ceramic bone susbtitution. The process of substitution is related to the particle size. The particle size acts on the recruitments of resorbing cells (monocytes, macrophages, osteoclasts) and on osteogenics cells like a scaffold. Addition of bone marrow increases the kinetics of resorption substitution process.[22]

This concept of bioactivity was also described for implant coating and Injectable Bone Substitute IBS™, an injectable non self hardening calcium phosphate composite.[23,24]

Calcium phosphate materials are also used as components or fillers in polymeric composites.[25,26] and in cements.[27] The hydraulic cement are not macroporous and numerous studies have demonstrated the necessity of macropores for bone osteo-conduction.[28]

The bioactive concept of BCP have been applied to a new composite associating hydrosoluble polymer and BCP granules. We have elaborated such injectable bone substitute ready to use and able to be largely invaded by osteo-conduction due to osteogenic cells.[29,30]

These materials are perfectly biocompatible and potentially resorbable, and thanks to their initial plasticity, they can fit bone defects very easily, without necessity to elaborate shaping of implantation site.

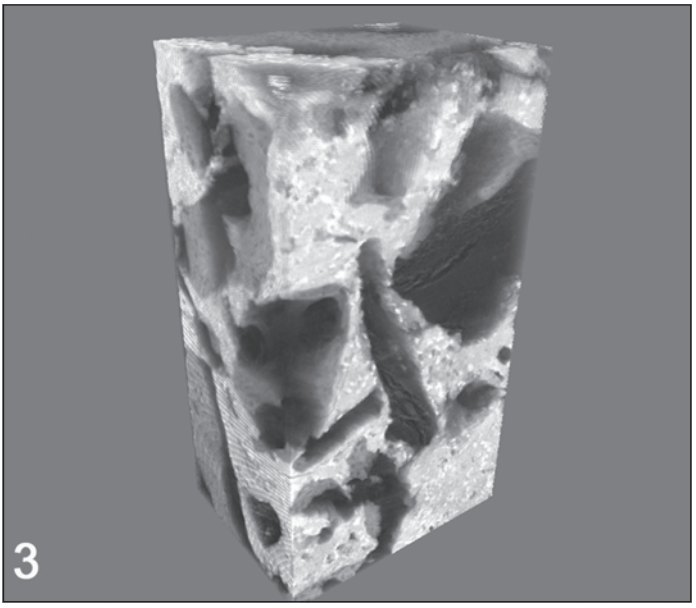

Figure 3. Bone ingrowth into MBCP® in rabbit implants after 6 weeks studied by microtomodensitometry (ESRF Grenoble, France).

The IBS cannot have mechanical properties like hydraulic bone cement able to have an hardening process.[27] However bone cells are able to invade the hydrated polymer and or the spaces released by the eventual disappearance of the polymer. Bone ingrowth take place all around and at the expense of the resorption of the BCP grains. In time, mechanical property could be observed due to the presence of bone.

Interest of Biphasic Calcium Phosphate for Tissue Engineering

The long bone reconstruction can be achieved by increasing the rate of bone formation by the implantation of mesenchymal stem cells in combination with bioactive ceramics, specifically biphasic calcium phosphates.

Generally a standard in vivo osteogenesis assay was developed in which MSCs were seeded onto HA/TCP and implanted subcutaneously on the back of immuno compromised mice.[31] Histological evaluation exhibited consistent bone formation in implants seeded with MSCs, whereas the cell-free implant rarely displayed significant bone formation. The potential feasibility of MSC-based bone regeneration was then established, and extensive experimentation has subsequently confirmed MSC-directed bone formation utilizing bioactive ceramics in many in vitro and in vivo models and applications.[32-34] Mesenchymal stem cells are found at a frequency of approximately 1/100,000 nucleated cells in bone marrow. Osiris Therapeutics has developed methods whereby this small, distinct population can be isolated and expanded, creating multiple therapeutic doses from one small aliquot of bone marrow.[35] This isolation and expansion procedure has been validated both with human subjects and animal donors including baboon, goat, canine, rabbit, and rat.

Porous HA/TCP (60% hydroxyapatite: 40% tricalcium phosphate) hollow were used after sterilization and fibronectin coating. Rat MSCs suspensions (7.5×10^6/mL) were used to load the cylinders. All implants were then placed in 8mm defects created in the diaphysis of Fischer 344 rats using plate fixation during 4 and 8 weeks implantation time. MSC-loaded implants

demonstrated a striking difference, with more than double the extent of bone formation compared to control without extended MSCs. With extensive bone formation between the host bone and implant, there was complete bridging of the gap, including periosteal callus development. Furthermore, complete vascularization of the new tissue demonstrated the abundant neovascularization that paralleled bone regeneration.

Confident that rat MSC-HA/TCP implants were effective in regenerating bone in this critically-sized model, subsequent studies were performed focusing on the use of human MSCs in an equivalent model.[36] To investigate human cells, Harlan Nude (Hsd:Rh-rnu) rats were subjected to the femoral gap described above. Osteogenesis was apparent at 4 weeks following implantation in the human MSC-loaded implants, but was absent in the cell-free implants. At 8 weeks, the MSC-loaded implants were well integrated with the host as evidenced in the radiodense bridging between the host and the MSC-implants. The bridging increased in radioopacity by 12 weeks and was concurrent with callus formation spanning the defect.

Similar studies have been performed in large animal models, to mimic human situations. Bone marrow samples were drawn from the iliac crest of large mongrel hounds; MSCs were then isolated and expanded from each donor.[37] The osteogenic potential of each donor was verified through subcutaneous implantation of MSCs loaded onto HA/TCP. The canine femoral gap defect was then used to demonstrate MSC-mediated bone formation in a large animal model. MSCs were loaded onto 60/40 HA/TCP porous scaffolds (MBCP® Biomatlante France) custom milled into hollow cylinders to closely approximate the defect dimensions. The implants were then shipped to the surgical facility for implantation. A 21mm osteoperiosteal defect was created unilaterally in the mongrel hounds. Implants were implanted in an autologous manner, with cell-free HA/TCP implants and autograft bone segments serving as controls. This study confirmed the feasibility of bone marrow harvesting, MSC isolation and bone formation for autologous MSC implantation. However limitations of the HA/TCP scaffold were (1) the lack of remodeling/resorption of the ceramic, which in turn limited the rate of bony integration, (2) its brittle mechanical properties. Others experiments were designed to investigate fully resorbable CaP scaffolds by varying the ratio of HA to TCP to increase the solubility of the BCP. The rate of degradation or resorption of HA/TCP ceramics in vivo can be accelerated by increasing the amount of the more soluble phase, TCP.[24] In order to design a scaffold more efficient for bone tissue engineering in combination with MSC a specific optimum balance between the more stable phase (HA) and the more soluble phase (TCP) must be achieved. The goal of this study was to determine the optimal HA/TCP ratio seeded with MSCs that promoted rapid and uniform bone formation in vivo. To this end, human MSCs were evaluated on various HA/TCP compositions in an ectopic model in immunocompromised mice.[38]

Six calcium phosphate ceramic compositions were examined: 100% Hydroxyapatite (100 HA), 100% β-tricalcium phosphate (100 TCP) and four formulations of HA/TCP (76/24, 63/37, 56/44 and 20/80, ratio of wt%HA/wt%TCP, Biomatlante, France). All compositions were manufactured with a porosity of 60%-70% and a pore size range of 300 to 600 μm, and 3mm cubic shaping. Bone marrow was collected from human donors and MSCs were isolated, culture expanded and cryopreserved. MSC-loaded implants and cell-free implants were implanted subcutaneously in the back of SCID mice. Implants were harvested at 6 and 12 weeks and processed for routine decalcified histology. More bone formed within the pores of MSC-loaded 20/80 HA/TCP than all other implants at 6 weeks. By 12 weeks, MSC-loaded 56/44 and 20/80 HA/TCP demonstrated the greatest amount of bone and were equivalent to each other. The least amount of bone formed within the pores of 100 TCP and 100 HA throughout the study. These results demonstrated the potential of using faster resorbing ceramics, 56/44 and 20/80 HA/TCP, in combination with MSCs for bone regeneration.[38,39] Future studies will test these results in more clinically relevant models. Use of this therapy in the clinic has begun in a Phase I clinical trial applying autologous MSCs to a 60/40 HA/TCP scaffold for

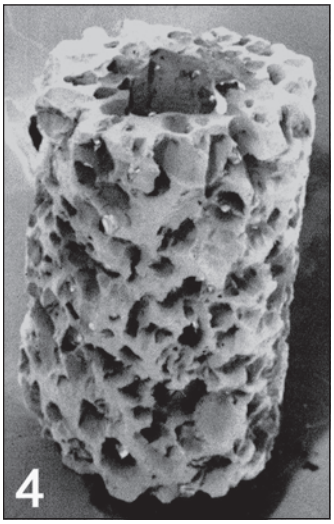

Figure 4. Hollow cylinders of MBCP® used in combination with MCS in maxillofacial surgery.

dental applications (Osiris Therapeutics Baltimore) using hollow cylinders of MBCP® (Fig. 4).

Conclusion

The bioactive concept based on the dissolution/transformation processes of HA and TCP can be applied to both Bulk, Coating and Injectable Biomaterials. The mixture of HA and β-TCP in the 3 different forms have the same evolution and adaptation to the tissues:

1. partial dissolution of the CaP ceramic macrocrystals cause an increase in the calcium and phosphate concentrations in the local microenvironment;
2. formation of CHA (either by direct precipitation or by transformation from one CaP phase on an other or by seeded growth) incorporating ions (principally, carbonate) from the biological fluid during its formation;
3. association of the carbonate-apatite crystals with an organic matrix; and
4. incorporation of these microcrystals with the collagenous matrix in the newly formed bone (in osseous sites). The events at the CaP biomaterial/bone interface represent a dynamic process, including physico-chemical processes, crystal/proteins interactions, cells and tissue colonization, bone remodelling, finally contributing to the unique strength of such interfaces.

Calcium Phosphate Drug Delivery System

Conventional means of administering therapeutic agents generally include oral medication, eye drops, ointments, intravenous injections and patches. However, the concept of targeted drug delivery to the site of action, remains of major interest to improve therapeutic efficiency while producing minimum systemic side effects.[41] Especially a considerable amount of attention is paid for improvement of drug delivery to sites dramatically limited in access such as bone tissue. In this context and with regards to their biological and physicochemical properties, synthetic bone substitutes have been contemplated as potential carriers for the local delivery of bioactive agents.

Calcium Phosphate Ceramics and Therapeutic Agents Association

The conventional CaP ceramic manufacturing process involves consolidation of powder by isostatic pressure followed by a sintering step (T>1000°C) to achieve fusion of particles of the

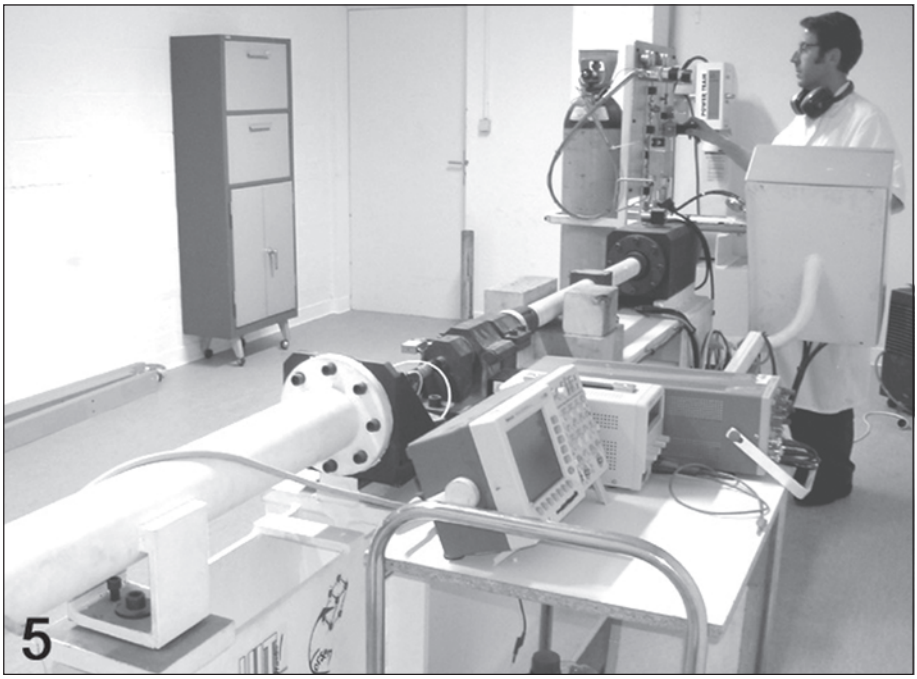

Figure 5. Dynamic compaction apparatus.

material. Heating is necessary to strengthen the material but unfortunately prevents mixing of therapeutic agents with CaP before consolidation. To overcome this problem, therapeutic agents have been loaded on CaP ceramics after consolidation by adsorption through electrostatic and reversible bound simply by incubating the ceramic block in a solution containing the drug. In addition, an innovative process, called "dynamic compaction", to consolidate CaP powder without the use of external heating, has recently been patented (Nantes University, patent n° 0 666 764). In this process, compaction is achieved by a shock wave produced by piston impact on the surface of CaP powders and subsequent consolidation by localized interparticle melting[42] (Fig. 5).

Pharmacokinetic Profile of Drug Released from CaP Ceramics

Drug release pattern from homogeneous drug-loaded solid matrix generally follows the square root of time relationship:[43,44]

$$M_t = A\, M_o\, [C_s \cdot (D_i\, \varepsilon/\tau)\, (2C_d - \varepsilon C_s) t]^{1/2}$$

$$K = A\, M_o\, [C_s \cdot (D_i\, \varepsilon/\tau)\, (2C_d - \varepsilon C_s)]^{1/2}$$

where M_t is the amount of drug released from the matrix at time *t*, K the Higuchi release rate constant, M_o the total amount of drug loaded, A the matrix surface area, D_i the drug diffusion coefficient, C_s the drug solubility, C_d the drug concentration, τ the matrix tortuosity, and ε the matrix porosity. The linear pattern for the square root of time obtained with CaP ceramic systems[5] indicates that initial release apparently involves a first-order diffusion-controlled mechanism, as described by Higuchi et al.[44] However, this theoretical analysis of the release rate of drugs dispersed in solid matrices concerns cases in which solid drug particles are dispersed in an homogeneous matrix. In this case, matrix acts as a diffusional support from which drugs are released by the leaching action of penetrating solvent. This description does not seem

to be totally appropriate to a drug solution adsorbed onto a solid degradable CaP matrix. Finally, even if drug release seems too complex to be described by a simple diffusion mechanism (with respect to the Higuchi equation), pharmacokinetic of drug release from CaP ceramic is also dependent on matrix tortuosity and porosity. Both of these ceramic parameters are affected by precipitation/dissolution process of CaP, which occurs when such ceramics are immersed in release solution or under the influence of the ceramic resorption/degradation process in bone sites. Thus, drug release in vivo is under the influence of both a diffusion mechanism and the ceramic resorption/degradation process. A precise knowledge of ceramic dissolution and resorption properties is therefore of importance to provide clinicians with a valuable CaP ceramic DDS for bone sites.

Therapeutic Applications of CaP Ceramic-Based Drug Delivery Systems

Preclinical and clinical trials have shown that CaP ceramics were able to induce new bone formation at their expenses leading to their total replacement by lamellar bone. However, osteoconductivity of CaP ceramics does not yet allow the filling of large bone defects. Today, one approach to improve the ceramic bone replacement consists in associating an osteogenic factor with the material. In this attempt, growth factors such as transforming growth factor, platelet-derived growth factor, bone morphogenetic proteins, growth hormone and insulin-like growth factor-1 have been successfully investigated. These promising results have encouraged the scientific community to consider CaP ceramics as supports for the release of others molecules potentially implicated in the local treatment of bone pathologies such as infections, bone tumors and pathological bone loss.

Infections

Therapy of bone infections (osteomyelitis) may easily last two years because of the poor accessibility of the infection site by commonly systemically administered antibiotics. This is mainly because bones are moderately perfused organs and likely because of a reduced blood supply associated to the formation of diffusional barriers in the infected bone tissues. In this context, to improve therapy, resorbable CaP materials have been contemplated as potential carriers for antibiotics. They release effective drug amount at the site of infection for several months, while the systemic drug concentration remains very low. Among the various antibiotics, vancomycin and gentamycin have been extensively investigated and proved efficacy in human osteomyelitis.[45]

Bone Tumors

A major attempt in treating bone and soft tissue tumors is to maintain local long acting and effective high concentrations of a chemotherapeutic drug at the site of tumors while producing minimum systemic side effects. Porous CaP ceramics have been demonstrated to be an efficient form of local DDS for methotrexate or cis-platinum and successfully used in the clinical treatment of mice osteosarcoma.[46,47] CaP ceramic could therefore play a major role in cancer chemotherapy in reducing the recurrence of tumors without risk of systemic toxicity.

Bone Loss

Osteoarticular disorders associated with increased osteoclastic bone resorption (as observed in osteoporosis, Paget's disease of bone, bone lytic tumors, periodontal disease…) often lead to pathological fractures. They are widely treated by systemic administration of Bisphosphonates, potent inhibitors of osteoclast activity. Association of CaP materials with bisphosphonates would allow to increase the efficiency of bisphosphonate by being locally released and decreasing significantly secondary effects (nephrotoxicity) observed after systemic treatments. In this objective, ceramic hydroxyapatite implants have been used in dental surgery. Denissen et al[48] have reported that bisphosphonates may be beneficial in preventing alveolar bone destruction associated with natural and experimental periodontal disease. These authors have demonstrated the efficiency of Bisphosphonate-complexed hydroxyapatite implants on the repair of alveolar bone.

Bone diseases, are in close relationship with population aging process and therefore their treatments are a great challenge for the scientific community. The development of bone targeted bioactive DDS using a bone substitute provides an attractive and efficient approach for the treatment of bone pathologies not only from clinical and scientific viewpoints but also at a social and economical level. Of course, the therapeutic window for CaP ceramic DDS is mainly based on in vitro results and animal studies do not always extend well to human situations, but this could be a good starting point for clinical trials. In addition, the approval of sustained release systems, such as those described here, may make possible the development of important innovative therapeutic-based strategies (vaccines or gene transfer) for the management of chronic bone affections in the near future.

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Chapter 7

Mechanical Modulation of Fracture Healing and Implications for Skeletal Tissue Engineering

Allen Goodship

Abstract

This chapter addresses the factors involved in the functional adaption of intact bone and the relevance of these to mechanical modulation of bone repair. The sensitivity of the repair process to the overall mechanical conditions imposed by the fixation system used is described. In addition the further modulation of the process of indirect bone repair by application of dynamic inter-fragmentary micro-movement. The putative common mechano-transduction pathways that may link functional adaptation in intact bone to the mechanical modulation of indirect bone repair and the relevance of these in optimisation of fracture healing are presented.

The process of indirect bone repair involving a cascade of connective tissue differentiation is discussed in relation to mechanical cues for selective specific tissue differentiation and the computer modelling of this process. Finally bone repair is discussed in relation to new concepts in skeletal tissue engineering and control of tissue responses in vivo.

Introduction

The mammalian skeleton provides structural support for the body, protection of some of the vital organs and a reserve of minerals. However, the mechanical aspects of the skeleton are paramount in enabling movement and locomotion with optimal energetic efficiency. The individual skeletal elements form a series of specific bones, identifiable from their specific morphological characteristics. The formation of these elements arises from a combination of a genetically determined template and the ability to mount a response to mechanical cues arising from the prevailing functional environment. Experimental evidence has been produced to demonstrate the refinement of certain morphological features of the genetically determined form by functional mechanical loading (Fig. 1) and the identification of morphological features determined by genetic influences and those arising from mechanical loading.[9,26]

During the changing mechanical demands of development and growth and throughout adult life the skeleton responds to mechanical input in a manner that optimises the functional and energetic performance of the individual.

Bone may be considered as a tissue, a structure and an organ. At the tissue level it is classified as a connective tissue in that it comprises cell populations and a composite extra-cellular matrix. This tissue is acutely sensitive to changes in mechanical environment in terms of mass and architecture. The cell populations respond to mechanical signals in a coordinated manner to optimise the distribution and mass of tissue within the structural skeletal elements to accommodate the magnitude and distribution of loads experienced at any particular time.[80]

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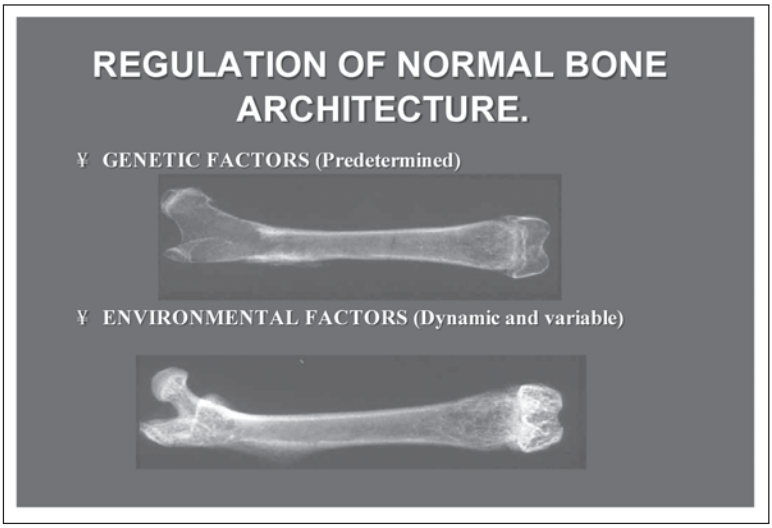

Figure 1. Two murine femora, one showing development from the cartilage anlage after transplantation to the spleen, devoid of mechanical loading and showing the form associated with the genetic template alone. The second shows the functionally related refinements to this template in a bone from the normal functional location.

Thus, persistent abnormal loading or change in patterns of loading will induce changes in the normal mass and architecture of the associated site of the skeleton. Changes in normal loading may be a consequence of abnormalities in muscle function or neural control and gravitational loading. The consequences of injury and subsequent orthopaedic procedures will also influence the loading of the skeleton both in terms of the presence of orthopaedic devices in relation to specific sites on specific bones, and also changes in load-bearing of the affected limb or region of the skeleton.

Gross overload resulting in fracture is followed by a repair process in which, under favourable conditions, bone undergoes a remarkable process of regeneration to restore the anatomical and functional capability of the damaged skeletal element. In contrast to many other tissues and organs this repair process does not involve scarring.

The process of repair is also modulated by the mechanical environment, initially to induce one of two distinct types of repair, either direct or primary repair under conditions of inter-fragmentary stability with rigid fixation, resulting in intra-cortical osteonal remodelling and no periosteal callus or, indirect or secondary repair, sometimes referred to as biological repair, in which the fixation methods allows greater inter-fragmentary motion that induces a periosteal callus and inter-fragmentary endochondral repair.

Perhaps it is now evident that this may be a continuum and in addition the process of indirect repair itself can be modulated by specific mechanical conditions at the fracture site, which may enhance or inhibit osseous union of the fracture fragments.

The process of indirect fracture repair replicates many of the processes of embryonic endochondral bone development. A fracture haematoma forms following disruption of the intra-medullary and periosteal blood supplies at the time of injury; this then organises into granulation tissue and progresses. Although a cascade of tissue differentiation through the entire spectrum of connective tissues, indicating the potential to regenerate these tissues even in the adult. The presence of mesenchymal stem cells within the medullary cavity together with the release of growth factors in the early "inflammatory" phase of the repair process needs to be understood not only to enhance the healing of fractures but also to explore the potential for engineering or regenerating other connective tissues.

This chapter will review the interaction of mechanical environment and the bone repair process together with the implications for in vivo regeneration of specific connective tissues using mechanical cues.

Adaptation of the Skeleton to Changes in Mechanical Conditions

Since the observations of Wolff, Culman, von Meyer and others, leading to the concepts that bone as a structure responds to mechanical loading to optimise mass and architecture, techniques to quantify the effects of load on the skeleton have only been developed relatively recently. Lanyon (1971)[48] and van Cochran succeeded in transferring engineering technology to enable bone strain to be measured in the living skeleton. Direct measurement of loads and stresses within the skeleton is not possible. However, the consequence of applying a load to a structure, is the deformation of that structure. The degree of deformation depends upon the magnitude of load and the structural and material properties of the bone. The ability to measure bone deformation not only in cadaveric bone in the laboratory but also in the living skeleton during functional loading was enabled by the advent of the cyano-acrylate adhesives that allowed foil strain gauges to be bonded to the bone surface in the living skeleton. This technique made it possible to quantify both the magnitude and direction of principal surface strains both during physiological activities and also during the magnitude and distribution of strains associated with imposed loading regimens to enable the investigation of the effects of specific loading regimens on tissue and structural morphology.

Following this breakthrough many experiments have been performed in which loads have been applied to the skeleton to perturb normal strain patterns and to relate the imposed strains to changes in bone tissue from the structural level of whole bones down to changes in gene expression in the bone cell populations of bone tissue in response to specific mechanical conditions.

The skeleton responds to changes in strain and adjusts mass and distribution of bone to restore the customary strain levels. Increase strains provoke an osteogenic response to increase skeletal mass and thus restore the optimal strain environment. Conversely, a reduction in strain evokes a cellular response that induces the removal of bone again to adjust the mass and structure of the bone to restore the optimal strain. This adaptive response, initially observed in qualitative terms, has now been validated experimentally and also modelled mathematically.[8,35,40,50,72]

The cascade of events following an episode of osteogenic cyclical strain has been elucidated in terms of effects on both the bone matrix and cell populations.

Initial responses to mechanical stimulation occur very rapidly in both the matrix following as few as 50 cycles of loading. There are changes in the orientation of proteoglycans that remain for twenty four hours after the imposition of the single train of loading cycles. This has been suggested as a "strain memory".[74] There is a concurrent change in metabolism of the osteocytes with elevation of G6PD levels and mRNA synthesis with changes in mediators such as prostanoids and nitric oxide occurring within a few minutes of the application of the stimulus. This is followed after a few hours by activation of the surface lining osteoblasts. Activation of gene expression and synthesis of bone matrix components by osteoblasts occurs within 24 hours.[66,75]

The coordination of the response of bone tissue to changes in loading is now known to be via the osteocyte cell population. These cells are well placed to respond to local changes in environment and to communicate not only with other osteocytes but also with the surface lining cells and active osteoblasts. The osteoblast has been shown to signal the osteoclasts and participate in the control of coupling between the two processes of bone resorption and bone formation that effect the changes in mass and distribution of matrix that are required to accommodate alterations in mechanical loading. This coupling is mediated by the RANK, RANKL and osteoprotegerin (OPG) system.[77]

The characteristics of an osteogenic mechanical stimulus are cyclical deformation rather than static loads, high rates of deformation, and diverse strain distributions. Recently the use of low magnitude strain at a specific frequency has been shown to be osteogenic, particularly in cancellous bone and in stimulating bone ingrowth into implants.[73] An interesting mediator of the mechano transduction pathway has been identified as the estrogen receptor.[12,21,22,23]

In the event of a decrease in loading bone mass is reduced. This is seen throughout the skeleton with bed rest and long term space flight. However, although there is a loss of bone throughout the level of loss is not uniform. Long term space flight induces a greater loss of bone mass from the lower limbs and in particular the distal bones of the lower limb. In the calcaneus some 5-10% of bone can be lost. Interestingly it appears that this loss can be modulated using short daily periods of mechanical stimulation based upon the heel strike transient, namely a high rate deformation.[32] Thus, these adaptive responses can be localized to regions of the skeleton or even a site within a single bone. This is particularly seen adjacent to joint replacement prostheses, where bone loss occurs at sites protected from normal functional strains by load sharing with the prosthetic component.[49]

This ability of the skeleton to adapt to changes in loading is very relevant to bone repair. The degree of injury resulting in fracture together with the fixation of the fracture both contribute to a reduction in the loading of the affected limb. Thus the normal mechanical environment for osteogenesis is changed to a scenario of reduced loading, associated with a drive to reduce bone mass. However, although there is indeed a loss of bone mass in the affected limb, the fractured bone does also undergo a repair process which involves bone formation. Thus an interesting paradox of reduced load and a simultaneous requirement for increased bone formation arises. An understanding of the interactions of mechanical environment and bone formation can provide an ability to enhance the process of fracture repair.

The aspects of mechanical stimulation that induce an osteogenic response are summarised as short periods of daily cyclical deformation, particularly at high strain rates and diverse strain distributions. The recent finding that the osteogenic potential of low magnitude sub-physiological strains at specific frequency, which can activate osteoblasts especially in cancellous bone,[73] was similar to that advocated by Weinbaum et al, 1994, as important in the mediation of osteogenic activity by streaming potentials. Streaming potentials are induced as a consequence of ionic fluid movement across a charged surface when bone is deformed. The role of streaming potentials as part of the strain related bone adaptation transduction mechanism has been explored by a number of authors[52,63,82] The mechanical characteristics that act to stimulate osteogenic activity in intact bone are also useful in the modulation of bone repair.

Fracture and Modes of Mechanical Failure

Bone may fracture as a consequence of a single episode of monotonic overload resulting in catastrophic fracture, or by fatigue failure from accumulation of micro-damage within the matrix associated with cyclical loading over a time period inadequate for an adaptive response.

Rapid cyclical loading of bone matrix over a short time period can induce fatigue damage within the matrix. This micro-damage, seen as micro-cracks within the matrix[3,57,85] may occur throughout life and is normally repaired by the process of intra-cortical secondary osteonal remodelling. The presence of micro-damage is accompanied by an increase in osteocyte apoptosis and the activation of osteoclastic resorption followed by osteoblasts forming concentric lamellae and a secondary osteon. An increased number of loading cycles over a short period of time, as is seen for example in some training protocols, can lead to accumulation of micro-damage at a rate greater than that of the repair process resulting in a painful condition. This scenario may be seen in ballet dancers, army recruits and racehorses in training.[61,62] Persistent loading can cause micro-cracks which can coalesce and result in a gross fracture of the affected bone.

Fracture can also occur under normal physiological loading conditions where bone strength is compromised. Such fractures occur in the elderly, particularly post-menopausal women with low bone mass. Specific sites of the skeleton are predilection sites for such fractures; these

include the femoral neck, distal radius and vertebral bodies. It is though there may be a genetic predisposition to these osteoporotic fractures, relating to a collagen gene polymorphisms.[70] With ageing population femoral neck fractures will increase in number and represent a greater burden for Healthcare systems than even current predictions indicate. Thus the prevention and treatment of these fractures is an important area of research. The biological process of bone repair in osteoporotic fractures is poorly documented. However, in this condition the rate of bone remodelling is increased although the net mass of bone is decreased.[13]

Fracture Repair

Direct Bone Healing

Following a fracture the fragments must be stabilised for the repair process to initiate the restoration of functional and morphological integrity. The method of treatment and post-operative patient activity determines the level of interfragmentary movement. In the late 1960s and early seventies knowledge of both the biology and biomechanics of fracture treatment and repair were advanced considerably by the AO Research Group. The concept of compression plating to provide rigid internal fixation and minimal interfragmentary movement was advocated to induce direct bone healing by osteonal remodelling across the fracture site.[69] The pattern of bone repair under these conditions was studied experimentally and shown to comprise osteonal remodelling across the fracture site at locations where the matrix of each fragment was compressed together. Where small gaps occurred, these were filled initially with woven bone that subsequently underwent osteonal remodelling. Osseointegration of the fragments was achieved by extensive secondary osteonal remodelling of the fracture site over a prolonged time period of months to years. In this type of repair process with minimal movement between fragments there is no evidence of external periosteal callus, however, internal endosteal callus within the medullary callus may be seen. The pattern of repair was initially termed "primary" fracture healing but more recently is termed direct bone repair.

The presence of a rigid fixation device, such as a plate, inevitably leads to a reduction in loading of the underlying bone, as a consequence of load sharing between the bone and the plate, and also affects the periosteal blood supply. The consequence of mechanical load protection is a reduction in strain levels of the cortical bone beneath the fracture plate with a consequent bone resorption.[2] The site of bone loss in a tubular diaphyseal region of a bone to maintain bending strength with minimal material would be to thin the cortical wall by endosteal resorption. The tubular structure of diaphyseal bone provides resistance to bending and torsional loading. Thus the removal of endosteal bone allows a minimal bone mass for a maximum resistance to bending. The effect of the presence of a plate on the formation of intra-cortical porosity was also initially attributed to the mechanical strain protection.[45] However, more recently this has been shown to be a temporary phenomenon and attributed to changes in haemodynamics as a consequence of the application of the plate to the periosteal surface of the bone.[67] The development of plates with reduced points of contact with the periosteum has reduced the initial intra-cortical porosity and pathed the way for fixation systems that do not compromise periosteal blood supply.[68] The location of a plate in relation to the pattern of loading of a bone is also important in providing optimal mechanical conditions at the fracture site. Again the interaction between the biomechanics of the skeleton and positioning of fracture plates formed part of the philosophy advocated by the AO Group. Application of plates to the tension surface of the bone allows compression to be increased across the fracture site during functional loading. Thus bones that are loaded in bending are ideally suited for the use of compression plates to induce direct bone healing. The major disadvantage of this pattern of healing is the time taken to remodel the fracture site and provide restoration of mechanical integrity without the need to retain the fixation device. The advantage is a restoration of anatomical integrity with minimal callus formation. The use of direct bone healing is ideal for intra-articular fractures where precise reduction is required with no callus formation.

Figure 2. The distribution of connective tissues in the mid phase of indirect bone healing.

Indirect Bone Repair

When less rigid methods of stabilisation are used ranging from casting and functional bracing to various configurations of external fixators and modalities of intra-medullary nailing, there is more interfragmentary motion, particularly in the early stages of the repair process. This initial mobility at the fracture site, induces a different pattern of tissue repair, called indirect, secondary or biological fracture repair. This was described elegantly by McKibbin, 1978.[55] In this complex biological response to damage there are several processes occurring in parallel. Periosteal proliferation occurs by intra-membranous ossification and initiates a bridging process between the fragments (Fig. 2). Within the fracture gap, the haematoma that forms as a consequence of the rupture of intra-medullary and periosteal vessels organises into granulation tissue, rich in blood vessels. With time the granulation tissue differentiated into fibro-cartilage and areas of hyaline cartilage which is avascular. The hyaline cartilage is then invaded by blood vessels and undergoes endochondral ossification, a process very similar to that seen in the embryonic development of the skeleton.

Thus, the fracture fragments undergo osseomechanical integration through both intra-membranous ossification and a cascade of connective tissue differentiation from haematoma through endochondral ossification to lamellar bone. Some reports also suggest a process within this cascade termed transdifferentiation,[76] in which cells of a defined phenotype such as chondrocytes express message for matrix proteins of a different tissue, for example, osteocalcin a typical marker of bone forming cells.

Indirect bone repair is also a rapid process in establishing osseous union, the large mass of reparative periosteal callus responsible for restoration of functional mechanical integrity then undergoing a prolonged period of remodelling toward restoration of anatomical integrity.

The process of indirect bone healing is sensitive to the mechanical environment at the fracture site. Both the inherent mechanical environment imposed by the combination of fixation device and patient activity and any imposed mechanical stimulation can modulate the rate and extent of the indirect bone healing process.

The Influence of Fixation Stiffness on Progression of Indirect Repair

The degree of motion may influence the rate of progression of healing and the volume and distribution of callus. The repair process is also affected by the size of the fracture gap. A critical size defect in either a clinical fracture with bone loss or an experimental osteotomy may prevent osseous unions leading to a nonunion or pseudarthrosis.[37] However, in many in vivo models of bone healing a noncritical osteotomy gap is used to allow a greater ability to investigate the influence of mechanical and biological stimuli on the repair process.

The level of motion at the fracture site is determined by the load applied through the fracture, the geometry of the fracture line and the stiffness of the fixations system. This micro motion is also an important factor in controlling the rate of progression of fracture healing. There also appear to be a relationship between initial load-bearing, fixation stiffness and progression of healing. High stiffness fixation devices induce high levels of initial load-bearing but reduce levels of micro-motion at the fracture site leading to a low rate of progression of repair. Conversely, a lower stiffness fixation device allows greater inter-fragmentary motion which stimulates callus formation but reduces initial ground reaction force. This suggests a mechano-sensory mechanism within the fracture callus which forms a biofeedback control.[30] It is possible that individual variability in such a control system may account for some of the variability in progression of repair in both experimental and clinical fracture healing.

Changes in the fixator stiffness influence the self regulated interfragmentary motion and changes in the magnitude of inter fragmentary motion in the initial stages of the repair process appear to influence the progression of repair. In a controlled study the use a low compliance single sided fixation system and an osteotomy gap of 3 mm, compared to an identical but higher compliance system, resulted in a greater magnitude of self imposed interfragmentary cyclical displacement in the early stages of repair. With the progression of repair the inter-fragmentary displacement reduced in both groups as a function of time becoming equivalent between groups at six weeks post-operatively and did not show any difference between the two groups up to the end point at ten weeks postoperatively. All other aspects of fixation were controlled between the two groups. However, the outcome in terms of both radiological and mechanical progression of healing was significantly greater in the low compliance fixator group. This suggests the importance of mechanical environment in terms of level of inter-fragmentary displacement in the early stages of the repair process.[29] Using a rigid fixation system in which the axial displacement induced by weight bearing was 0.06 mm in an osteotomy gap of 0.6 mm, a defined displacement, activated by weight bearing, of 0.15-0.34 mm, was permitted in the dynamised group and compared to the control nondynamised group Claes et al (1995),[17] showed the healing of a transverse osteotomy was enhanced by dynamisation.

The degree of compliance can also affect the progression of tissue differentiation in terms of the distribution of tissue types and the progression of angiogenesis. Claes et al (2002),[14] compared two groups of sheep in which the fixators allowed a displacement of 1mm and 0.5 mm with an osteotomy gap of 2 mm. At the end point of nine weeks there was a greater amount of fibrocartilage and a reduced amount of bone with the larger displacement group. Thus the small difference in level of inter-fragmentary motion modulated the progression of indirect bone repair. The influence of fixator stiffness and thus inter-fragmentary motion on bone healing has also been related to consolidation of the regenerate bone following experimental distraction osteogenesis. Here the fixator stiffness in relation to development of increased regenerate bone density was examined and it was found that an optimal level of inter-fragmentary displacement was evident with greater levels inducing an appearance of hypertrophic nonunion.[15]

The evidence that micro motion at the fracture site is important in stimulating the biological process has been apparent for some time. The strength of the fixation system may, therefore, not relate to the optimum environment to stimulate effective healing although a strong fixation does provide short term functional activity. A study comparing the relation between strength of fixation and progression of repair in a triple osteotomy model, supported the use of a

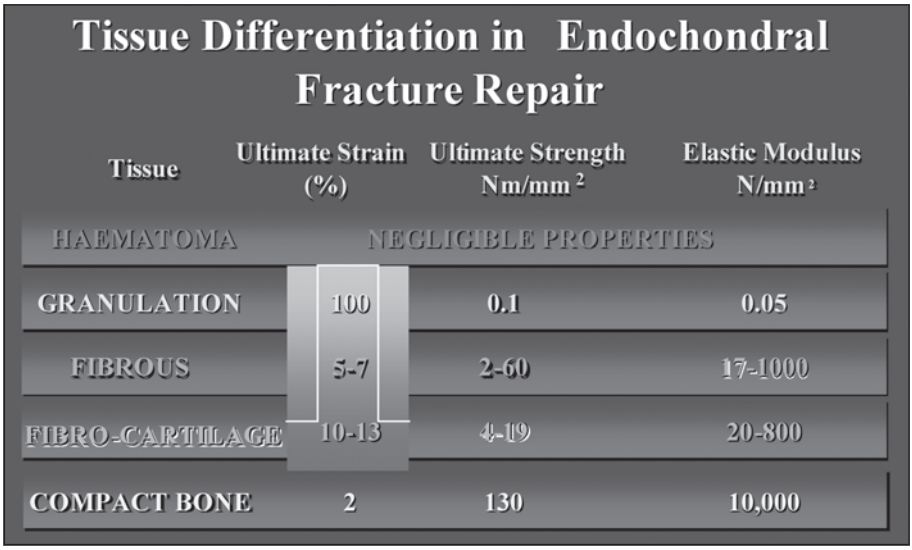

Figure 3. The material properties of tissues occurring during the process of indirect bone repair.

bridging fixation that induced indirect bone healing as the most effective fixation in stimulating repair.[38]

Fracture Geometry

The geometry of the fracture or osteotomy site may influence the distribution of strains that occur with a given method of fixation. The absolute size of the fracture gap may be critical in a number of ways. Perren indicated that in rigid fixation of fractures with good reduction any small gaps could generate very high levels of strain if the fixation was not absolutely rigid. This was tested experimentally[10] where lower strain field was found to facilitate bone union.

The process of repair involves the synthesis of connective tissue matrices, ultimately lamellar bone. Each of the tissues that differentiates in this process has a range of material properties (Fig. 3). The ultimate strain for each of connective tissue has a specific value, for example bone can only withstand a strain of 2% before damage occurs, yet fibrous tissue can tolerate around 12-15%. Thus if the mechanical environment at the fracture site generates an inter-fragmentary strain of 12% it is impossible for bone to exist unless the load is reduced so that with tissue differentiation the level of strain decreases with the increased stiffness of the inter-fragmentary tissues.

Another aspect of the fracture site geometry is the level of shear strain. Traditionally it has been thought that shear per se is disadvantageous to bone repair. However, when a load is applied to a structure or material strains are induced. The principal tensile and compressive strains are orthogonal to each other and a maximum shear strain occurs at 45 degrees to these principal strains, thus shear strains do exist. Some workers have used oblique osteotomies to investigate the effects of increased shear when compared to a transverse osteotomy,[1] in this model there was a reduction in early weight bearing on the oblique osteotomies and the mechanical properties of the healed osteotomies were greater in the transverse osteotomies. Interestingly, in this external fixation model the pin loosening was higher in the oblique osteotomies despite the initial reduction in weight bearing. The amount of inter-fragmentary displacement was not measured in this study, however, the stiffness of fixation in the oblique osteotomies was only 45% of that in the transverse osteotomy constructs. The stiffness of the fixation construct has been shown to influence both the initial weight bearing and the progression of healing,

suggesting a mechanosensory mechanism.[30] This could regulate inter-fragmentary displacement in a "stroke control" feed-back loop within a window of applied loads. Park et al (1998),[64] also investigated the effect of an oblique osteotomy compared to a transverse in a rabbit model using both locked and telescoping fixators. They also reported on the relationship between magnitude of inter-fragmentary motion recoded in vivo and the pattern of healing. With a transverse osteotomy and a telescoping fixator there was a high level of inter-fragmentary displacement initially but this reduced over the first week, whereas with an oblique osteotomy the level of inter-fragmentary displacement was high throughout the early stages of healing The oblique osteotomy group showed not only a greater inter-fragmentary movement but also a greater amount of periosteal callus and higher mechanical properties than the other groups. The authors concluded that the sliding related shear promoted greater cartilage differentiation and callus stimulation than axial motion or the locked configurations. This effect is difficult to relate to the micro-mechanics at the fracture site but does indicate a role for early cyclical deformation in enhancing callus differentiation and size to restore mechanical integrity of the fractured bone. Issues still remain to determine if these effects are the type of strain or merely reflecting magnitudes of deformation. The loads acting through the fracture or osteotomy site will also influence the magnitude of inter-fragmentary strain. These are influenced by the load applied to the limb and the load sharing of the fixation device.

The properties of the fixation device are constant, the loads applied by the patient being regulated be many factors including the fixation device stiffness, but also many undefined influences ranging from psychological reaction to the fracture and the fixation device through the direct and indirect biomechanical effects of the combined fracture and the fixation system, to systemic effects such as smoking, concurrent pharmacological treatment for pain and oedema. Thus the control of the inter-fragmentary motion is complex and the influence of this mechanical movement on the progression of healing may not be optimal for these reasons. A potential solution to this problem is to provide an applied dynamisation of the healing fracture.

Application of Cyclical Mechanical Stimulation to Modulate Bone Repair

In the development of external fixation for the repair of fractures and control of osteotomies a number of advantages have been identified in relation to a variable control of fracture site mechanics. These are to some extent offset by the disadvantage of the percutaneous fixation pins and associated care issues. However, the ability to both control and modify the stiffness provides the means to both monitor and influence the progression of healing in individual patients.

In many cases of fracture there are concurrent injuries that impede ambulation and consequent loading of a fracture. In such cases mechanical stimulation can be applied via an external fixator.

A very rigid fixator will act as a load sharing device and strain protect the fracture. However, a low stiffness fixator while reducing strain protection and providing mechanical stimulation to the fracture site will also increase the stresses at the pin/bone interface. Using the information from studies in intact bone, that short periods of cyclical deformation provide a potent osteogenic stimulus, a similar mechanical stimulus was applied to a healing osteotomy using a pneumatic actuator on a rigid external fixator.[34] The application of short periods of inter-fragmentary cyclical deformation was shown to modulate the progression of healing of a standard mid-diaphyseal tibial osteotomy. The magnitude of the initial deformation in relation to the osteotomy gap can provide either an enhancing or inhibitory effect compared to a nonstimulated control. An initial deformation in a 3 mm gap of 0.5 mm increased the rate of increase in both mineralisation of the osteotomy gap and in stiffness index of the healing osteotomy, whereas an initial 2 mm displacement induced an inhibition in the time related increases of these parameters, although the larger initial displacements induced a greater proliferation of callus (Fig. 4).

A similar observation that callus proliferation is related to the magnitude of inter-fragmentary displacement was made by Wolf et al (1998).[79] These authors also implied an optimal

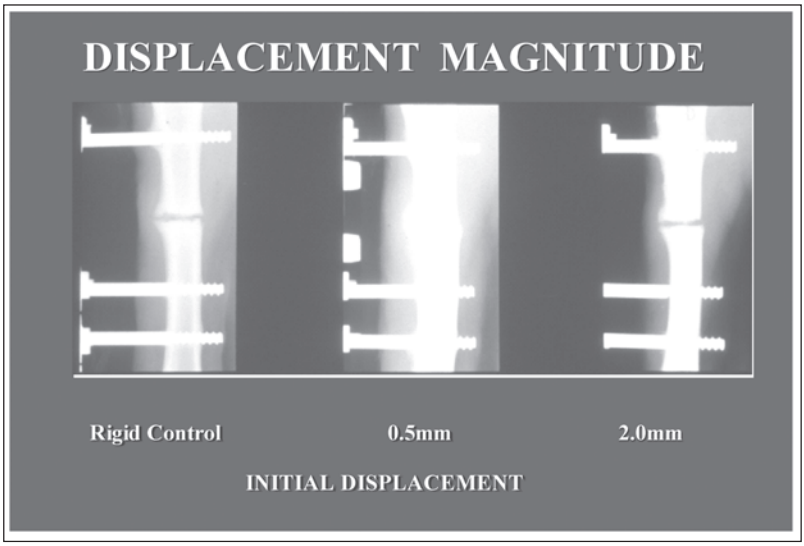

Figure 4. Effects of short periods of micro-movement on callus distribution in bone healing.

inter-fragmentary displacement of 0.5 mm in a similar model but could not show a statistically significant effect. They concluded that externally applied mechanical stimulation could have a role in stimulation of healing in patients prevented from normal weight bearing, avoiding a delayed union.

The Characteristics of Applied Mechanical Stimulation

These studies using in vivo models demonstrate how sensitive the repair process is to local mechanical conditions. However, the relevance of the role of mechanical environment in relation to human clinical fractures has also been demonstrated. The application of a mechanical stimulus increased the rate of healing with respect to an objective end point of fracture stiffness.[43,71] Using similar methods it is possible to both stimulate and monitor the progression of fracture repair in terms of restoration of mechanical integrity.

The process of bone repair, however, can be enhanced or inhibited by very subtle changes in the type and timing of the mechanical stimulation. Thus, there is potential for an optimal level and type of stimulus. The experimental studies can provide guidelines for clinical fracture management. However, each patient may require "fine tuning" of these principles. One major advantage of the system of external fixation is the ability to modify the system stiffness in response to the healing progress in that particular individual. Unfortunately, this system of fracture management is often regarded as labour intensive in the short term and may also have lower patient compliance than systems such as intra-medullary nailing.

Subsequent studies have indicated the characteristics of the stimulus that can be used to modulate bone healing.

These studies have shown significant effects in modulation of the healing process as a consequence of relatively small movements between fracture fragments. Recently, it has been shown that even sub-physiological strains can be potent osteogenic signals in intact bone. The application of these to healing osteotomies has also shown the potential to enhance indirect bone repair. Using the ovine tibial mid-diaphyseal osteotomy model with an external fixator, a 30 Hz cyclical stimulation was applied to a 3 mm osteotomy gap for a short period each day. The magnitude of the applied displacement was initially only 250 microns, while the inter-fragmentary displacement induced by walking was of the order of 1 mm. The effect of

this short term low magnitude specific frequency inter-fragmentary stimulation was to enhance the progression of healing, particularly the formation of periosteal callus. This resulted in a significantly greater torsional stiffness and strength compared to the non stimulated control. In this study the stimulus was delivered via the external fixator and the inter-fragmentary displacement measured at the osteotomy site from accessory half pins.[31] In another study by Wolff et al (2001),[31a] using a ground vibration plate to induce 200 microns at 20 Hz no significant effect was seen, although these authors did report 11% greater callus in the stimulated group compared to controls.

Chen at al (1994)[11] used a rabbit model to evaluate the effects of different frequencies of mechanical vibration on the repair of a fracture of the radius. They found that all vibration stimuli enhanced repair compared to the nonstimulated control. The most effective of the vibration frequencies was found to be 25 and 50 Hz.

This suggests a frequency dependent relationship with displacement magnitude, again resembling the effects seen in intact bone. This type of stimulus would have advantages in stimulation of a biological effect with minimal detriment to implants and the tissues occurring during the process of endochondral bone repair as a consequence of the very low strain magnitudes required.

The deformations applied at these specific frequencies were imposed as a sine wave. However, the early application of cyclical micromotion reported by Goodship and Kenwright (1985)[34] were applied using a ramped square wave, thus in both situations the rate of applied deformation was relatively high. In intact bone high strain rate is an important osteogenic mechanical signal. Using the same ovine model the importance of high strain rate was shown by Goodship et al (1998),[32] osteotomies stimulated with short periods of daily micromovement with a high rate of deformation showing a statistically significant increase in the rate of mineralisation and increase in fracture stiffness compared to both non stimulated controls and groups stimulated with a low rate of deformation. The effect of rate of deformation may be related to the nature of the healing callus, this being a heterogeneous mix of connective tissues, the nonosseus tissues in particular exhibiting viscoelastic properties. The modulus of viscoelastic materials changes as a function of strain rate. At high rates of deformation these materials exhibit a higher stiffness, thus in the healing fracture it is possible that significant strains could be imposed on the fracture fragments via the callus, whereas at lower rates of deformation the viscoelastic nature of the callus would prevent such stimuli occurring on the fracture fragments. Thus at high rates of deformation an osteogenic signal could be generated in the bone adjacent to the fracture.

The amount of deformation can be limited by the device stiffness in relation to the forces applied either by the patient or by the stimulation system. The stage of tissue differentiation will also control the level of inter-fragmentary displacement in relation to the applied force. Thus if the applied force is high the strains may exceed the ultimate strain value for the differentiating tissues and induce further damage. This may in turn lead to a delay or failure in healing. Provided the inter-fragmentary displacements are imposed with a low force the callus tissue differentiation will proceed from the fracture haematoma to lamellar bone.

As the tissues change during healing the material properties also change with an increase in stiffness, thus the inter-fragmentary displacement decreases with time, whereas the loading of the limb increased with time. Thus there is a complex control of loading of the healing fracture, subsequent to the acute period where pain associated with inter-fragmentary motion will limit the loading of the fracture. Experimental studies have also shown that even for very short periods of daily stimulation the application of a high load to achieve inter-fragmentary displacement can result in an inhibition to healing.[42] Goodman and Aspenberg (1993)[28] also suggested the exogenous application of loads could provide a stimulus for bone healing and ingrowth into porous materials by utilising the principles of Wolff's observation as early as 1892 that bone was a dynamic material responsive to mechanical environment.

The increased progression of healing of an appropriate level of initial deformation applied as a cyclical mechanical stimulus can be enhanced further by reducing the magnitude of the displacement as a function of time. This subtle programming of the mechanical input significantly improved the progression of healing in the ovine tibial osteotomy model.[18]

This type of stimulus mimics the normal reduction in inter-fragmentary displacement that occurs in normal healing and may stimulate tissue differentiation through a mechanobiologically mediated pathway. The reduction in inter-fragmentary motion could be attributed to the increased material stiffness of the different tissues present in the differentiation cascade; any adverse loading that damages these tissues would inhibit the cascade and delay differentiation. Alternatively the reduction in motion may be mediated by other mechanisms, possible mechanosensory pathways, and the consequence of the reduced motion drives the tissue differentiation. Thus the active time related reduction in displacement[18] demonstrated that this additional refinement to the healing environment did have a further significant effect.

These studies using in vivo models have demonstrated the extreme sensitivity of the process of bone healing in response to local mechanical environment, and the potential for individual control and monitoring through appropriate fixation systems.

The Time of Application of Imposed Mechanical Stimulation

The critical period for assuring rapid progression of fracture healing is in the early stages of the repair process. Delay in providing the correct environment for healing can lead to a delay or even failure of the process to occur at all, resulting in a nonunion (McKibbin, personal communication).

Although some regimens of applied micro-movement have been shown to enhance the rate of repair in osteotomy models, when applied at a later stage of healing these regimens can adversely affect the progression of healing. The regimen of a high displacement rate applied at the onset of healing significantly increased the rate of both mineralisation and fracture stiffness index. However, if the same regimen was applied at the time of callus bridging, the effect was to inhibit the progression of healing. The stimulus was only applied for approximately 17 minutes per day, thus healing can be adversely affected by changes in mechanical environment during the period of repair and these changes may only be for a short time.[32]

The study by Lawes et al (1996),[32a] in which fixators of two differing levels of compliance were used, showed the difference between the two groups in terms of inter-fragmentary displacement was seen only in the early stages of the repair process. By six weeks post-osteotomy levels of inter-fragmentary displacement were similar in each group and remained so declining in both groups to the end of the healing period. However, the progression and outcome of healing were significantly greater in the groups with the low compliance fixator, which induced a greater magnitude of inter-fragmentary displacement in the very early stages of repair. This also provides evidence that stimulation in the early stages of healing could enhance the process. Indeed this was confirmatory of a trial in human patients by Kershaw et al (1993)[44] in which the magnitude of inter-fragmentary motion was determined in the early stages of healing and found to be in the order of 0.6mm, this was increased by 50% using an externally attached actuator. A significant reduction in healing time was obtained in a prospective trial using micromotion applied in the early stages of the healing process.

In an ovine experimental study with a 3 mm tibial osteotomy Hente et al (1999),[39] were unable to show an effect of axial dynamisation. However, in this study the dynamisation was not applied until the fourth week post osteotomy, thus the lack of modulation of the repair process could be a consequence of missing the early phase of healing or alternatively an inappropriate dynamisation regimen. As stated previously the characteristics of any applied mechanical stimulus will influence the type of modulation compared to the nonstimulated pattern of repair.

Transduction Mechanisms

The effects of different mechanical conditions at the fracture site have been discussed in relation to the material properties of the matrices of the range of connective tissues that are induced and differentiate in the process of indirect bone repair as a consequence of fracture or osteotomy. Indeed increasingly the effect of periods of cyclical mechanical strain are being found to modified the various tissues that also appear in bone healing. For instance the development of sutures, which are fibrous joints between some of the bones of the skull, is modified by cyclical strain which significantly enhanced sutural growth.[47] However, the connective tissue matrices are produced, maintained or modified as a function of cell activity. The cells respond to mechanical cues or signals which are related to the loading of the limb and local material conditions of the adjacent matrix.

The transduction mechanisms for both mechanical and biological cell signalling provide potential pathways to modify the process of healing in a controlled manner, for example in limb lengthening where soft tissue extension requires long periods of slow distraction that are impeded by the potential for osteotomies to heal. For instance, it may be possible to superimpose a cyclical micro-movement regimen that reduces the rate of healing during the distraction phase.

Direct deformation of cells can induce changes in cell metabolism. Various studies in vitro have shown that different magnitudes of strain will influence proliferation and cell metabolism.[59] This group also demonstrated that the response of bone cells to direct strain in vitro was modulated in skeletal conditions that also down-regulated the response of the skeleton to mechanical loading in vivo. They found that osteoblasts from osteoporotic patients responded to in vitro cyclical strain with a reduced proliferation and TGFβ synthesis compared to osteoblasts from normal subjects (Neidlinger-Wilke et al, 1995)[58] suggesting that in vitro response to direct mechanical stimulation is representative of the whole body response. The cells may also respond to fluid shear stresses and the electrical effects of streaming potentials as ionic fluids flow both within the cells and matrix as a consequence of mechanical deformation of skeletal structures.[52,46] The effect of high rate micro-motion could therefore be a direct effect of strain at the cellular level or alternatively mediated indirectly as a consequence of fluid movement and streaming potentials.

The transduction pathway of mechanical strain in bone has been shown to involve the prostaglandins,[81] inhibitors of prostaglandin synthesis such as indomethacin which blocks both constitutive and inducible cyclo-oxygenases down-regulate the adaptive response of bone to mechanical stimulation.[60,65] The prostaglandins also play a role in bone repair, the effects of mechanical stimulation using applied cyclical inter-fragmentary micromotion involve the presence of endogenous prostaglandins. Different regimens of micro-motion were shown to be associated with different levels of prostaglandins in the callus at a very early stage of the repair process,[30] this may also indicate the importance of providing an appropriate mechanical stimulus in the early stages of the repair process. The role of prostaglandins in bone repair should also be considered with respect to the inhibitors of cyclo-oxygenases that might be used clinically and could have an adverse impact on healing by blocking an important transduction pathway in the critical stages of healing.[24]

Recently it has been found that mechanical strain acts via the estrogen receptor and thus this receptor provides a common pathway for mechanical and hormonal activation of bone cells.[21,41] Estrogen receptor mRNA has also been found in fracture callus[5] and thus could represent part of the transduction pathway for mechanical modulation of bone healing. Further, evidence suggests that estrogen receptors are only found in the callus tissues present in the early stages of healing and absent in the latter stages.[56] This may also be related to the importance of imposing the correct mechanical stimulus in the early stages of repair. Thus manipulation of estrogen related pathways also has the potential to modulate the progression of bone healing.

Another component of the pathway, again common with bone adaptation, is nitric oxide. This mediator has been shown to be present in the cascade of indirect repair, suppression of this messenger molecule being inhibitory to the repair process.[20] Additionally this mediate has been shown to be most prevalent in the early stages of repair[19] with specific isoforms occurring in association with progression though the early tissue types.[84] Thus the interaction of the different isoforms with time in the repair process could have implications for modulating the progress of the repair process using inhibitors or stimulators at specific times after injury.[83]

Despite the identification of these potential transduction pathways further work is required to identify the critical time related interactions and thus optimise conditions in specific fractures for a maximal rate of restoration of mechanical integrity.

The Process of Bone Repair as an in Vivo Tissue Regeneration Scenario and Implications for Tissue Engineering

Interestingly, the fracture repair process seen in indirect or endochondral repair involves the formation de novo within the adult osseous skeleton of the entire range of connective tissues, from blood in the immediate post-fracture period forming the fracture haematoma, through fibrous and cartilaginous connective tissues to lamellar bone. Additionally the endochondral element of the repair process resembles a repeat of embryological bone development. This indicates the potential for the body to regenerate connective tissues given the appropriate mechanical and biological signals. Thus a delayed or nonunion is a complication for fracture healing but an in vivo bioreactor to generate nonosseous connective tissues.

In hypertrophic nonunions hyaline cartilage is formed on a bone surface, this biological response could provide a biological solution to resurfacing degenerate joints. Theoretical models of tissue formation in fracture healing have been proposed based upon the influence of stress and strain in relation to tissue differentiation. Claes and Heigele (1999)[16] developed a model that suggests influences that might indicate the conditions inducing specific tissue differentiation from mesenchymal stem cells in the process of bone repair. This model suggests that low values of strain and hydrostatic pressure induce intra-membranous bone formation, whereas high values of hydrostatic stress with low strains induce cartilage and high hydrostatic stress with high strains leads to fibrocartilage. This work built on previous modelling of both bone development and bone healing that indicated a potential to control tissue formation solely by imposed mechanical environment and thus set the scene for the role of mechanobiology in skeletal tissue engineering.[4,6,7]

As has been discussed above the application of specific regimens of inter-fragmentary movement can lead to enhancement or inhibition of the progression of bony union. Some regimens will induce formation and persistence of nonosseus tissues. In addition the size of an osteotomy gap will also influence the progression of healing and large gaps form a "critical size defect" that inhibits bone union resulting in a persistence of fibrous fibro-cartilaginous or cartilaginous tissue. The interaction of biological and mechanical signals in engineering differentiation of specific connective tissues requires a mechanically defined model, the use of rodent species allowing biological techniques to be used that can identify the associated molecular mechanisms,[37] With such a model where mechanical conditions can be determined to some extent, the effects of biological cues can be examined under more controlled conditions.

The models that have been proposed in relation to the mechanobiology of bone formation and connective tissue differentiation in bone healing need to be cross referenced to the emerging field of tissue engineering.

An understanding of the mechanical and biological interactions in bone repair contributes to the requirements to engineer skeletal tissues both using bioreactors in vitro and manipulation of tissues in vivo. Additionally these mechanisms can be applied to improve osseointegration of current synthetic skeletal prostheses.

Fracture healing should be viewed not only as a clinical challenge but also as one of the most fascinating tissue repair and regeneration processes in the body.

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CHAPTER 8

Experimental Animal Models for Tissue-Engineered Bone Regeneration

Véronique Viateau and Geneviève Guillemin

Abstract

Choosing an appropriate experimental model for the study of bone repair necessitates to clearly identify the problem to be solved in order to obtain the right answer to the right question. Animal tests constitute a step midway between in vitro studies and human clinical applications. Experimental designs allow selection of the species and standardised operative and analysis procedures. This paper reviews the different animal models used for studying large bone defects repair in human clinical situations. Relevance, objectivity and reproducibility of the models as well as genetical status of the animal, financial and ethical factors influencing the decision are discussed.

Biomaterials have to be evaluated in experimental animal models before being used in human clinics. Animal tests constitute a step midway between in vitro studies and human clinical applications. Experimental designs allow selection of the species and standardised operative and analysis procedures. The model must be relevant, reproducible, cost-effective and must demonstrate low morbidity

Several animal models have been developed to test bone healing, making it difficult to choose the most appropriate. The present paper reviews the most commonly used models in bone tissue engineering and offers a comprehensive decision-making approach.

Factors Involved in Choosing a Model

Before deciding on an animal model, the problem to be solved has to be correctly identified in order to obtain the right answer to the right question.[1] The animal species to be used and the experimental design to be selected will depend upon the question asked. While model relevance is the most important factor, experimental design reproducibility and morbidity, and objectivity of data analysis, are elements of major importance. In addition, technical and financial limits may modulate the final choice.

Model Relevance

A model is relevant if experimental conditions and generated effects are linked. The experimental design must therefore include the innovating technique as well as negative and positive controls guaranteeing valid comparisons.

As far as tissue engineering in bone regeneration is concerned, experimental objectives fall into two different categories depending on whether the bone replacement material (BRM) is submitted to a preliminary evaluation to assess its biofunctionality or is evaluated in a preclinical setting. Biofunctionality does not need to be evaluated in a complex biological and biomechanical environment reproducing clinical-like situations. Simple tests such as animal

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implantation in ectopic (sub-cutaneous or intra-muscular) and orthotopic (calvaria) sites allow in vivo evaluation of biocompatibility, biodegradability, osseointegration, osteoconductivity, osteoinductive and osteogenic potential. On the contrary preclinical evaluations rely on animal models simulating the clinical situation in which the BRM will be used. Because the main application of BRM in orthopaedic surgery is bone loss replacement, the most commonly used designs are surgically induced bone defects that are known to progress to nonunion if not replaced. The notion of a critical size defect (CSD) was firstly described by Schmitz as "the smallest intraosseous wound that does not heal by bone formation during the lifetime of the animal".[2] Hollinger et al further defined a CSD as "a defect which has less than 10% of bony regeneration occurring within the lifetime of the animal".[3] The CSD must be created under conditions of optimal mechanical stability in order to guarantee that nonunion results exclusively from bone loss. Periosteum should also be resected because leaving the periosteum intact when performing resection of the bone enhances bone healing.

Experimental design and associated effects must not only be correlated in the laboratory animal but also in human surgical applications. Animal anatomy, bone healing and remodelling specifications, as well as immunological and genetic status, condition model relevance.

Anatomical Features

Specific anatomical characteristics may condition the feasibility of surgical procedures in preclinical tests. For example, mandibular defects, currently used in maxillofacial experimental surgery to evaluate BRM, are challenging to perform in rodents and rabbits as mouth opening is very limited in these species. BRM stability is also difficult to obtain in such very small defects.[3] Bones shapes and sizes also condition surgical technical feasibility. Rodents and rabbits have small-sized long bones, with thin and fragile cortices, thus requiring delicate surgical technique and custom-made implants for bone fixation.[4,5] Cats, dogs, sheep and pigs have larger bones, allowing the use of surgical techniques and implants designed for humans.

Lastly, the type of bony tissue in which the material is tested will be different depending on the future applications of the BRM: calvarias (i.e., membranous bone) will be preferred if the material is to be used in cranioplasties while metaphyseal extremities (i.e., trabecular bone) or diaphysis (i.e., cortical bone) will be preferred if the material is to be used for filling defects or segmental loss in long bones.

Bone Healing and Remodelling Characteristics

Bone healing and remodelling characteristics in the laboratory animal depend on many variables amongst which some are species or age dependent. Bone blood supply and mechanical loading also affect healing.

Order along the phylogenetic scale inversely correlates with the rate of bone repair: bone healing capacity is higher in rodents and rabbits than in other species.[2] The type and rate of bone remodelling also differ amongst species. Whereas large animal (rabbits, cats, dogs, pigs, and nonhuman primates (NHP)) show Haversian type remodelling in cortical bone, rodents do not.[6] Cortical bone remodelling in rabbits is also twice as fast as in dogs and three times as fast as in humans.[7] These features must be taken into account as they may affect material resorption: natural coral resorption is for example slower in sheep than in pigs.[8] Results in rodents and rabbits must therefore be considered with caution and validated in other species higher on the phylogenetic scale before attempting extrapolation to humans.

Immature animals have higher bone healing capacity and regeneration compared with adults. The magnitude of a critical size defect is therefore inversely related to the age of the animal and bone substitutes must be evaluated in adult animals in which closed epiphyseal plates are documented with radiographs.[9] Mice and rats remain exceptions to that rule as bone growth is constant throughout life in these species.[6]

Local blood flow and mechanical loading should be critically assessed when studying augmenting bone healing procedures. Kusumuto[10] has recently noted the importance of blood supply in promoting heterotopic osteoinduction by rhBMP-2. Skeletal unloading in weight

bearing bones decreases osteoblast number, bone formation rate, bone mass, bone maturation and mechanical strength.[11]

Biomechanical Features

Variations observed in the mechanical properties of bone arise from differences in cross-sectional geometry, relative proportions of trabecular and cortical bone, amount of mineralisation, degree of porosity and Haversian remodelling. Specific biomechanical behaviour therefore correlates more with the particular shape and function of the bone, the size of the animal and its lifestyle than with its taxonomic position. Specific bone loading conditions must be taken into consideration when choosing an experimental design. As emphasised by Van der Meulen,[12] conclusions regarding bone mechanical function based solely on geometry or bone mineral content are inappropriate: when performing biomechanical assays one must remember that there is no alternative to testing whole bone strength.

Immunological Status

An assessment of allografts or the osteogenic potential of various allogenic or xenogenic cellular components may benefit from implantation in immunocompromised animals. For these reasons, immunocompromised nude or SCID mice are currently used.

Physiological Characteristics

Bone physiology is partly conditioned by oestrogenic metabolism in females. Augmenting bone healing procedures are therefore usually performed in males. Ovariectomy-dependent bone loss models developed to mimic human osteoporosis are exceptions to that rule. Mice, rats and NHP[13] are currently used for this purpose. Preliminary studies have indicated that ovariectomised ferret[14] or sheep[6] could also be interesting models for human osteoporosis. The degree of osteopenia obtained is species dependent: osteopenia in ovariectomised monkeys is mild compared with the profound cancellous osteopenia observed in ovariectomized rats. NHP models do mimic early post-menopausal changes in skeletal biology but not the disease of post-menopausal osteoporosis.

Genetic Status

Unless specifically required, genetic variations are not usually taken into consideration and experimental trials are currently performed in pure breed animals (i.e., undefined genetic homozygosity) or "mongrels" (i.e., unknown genotype). Genetic variations in bone regenerative capacity nevertheless do exist as demonstrated by Li.[15]

Genetic uniformity is required for cell or tissue transplantation trials: hybrid rodents, rabbits (New-Zealand White rabbit), micropigs (Yucatan Micropig) or immunocompromised inbred mice (SCID or nude mice) are used for this purpose. Genetic selection of laboratory animals in bone engineering can also be performed to develop specific bone diseases (i.e., SAM mice developing osteoporosis).

Model Objectivity and Reproducibility

Bone regeneration must not only be evaluated in animals treated with the innovating technique but also in sham-operated animals (negative controls) and in animals treated with autologous corticocancellous bone grafts (positive controls) which remains the gold standard material in bone tissue engineering. Each group should include at least 5 animals to allow analysis of statistical significance.

Accurate follow up and assessment of bone regeneration both through conventional radiographic, microradiographic, histologic and ideally biomechanical data must be achieved. Recent availability of imaging techniques such as micro-computed tomography opens up new perspectives.[16] Quantitative methods allow objective comparison of bone healing between groups and are preferred over semi-quantative techniques. Quantitative assessment of bone healing is possible provided that animals are of similar sizes and breeds, that bone sizes and curvatures as

Table 1. Analgesics used in laboratory animals

	Mouse	Rat	Guinea Pig	Rabbit	Cat	Dog	Sheep	Pig
Aspirin (mg/kg)	120 p.o. every 4 h	100-150 p.o. every 4 h	50-100 p.o. every 4 h	100 p.o. every 8 h	10 p.o. every 48 h	20 p.o. every 12 h	-	-
Carprofen (mg/kg)	-	5-10 p.o.	-	2.2 p.o. every 12 h	1-2 p.o. every 12 h	4 p.o., SC every 24 h	-	-
Meloxicam (mg/kg)	-	-	-	-	0.1 sid	0.2 SC first day; 0.1 p.o. 1-3 w	0.5 SC or IV once	-
Ketoprofen (mg/kg)	5 every 24 h IM, SC	1-3 every 12 h IM	-	1-3 IM every 12-24 h	2 every 24 h; 1-3 days SC, IV	2 every 24 h; 1-3 days SC, IV	3 every 24 h; 3 days IV IM	3 every 24 h; 1 day IV, IM
Flunixin meglumin (mg/kg)	2.5 every 12-24 h SC	2.5 every 12-24 h SC	2.5 every 12-24 h SC	1-2 SC IM every 24 h	1 SC IM every 24 h	1.1 IM once	2.2 IV	0.5-1 SC every 12-24 h
Morphine (mg/kg)	2-5 SC every 2-4 h	2-5 SC every 2-4 h	2-5 SC every 2-4 h	2-5 SC every 2-4 h	0.1 SC, IM every 6 hours	0.2-5 SC, IM every 4 hours	-	0.2 IM SC every 4 h (< 20 total)
Fentanyl transdermal patch (μg/h)	-	-	-	Half a 25 patch every 72 h	25 every 96 h	10-20 kg:50 20-30 kg:75 >30 kg:100 every 72 h	-	-

well as shapes and surfaces of defects are reproducible. Straight bones (metatarsus, mid-femur or tiba) allow good reproducibility in data acquisition for histomorphometry and biomechanical assays whereas curved bone (radius or ulna) do not. In such situations only semi-quantitative methods can be used.

Model-Associated Morbidity

High morbidity in animal models often reflects poor surgical procedure design. When selecting a model, associated morbidity should be critically assessed and designs with high morbidity should be discarded. Models developed in the oral cavity and models using external fixation are more prone to post-operative complications.

Unless contraindicated, pain control must systematically be provided through the peroperative and early post-operative periods (Table 1). Amongst the most commonly described experimental procedures, experimental fractures, bone transfers, massive corticocancellous graft collections and bilaterally performed orthopaedic procedures are extremely painful and provide major subject discomfort and stress. External fixation provides some degree of permanent discomfort. Transdermal fentanyl patches can be used in conjunction with conventional

antiinflammatory medication to ensure satisfactory pain control in cats and dogs in these painful procedures.[17]

Ethical Considerations

In vivo experiments should only be done when alternative techniques (cell or organ cultures) fail to solve the problem. When experimenting a new design, preliminary assays should be performed on a small number of animals and models with the lowest morbidity. All the animal experiments must be done by experienced and specially trained personnel licensed for animal experimentation. Protocols must be evaluated by an ethics committee and tests must be performed in accordance with the usual regulations of the country. In order to prevent unnecessary animal usage, the scientific community should be informed of negative results in publications to minimize future repetition of unfruitful experimentation.

Financial Considerations

Models developed in rodents and rabbits are cost-effective and the volume of material to be tested is not great. A large number of animals can therefore be operated allowing preliminary studies performance at low expense. Models using medium-sized to large-sized animals are expensive and are more demanding as far as housing facilities and support staff is concerned. They are essentially used in preclinical trials.

Technical Limitations

The choice of laboratory animal must take into consideration the ease of handling as well as feasibility of postoperative care. When a large animal is needed, sheep are preferred over pigs which are challenging to manipulate and confine. External fixation will also preferably be performed in dogs which are housed in a clean environment.

The choice of the experimental design is conditioned by individual technical skills and surgical facilities: whereas calvarial defects, subcutaneous, intramuscular and intraperitoneal implantations in rodents or rabbits can be performed without specific surgical skills and specialised instrumentation, segmental bone defects, spinal fusions do require surgical experience and specific equipment. The experimental design must also take into account the physical characteristics of the biomaterial. Pastes or liquid must be injected in a closed cavity to prevent leakage (i.e., sealed metaphyseal defects) and particulate or massive materials often require additional restraints to remain under stable biomechanical conditions. Lastly, the experimental design must consider the analysing techniques that will be applied and ensure its compatibility with them (i.e., presence of metallic implants preclude the use of imaging techniques such as CT or IRM).

Decision Making: Current Animal Models Used in BRM Evaluation

As stated previously one must identify the setting in which experiments are conducted. Evaluation of a BRM's biofonctionality is independent of its future field of application and is always performed in the same animal models. On the contrary preclinical evaluations must be performed in an animal model that reflects a specific human clinical setting.

Preliminary Evaluation of the Biofonctionality of a New Biomaterial

Heterotopic Implantation

Subcutaneous or intramuscular implantations are the gold standards for in vivo evaluation of biocompatibility as well as osteogenic and osteoinductive potentials. These simple surgical procedures are mostly performed in rats and lagomorphs for financial reasons and technical simplicity but have also been described in larger species. The material is usually directly implanted in the recipient bed. Culture cells that are not loaded on a vehicle are inoculated in diffusion chambers prior to implantation. The nucleopore filters of diffusion chambers prevent invasion of implanted material by host's cells while allowing the diffusion of nutrients and cell

contacts through the pores. Intraperitoneal implantation of these chambers is also currently performed.[18-20]

Orthotopic Implantation

Orthotopic implantation allows evaluation of osteoconductivity and osseointegration of the material as well as its biodegradability in a bony environment.

Rat calvaria is the gold standard model for orthotopic implantation. Calvaria develops from a membrane precursor, has poor blood supply and a relative deficiency of bone marrow. Calvarial defects therefore create a hostile environment for bone healing. Single midsagittal 8 mm circular defects[2] and bilateral 5 mm parietal defects[21] can be performed in rats: both create CSD as they give rise to a fibrous nonunion when bone loss is not replaced. Craniotomies are easy to perform, have low morbidity and are highly reproducible if created with a circular trephine. All forms of material can be evaluated in that location which is particularly well suited for granular or paste-like materials (for illustrations see paper by Logeart-Avramoglou). A large number of animals can be operated on allowing significant statistical analysis. Laterally performed craniotomies have the advantage of allowing paired design and minimised morbidity while avoiding accidental damage to the midsagittal sinus. The close vicinity of adjacent defects may nevertheless allow substance diffusion and impair model relevance.[22]

Preclinical Evaluations of Augmenting Bone Healing Properties in Bone Replacement Procedures

Cranial Surgery

Calvarial, parietal or skull defects have been described in rabbit, dog, sheep, minipig and NHP.[2,3,22,23] They are easy to perform, are highly reproducible and have low morbidity. The experimental design correctly reflects the clinical setting in which cranioplasty is performed although calvaria regenerative capacity in animals encompasses that of humans in whom calvaria is devoid of muscular insertions. Several defects can be performed in the same animal allowing comparisons of several biomaterials, and negative and positive controls in the same animal. Up to twelve 22-mm defects per animal have for example been performed by Viljanen in sheep.[22]

Maxillo-Facial Surgery

Filling Defects

Filling defects in maxillofacial surgery often result from alveolar bone resorption at tooth extraction sites. Such models have been developed in the dog by mandibular and maxillary premolar extractions.[24] Bone fillers are also currently evaluated in uni or bicortical mandibular filling defects, developed in the dog,[3] miniature pig,[25] and sheep.[7] Mandibular defects have several advantages over periodontal wounds:[7] (i) they can be standardised in size and shape, (ii) they involve a closed wound rather than an open wound in the mouth, (iii) the only tissue regenerated is bone. Calvarial defects like those described in rabbits, dogs and NHP are also currently used because of anatomic similarities with the mandible (two cortical plates with intervening cancellous bone).[2] A nasal critical-size defect has been described in large size Sprague Dawley rats.[26] The later has the advantage of having been developed in an endochondral type bone whereas calvarial defects are developed in a membranous type bone.

Segmental Bone Defects

Mandibular discontinuity defects are created for experimental trials to mimic segmental bone resections that are currently performed in oncologic maxillofacial surgery. These designs partly reproduce the adverse clinical setting in which bone replacement usually takes place in maxillofacial surgery: (i) bone loading requires segment motion neutralisation by additional bone fixation when extensive bone resection is performed, (ii) replacement is performed in the

vicinity of a potentially highly contaminated site. Masticatory stresses in human and animals are nevertheless different and vary from one species to another according to their nutritional habits. Models have been described in the rat, rabbit, dog, sheep and NHP.[3,27] As previously stated by Hollinger and Kleinschmidt,[3] mandibular bone is very thin in rodents and rabbits and retention of implants is challenging. We do not recommend mandibular resections in these species. Mandibular discontinuity defects are preferably performed in dogs, sheep or NHP in which they are easier to perform. Although objective information is lacking on the subject, mandibular CSD is, according to Schmitz and Hollinger[2] probably as great as 40 mm long in adult foxhounds and up to 25% the length of the mandible in NHP. Mandibular resections are prone to postoperative infections and, when extensive, are challenging to stabilise. Complications are minimised by (i) preventive extraction of teeth bordering the defect (maxillary teeth included) 2 weeks before bone resection,[3] (ii) submandibular approaches to avoid intraoral perforations, (iii) mandibular bone fixation with bone plates.

Orbital wall defects have been performed in sheep.[28] Model reproducibility may be superior to mandibular defects and morbidity lower but as they are located in nonloading bones this model should only be used for material to be used in unloaded bones (Table 2).

Orthopaedic Surgery

Filling Defects

Many BRM are used as "bone fillers" in defects resulting either from bone sampling for grafting procedures, from metaphyseal traumas, from surgical removal of benign or malignant tumours. Bone fillers are engineered as paste-like, granular or massive (preset blocks) materials and experimental defects must be compatible with these specific presentations.

Standardised metaphyseal defects have been described in rabbit[34] (Fig. 1) and sheep[35] distal femoral condyles and in the dog proximal tibia.[36] They do not require additional specific immobilisation and are highly reproducible thus allowing quantitative statistical analysis of histological data. Defect lumens are sealed either with a bone flap or with methylmetacrylate cement to prevent material leakage. As they are located in loaded bones, they are more interesting than the CS Ilial defect recently described in the goat.[37]

Segmental Bone Defects

Keys has shown that under experimental conditions, a segmental long bone defect 1.5 times the diaphyseal diameter, exceeds the regenerative capacities of bone in skeletally mature dogs and results in nonunion when bone loss is not replaced.[9] The length of bone to be resected to obtain nonunion is species and bone-dependent and must therefore be established each time a new model is designed (negative controls). Key's observations nevertheless roughly apply to many species (cats, pigs, sheep, NHP) and can serve as guidelines for new designs (Table 3). Rodents and rabbits remain exceptions to that rule as comparatively longer resections are needed to generate nonunion in these species.

As stated previously, segmental defects must remain in a perfectly stable biomechanical environment while healing occurs. Unlike in humans, weight bearing is difficult to prevent in animals. Achille's tenotomy is not a reliable adjunctive technique as it only prevents weight-bearing for a short time.[38] Models in which the bone fixation technique correctly neutralises forces (locked intramedullary nailing, bone plating) are therefore preferred over unlocked intramedullary nailing or external coaptation by plaster or casts which do not neutralise compression, traction and rotation forces. Although satisfactory from a biomechanical point of view, external fixation has several limitations: (i) weight bearing is inconsistent, (ii) bone lysis around the pins occurs over time thus compromising stability, reducing model reproducibility and relevance.[39,40]

Femoral resections have been performed in rats by many authors: 5 to 8 mm long bone resections (20 to 25% of femoral bone length) stabilised by custom made bone plates[4] or external fixators[5] generate nonunion in approximately 12 weeks.

Table 2. Currently performed animal models for pre-clinical evaluation in cranial and maxillofacial surgery

Animal Species	Author	Bone	Diameter or Length (mm)	Discontinuity Fixation Technique	Bone Observation Length (w)	Negative Controls	CSD	Positive Controls	Morbidity
Rat	Bosch[21]	Calvaria	8	-	34-52	+	+	-	+
	Lindsey[26]	Nasal bone	20 x 8	-	24	+	+	-	0
Rabbit	Frame[29]	Calvaria	15	-	36	+	+	-	0
Dog	Prolo[30]	Calvaria	20	-	24	+	+	-	0
	Hjorting-Hansen[31]	Mandible	8	-	16	+		-	0
	Leake[32]	Mandible	30	Alloplastic trays	24	+		-	++
Pig	Lindholm[33]	Skull		-					
Sheep	Viljanen[22]	Calvaria	22	-		+	+	-	0
	Salmon[7]	Mandible	9	-	6-12	+		-	0
	Schliephake[27]	Mandible	unicortical	-		+	+	-	0
	Hanson[28]	Orbital wall	- 15 x20	-	24	-		-	0
NHP	Hollinger[3]	Calvaria	15	-	8	+		-	0

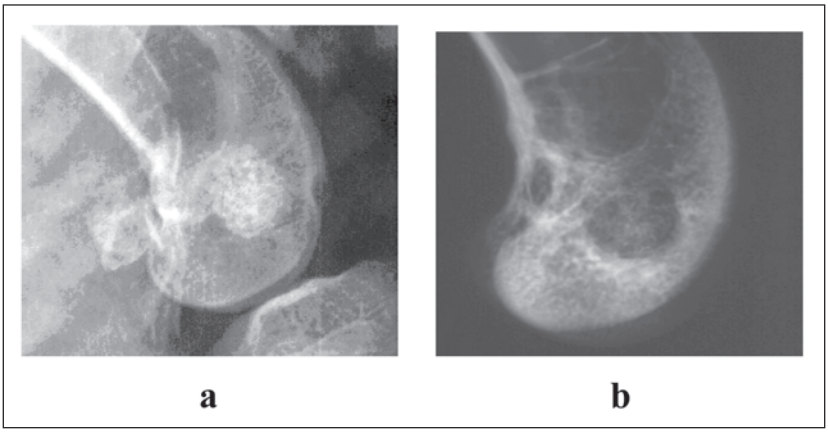

Figure 1. Distal femoral filling defects in rabbit. a) filled with coral alone; b) filled with a mixture of fibrin sealant and coral.

Rabbit ulna is one of the most currently used segmental long bone resection models.[41,42] The surgical procedure can be performed bilaterally,[43] is easy to perform and internal fixation is not theorically required as ulnar stability is afforded by radio-ulnar synostosis. The ulna is a weight bearing bone which allows implant loading and morbidity is low. Several limitations must nevertheless be pointed out: (i) spontaneous bone healing occurs in 30% of animals after 10 mm long resections and some authors have observed bone healing with 20 mm long ostectomies making this model more a model for delayed bone healing than a CSD,[44] (ii) bone curvature and synostosis makes implant positioning and stabilisation challenging for massive materials, (iii) individual variations in bone curvatures impair reproducibility of bone sampling for histologic analysis and biomechanical assays (Fig. 2). Radial resections have been performed in rabbits and carry the same disadvantages.[45] Tibial resections stabilised by unlocked intramedullary nailing have been associated with rotational instability and high morbidity which preclude their use.[46]

A highly reproducible CSD model has been developed in the cat tibia.[9] Femoral,[42,47,48] tibial,[49] radial[40] or ulnar[50] resections have been performed in dogs and were either stabilised by bone plating[47,48] or external fixation.[40,49] Some of these models have shown spontaneous bone healing and are not thus CSD.[40] 25 mm long ulnar resections have been performed in dogs and carry the same advantages and disadvantages as in the rabbit. Radial synostosis is nevertheless more localised than in the rabbit which simplifies implant positioning and stabilisation.

Femoral resections stabilised by bone plates and tibial resections either stabilised by bone plates[51] or an intramedullary interlocking nail[52] have been performed in sheep. The recently described metatarsal resection[53] carries many advantages: (i) it is a true CSD as fibrous nonunion occurs for 25 mm long bone defects after 4 months observation, (ii) it is performed in a straight bone allowing reproducible histomorphometric and biomechanical assays, (iii) it has a low morbidity (Fig. 3).

Post-operative handling of pigs is often difficult making orthopaedic surgery challenging. A radial defect has been described in the miniature pig with minimal morbidity.[54] Femoral defects stabilised either by bone plate or external fixation have been proposed by Sendowski in Pitmann Moore minipigs and Large White pigs. Morbidity with these models has been high: external fixators have failed in both sized animals and bone plates have been satisfactory in minipigs only.[55]

Femoral, tibial, ulnar and humeral resections have been performed in NHP.[56-58] Although developed in a species close to man as far as bone healing and biomechanical loading are concerned, the experimental designs suffer from major drawbacks: (i) lack of negative controls[56-58]

Table 3. Animal models for pre-clinical evaluation for long bone resection in orthopaedic surgery

Animal Species	Author	Bone	Resection Length (mm)	Bone Fixation	Observation Period(w) Technique	Negative Controls	CSD	Positive Controls	Morbidity
Rat	Werntz[60]	Femur	5	Bone plate	12	+	+	-	ND
	Einhorn[61]	Femur	6	External fixator	12	+	+	-	ND
	Wolff[4]	Femur	8	Bone plate	8-16	-	ND	-	ND
Rabbit	Dahners[62]	Ulna	10	-	6	+	-	+	ND
	Gaullier[63]	Ulna	20	IM Pin	8	+	-	-	-
	Bolander[42]	Ulna	20	-	12	+	+	+	-
	Wittbjer[45]	Radius	12	-	4	-	ND	-	-
	Kitsugi [46]	Tibia	16	IM pin	25	ND	-	-	+++
Dog	Johnson[40]	Radius	25	External fixator	24	+	-	+	+
	Johnson[50]	Ulna	35	Bone plate	14	-	ND	+	ND
	Bruder[48]	Femur	20	Bone plate	16	+	+	-	-
	Cong[47]	Femur	15	Bone plate	8-24	-	ND	-	ND
	Johnson[49]	Tibia	15	External fixator	12	+	-	+	+
Cat	Toombs[9,64]	Tibia	10	Bone plate	12	+	+	+	-

Table continued on next page

Table 3. Continued

Animal Species	Author	Bone	Resection Length (mm)	Bone Fixation	Observation Period(w) Technique	Negative Controls	CSD	Positive Controls	Morbidity
Pig	Sendowski[55]	Femur	25	External fixator or bone plate	24	-	ND	-	+++
Minipig	Sendowski[55]	Femur	25	-	24	-	ND	-	+++
Minipig	Meinig[54]	Radius	30	Bone plate	12	+	-	-	-
Sheep	Ehrnberg[65]	Femur	40	External fixator	16	+	+	-	+
	Gerhart[66]	Femur	25	Bone plate	12	+	+	+	+
	Wippermann[38]	Tibia	20	Bone plate	12	+	-	+	+
	DenBoer[67]	Tibia	30	Locked IM pin	12	+	-	+	-
	Muir[52]	Tibia	50	Locked IM pin	14	-	ND	+	-
	Gao[51]	Tibia	25	2 Bone plates	16	-	ND	-	-
	Viateau[53]	Metatarsus	25	Bone plate	16	+	+	+	-
NHP	Andersson[56,57]	Humerus	50	Bone Plate	24-240	-	ND	-	-
	Andersson[56,57]	Femur	76	Bone Plate	24-240	-	ND	-	-
	Andersson[56,57]	Tibia	63	Bone Plate	24-240	-	ND	-	-
	Cook[58]	Ulna	20	External coaptation	20	-	ND	-	-
	Cook[58]	Tibia	20	IM pin + external coaptation	20	-	ND	-	-

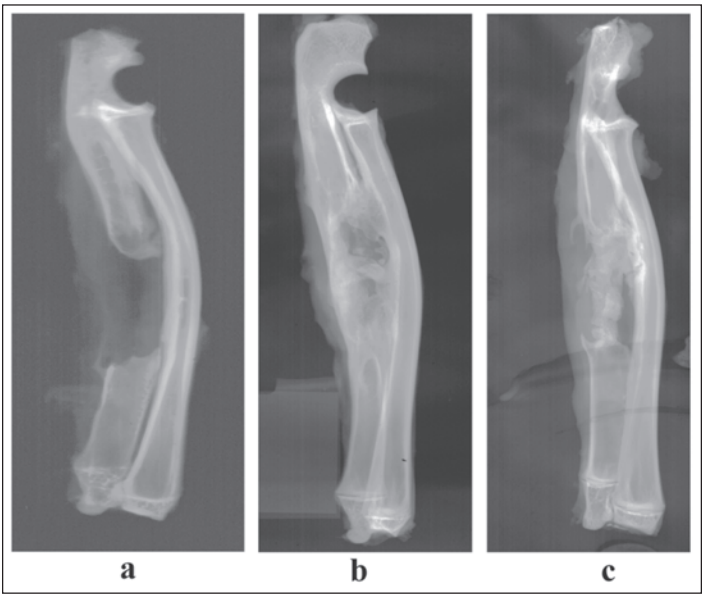

Figure 2. 2 months postoperative radiographs of 20mm long ulnar resections in rabbit. a) without any bone filling biomaterial b and c) filled with coral.

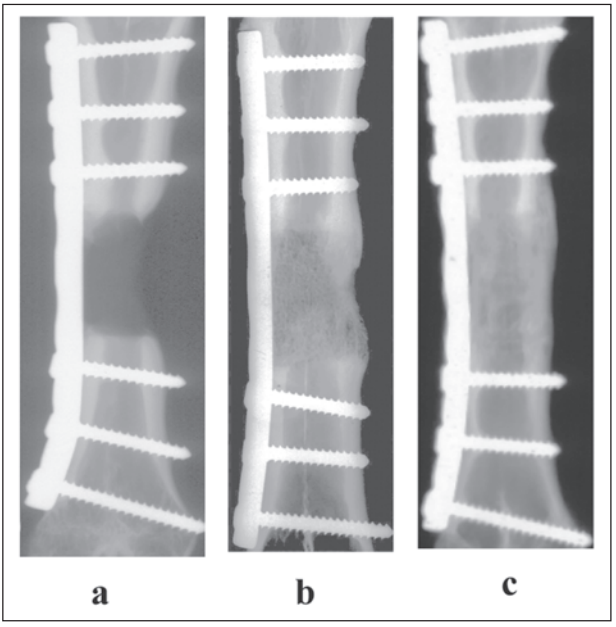

Figure 3. 25mm long resections in sheep metatarsus diaphysis. Four months post-operative radiographs. a) negative control, b) positive control c) replaced with a coral implant seeded with mesenchymal stem cells.

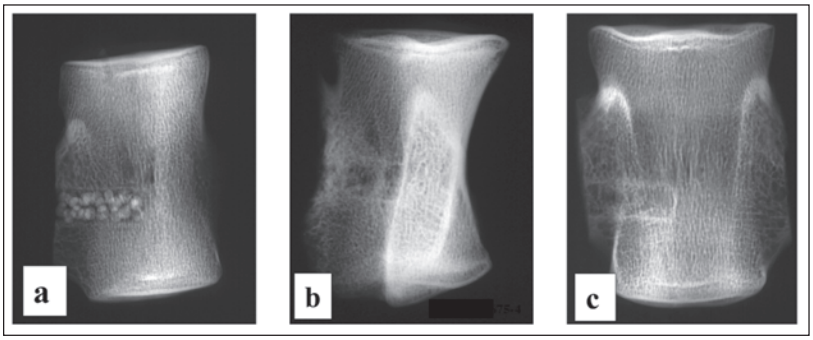

Figure 4. Postoperative radiographs of vertebral defects filled with coral granules. a) Immediate post-implantation b) after 2 months c) after 4 months.

(ii) the inherent excellent mechanical strength of employed BRM which were massive metallic implants.[56,57]

Spinal Surgery

BRM have two main applications in spinal surgery: (i) as bone fillers in vertebroplasties to maintain or augment vertebral body volume when bone loss results from tumour or osteoporosis, (ii) as an augmenting bone healing procedure in spinal fusions.

Vertebroplasty

A model of vertebral bone loss has been described in sheep.[59] A defect is surgically created in the lumbar vertebral body, left intact (negative controls) or filled with coral (innovating technique) (Fig. 4) or an autologous corticocancellous graft (positive controls).

Spinal Fusions

A critical and extensive review of animals models used in spinal fusion preclinical trials has been proposed by Schimandle.[68] Anterior or posterior body spinous process fusions as well as laminar, facet and postero-lateral intertransverse process fusion methods have been proposed in the rat, guinea pig, rabbit and dog. These models have several limitations amongst which: (i) experimental fusion techniques differ from those commonly performed in humans (posterolateral intertransverse process fusion) and results cannot be extrapolated from one technique to another, (ii) animals have increased propensity to fuse compared with humans even after decortication alone, (iii) many models compare the BRM with autografts (positive controls) while negative controls are rarely performed.

Conclusions

The appropriate choice of an experimental model is essential to obtain relevant results. A complete bibliography review and a sharp critical analysis of previously developed models is an essential preliminary step. Results become more reliable with decreasing variability in experimental conditions. Ethical considerations must be considered and one should always choose the animal model with the lowest morbidity and the least painful procedures. In some instances, previously designed models are not relevant enough, requiring a custom-made experimental design. Preliminary trials must then be performed on a few animals to assess feasibility and morbidity.

The choice of an experimental model depends on many variables. The following guidelines can nevertheless be proposed: (i) when experimenting a new BRM, preliminary studies on biocompatibility may be performed in rat by subcutaneous or intramuscular implantation followed by calvarial CSD, (ii) preclinical studies must be performed in a model suited to the

future field of application (i.e., dog or NHP mandibular CSD if the BRM is to be used for maxillofacial surgery, rabbit ulnar CSD followed by sheep metatarsal CSD if it is to be used in orthopaedic surgery for segmental long bone replacement). Due to the differences in bone regeneration between species, any innovating technique should be performed in at least one large animal model before attempting clinical use. Whichever model is chosen, all results must be interpreted in light of the experimental model and care must be taken before valid extrapolations to humans can be made.

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Part III
Tissue Engineering of Bone

Chapter 9

Delivery of Osteogenic Regulatory Growth Factors

Delphine Logeart-Avramoglou

Abstract

Growth factors play an important role in both physiological and therapeutic tissue regeneration. In this review we will focus on skeletal regeneration and give a special emphasis to the delivery of bone morphogenetic proteins (BMPs). Although it is now possible to generate large amounts of recombinant human (rh) BMPs for clinical use, we will argue that the major challenge remains the development of optimal local delivery systems for these proteins. In fact, BMP efficacy is highly dependent upon the carrier that acts both as a drug delivery system and a substratum for osteoprogenitor attachment, proliferation and differentiation. In this review, we will give the rationale for designing an effective BMP carrier and will review the carriers currently used to locally deliver BMPs.

Introduction

Growth factors are polypeptides that influence cellular activities through biochemical signalling. Depending on the targeted cells, they modulate cell migration, division, and differentiation through gene expression.[1] Growth factors can also either stimulate or inhibit the receptor expression and/or function of other growth factors in a concentration-dependent manner. They are synthesised as a partially or fully inactive precursor protein that require proteolytic activation, and they need to bind to matrix molecules for activity or stabilisation. Under physiological conditions, they are entrapped in the extracellular matrix that plays a key role in their display and release.

Growth factors are currently developed either as therapeutics in their own right or as key biomaterial components to enhance tissue repair.[2] Their biological activity depends not only upon their identity, but also on how and when they are presented to the cells. For example, some cytokines are more effective when provided by a sustained release process, whereas others are more effective when presented as a bolus.[3] This difference in efficacy may be related to how the cells circulate and recycle their growth factors receptors.

Bone formation and repair is known to be under cellular control whose activity is regulated by cytokines.[4-6] The main growth factors involved in this process are beta transforming growth factors (TGFβs), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), insulin like growth factors (IGFs), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF). However, BMPs differ from all other growth factors in that they are osteoinductive (defined as the ability to induce bone formation at an ectopic (non osseous) site.[7]) and have been successfully used to promote bone healing of critical-sized defects in rabbit, sheep and dogs[8-10] as well as spinal fusion in dogs, monkeys,[11-13] and even in humans.[14,15]

This chapter outlines the fundamentals behind growth factor delivery with a special emphasis on BMPs.

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Rationale for Designing an Effective Growth Factor Carrier

One of the most important advances in biotechnology has been the production of large quantities of highly purified proteins by recombinant cDNA technology. However, further progress is needed as direct injection of growth factors has limited efficacy and a prolonged local delivery of growth factors is often needed to incur a biological response. Such a delivery can only be achieved through the use of controlled release systems due to the short half-life of growth factors, usually in the order of minutes.

As early as 1982, Urist et al then Lucas et al demonstrated the necessity for carrier material that can deliver the water-soluble osteoinductive factors. When implanted alone, the proteins were completely unable to induce the osteogenic cascade into a muscle pouch although capable of inducing cartilage in in-vitro assay systems.[16] The authors postulated that these proteins diffused too rapidly for induction to occur.[17,18] The combination of a carrier system with the growth factor appeared to be essential for the regeneration and ingrowth of bone tissue.

Supra-physiological doses of single factors are generally required to induce bone: in the order of milligrams for endogeneously extracted BMPs and micrograms for recombinant BMPs.[19] These amounts are several orders of magnitude higher than that which are present in bone (typically μg quantities per kg of bone).[20] An optimised delivery system might be able to improve the osteopotency of the device while reducing the amount of implanted BMP. This fact is of major importance when considering the clinical consequences of using very large amounts of growth factor. Very little is known at this moment about the potentially adverse effects of systemic diffusion of BMPs and thus, it is clinically prudent to minimise therapeutic quantity. Obviously, another advantage in decreasing BMP dosage is to reduce the cost of the implanted device.

Controlled release devices for drug delivery are well known in pharmacology. These systems should allow a local release at therapeutic levels over periods long enough to allow bone tissue regeneration. Properties of a BMP carrier system will comprise numerous specifications. Successful delivery of inductive proteins require all the following features: (1) a controlled release, (2) an adequate amount delivered to the inducible cells, (3) a rapid ingrowth of host tissue into the carrier, (4) biocompatibility and safety of the implant material, (5) adequate resorption of the implant and (6) reproducibility of the carrier properties. Additionally, the ideal matrix should also be malleable, sterilizable and easily manufactured.

From the preclinical studies, it appears that a sustained release of the osteoinductive proteins may be the most satisfactory means and poses the least risk from unanticipated side effects. Indeed, BMPs are bone cell differentiation factors that are appropriately classified as morphogens.[21] In this respect, BMPs obey the laws of diffusion and form concentration gradients with patterns that are tissue specific. The development of BMP delivery systems capable of precisely and predictably releasing the morphogen in effective concentration gradients over the duration of wound healing is quite essential for the implantation of BMP in patients.

Selecting a matrix which has most or even some of these desirable properties has been the principal problem faced by pharmaceutical companies developing BMPs for clinical application. As a result, extracted and recombinant BMPs have been evaluated using a large variety of carriers that are made of natural and synthetic materials.

Herein, this chapter reviews some of the major properties of the " ideal " controlled delivery system.

Scaffold Properties

BMP induced osteogenesis and chondrogenesis is highly dependent upon the carrier: the carrier acts not only as a drug delivery system but also as a permissive environment into which bone cells would migrate, proliferate, differentiate and begin the process of depositing bone matrix (i.e., osteoconduction).[22] A cell substratum requires specific biochemical (molecules of the extracellular matrix), physicochemical (surface free energy, charge and hydrophobicity) as well as geometrical properties (three dimensional, interconnected porosity).[23] Numerous

authors have demonstrated the effects of these parameters on bone formation.[24,25] Three dimensional-systems for bone tissue regeneration imply a specific internal microarchitecture of the materials used. Microarchitecture entails having sufficiently large porosity (> 100 μm) and interconnections between the pores for diffusion of nutrients, clearance of wastes, and infiltration of progenitor cells. In addition to osteoprogenitor cells, endothelial cells must be able to migrate into or near the matrix and develop vascular beds to nourish the newly formed tissue. Interestingly, Jin et al have shown that the length and continuity of pores as well as the surrounding spaces controlled the capillary penetration and thus influenced bone formation.[23] They observed that a porous structure such as hydroxyapatite particles or fibrous collagen, which are favourable for vascularisation, allowed rapid differentiation leading to direct osteogenesis without an observable chondrogenesis phase. Early in 1961, Bassett showed that oxygen pressure was one of the reasons vasculature is essential to osteogenesis but not to chondrogenesis. He reported that high oxygen pressure favours osteogenesis whereas low oxygen pressure favours chondrogenesis in an in vitro culture system.

The mechanical stability of the scaffold as well as the mechanical load transfer might also play a significant role in the success of a device. For this reason, osteoconductive biomaterials can serve as a starting point for the design of BMP carriers.

Degradation of the Carrier

Biodegradable materials are preferred for several reasons: Firstly, they disappear from the body, leaving space for the newly formed bone. Secondly, they obviate concerns about long-term biocompatibility. Thirdly, while diffusion-controlled release is an excellent means of achieving predefined rates of drug delivery, it is limited by the material permeability and the characteristics of the drug.[26] Thus, complete degradation of the scaffold allows a more efficient and complete drug delivery.

A promising scaffold used to deliver osteoinductive growth factor at the defect site should not only allow an appropriate delivery of the growth factor but also assist local bone formation. The important point is that the rate of biodegradation must be commensurate to the rate of bone formation, otherwise bone repair will be delayed, inhibited, or corrupted.[27] When the rate of carrier degradation is too slow compared to the rate of bone formation at the implant site, the remaining carrier will physically impede bone growth in the defect. Inversely, when the rate of the carrier degradation is too fast, it will fail to prolong the in vivo retention of growth factors and may promote the ingrowth of soft tissues. It is known that the infiltration of fibrous tissue into a large size defect will interfere with bone regeneration.

Interactions between Carriers and Growth Factors

The primary role of a delivery system is to provide stabilisation and preservation of the growth factor until in vivo delivery.[28] The interaction between carriers and growth factors should delay the rapid dispersion of BMPs from the implant site. Without such protection, BMPs would rapidly degrade due to cellular proteolysis.

Essentially, the interactions between the carrier and the released growth factor depend on ionic and hydrophobic interactions. Uludag et al demonstrated that rhBMPs differentially bound to various carriers as a function of their isoelectric point (pI).[29] The protein retention in the carrier decreased with the protein pI (rhBMP-2 ~ rhBMP-6 > rhBMP-4 > acetylated/succinylated rhBMP-2) and this phenomenon was observed irrespective of the physicochemical nature of the carrier (collagen sponge, poly (glycolic acid) mesh, bovine bone mineral, and human demineralized bone matrix (DBM)) (Fig. 1). Since proteins with a lower pI have a higher fraction of negatively charged residues, the faster rate of BMP loss might be attributed to a lower affinity of the protein for the carrier and/or higher solubility of the molecule in the biological medium. In order to maximise the in vivo osteoinductive potency of BMPs, these experiments emphasised the necessity to optimise their initial retention resulting from a good growth factor-carrier affinity.

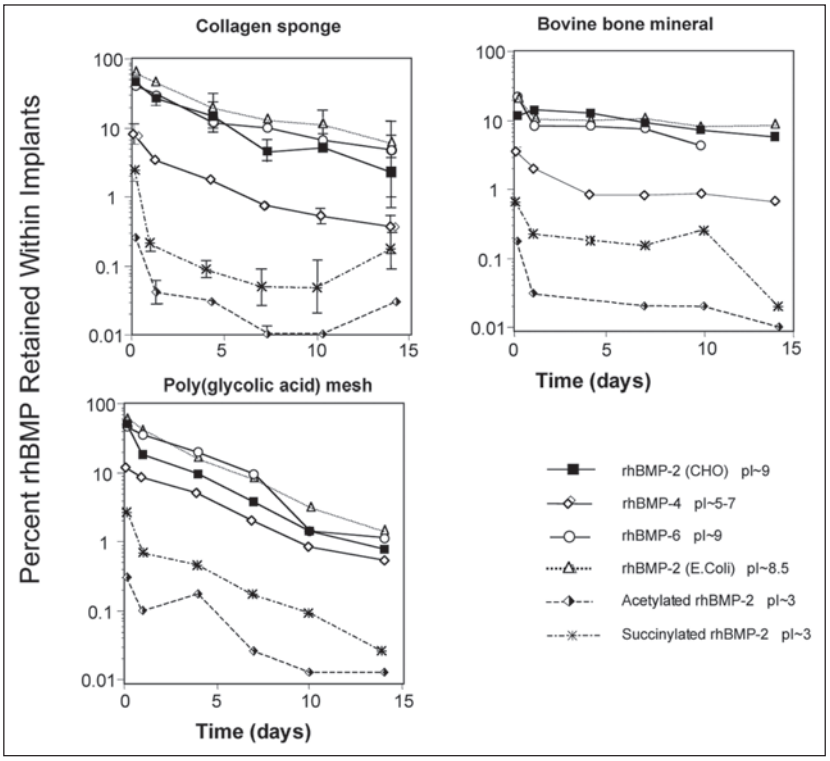

Figure 1. Pharmacokinetics of various rhBMPs combined in three different carriers in the rat ectopic assay. 10 μg of iodinated rhBMP were implanted for 3h, 1, 4, 7, 10 and 14 days. The protein-bound radioactivity from the explants was then measured to determine the percent retention of the implanted rhBMP dose. Figure adapted from Ref .29. Copyright© 2000 John Wiley & Sons. Reprinted by permission of John Wiley & Sons, Inc.

The presence of specific chemical binding groups like heparin-binding domain might influence the binding of growth factors to a carrier. Heparin as well as heparan sulfate proteoglycans in the extracellular matrix bind to numerous polypeptide growth factors with high affinity. These growth factors, usually named "heparin-binding growth factors" include BMPs, FGFs, IGF, EGF and VEGF. Physiologically, this binding activity is important in sequestering growth factors in the extracellular matrix, allowing the localisation of growth factor activity, the prevention of growth factor degradation, and in some cases, the enhancement of growth factor binding to cell surface receptors.[30-32] To exploit this binding affinity, some investigators combined growth factors to heparin to stabilise the proteins.[33] Heparin was also immobilised to a matrix, acting as an affinity site to bind and slowly release growth factors in the healing site. This drug delivery system consisted of the combination of growth factor, heparin and heparin-binding peptides covalently immobilised within a fibrin matrix.[34]

Kinetics of Growth Factors Released from the Carrier

There are two major mechanisms of growth factor release: material degradation and carrier diffusion. The mechanism is highly dependent on the choice of material and the method of loading the growth factor into the matrix (e.g., soaking in the carrier or mixing with the carrier substance before moulding). Physical hindrance and chemical interaction are two means by which matrices could delay growth factor release and diffusion.[35-37] These properties serve to

prolong exposure of the proteins to stem cells and may allow their interaction with other growth and differentiating factors, setting the stage for synergistic activity. However, in many studies, authors point out the difficulty in maintaining growth factor release over the rather long duration of bone regeneration. It seems essential that the delivery system provides a prolonged release of the active growth factor over the full duration of wound healing.

The observations made on in vitro studies as well as on retrieved implants in the rat ectopic model indicate that pharmacokinetic profiles of rhBMP could be influenced by the chemical properties of the carrier system.[36,38] The typical in vivo release profile consists of an initial burst release within the first few hours of implantation, which appears to be carrier dependent, followed by a secondary release characterised by a half-life of 1 to 10 days (Fig. 1). The differences in the initial rhBMP-2 release between carriers (range 75-10%) are presumably due to differences in their available surface areas and/or differences in binding affinities with the growth factor. Collagen based carriers demonstrate a sustained release which parallels the gradual degradation of the material. Conversely, mineral based carriers show a burst in initial release, but thereafter, seem to irreversibly bind a fraction of rhBMP-2 within the implants.[36] The reason for this behaviour is not clearly understood and would need a more precise understanding of the interaction between the carrier surface and the growth factors. Recently, Ziegler et al observed an alteration of the molecular structure of released growth factors in vitro from various biodegradable carriers (TCP, PLG/ceramic glass, ceramic glass and dehydrated bone).[38] This observation emphasises the need to check the biological activity of the released growth factor after its association with the carrier. To impede the potential degradation of the protein, some authors protect the growth factor molecule with material comprising similar properties to those of heparin.[33]

It has been suggested that the initial burst release of growth factor from the implants might serve as a chemotactic signal for the local recruitment of stem cells. Thereafter, the slow release of residual BMP retained in the implant might serve to differentiate recruited stem cells towards an osteoblast phenotype.[36] This is important in the treatment of large defects, where undifferentiated cells have to migrate long distances for proliferation and differentiation into hypovascularized tissues.

Interestingly, different in vivo pharmacokinetics have demonstrated a positive correlation between the retention of rhBMP upon implantation and the osteoinductive activity, i.e., a higher growth factor retention within a scaffold resulted in significantly higher bone scores. For instance, despite similar in vitro specific activities, rhBMP-2 has been shown to be more effectively retained at the implant site and more osteoinductive than rhBMP-4. This phenomenon was observed for both collagen and lactide/glycolide-based delivery systems[37](Fig. 2). A high local BMP concentration in the implant might allow a larger proportion of uncommitted cells to differentiate into osteoblasts.

In conclusion, all these investigations demonstrate that pharmacokinetics of BMP-release is a key factor for good bone regeneration. However several important questions remain:

- Do the rhBMP pharmacokinetics obtained at a heterotopic site correspond to the ones obtained in an intraosseous site?
- What are the local needs for various growth factors and their doses during human bone regeneration?
- What is the optimal time period for the most effective growth factor delivery?

Carrier Matrices

This chapter gives an overview of the major organic, inorganic, and composite carriers that have been used in preclinical experiments, or promising ones, which are still at an experimental stage. It summarises the relevant experiments with these materials as growth factor delivery systems and highlights the advantages and drawbacks of these materials (see Table 1).

However, the comparison of these scaffolds as BMPs delivery systems is a difficult task due to variations in either preclinical models (differences in animal species, age and anatomical sites of implantation, etc.) or BMP sources (especially for purified BMP preparations).

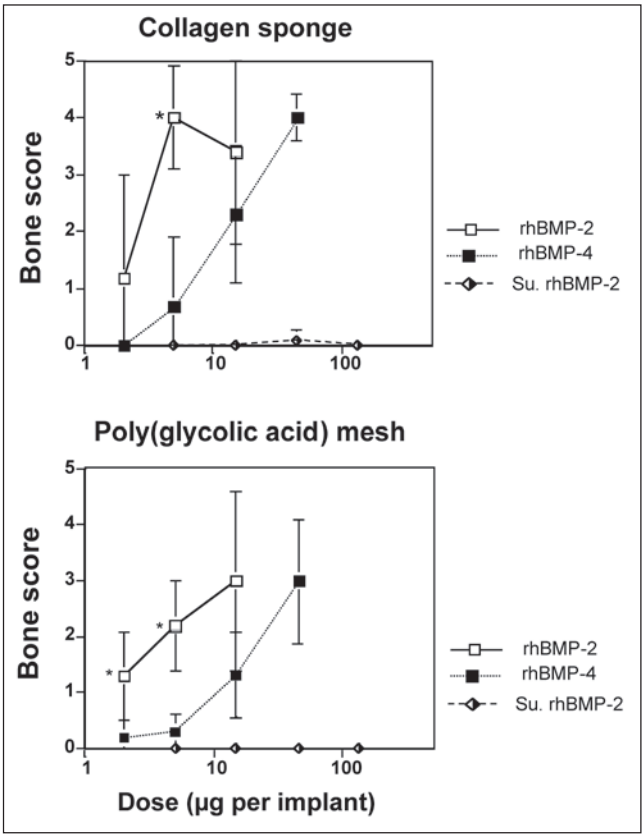

Figure 2. Bone scores for rhBMP-2, rhBMP-4 and succinylated rhBMP-2 implanted with collagen sponge and poly (glycolic acid) mesh. 0, 2, 5, 15 μg for rhBMP-2, 0, 2, 5, 15, 45 μg for rhBMP-4 and 0, 5, 15, 45, 135 μg for succinylated rhBMP-2 were implanted in a rat ectopic site for 14 days. The bone score of 0, 0.5, 1, 2, 3, 4 and 5 corresponded to implants containing 0%, < 10%, 10-20%, 20-40%, 40-60% and 80-100% of newly formed bone, respectively. Figure adapted from Ref. 29. Copyright© 2000 John Wiley & Sons. Reprinted by permission of John Wiley & Sons, Inc.

Inorganic Matrices

Inorganic matrices offer the advantages of being structurally strong, immunologically inert, variably biodegradable and osteoconductive i.e., providing a favourable scaffolding for ingrowth of bone tissue. A variety of ceramics (e.g., coralline, hydroxyapatite (HAP), tricalcium phosphate (TCP)), calcium sulfates, bioactive glass, and metals have been used as inorganic carrier devices for the BMPs.[39,40]

Hydroxyapatite and Tricalcium Phosphate Ceramics

Ceramic implants made of hydroxyapatite (HAP) and tricalcium phosphate (TCP) have been investigated extensively because they are composed of minerals similar to natural tissue.[41-43] It has been clearly established that incorporation of an osteoinductive protein (BMP) in HAP/TCP ceramics greatly accelerates bone formation in various models of bone healing.[44-48]

HAP is the major mineral component of bone with the chemical formula, Ca10(PO4)6(OH)2. This material can be hydrothermally converted from coral or synthetically manufactured. HAP can be supplied as a powder, granule, disk or block. Its geometry appeared to significantly affect the osteoinductive ability of BMPs with the key parameter being the porosity of the

Table 1. Major classes of materials used as carriers for the delivery of osteogenic growth factors

Carrier	Advantages	Drawbacks	Model	Selected References
Organic carriers	• Biocompatible • Biodegradable	• Potential pathogen transmission	Non union fracture defects	91,144
DBM/Collagen/Gelatin	• Functionalizable		Long bone defects	9,84
Fibrin glue	• Moldable		Dental/maxillofacial	85,88,
Hyaluronic acid	• Natural subtratum for cells		defects	101,146
Chitosan			Spinal fusion	12,15,89
Inorganic carriers	• Biocompatible	• Brittle	Non union fracture	–
Tricalcium phosphate	• Osteoconductive	• Difficult to mold	defects	
Hydroxyapatite	• Structurally rigid		Long bone defects	61,62,147
Coral			Dental/maxillofacial	63,148
Ceramic glass			defects	
			Spinal fusion	13
Synthetic polymers	• Ease to manufacture	• Additives may lead to adverse tissue responses	Non union fracture defects	149
Poly(α-hydroxy acids)	• Cost efficient		Long bone defects	
Polyanhydrides	• Free of contamination	• Break-down products may lead to inflammatory reaction	Dental/maxillofacial defects	
Poloxamers			Spinal fusion	
Methacrylate-derived polymers	• Ease to sterilize • Functionalizable			

carrier.[49] Highly porous HAP has a larger surface area enabling an increased amount of BMP to be entrapped throughout the scaffold. A comparison among porous blocks of HAP with different pore sizes demonstrated that pores sizes of 300-400 μm most favours bone formation.[50] In honeycomb-shaped hydroxyapatite with straight tunnel structures of various diameters, tunnels with smaller diameters (90-120 μm) induced cartilage followed by bone formation, whereas those with larger diameters (350 μm) induced bone formation directly within the tunnels.[51] In the rat ectopic assay, significant bone induction was observed when osteogenin (i.e., BMP-3) was combined with a disc-shaped HAP carrier, but no osteogenesis was observed when the same factor was combined with HAP in the form of granules.[24] More recently, Jin et al have shown that the geometry of HAP clearly controlled vascular capillary invasion and modulated cell growth and differentiation.[23] The main geometrical factors involved in initiating rapid osteogenesis are the porosity and its interconnectivity within the implant, which give rise to easy access of oxygen-carrying capillaries.

However, HAP is rigid, brittle and difficult to shape. This limits its use as a bone substitute. On the contrary, tricalcium phosphates can be prepared in a paste form that can be moulded to a desired shape prior to setting. In 1984, Urist et al reported that a composite made of bovine BMP and β-TCP produced 12 times more bone than BMP alone in a rodent assay system.[47] When implanted in adult dogs with skull defects of critical size, this composite induced an almost complete repair of the defect (91%-100%) by deposits of new bone.[52] However, the use of Tricalcium phosphates in repairing skeletal defects has not proven satisfactory because of their too rapid rate of resorption which is not always commensurate to bone formation.[41] Indeed, the TCP absorptive rate in vivo was 60-80% in 6 months, whereas HAP is poorly degraded in vivo (several μm thick after 18 months).[53-57]

Consequently, mixtures of HAP and TCP named biphasic calcium phosphate (BCP) with different ratios of each components have been prepared to obtain ceramics with an intermediate range of resorption rates. Ceramics particles composed of 25%HAP-75%TCP impregnated with rhBMP-2 exhibited the best results in term of bone formation and resorption when implanted under the rat pericranium.[58]

These ceramic materials have been also used as a delivery vehicle for BMP in combination with other matrices like collagen[59] or fibrin sealant.[60]

Other Inorganic Matrices

Other inorganic matrices such as natural calcium carbonate -coral exoskeleton,[61-63] calcium sulfate - plaster of Paris,[64] sintered bone,[65] bioactive glass,[66,67] and apatite-wollastonite-containing glass ceramic[68] have been evaluated as BMP or TGFβ1 vehicle.

Coral is an osteoconductive, bioresorbable ceramic composed of 97-99% calcium carbonate with good mechanical properties. Its porosity is similar to cancellous bone.[69] Coral particles have been used as a delivery system for the sustained release of TGFβ1. The release rate may be modulated through modification of adsorption (pH and coabsorbants) and coral particle size.[70]

Organic Matrices

Naturally-Derived Polymers

Collagen and Derivatives

Demineralised bone matrix (DBM), fibrillar collagen and gelatin are type I collagen based scaffolds. DBM, from which BMPs were originally isolated was the first carrier used for delivering BMPs.[71,72] This matrix is prepared either by HCl demineralisation alone or followed by guanidine/urea bone extraction. The guanidine procedure removes all bone-inductive proteins from DBM powder and leaves an osteoconductive, inactive, insoluble collagenous bone matrix (ICBM), essentially made of insoluble, highly cross-linked type I collagen.[73] Combination of aqueous BMP with ICBM results in a bone repair material comparable and even better in consistency and activity than DBM.[74,75] The presence of proteins such as osteopontin and related phosphorylated sialoproteins in DBM preparations may exert potentiating effects on BMP's biological activity.[76] Encouraging results were obtained with DBM and ICBM in association with purified and recombinant BMPs which were frequently tested in ectopic sites[36,77,78] as well as in critically sized defect models.[75,79]

Although DBM/ICBM are effective substrates for delivering BMP, many practical difficulties remain for their use in a clinical setting. Firstly, there is the possibility of an immune response, which may lead to rejection and/or resorption of the implant.[80] The removal of collagen telopeptides (ateloization) decreases its antigenicity but solubilises the molecule[81] Secondly, collagen-based matrices require a secondary rigid scaffold due to its weak mechanical properties for their use in weight-bearing bone. Other potential disadvantages include standardising collagen extraction and eliminating pathogen transmission. All these drawbacks complicate the selection for allogeneic or xenogeneic ICBM as delivery systems and encourage the development of other alternatives.

Thus, other collagen formats have been developed in porous sponge or gel forms. The use of type I collagen as a biomaterial in these forms is currently undergoing renaissance in the tissue engineering field. It is generally derived from bovine or porcine bone, skin or tendon. The current purification procedures eliminate the immunogenic telopeptides. This natural polymer represents the major component and fibrous backbone of the extracellular matrix (ECM).[82] Thus, its use as scaffold is of major interest since it is biocompatible, biodegradable and is a natural substrate onto which cells can adhere and proliferate.[83]

Absorbable collagen sponge, which is the most tested delivery matrix, is manufactured from lyophilised fibrillar collagen crosslinked by a chemical (formaldehyde) or a dehydrothermal

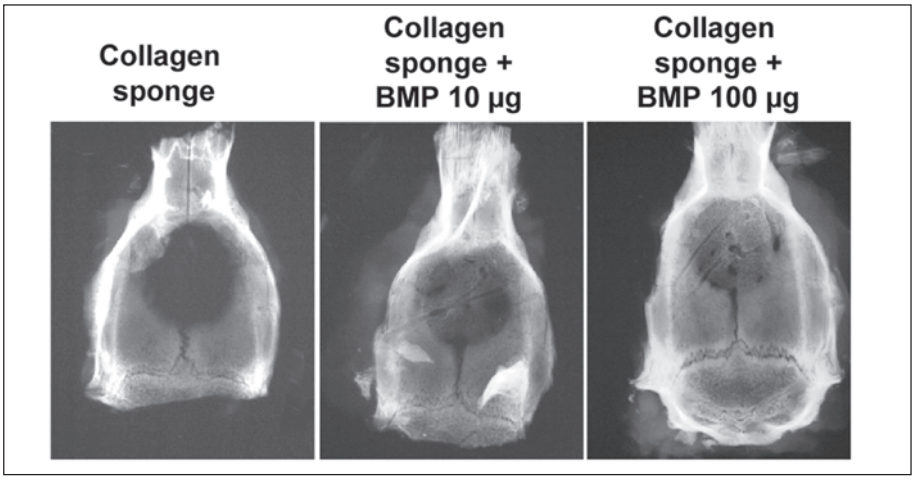

Figure 3. Radiographs of rat calvarial defects treated with collagen sponge with 0, 10 or 100 µg of BMP at one month post-implantation.

process. It has been tested quite extensively with BMP (Fig. 3) in numerous animal models of long bone healing,[9,84] maxillofacial reconstruction,[85-88] and spinal fusion.[12,89] Currently, it is almost the only BMP delivery system used in clinical trials.[15,90,91]

Friess et al looked at the interactions between rh-BMP-2 and absorbable collagen sponge and the effects of the chemical and physicochemical properties of the carrier on the growth factor binding.[92] From this study, it appears that rhBMP-2 binds to the collagen sponge efficiently. The protein incorporation can be modulated depending on several parameters such as material mass, soaking waiting time, liquid pH, and anion concentration.

As mentioned above, some investigators characterised the local pharmacokinetics of iodinated BMP-2 released from collagenous carriers in the rat ectopic model. The results are clearly presented in the review written by Winn et al.[93] As with most of the carriers, the pharmacokinetics of BMPs are biphasic. Some processes such as crosslinking with formaldehyde might lead to a slight increase in in-vivo persistence, which may be related to slower degradation. However, the type of sponge crosslinking process (chemical vs. dehydrothermal) as well as ethylene oxide sterilisation do not affect the biphasic pharmacokinetics.[92]

Gelatin is prepared through chemical denaturization of type I collagen. An interesting variant of gelatin hydrogels has been prepared through chemical crosslinking of acidic gelatin with an isoelectrical point of 5. Sustained release of basic growth factors was obtained based on polyion complexity.[94] Incorporation of rhBMP-2 or TGFβ1 into acidic gelatin hydrogels enhance their bone regenerative potency significantly in a skull defect site in rabbits.[95,96]

The use of collagen as a drug release system presents various advantages. It is available in abundance, is biodegradable, hemostatic, and can be prepared in a number of different forms including strips, sheets, sponges and beads. Functional groups of collagen side chains can be chemically modified allowing the coupling of therapeutical agents such as antibiotics to eventually improve the healing process.[97] However, biodegradation of collagen is a combination of a hydrolysis and an enzymatic process, which increases the difficulties in predicting its in vivo behaviour. Standardisation of collagen extraction still remains a challenge. Additionally, an important disadvantage of collagen products consists of the constant risk of pathogenic transmission.

Hyaluronic Acid

Hyaluronic acid (HA) is a polysaccharide which exists naturally in the ECM and plays an important role in proteoglycan organisation, cell hydration, cell differentiation, and wound healing.[98] Hyaluronan is easily derivatized by the introduction of functional groups onto the glucuronic acid residue and easily crosslinked yielding biocompatible and biodegradable hydrogels, films, or sponges. Some derivatized hyaluronic acid scaffolds were investigated as rhBMP-2 delivery vehicles. Kim et al obtained a matrix that demonstrated in vitro a slow and sustained rhBMP-2 release during one month. This retention level was much greater than that from collagen gels, which released most of the initial load of BMP within 14 days in same experience.[99] This result suggested a good rhBMP-2 affinity towards the HA scaffold, probably due to an electrostatic interaction between the basic growth factor and the acidic, carboxylic acid residues of the polysaccharide. However, this scaffold has not yet been evaluated in vivo with BMP. Others have formed high-molecular-weight HA-derived hydrogels that were easily infiltrated by cells and supported growth factor-induced bone tissue formation and remodeling in the rat ectopic bone formation model.[100] Recently, HA sponges have been shown to be suitable rhBMP-2 carrier for the reconstruction of advanced alveolar ridge defects in dogs.[101]

Fibrin Matrix

Fibrin matrix derived from human plasma combine numerous properties such as hemostatic ability, resorbability, angiotropicity, and provides an excellent three-dimensional cell infiltration scaffold. They mimic the last step of the physiological coagulation by the mixture of fibrinogen, factor XIII, thrombin and Ca^{2+} ions. However, because of the lack of solid evidence of safety and efficacy, commercial fibrin sealants were not approved by the FDA in United States for many years, which might explain the lack of data with this material. Only recently commercial products have been developed and are now the object of clinical trials in this country.[102]

Very few experiments reported the implantation of human fibrin as delivery vehicles for BMP. After three weeks of implantation in ectopic sites, correlated microradiographic and histologic observations showed that a BMP/fibrin composite produced larger volumes of new bone than the BMP control group.[103] The authors suggested that the fibrin may cause a synergistic effect, stabilising the BMP while enhancing cell proliferation. More recently, fibrin carriers have been used in association with DBM and FGF-1 in a critical-sized defect in rabbit radius. Interestingly, the combination fibrin/FGF-1 also promoted a significant increase in bone formation.[104]

Sakiyama-Elbert et al developed a novel approach to deliver growth factor from a fibrin matrix based on a localised release of bound growth factors from the matrix in response to cellular activity during healing.[34] The fibrin matrix was modified to contain immobilised heparin that served to sequester exogenous heparin-binding growth factors within the scaffold. The proteins can be released by enzymatic factors such as heparinase or plasmin. These matrices have been tested with FGF-2 as therapeutic materials to enhance nerve regeneration in vitro. Although this sustained delivery system needs to be validated in vivo, we can hypothesise that similar matrices might apply for bone regeneration by locally delivering BMPs, which are also, heparin-binding growth factors.

Synthetic Polymers

Materials that are well characterised, easy to manufacture, cost-efficient, free of potential contamination and easy to sterilise are synthetic polymers. Over the past two decades, the use of polymeric materials as biomedical devices has increased dramatically, particularly in the areas of controlled drug delivery systems.[105] The pioneering studies in the field of controlled subdermal drug delivery began in 1960s, and used biostable commercial polymers such as polyethylene and silicon rubber.[106] The release rate of the drug from the polymeric matrix or reservoir device was determined solely by diffusion.

Many reviews have been written in this field.[26,107] As a matter of convenience, we will essentially focus on biodegradable or bioerodible polymers that seem the most convenient for our purpose.

A great number of parameters will contribute to the rate of degradation:

- water permeability which determines the rate of hydrolysis and whether bulk or surface hydrolytic degradation occurs
- chemical composition, molecular weight and additives
- physical dimensions of the device (size, surface-to-volume ratio) as well as its site of implantation

Poly (α-Hydroxy Acids) PHAs

Among the few polymers which have been recognised as degradable in a mammalian organism, polymers derived from glycolic acid (PG) and from D- and L- lactic acid (PL) enantiomers are presently the most attractive compounds. This is largely due to their biocompatibility and to their resorbability through natural pathways. This is also related to the fact that copolymers issued from glycolic acid and lactic acid (PLG) polymers are approved by the FDA and are now commercially available. PLG have been the most widely bioerodible systems for controlled release and have a thirty year history of clinical efficacy and safety as sutures.[108]

Lactide and glycolide-based polymers are most commonly synthesised by ring-opening melt polymerisation of lactide and glycolide using a catalyst.[109,110] However, as residuals of the polymerization reaction, these additives as well as solvents may lead to adverse tissue responses during polymer degradation.[111] PLG is a general acronym, which corresponds to a large family of compounds. It is almost impossible to produce the same member of a family twice because of batch and statistics dependencies of macromolecular structures. Although this latter remark is applicable to all polymeric compounds, it appeared more critical in the case of degradable polymeric materials because degradation and bioresorption characteristics are very much dependent on chain characteristics and solid morphology.[112] The polymers degrade by bulk hydrolysis of the ester bonds that result in a decrease in molecular weight. Lactic and glycolic acid resulting from the hydrolytic cleavage are natural molecules that can be metabolised by endogenous systems. The rate of hydrolysis is modulated by crystallinity and hydrophobicity of the monomer components. Crystalline regions are more resistant to hydrolysis than are amorphous regions.[112] In addition, PG is more hydrophilic than PL and its biodegradation is more rapid: PG is resorbed within 3-6 months as PL is resorbed within 6-36 months in vivo. Thus, PLG copolymers will biodegrade according to the PL and PG constituents' molar ratio.[113] Unfortunately, PLG copolymers synthesised from monomeric constituents can yield to copolymers with various combinations of monomeric sequences: from blocky regions of polymers to orderly and alternating ones. Consequently, devices fashioned from copolymers may have nonuniform rates of hydrolysis, which therefore impact the diversity of host responses. Some authors highlighted the fact that inadequately characterised homopolymers or copolymers might contribute to discrepancies of results in spite of apparently similar clinical experimental conditions.[111,112,114] For this reason, numerous researchers emphasised homopolymers (usually polylactic acid) for designing carrier systems, which should result in a more homogeneous and predictable outcome.[111,115]

Despite of its known biocompatibility, numerous studies have mentioned the presence of an adverse host tissue reactions from bulk PHA devices as fixation plates and screws.[116] Histological examination showed a typical nonspecific foreign-body reaction with abundant giant cells. Vert explained this phenomenon by a heterogeneous degradation of large size devices, the rate of degradation being greater inside than at the surface of the device.[117] Consequently, the entrapped carboxylic moieties accumulate within the centre, accelerating internal degradation of the device until the central mass breaks the outer layer. This contributes to a decrease in local pH manifesting as an aseptic sinus.[116] Unlike large sized devices, microspheres less than 300 μm in diameter undergo a homogeneous degradation of the core being equivalent to that at the

surface.[118] It seems that degradation and resorption reduce the size of microspheres larger than 10 μm to a point where they become susceptible to phagocytosis by macrophages and foreign body giant cells.[119,120]

The design of PHA carriers for bone regeneration must integrate a number of features required for cells to attach, differentiate and express the set of appropriate gene products leading to tissue regeneration.

As with ceramics, the porosity of the device appears to be an essential parameter. Porosity is defined by the pore size, range, void volume and pore distribution. These features contribute to the macromolecular internal space available for bone ingrowth. Poly (lactic-coglycolic acid) particles with pore sizes of 150 to 300 μm diameter which corresponds to the porosity of human haversian bone, were manufactured to allow for cell ingression into pores.[111] One study has shown that disk implant with a high pore size (300-350 μm) demonstrated the best osteoconductive ability in a standard intraosseous calvarial wound.[121]

Moreover, the carrier shape will have an impact on the rate of polymer degradation and influence the pharmacokinetics for drug delivery. Block devices do not allow easy host cellular infiltration and apparently elicit significant foreign body reactions. Microparticulate PLG copolymer were therefore preferred because they present numerous advantages. First, they provide a delivery system with a high exchange surface, which potentially enhance the growth factor availability. Second, they resorb rapidly with minimal foreign body reactions and can be incorporated into mouldable implant. However, to provide a surgically convenient composite suitable for implantation into irregularly shaped skeletal defects, a third component is necessary to yield a semi-solid paste. Different biopolymer solutions have been used: blood clot, calcium cross-linked alginate, cellulose and its derivatives.

The rhBMP-2 release from porous microspheres made of 50:50 PLG (50PL/50PG) was evaluated in vitro and indicated a triphasic process.[122,123] These porous matrices retained BMP in both « free » and « bound » forms. The initial phase represented loss of free protein from the porous particles over 3-4 days (growth factor present on the surface and within the pores of the PLG particles). A lag period followed during which bound rh-BMP-2 is retained until 14-21 days. During the third phase that began after 3 weeks, mass loss of the bioerodible particles began and consequently resulted in renewed release of the bound protein. The combination of PLG with carboxymethylcellulose (CMC) or methylcellulose (CM) polymer delayed the BMP release.

Particulate PLG combined with various « thickening agents » have been tested with BMP to accelerate osseous regeneration in various critical size defect models: calvaria,[124-126] femur, radius,[111,125,127] maxillary cleft,[128] and spinal fusion.[11,129] The addition of thickening agents to PLA particulates probably influenced the effectiveness of the osseous regeneration and it remained difficult to evaluate the exact role played by each component, especially when the agent was an autologous blood clot. Blood clot contained cells and platelets containing numerous growth factors such as TGFb1 and PDGF. All these in vivo experiments with particulate delivery systems gave promising results without major adverse tissue responses. However, in some cases, the authors observed a dislocation of the carrier coming from soft tissue movements and blood ooze from the recipient bed.[111] In order to improve the plasticity of the material (i.e., the ability to mould to the bone defect), Saito et al synthesised polylactic acid-polyethylene glycol block copolymers (PL-PEG). They prepared diverse copolymers with various sizes of PLA and PEG and tested them as BMP-carrier on a rat ectopic model.[115,130-132] It appeared that the ratio of PL size to PEG size and the total molecular size are essential factors for a good carrier and influence degradation rate, swelling ratio, and implant hardness. To improve the resorption of the polymer and thereby optimise the delivery system of BMPs, the same authors synthesised a new copolymer based on a random insertion of p-dioxanone (DX) in the PL segment. This PL-DX-PEG polymer exhibited good degradation characteristics, and when mixed with rhBMP-2, demonstrated ectopic bone formation and healing of large bone defects.[133]

Others

Other synthetic materials that have been tested as BMP carrier include polyanhydrides,[134] poloxamers,[135] polyphosphate,[136] and methacrylate-based polymers.[137]

Recently, Uludag and Gao explored a novel way to sequester BMP locally and investigated synthetic thermoreversible polymers (i.e., polymers that exhibit a temperaturedependent solubility in aqueous medium).[138,139] These polymers, based on N-isopropyl-acrylamide and ethyl methacrylate, were engineered by incorporating a protein reactive group for controlled drug delivery. In the soluble phase, an injectable solution is obtained which can conjugate with BMP through the functional groups. Then, by simple transition of the temperature, the polymer solution turns into insoluble hydrogel at the application site. A local retention of rhBMP-2 was observed using these thermoreversible polymers, although their in vivo osteoinductive activity has to be demonstrated. Moreover, at this moment the biocompatibility and the nonresorption of the polymer are critical issues that need to be evaluated for the use of this new and promising BMP delivery system.

Composites Materials

As most of the carriers did not fulfil all the requirements of the ideal BMP delivery system, much attention has been focused on the synthesis of composites of different materials combining the properties of both constituents.

On the one hand, osteoconductive materials with interesting physical characteristics such as high porosity and large continuous macropores are typically unable to hold a large reservoir of protein and are not capable of retaining proteins locally for a prolonged period of time. On the other hand, materials with controllable local retention and release of growth factors usually have weak mechanical properties as well as uncontrollable porosity. The association of complementary materials in term of properties will likely lead to materials of the next generation.

Some composite materials have already been tested as BMP delivery systems. For instance, interesting results were obtained by the association of PLG polymers[140] or TCP and HA ceramics with collagen or gelatin.[59,141-143]

Conclusion

As early as 1982, upon isolating BMP, Urist et al realised the necessity of a suitable carrier to decrease the rate of BMP diffusion and allow the induction process to proceed.[17] Recombinant technology has enabled the cloning and expression of human BMPs in sufficient quantities for therapeutic applications in preclinical studies. Many carriers have been tested as growth factor delivery systems. Some of them have been evaluated more intensively such as collagen sponges which have been tested in various animal models and in clinical trials for bone repair,[91,144] spine fusion[15,90] as well as in oral applications.[145] However, it seems unlikely that one single delivery system for BMP will achieve optimal effects to suit all clinical needs. Different anatomical sites do not heal in the same manner, and thus require carriers demonstrating different features in terms of degradation rate, release kinetics and amount of implanted growth factor.

The challenge for researchers today is to deliver these factors in ways to ensure consistent and safe success in humans. In the next decade, the delivery system should be improved and delivery of a therapeutic dose of a drug is pivotal to its success. Thus, the carrier should retain the optimal dose of the entrapped growth factor within the recipient bed.

Furthermore, we know that the bone repair process requires several growth and differentiation factors, which act in a spacio-temporale manner. Novel carriers will combine various polymers that release each growth factor with suitable kinetics in order to mimic the natural bone healing process. These manifold delivery systems would improve the osteoinductive potency as well as decrease the amount of factor within the site. They likely represent carriers of the future.

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Chapter 10

Gene Therapy for the Enhancement of Fracture Healing

Charles Sfeir, Hannjörg Koch, Julie Jadlowiec, Paul Robbins and Jeffrey O. Hollinger

The ability to introduce exogenous DNA into cells is a powerful tool for researchers in the quest to improve treatment options. Recent years have witnessed increased enthusiasm for gene therapy and the use of this technology to enhance bone repair is an obvious choice. To gain therapeutic value from gene transfer into a cell, two main technical requirements have to be met. One is to determine the most efficient delivery vehicle. The other is transgene choice for local, therapeutic production in a sustained fashion.

To introduce exogenous DNA into the cell and more specifically into the nucleus where the transcriptional machinery resides, vectors are the delivery vehicles. These vectors could be viral or nonviral. Each delivery system has advantages and disadvantages. Vector optimization remains an active area of research to achieve the most efficient gene delivery system.

The first part of this chapter will give an overview of the gene delivery technology currently available to transfer genetic sequences into mammalian cells. Gene therapy applications to bone will be discussed in the second part.

Gene Delivery Vectors

Vectors are defined as vehicles to enhance DNA entry and subsequent expression in a host cells. Vectors are grossly divided into two types: viral and nonviral.[1] Although the ideal vector is yet to be found or designed, certain features of different vectors may be appropriate for diverse therapeutic approaches. The ideal vector should be safe for the recipient and should not induce inflammation. It should be stable and easily produced.[2] Another very important feature of the ideal vector relates to the regulation of gene expression. Following gene transfer to host cells, control of gene expression is particularly advantageous.[3] This represents an ability to turn gene expression on and off with discretion and ideally integrate it into a feed-back control system. However, apart from these general characteristics, ideal vectors for gene transfer must be tailored to address the unique physiology of each disease state. These variables include specificity to target cells and duration of protein expression. In most cases, a vector's ability to transduce both dividing and nondividing cells is desirable. However, for some types of cancers, it might be beneficial to distinguish between dividing and nondividing cells for targeted gene transfer.[4,5] Enhancement of fracture healing and fusions, and even treatment of nonunions is most often accomplished after a significantly shorter period of protein expression than successfully completed treatment of large critical bone defects. However, if the pathophysiology of healing is disturbed by an inappropriately prolonged expression of therapeutic proteins, this could be detrimental to the bone healing process. Finally, a chronic disease will need a prolonged therapy with consistently stable protein expression, regulated by a feed back mechanism.[6]

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Gene therapy vectors for bone fracture healing that have shown promise are retroviral, adenoviral and adeno-associated viral vectors. These vectors will be discussed below. Lentivirus might show promise for the development as a vector in gene therapy. However, this is still not yet ready to be used in a clinical setting.[7,8]

Viral Gene Delivery

Retroviral Vectors

Retroviruses are viral vectors that have been used most extensively for gene transfer.[4,9-14] These were the first viral vectors used in human clinical trials.[15] In the year 2000, it was reported that 40% of clinical gene therapy protocols involved recombinant retrovirus particles or retrovirus producing cells for gene transfer.[16] Retroviruses are small RNA viruses that infect target cells through a specific interaction between the viral envelope protein and a cell surface "receptor" on the target cell.

For many types of retroviruses such as derivatives of Moloney murine leukaemia virus (MoMuLV), mitosis, and thus, breakdown of the nuclear envelope is required for the viral genome to reach the nucleus.[4,17] Furthermore, derivatives of MoMuLV are able to carry eight kilobases of additional genetic information which is a rather small capacity.[3]

Retroviruses, in contrast to adenovirus, integrate their genomes into the chromosomal DNA of the host. Hence, a potential advantage is that theoretically, this additional genetic information is preserved throughout every cell division and propagated to the next generation of cells. To demonstrate the feasibility of treating arthritis and other joint disorders with gene therapy Bandara and coworkers transduced rabbit synoviocytes in culture by a retrovirus encoding for the human interleukin-1 receptor antagonist (IL-1ra). The genetically modified synovial cells were then transplanted by intraarticular injection into the knee joints of rabbits, where they efficiently colonized the synovium. Assay of joint lavages confirmed the in vivo expression of biologically active human IL-1ra. However, the detectable amount of the transgene decreased and lasted for only 6 weeks.[18]

Inflammatory response is a particular concern for adenoviral vectors due to persistent viral protein expression.[3,19,20] Vector immunogenicity is an important consideration for adenoviral based strategies since viral proteins are present on the surfaces of host cells after digestion in the cell's cytoplasm. In contrast, retrovirally infected cells do not express viral proteins and thus are not immunogenic.[18]

Last but not least there is concern about the site in the host's DNA where the transferred genetic information integrates. Because integration of the retroviral genome happens at random locations in the host's DNA it is unlikely, but possible, that functional deletion of a tumor-suppressor gene or activation of an oncogene could occur, due to interactions with house-keeping genes, leading to uncontrolled and malignant cell growth.[3]

Retroviral Vectors in Gene Therapy

Retroviral vectors currently used for gene therapy are predominantly derived from MoMuLV and possess the classical properties of a retrovirus.[10,16] MoMuLV derivatives can be generated that are amphotropic and therefore, are able to infect several species of cells including murine and human cells. According to Walther and Stein more than 150 clinical studies were underway in the year 2000, using retroviral vectors for gene transfer and more than 20 studies using virus-producing cells for gene transfer. These clinical studies included more than 1500 patients.[16]

Retroviral gene transfer as a therapeutic approach was effectively performed in several diseases, including adenosine deaminase (ADA) deficiency,[21,22] Gaucher's disease, a lysosomal storage disorder affecting the glucocerebrosidase enzyme,[23] plus many more, especially in the field of oncology. In a clinical study, gene therapy strategies are being used to treat rheumatoid arthritis. This is the first non-life-threatening disease in the usual sense, which was treated by gene therapy in a clinical study. A retroviral vector was used in an ex vivo approach.[24-27]

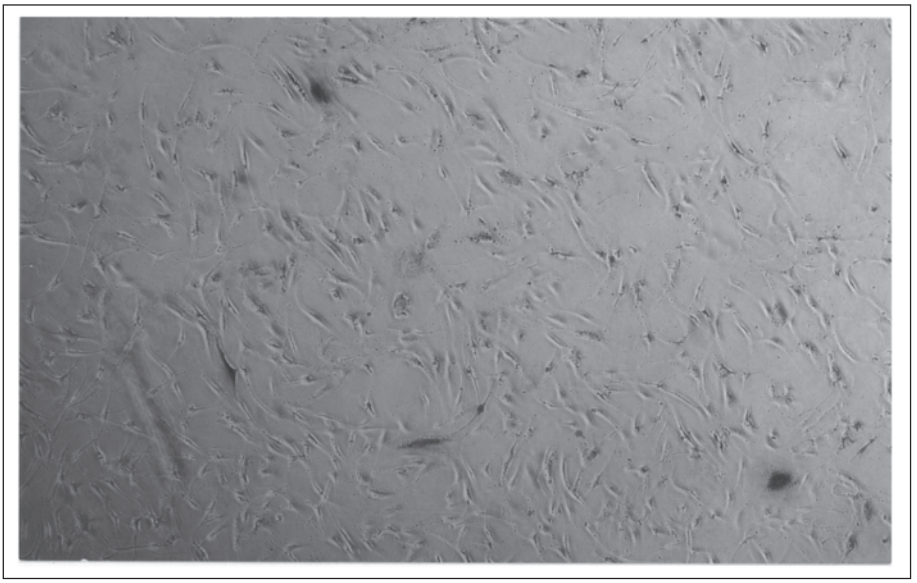

Figure 1. Human osteoblast like cells retrovirally transduced with LacZ. Transduction was enhanced only by the cationic polymer Polybrene®. X-gal staining. Approximately 10% blue cells. Micrograph courtesy of Dr. Axel Baltzer. Reprinted with permission from Baltzer A: Tierexperimentelle Untersuchungen zur Entwicklung einer Gentherapie für die Behandlung von Knochenerkrankungen. Monograph. Shaker Verlag, Aachen 2000.

Application of Retroviral Vectors to Bone Healing

There are only a few studies published on gene therapy using retroviral vectors for bone and fracture healing specifically. Reinecke and coworkers reported an ex vivo approach to gene transfer to cells of disc, spinal nerve root, and vertebral bone of the lumbar spine of rats.[28] The cDNA of human interleukin-1 receptor antagonist (IL-1ra) was transferred via retroviral vector into cells isolated from vertebral bone, resulting in a mean protein expression of 33.5 pg/ml/10^3 cells/48 hrs (SD 11.1). Baltzer and coworkers were able to transduce human osteoblast cells in vitro using a retroviral vector.[29] Again, the cDNA of human IL-1ra and the β-galactosidase (LacZ) gene were introduced into the isolated osteoblasts. As previously mentioned, poor transduction efficiency of target cells may lead to few retroviral particles that actually bind the appropriate receptors on the target cell surface. Baltzer and coworkers attempted to alleviate these effects by centrifugation at 500 rpm and lowering of cell culture temperature to room temperature during the first hour of transduction. By these means it was possible to increase the initial transduction efficiency from 10% to approximately 60%[29] (Figs. 1 and 2).

Mason, Breitbart and coworkers published several studies using cultured periosteal cells transduced retrovirally with the BMP-7 gene for bone healing applications.[30,31] Periosteum-derived cells were transduced ex vivo via retroviral vector encoding BMP-7 cDNA and seeded onto poly-(glycolic acid) (PGA) matrices and implanted to treat an osteochondral defect in a rabbit knee. Completed or nearly completed bone and articular cartilage regeneration was observed. Since bone was appropriately regenerated only in the subchondral region and articular cartilage only in the region of overlying cartilage, the authors conclude that BMP-7 may not act alone in signaling during regeneration. Therefore, it may be likely that BMP-7 has paracrine regulatory effects on local populations of cells.[31]

In a similar approach, experiments have been performed on bone marrow-derived cells which may not only serve as a vehicle for the delivery of therapeutic proteins, but also may differentiate into osteoprogenitor cells. A fraction of bone marrow-derived cells is considered

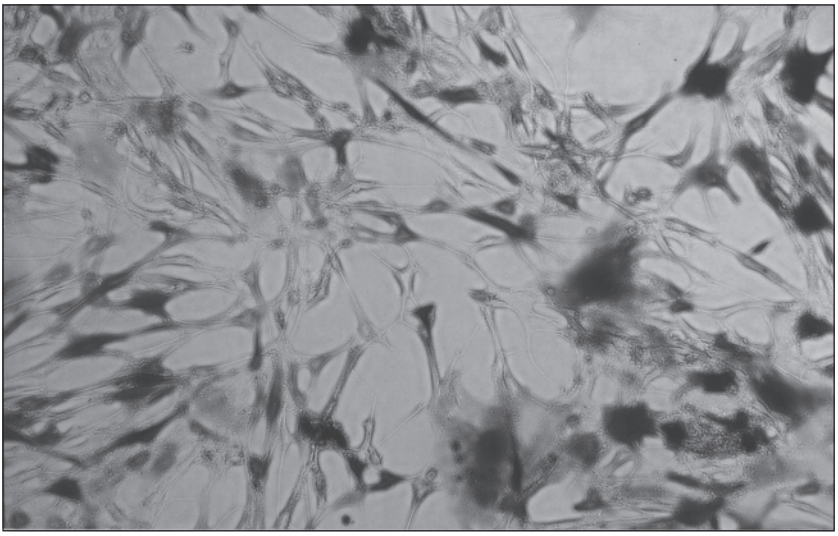

Figure 2. Human osteoblast like cells retrovirally transduced with LacZ. Transduction was enhanced by the cationic polymer Polybrene®, by temporary lowering of the temperature, and by mild centrifugation with 500 rpm. These measures were taken to improve contact of retroviral vectors with the cells. X-gal staining. Compared to fig 1 there is an increase in the number of blue cells to approximately 60%. Micrograph courtesy of Dr. Axel Baltzer. Reprinted with permission from Baltzer A et al: In vitro Transduktion humaner osteoblastärer Zellpopulationen mit retroviralen Vektoren [In vitro transduction of human osteoblastic cells using retroviral vectors]. Z Rheumatol 1999; 58: 88-94.

pluripotent and also contains osteoprogenitor cells.[32] These cells can act in paracrine and autocrine mechanisms to enhance fracture healing. Balk and coworkers demonstrated that mouse stromal cells can be retrovirally transduced.[33] Cells were transduced with a reporter gene and cells maintained expression of osteoblast-specific marker genes in vitro following supplementation with known osteogenic factors in the cell culture media. Similarly, Allay and coworkers demonstrated that human bone marrow-derived mesenchymal progenitor cells are susceptible to retroviral transduction. Cells retained their osteogenic potential and formed bone in vivo after being seeded into porous calcium phosphate ceramic cubes and implanted subcutaneously into SCID mice.[34] Another interesting example of retroviral gene transfer is from Oyama and coworkers. The authors retrovirally transduced murine stromal cells for a marker gene ex vivo, and returned the genetically modified cells to syngeneic mice homozygous for the osteogenesis imperfecta murine (oim) mutation by intramedullary injection. Interestingly, these cells could be detected in both the marrow of the injected bones and also, remarkably, in noninjected bones of the same animal. The authors concluded that the cells were able to traffic between different bone marrow compartments. Furthermore, it was reported that the cells adhered to the trabecular surfaces of the bone and appeared to be in the process of differentiating into osteoblasts in vivo.[35]

In conclusion, retroviruses integrate their genomes into the chromosomal DNA of the host cell. Although long term expression must be considered variable for retroviruses,[3] they may still be advantageous for diseases that require prolonged expression of therapeutic proteins other than adenoviral vectors, which integrate episomally. However, treatment of fractures and the achievement of solid fusions in the spine or peripheral joints should not take longer than a few weeks or a couple of months. Within this crucial time span, an increased expression of therapeutic agents may be of utmost benefit. Hence in these cases, gene expression following transfer via adenoviral vector, which is typically high and transient, may be more appropriate.[36] In contrast, prolonged delivery of beneficial drugs may even cause harm to the patient.

Retroviral vectors have specific advantages for ex vivo gene therapy, and in fact retroviruses are unfavorable for in vivo approaches. By ex vivo methods, cells can be isolated and propagated in culture, genetically modified by retroviral transduction and finally transplanted into a recipient.[4,37] Cited studies demonstrate feasibility of retrovirus mediated ex vivo approaches to bone and fracture healing. The use of progenitor or stem cells, which must be harvested and isolated prior to delivery, opens exciting avenues for ex vivo gene therapy approaches. Further studies are required to determine the ideal vector systems that work best for each individual application. Understanding the workable mechanisms for gene expression regulation will have major impacts on the future of gene therapy. Research must focus on the basic biology of the mechanisms of action for target gene and the vector systems used to deliver the genes to cells. In contrast to retroviral vectors, there is much more literature describing the successes of adenoviral gene therapy strategies for orthopaedic applications.

Adenoviral Vectors

Adenoviruses

Adenoviruses are a class of double stranded, linear DNA viruses of approximately 35 kilobases in length (reviewed by ref. 38). There are more than 40 serotypes of adenoviruses with serotypes 5 and 2 (Ad.5 and Ad.2) being the most utilized as vectors for gene transfer. The advantages to adenoviral vectors are that they can infect a wide variety of both dividing and nondividing cells, resulting in high, transient levels of gene expression. Thus, adenoviral vectors are well suited for in vivo gene transfer where short-term gene expression is required. The disadvantages to adenoviral vectors are that the vectors are toxic to some cell types at a high multiplicity of infection and can stimulate an immune response to the infected cells.

Adenoviruses infect cells through an interaction of the viral fiber protein with its receptor, CAR. An interaction between a RGD protein motif in the penton base of the virus with a specific integrin then allows for fusion of the adenovirus to the cell and entry of the linear viral DNA into cells. Expression of the E1 region of the virus, containing the E1A and E1B open reading frames, from the adenoviral promoter results in subsequent E1A dependent expression of the E2, E3 and E4 open reading frames. Finally, following expression of the early genes important for DNA replication, expression of the viral late genes allows for packaging of the DNA into the viral capsid and release from the cell by lysis.

Adenovirus Vectors

Given that the temporal order of viral gene expression requires E1A for subsequent expression of the viral genes needed for viral DNA replication and packaging, deletion of the E1 region results in a replication defective virus. However, the replication defective ΔE1 virus can be propagated by gene complementation of E1A and E1B in trans. In particular, the human embryonic kidney cell line 293, stably transfected with E1A and E1B, allows for propagation of the E1 deficient viruses. Thus first generation Ad.2 or Ad.5 adenoviral vectors are always deficient in E1, with the therapeutic or marker transgenes usually inserted into the E1 region of the virus, under the regulation of a heterologous promoter. In addition, many of the vectors are deficient in E3 since proteins expressed from differentially spliced E3 transcripts are involved in blocking the immune response to virally infected cells as well as inhibiting apoptosis. Deletion of the E3 region, in conjunction with deletion of the E1 region results in a larger capacity of the vector for therapeutic genes. These first generation vectors have been used extensively for both preclinical studies as well as in clinical trials. However, following infection with the E1 and E3 deleted vectors, there is still a low level of expression of the E2 and E4 regions of the virus, resulting in toxicity at high viral doses and the induction of an immune response to the virally infected cells.

In order to reduce the toxicity and immunogenicity of the virus following infection, expression from the E4 region has been eliminated in second generation vectors. The E4 region encodes for 7 different open reading frames with Orf3 appearing to be the most important for

viral replication. In order to grow the ΔE1 and E4 adenoviral vectors, cell lines stably expressing both E1 and E4 have been generated. Given that E4 expression is toxic to cells, these stable cell lines have to express the E4 region from an inducible promoter. Although second generation viruses can be propagated, their titers are usually lower than first generation viruses. Analysis of gene transfer in vivo suggests that the toxicity and immunogenicity of the second generation vectors has been reduced, allowing for longer term gene expression. However, the level of transgene expression also appears to be reduced.

To generate adenoviral vectors that are even less immunogenic and toxic, third generation, gutted vectors have been generated where all viral genes have been removed, leaving only the viral terminal repeats and the packaging site needed in cis for viral replication and packaging. In order to propagate the gutted vectors, a helper virus carrying the E2, E4 and late genes is required. The difficulty with this type of vector is how to separate the recombinant gutted vector from the helper viruses after propagation in the helper cell line. The use of packaging compromised mutants in the helper virus have allowed for production of 3-4 logs more gutted vector than helper virus, but more optimal production methods are still required.

Applications of Adenoviral Vectors to Bone Healing

Adenoviral vectors have been used to deliver genes encoding proteins able to enhance bone healing in several different types of models. In addition, adenovirus has been used for both in vivo gene transfer at the site of defect or ex vivo to modify cells in culture prior to implantation into the defect. Currently, most studies have been performed with an adenovirus expressing human bone morphogenetic protein (BMP) 2. Infection of a bone marrow stromal cell line with Ad.BMP-2 followed by injection into large femoral defects resulted in enhanced and improved healing.[39] Similarly, Ad.BMP-2 was used to infect muscle-derived stem cells that were implanted into 5 mm diameter critical sized skull defect in the mouse, using a collagen sponge. Expression of BMP-2 in the stem cells resulted in their differentiation into osteogenic cells and improved healing of the critical sized defect.[40] In contrast to the modification of cells in culture that are then implanted, several groups have directly injected the adenoviral vector. Injection of an adenovirus carrying the luciferase marker gene into a segmental defect in a rabbit femur resulted in expression only at the site of the defect and adjacent soft tissue.[41] Expression within the defect and surrounding muscle persisted for at least 6 weeks with no expression observed in other tissues such as lung, liver and spleen. Injection of Ad.BMP-2 resulted in significant differences in healing as determined by radiological criteria, histology, and biomechanical testing.[42] Taken together, these results suggest that adenoviral vectors are well suited for transient delivery of therapeutic agents to either nondividing stem cells in culture or to cells within the defect in vivo. At least in animal models, there appears to be only limited inflammation associated with direct injection of low doses of Ad.BMP-2. Thus adenoviral vectors may be well suited for orthopaedic applications where only transient, localized gene expression is required to provide enhancement of the normal healing process.

Adeno-Associated Viral Vectors

Adeno-associated virus type 2 (AAV2) is a single-stranded DNA, nonenveloped, parvovirus that has gathered much attention in the viral gene delivery arena. The only cis components required to generate AAV2 vectors are the two 145-nucleotide inverted terminal repeats (for review, see ref. 43).

AAV vectors have many advantages for gene therapy. First, they have no proven pathogenicity in humans.[44] Secondly, they possess the capability of transducing a broad host of tissues,[45] although this could also be viewed as a liability in a bone fracture healing model. AAV vectors transduce both proliferating and nonproliferating cells;[46,47] the DNA can be stably integrated into cellular DNA and stable expression of the transgene was shown in animal models.[48] Low risk of insertional mutagenesis, due to the weak activation of downstream genomic sequences or intrinsic promoters by the inverted terminal repeats (ITR) was also noted.[44]

The immune response against AAV type 2-based vectors is an important consideration for use in gene therapy. Determining the preexisting immunity due to naturally acquired infections is essential. Studies using seropositive antibodies against AAV-2A were determined to be 50-96%, with 18-67.5% being neutralized.[49-51] Also, in most therapeutic applications it is necessary to readminister the vector. Chirmule et al[52] reported the efficacy of vector readministration in skeletal muscle of mice and rhesus monkeys, even though neutralizing antibodies were produced.[52] Other papers described animal experiments where neutralizing antibodies were shown to eliminate[46,53] or greatly reduce[52,54] the levels of transgene expression of the readministered vector. The variability of the outcome underscores the need to better understand the immune response against AAV-2.

A major disadvantage of the AAV type 2 as a gene therapy vector is its large-scale production. Recently, methods for producing a high titer[55] and purification[56] were published. These advances will allow further studies using AAV vectors and make it available for clinical trials. Another important characteristic and consideration for the use of AAV as a vector system, is its natural tropism, which is predominantly skeletal muscle, neurons, and hepatocytes.[57] The primary AAV type 2 (AAV-2) receptor was recently shown to be membrane-associated heparan sulphate proteoglycan (HSPG), mediating both AAV attachment to and transduction of target cells.[58] The receptor characterization explains the broad host attachment and transduction. It also should be noted that the transduction efficiency of AAV vectors varies greatly among different cells and between tissues in vitro and in vivo, suggesting coreceptor involvement. Human fibroblast growth factor receptor 1 was identified as a coreceptor for successful viral entry into the host cell.[59] Also, $\alpha v\beta 5$ integrin was also identified as a coreceptor for virus purification and this provides critical information for in vivo distribution of AAV vectors.[60] The tissue tropism of AAV-2 could be viewed as a liability and many researchers are investigating approaches for targeting the vector to a specific cell type. As of yet, two approaches have been used to modify the AAV's virion: chemically cross-linking bifunctional antibodies and genetically manipulating the capsid gene.

Bartlett et al 1999, demonstrated that chemically cross-linked monoclonal antibodies such as a bispecific $F(ab'\gamma)_2$ that mediate an interaction between the AAV vector and a specific cell surface receptor expressed on human megakaryocytes could target the AAV vectors to transduce megakaryocyte cell lines. The data indicated that AAV vectors can be targeted to a specific cell population and that transduction can be achieved by circumventing the normal virus receptor.

The second approach of altering the AAV-2 tropism using genetic manipulation of the capsid gene is more challenging; a requirement for this to be successful is to determine the surface amino acids and epitopes for manipulation. Moskalenko et al[49] defined peptide pools that represent immunogenic regions on the virion surface. Rabinowitz et al[61] used random linker insertion mutagenesis to also define the surface epitopes. In two other studies, using the amino acid sequence alignment in conjunction with the crystal structure, both groups were able to predict similar regions of the virion capsid that would be surface-localized.[62,63] These various approaches will eliminate the AAV-2 tropism and once this is achieved, the focus will turn to targeting the virion to specific tissues.

AAV Vectors in Gene Therapy

The adeno-associated virus (AAV) therapeutic applications are widening. Enthusiasm for AAV is due not only to the relative safety of these vectors, but also to advances in the understanding of the unique biology of this virus. Compared to retrovirus, AAV vectors are able to transduce nondividing cells in vivo including muscle, liver, and brain.[64-66] The broad host range, low level of immune response, and longevity of gene expression observed with these vectors in numerous disease paradigms has enabled the initiation of a number of clinical trials using this gene delivery system. So far however, reports have been published only on cystic fibrosis and hemophilia B.

In humans, two phase 1 clinical trials for gene therapy of cystic fibrosis have been undertaken.[67,68] AAV is of relatively small size. Therefore, all viral genes are deleted in the production of AAV vectors for the treatment of cystic fibrosis. This means that the least amount of immunogenic material remains. However, this intervention leaves little space for an optimal promoter sequence. Site specificity of the integration is lost with the deletion of the AAV *rep* gene, required for replication. However, it has been shown that even with the *rep* deletion, AAV vectors have been capable of long-term persistence through random-site integration and episomal persistence.[69]

Preliminary results of a not yet completed dose-escalation study including 19 subjects with mild cystic fibrosis lung disease have shown that administration of AAV vectors does not induce inflammatory responses over a wide range of doses.[67]

In a second study, administration of AAV vectors to the paranasal sinuses of cystic fibrosis patients resulted in gene transfer to the epithelium at a rate of one copy of the gene for every 10 cells. This level of transfer appeared to be stable for up to 10 weeks.[68]

Kay et al[70] reported on a clinical trial of gene therapy in severe hemophilia B. Based on preclinical studies demonstrating efficacy and absence of vector-related toxicity in hemophilic mice and dog models, an AAV vector encoding for human F IX was injected intramuscular in adults. Results regarding safety, gene transfer and expression in the first three patients were encouraging and gave no evidence of toxicity, transfer in semen or formation of inhibitory antibodies against F IX.

Orthopedic Gene Therapy with Recombinant Adeno-Associated Virus Vectors

An inflammatory arthritis rodent model was used to evaluate the efficacy of the recombinant AAV vectors for orthopedics therapy. Two studies using the same model described their findings; Pan et al[71] reported 95% of inflamed synoviocytes expressed the beta-galactosidase driven by a cytomegalovirus promoter (AAV-CMV-LacZ). Goater et al[72] also showed transgene expression of beta-galactosidase in arthritic knees. The data indicated a 10-fold increase in the reporter gene expression in the arthritic knee compared to the control. The preliminary work with AAV vectors for gene therapy is encouraging and we anticipate a larger number of future studies especially since methods for producing a high titer[54] and purification[56] have regularly been published. These advances will allow further studies using AAV vectors. In general, AAV vectors hold promise for gene therapy as they appear to be safe and have superior duration profiles.[73,74]

Nonviral Gene Therapy

The limitations of viral vectors, in particular their relatively small capacity for therapeutic DNA, safety concerns, (such as insertional mutagenesis and toxicity problems), and difficulty in targeting to specific cell types have led to the evaluation and development of alternative vectors based on chemically nonviral systems. Advantages of nonviral methods of DNA transfer include their capability of transporting large size DNA inserts, their safety regarding mutagenic issues, and their lesser immunogenicity. Furthermore, using pharmaceutical techniques large scale production should be possible. Disadvantages of non viral vectors comprise their poor transfection efficiencies, which in part is due to endolysosomal degradation[75] and only transient expression due to the lack of integration in the host genome. Although, in certain circumstances a transient expression is desired.

To develop successful non viral vectors, researchers have to understand and optimize the mechanisms involved in the traffic of DNA to its target destination. The three barriers that the DNA has to overcome are: (1) The DNA has to be transported to the cytoplasm, either directly through the plasma membrane or by escape from the endosome; (2) The DNA has to be translocated to the nucleus; or (3) For many applications the DNA has either to be integrated into the genome or extrachromosomally replicated. Many approaches were investigated to efficiently transition the DNA through these steps. Below we will discuss the different approaches to transfer non viral DNA.

Naked DNA (in the form of a plasmid) can be directly injected into certain tissues, particularly muscle. Direct muscle injection was shown[76] to be feasible and produces surprisingly high levels of gene expression. In addition to the muscle, direct injection was successful in other tissues such as liver,[77] pancreas,[78] thyroid,[79] myocard,[80] brain,[81] urological organs[82] and blood vessel development.[83] Enhancement of the transgene expression levels were noted following either the use of a number of muscle-specific promoters and a myosin light chain[84] or the modifications to the polyadenylation and transcriptional termination.[85]

The simplicity of this approach has led to its adoption in a number of clinical protocols. In particular, this approach has been applied to the gene therapy of cancer where the DNA can be injected either directly into the tumor and the DNA expression is high enough to elicit a therapeutic response[86,87] or into muscle cells in order to express tumor antigens that might function as a cancer vaccine. Gene expression of direct DNA injection can persist for several months after intramuscular injection,[88,89] and does not usually undergo chromosomal integration.

Direct injection of naked DNA into the blood stream did not result in gene expression in major organs.[90] This was due to the presence of nucleases responsible for DNA degradation and a clearance system performed by mononuclear phagocyte with a half-life of less than 5 minutes.

Several studies have reported uptake of naked DNA in several organs but mainly the liver when they either rapidly injected large volumes of fluids/naked DNA into the vasculature system in order to increase the osmotic and hydrostatic pressure or occluded blood vessels to again achieve increased pressure.[91-94] This approach of DNA introduction appears to benefit from the use of cationic liposomes to protect the DNA from degradation and also from increased osmotic and hydrostatic pressure. A better understanding of the mechanism of uptake of DNA by these approaches might yield more enthusiasm for its use clinically.

The Gene Gun approach uses gold particles (1-3 μm) that are attached to DNA that are cannonaded into the tissue.[95,96] The prolonged and sustained low level expression of this technique has made it one of the methods of choice for the development of DNA vaccines. DNA vaccines promise to be valuable where responses to agents are inappropriate, ineffective or even lacking, for example, HIV and influenza.[97,98] Some of the advantages of the DNA vaccines compared to conventional attenuated and protein based vaccines are the relatively low cost to produce, and the ability to introduce multiple pathogens by using a single plasmid. Moreover, DNA vaccines are unaffected by preexisting immunity.[99] An interesting aspect of this method is the likely means by which DNA vaccines induce cytotoxic T lymphocytes (CTL) and that the type of immunity induced can be modified. This might be a practical approach to manipulate undesired immune response.[99]

Electroporation has been widely used to transfect DNA to cells in tissue culture.[100] This technique relies on the fact that electric pulses can induce opening up the pores in the cell membrane through which DNA can pass down a concentration gradient into the interior of the cell.

In vivo, naked plasmid DNA is usually injected into the interstitial space of the tissue and then electric pulses are applied with needle or caliper-type electrodes to transfect the DNA intracellularly. So far, this technique has been applied to introduce plasmid DNA into the following tissues: skin,[101] liver,[102] melanoma,[103] and muscle.[104]

Cationic liposomes are lipid bilayers entrapping a fraction of aqueous fluid. Cationic lipids are widely used in vitro and the majority of cell lines may be transfected. The negatively charged nucleic acids interact with the positively charged macromolecules, induce a condensation of the DNA and a physical association with the lipid. Several kinds of cationic lipid, such as dioctadecylamido-glycylspermine (DOGS, Transfectam™)[105] and Lipofectin™,[106-108] have been developed. In addition a neutral lipid such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol is generally added to facilitate the release of plasmid DNA from the endosome following endocytic uptake of the complex.[109] The exact mechanism of the cellular membrane interaction with the complex and subsequent cell entry is unknown. But it is thought that

endocytosis, phagocytosis, pinocytosis and direct fusion may all contribute to the liposome-DNA crossing the cellular barrier.[110] To facilitate uptake into the cell as endosomes, targeting proteins have been included in liposomes, for example, anti-MHC antibody[111] and transferrin.[112]

This method of introducing DNA has been used in clinical trials for the treatment of cancer and cystic fibrosis,[113,114] lung,[115] brain,[116] tumor,[117,118] and skin[119] by local administration or to vascular endothelial cells[120,121] after systemic, intravenous injection.

Cationic polymers include poly-L-lysine (PLL), poly-L-ornithine, polyethylenimine (PEI), chitosan, and starburst dendrimer. In addition to the cationic nature of these polymers they complex the DNA and condense it, both of these characteristics facilitate the endocytic uptake of the DNA and increase the transfection efficiencies.

The low molecular weight PEI showed favorable results in in vitro and in vivo applications and with the least toxicity among the cationic polymers group. It has been used successfully in vivo to transfer genes in mice brain[122] and rat kidney.[123] Another desirable characteristic of using PEI as a gene delivery vehicle is the capability of buffering the endosome/lysosome compartment, thus avoiding DNA degradation. Many parameters have been modified to optimize the PEI transfection efficiency. Some of these parameters include: different PEI concentration,[124] PEI nitrogen:DNA phosphate ratios,[123] PEI molecular weight[125] and improved packing of PEI.[126]

Immunogenicity of Plasmid DNA

Plasmid DNA complex can elicit an immune response and cells of the immune system recognize the DNA as an extrinsic antigen. Phagocytosis by the mononuclear phagocytes appears to be the primary defense mechanism. Krieg et al[127] has reported that an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines induces immune reactions. This property has been used to improve genetic vaccination. However, when the purpose of plasmid DNA delivery is to produce a therapeutic effect, the immune reaction to the unmethylated CpG is a critical obstacle to the success of nonviral gene therapy. Efforts are underway to engineer vectors that minimize the presence of the CpG motif.

Applications of Nonviral Vectors to Bone Healing

In vivo direct plasmid DNA delivery was successfully reported by Fang et al and Bonadio et al.[128,129] The investigators coined the term gene activated matrices (GAM) for the methodology they used. GAM consists of a biodegradable scaffold where the plasmid DNA is entrapped and locally delivered to the wound site in a sustained manner. The released DNA will transfect the recruited cells during the granulation phase of bone repair. The newly transfected cells will regulate the expression of the transfect gene. To stimulate or enhance bone formation, the choice of an osteoinductive gene is critical. Fang et al[128] delivered either a bone morphogenetic protein-4 (BMP-4) plasmid or a plasmid coding for a fragment of parathyroid hormone (amino acids 1-34) ($PTH_{1\text{-}34}$) in both cases, a biological response was shown. In addition, it appears that when both BMP-4 and $PTH_{1\text{-}34}$ are delivered, bone formation occurs at more rapid rate than either factor alone. Bonadio et al[129] used a tibial defect model where collagen and $PTH_{1\text{-}34}$ were delivered. The local delivery of $PTH_{1\text{-}34}$ was shown to substantially increase bone formation whereas the control containing only the matrix produced no bone.

Local gene delivery of plasmid DNA was also used in a spinal fusion model with $PTH_{1\text{-}34}$ as the gene of choice. The authors reported significant bone infiltration in the experimental group whereas the control showed moderate bone fill. Five of the seven specimens analyzed showed anterior fusion mass.

In conclusion, non-viral gene delivery is showing great promise for therapeutic purposes. Much research is underway to optimize the transfection efficiency. As mentioned above, there are applications for which nonviral vector would be better suited. In particular, applications that require a transient expression and repetitive administration of vectors. Repetitive administration of viral vectors poses the risk of developing an immune response against the viral proteins and, hence, the vector. Nonviral DNA delivery vehicles offer—at least in principle—a nonimmunogenic alternative.

Gene Therapy Applications to Bone Fracture Healing and Tissue Engineering

Gene Therapy for Bone Healing

The success of gene therapy strategies depends on the basic biology and physiology of the target tissues, the nature of the defect and the limitations of each specific gene therapy technique. Although gene therapy was initially envisioned to treat heritable genetic disorders, major advances in somatic cell gene therapy have indicated an ability to revolutionize modern medicine. As we further our understanding of the basic biology of cell differentiation to form tissues and organs of the body, we can design new therapeutic strategies to combat disease and injury. The microenvironment in which a cell resides is an intricate and dynamic network composed of soluble and immobilized growth factors and extracellular matrix (ECM) proteins.[130] Characterizing genes that participate in tissue development, maintenance, repair and degradation leads to an understanding of the unique microenvironment cells require to form specific tissues in the body. Further, it is known that for bone and other systems, the ECM participates in signaling for proliferation, differentiation and survival.[131] Bone represents an ideal system in which to employ gene therapy in tissue engineering to repair defects due to disease, trauma or aging. Bone has the inherent capacity to regenerate itself. We must use the known basic biology of the bone microenvironment to reengineer bone. Gene therapy can help recreate the specific microenvironment necessary for successful bone healing, by providing the correct cues to cells, enabling them to participate in new bone formation. Traditional treatments for complications associated with fracture healing fall short of mimicking a bone-favorable microenvironment, have varying success and many disadvantages.

Gene therapy can be used to overcome the complications of conventional treatments for bony defects. In this section, we will address examples of the successes and failures of gene therapy for fracture healing. Future directions in bone tissue engineering using gene therapy will be suggested and its eventual success as a clinical therapy will be evident.

Gene Therapy Strategies for Bone Tissue Engineering

The ideal delivery system must effectively transport a functional protein (or coding DNA) to the site of the wound and maintain localization of that growth factor with controlled expression. The DNA transfer must remain functional during transport, be replicable, transcribable and made into a properly folded, functional protein. Proper delivery of osteoinductive factors will provide molecular signals to host cells, recruit potential osteoprogenitor cells and enhance or help recreate the natural microenvironment for bone regeneration. The three main strategies for growth factor delivery to bone wound sites are shown in Figure 3:

1. Gene therapy: the direct delivery of DNA encoding a growth factor to the patient
2. Cell therapy (ex vivo gene therapy): in vitro transfer of growth factor DNA to cultured cells, followed by implantation into the would site
3. Protein therapy: direct injection of growth factor to patient via carrier matrix

All three delivery strategies usually make use of a carrier matrix (i.e., polymer) for effective delivery of DNA, cells or proteins to the wound site. In the case of gene therapy or cell therapy the vector (plasmid or viral) or cell could serve as the carrier. However, the addition of a polymer scaffold as a carrier matrix could offer several additional levels of protection and release control. DNA for gene therapy or cultured cells transfected in vitro can be seeded onto scaffolds which resemble the natural porous structure of bone. The ideal scaffold mimics the target tissue, optimizes the activity of the growth factor, stability in vivo, yet biodegradable. Additional desirable attributes include controlled release of the growth factor or gene and cell growth conducive. These characteristics often conflict with each other and lead to difficulty finding the optimal scaffold. Clinicians prefer a material that can be injected percutaneously, however such a substance would not be cohesive enough to stay localized to the defect. Obviously, it would be advantageous to use a scaffold of natural origin such as collagen or hydroxyapatite.

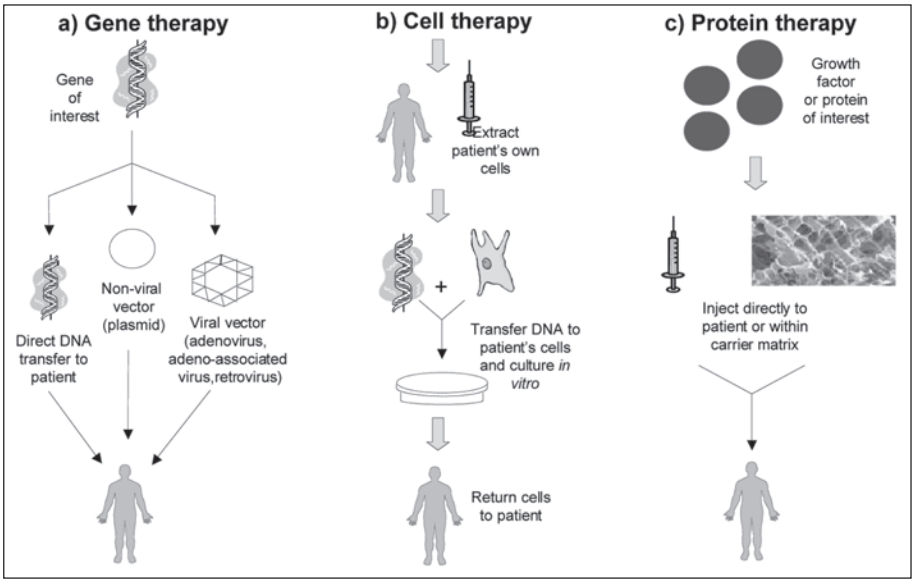

Figure 3. The three main strategies for growth factor delivery to cells. a) Gene therapy: The gene of interest is transferred directly to the body either alone or with a non-viral or viral vector. b) Cell therapy: DNA is transferred to the cells in vitro and cultured before transplantation back into the patient. c) Protein therapy: The growth factor is directly injected into the patient or delivered via a carrier matrix.

These are primary constituents of bone matrix and are known to naturally bind BMPs and other potential osteoinductive factors within the ECM.[132] Unfortunately, the most cost-efficient, easy to manufacture, well-characterized materials are not natural; they are often easily-sterilized synthetic polymers. Examples of matrix materials and their properties are shown in Table 1.

Scaffolds further contribute to the re-creation of the "natural microenvironment" of bone formation. Host or seeded cultured cells infiltrate the scaffold and respond to the endogenous molecular cues or those enhanced by gene, cell or protein therapy. The proper combination of cells, signals and matrices promotes effective and successful bone healing. Much more research must be done to fully characterize the basic biology behind bone formation. However, since we know that certain growth factors exist that promote bone regeneration (TGF-β, FGF, PDGF, IGF, BMPs); we can begin to focus on mimicking Mother Nature's design of the bone microenvironment using the information known. Protein therapy (especially with BMPs) using carrier matrices is the most widely used and researched treatment; however the many disadvantages mentioned above lead scientists towards gene and cell therapy techniques for bone tissue engineering. Growth factor delivery to the wound site via gene or cell therapy overcomes many of the obstacles faced with protein therapy. The potential success of gene therapy strategies in a future clinical trials are evidenced in studies conducted both in vitro and in animal models for bone defects. Attempts to heal common bone defects using gene therapy are discussed below. The relative successes and failures of gene therapy for bone healing are addressed. The future of gene therapy and the ultimate healing of bone defects are dependent on scientists' abilities to overcome the obstacles faced when attempting to heal bone defects. Reengineering the bone microenvironment can be achieved through gene therapy by first understanding the fundamental biology of bone, the ECM and limitations of gene therapy. However, with each bone defect or disease, a unique situation exists, complete with different physiologies, pathologies and dynamics which directly influence the success of the gene therapy methods employed.

Table 1. Common carrier matrices employed for bone tissue engineering

Carrier	Advantages	Disadvantages	Examples	References
Inorganic materials	Resembles natural structure of bone (calcium phosphates Resorbable or nonresorbable Binds BMP's	Brittle Difficult to mold Exothermic	Hydroxyapaptite, porous coralline, hyaluronic acid, calcium phosphate cements, metals, and calcium sulfate	133-137
Synthetic polymers	Reproducible manufacture Controlled release properties Easy to sterilize	Degradation products may be toxic Cell recognition Solvents or crosslinkers may damage proteins	Poly (α-hydroxy acids), polypropylene fumarate, polyanhydrides, polyphosphazenes, polyethylene glycol and poloxamers	133,138-145
Naturally-derived polymers	Extremely biocompatible Possible natural affinity for growth factors	Disease transmission Difficult to sterilze Immune response	Fibrin glue, collagen, chitosan and hyaluronic acid	133,146-149
Composite materials	Benefits depend on material exploited	Complex to manufacture	Collagen-TCP, collagen-HA and TCP cellulose	133,150-153

Applications

Fracture Healing

A large number of fractures are classified as high risk for potential nonunions or incomplete unions.[154,155] There are many factors contributing to poor healing risk factors such as significant soft tissue injury, anatomic location, velocity of impact plus others.[156-158] Poor fracture healing is linked to chronic pain and prolonged ambulatory impairment and must often be treated with an invasive operative procedure.[159] Currently, there are many treatments used to enhance bone healing including autogenous or allographic bone grafts and various fixation devices as mentioned previously.[154,156,160] Despite proper treatment being currently available, lack of viable bone at the wound site and other adverse conditions may lead to structural instability, possible infection and bone erosion. Current orthopedic treatments for defects of a critical-size or severe complications that arise from common fractures lack an effective therapy for complete healing in terms of restitutio ad integrum, evidenced by the frequent reported complications. Recent progress has indicated that future enhancements will rely on applications of recombinant factors such as growth factors or cytokines known to stimulate bone repair, and the use of ex vivo or in vivo delivery of these factors by gene therapy.[159] Although, the use of recombinant proteins has been evaluated in a few human clinical trials, the use of local gene therapy to deliver growth factors to the wound site has been limited to animal models.[129,149,161-164] Recombinant BMPs (BMP-2, BMP-3, BMP-4, BMP-5, BMP-6) and TGF-β can promote osteogenesis and bone healing in animal models and humans as shown in a few, limited studies.[159] However, large doses (milligram amounts) of protein must be delivered to the wound site to achieve healing. This raises concern that therapeutic doses for humans would be expensive and include the additional risk of toxicity. Gene therapy for delivery of growth factors to the defect site represents a more cost-effective solution. Further, cell-mediated in vivo production of the protein will enhance protein functionality and localize expression to

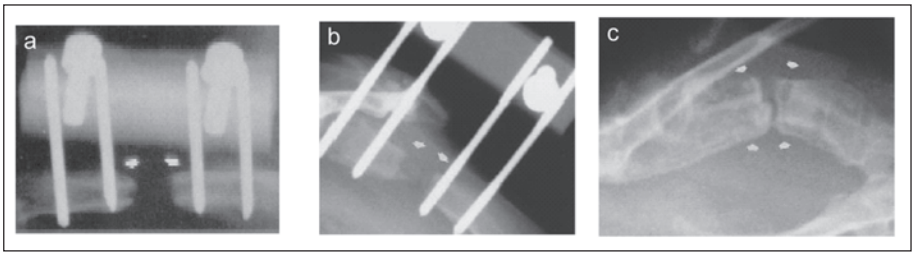

Figure 4. Delivery of BMP-4 and PTH 1-34 cDNA via plasmid vectors embedded in a collagen sponge results in bone formation in a rat femur critical-sized defect model. a) Plain x-ray film of control rat femur osteotomy 9 weeks post-surgery showing no healing of the defect. b) Plain x-ray film showing new bone bridging the gap of a 5-mm defect four weeks post-implantation. c) The same gap as in (b) without the external fixator, 17 weeks post-surgery. Arrows point to sites of original defect which is now filled with radiodense tissue in (b) and (c). Plasmid delivery of BMP-4 and PTH 1-34 cDNA in a collagen sponge was able to heal a rat femur critical-sized defect.

the wound site. Examples of initial animal studies examined the use of both plasmid and viral vectors to deliver osteoinductive growth factors to defect sites in bone.

BMP-4 and PTH 1-34 cDNA was delivered to the site of 5 mm midshaft osteotomies in rats using direct gene transfer with a plasmid vector.[128] A collagen sponge served as the carrier matrix for the plasmid DNA. New bone formation was stimulated at the site of the defect where in controls, fibrous tissue formed. Bone induction resulted in functional union of large segmental defects in the rat femur (Fig. 4). The rates of new bone formation paralleled results from previous studies which used direct protein therapy to administer BMP-2, OP-1 and TGF-β to the wound site.[165,166] Therefore, plasmid DNA can be used to transfer growth factor cDNA to repair cells at the wound site. The amount of new bone formation is comparable to that achieved using direct delivery of large doses of protein. This represents a simple way to simulate mammalian bone growth by controlling transient, local, over-expression of osteogenic factors. Further, gene transfer may be an advantageous strategy for drug delivery for molecules that normally require post-translational modifications. A relevant example is TGF-β, which has been difficult to develop as a recombinant bone growth therapy despite many in vivo studies supporting its role in osteogenesis. Given the large number of known osteoinductive factors, others should be examined for gene therapy strategies either alone or in combination. The method of delivery for each factor depends on its function, expression level requirements, and mechanism of action. Further, since plasmid vectors are known to have relatively low gene transfer efficiency, the use of viral vectors for the delivery of osteoinductive growth factors is often warranted.

Adenoviral vectors have also been used to deliver growth factors to a site of fractured bone. By directly injecting adenoviral vectors into rabbits, in vivo gene therapy provides a minimally invasive treatment with little damage to local blood circulation. Marker gene transfer (LacZ) was shown to persist locally for up to six weeks at a declining rate.[41] For the treatment of complications following fracture, this duration may be suitable. It was also shown that bone marrow cells, expressing BMP-2 via viral vector can heal a segmental femoral defect.[167] The results show the feasibility of an ex vivo gene therapy technique with short-term cultures of bone marrow cells. These studies lead to perhaps the most exciting results; those in which gene therapy enhance tissue engineering approaches to bone healing.

Cultured rabbit periosteal cells transduced retrovirally with BMP-7 cDNA were shown to induce new bone formation and repair bone defects. Further, TGF-β cDNA can be effectively transferred to osteoblasts and osteocytes in vivo. This treatment can be administered percutaneously. These techniques may be superior to the use of growth factor proteins alone; overcoming problems of protein instability, localization and duration of expression. Local gene therapy

for fracture healing is an attractive technique. Osteogenic genes can be delivered to the correct anatomical site and the duration of expression can be controlled by the choice of vector. Perhaps the greatest advantage of gene therapy for fracture healing is the fact that long-term transgene expression is not necessary. Once the bone is healed, protein over-expression is no longer needed, and may harm the patient.

Although gene therapy for fracture healing shows much promise for clinical applications, there have been no published human clinical trials. There are several factors to consider prior to treatment for humans. First, the physician must consider the condition of the host bed, including the quality of the bone present. The degree of vascularity to the soft tissue and the location of the defect have a significant impact on the success of a gene therapy treatment to the wound site. Other existing systemic factors such as osteoporosis, diabetes, peripheral vascular disease, medications and smoking will all have an effect on the effectiveness of a gene therapy application. Physicians must find a balance between safety, efficacy and cost to determine in every individual case if gene transfer is a suitable additional treatment option for fracture healing. Apart from infection, the major risk factor contributing to fractures and insufficient fracture healing is osteoporosis.

Osteoporosis is now recognized as a major public health concern. Low bone density, or osteopenia, caused by either increased osteoclast production or decreased osteoblast production or both results in osteoporosis. This causes fatigue degradation, leading to a significant clinical risk for fractures from minimal trauma. The most common fractures due to osteoporosis occur in the wrist, spine and hip, totalling 1.7 million fractures per year in the United States. (In comparison, there are 1.2 million new cancer cases each year).[168] Hip fractures account for the majority of morbidity, mortality and costs associated with osteoporotic fractures.[168] In the United States, the number of individuals over age 65 is expected to rise from 32 to 69 million between 1990 and 2050, and the risk for osteoporotic hip fractures increases exponentially with age.[169] Therefore, if current trends continue, the number of hip fractures in the U.S. could total 840,000 by 2040.[170] The treatment of choice for hip fractures in the elderly is most often operative management. The ideal result is fracture fixation that promotes a return to normal weight-bearing mobility. For most patients, fracture fixation and healing is achieved with the use of an orthopaedic device made of titanium alloy or stainless steel. In some cases where joint function cannot be restored or degenerative changes are advanced, instead of an anatomical reconstruction of the bone, a total joint replacement is performed. Osteopenia at the fracture site contributes to a less stable hip and complicates the success of this common treatment. The inability of screws, pins and prosthetic implants to gain adequate access to the bone stock causes failure in fracture healing and bone regeneration.[168] The most common complications are fragment displacement, malrotation deformity, nonunion, and periprosthetic fracture and early aseptic loosening.[168] Other complications not related to osteoporosis may include breaking or bending of the implant, screw penetration into the joint, and disassociation of the plate from the shaft.[168] It is feasible to suspect that 5-20% of hip fracture patients suffer at least one of the above complications; however there is no acceptable method to evaluate fracture healing.[168] Fracture healing for osteoporotic patients is obviously in need of improvement. Gene therapy to stimulate bone formation and create denser bone mass is an attractive possibility. Although there have been no clinical trials published thus far. However, the mostly likely choice for the first human subjects would be Mat-100, a single application, plasmid gene therapy for bone fracture repair in osteoporotic elderly patients.[168] In vitro and animal studies have contributed evidence to support possible success for Mat-100 in humans as a treatment for osteoporotic fractures.[168] PTH 1-34 peptide is the best studied human bone growth factor in terms of its anabolic effects on the osteoporotic skeleton.[168,171-174] As stated, there is an unmet clinical need associated with osteoporotic fractures in the elderly. PTH 1-34 cDNA as the plasmid DNA component of Mat-100 shows promise as a future clinical therapy.

A canine study in which Mat-100 prototypes were used to fill surgical defects in tibiae of intact, skeletally mature beagles that mimic acute bone fracture in humans suggests potential for hPTH 1-34 peptide as a therapy.[129] New bone filled these defects and persisted at least 2

years post surgery without any evidence of bone loss.[129] The optimal dosage range of hPTH 1-34 must be identified; however, hPTH 1-34 is a reproducibly effective and a safe anabolic agent for fracture repair. One important limitation to this study, which must be considered, is that these beagles were young, metabolically intact adults. This makes it difficult to predict if such a therapy would be effective in an osteoporotic elderly human. Fortunately, the dose response for hPTH 1-34 peptide and new bone formation has been well documented in the literature.[171,173,174] The most common effective dose for rats was 80.0 μg/kg, administered subcutaneously three times per week.[168] Lower doses (30.0 μg/kg-60.0 μg/kg) have also produced consistently positive results.[173,174] A low dose of hPTH 1-34 peptide has be used in human clinical trials as a protein therapy, and an equivalent dose (2.5 μg/kg) consistent with these has been used in osteoporotic rats.[175,176] This low dose was able to stimulate bone formation partially in the osteoporotic rats and restore bone that was surgically removed.[174] Studies including high doses of hPTH 1-34 (400 μg/kg-1000 μg/kg) have shown strong anabolic response but some of the new bone is woven rather than lamellar and marrow fibrosis (undesirable side effect).[168] More recently, studies in other animal species, including monkeys has shown that hPTH 1-34 induced a rapid and significant increase in bone mass at the spine and hip and increased biomechanical strength.[177] Two doses of hPTH 1-34 were administered (1 and 5 μg/kg) as a daily cutaneous injection for one year. A dose response was observed with no safety risks. Current and ongoing clinical trials indicate that hPTH 1-34 is rapidly, significantly and reproducibly anabolic for both cortical and trabecular bone.[168,175,176] Higher doses will persist years after plasmid DNA delivery to the wound site and shown to be safe and effective.

It is well established that hPTH 1-34 anabolic effects are enhanced when the peptide is administered intermittently.[168,174] Continuous administration increases bone formation in intact rats, however, resorption is increased to roughly the same level as formation so that overall bone density/amount is unchanged.[172,178] Intermittent treatment stimulates new bone formation, total amount and density.[171] There is no major evidence for gene therapy success in wound healing. For a gene therapy application, the administration of hPTH 1-34 from a gene-activated matrix to the wound site is the most important factor. Local gene delivery is the best strategy to achieve dose-dependent, reproducible and safe tissue regeneration. Given the evidence from rat, canine and nonhuman primate studies, it is feasible that hPTH 1-34 would be effective as a gene therapy for bone healing. A viral vector for hPTH 1-34 delivery to the wound site would be advantageous due to its ability to promote long term expression. For an osteoporotic patient, long term expression of an osteoinductive factor delivered to the site is desirable, however, not necessarily required for the healing of fractures in younger patients. The specifics of each case, risk factors contributed by the patient and nature of the defect all play a role in the success of a gene therapy technique.

Spinal Fusion

Spinal fusion is a relatively common surgical procedure. Internal fixation devices currently used keep the fusion site stable for the time that is usually necessary for the bone to achieve fusion. However, solid bone fusion failure can reach 45%.[179] Autogenous grafts are severely limited in volume and harvest from the iliac crest has shown a high incidence of morbidity at the donor site.[159] Allograft bone is an alternative; however it has also shown antigenic potential and disease transmission.[159] Methods of sterilization or "bio-cleansing" for allograft materials are under investigation but current knowledge is sufficiently lacking in this area. Therefore, the focus has shifted towards enhancing growth factor activity to stimulate osteogenesis for successful spinal fusion.

Osteogenic proteins such as the BMPs represent an attractive alternative to autografting. Delivering BMP to the site of the wound can contribute to the recruitment of host osteoprogenitor cells and stimulate bone cell formation locally to promote fusion. BMPs are secreted signaling molecules. They form dimers after secretion and bind to a complex of transmembrane receptors. This event triggers a phosphorylation cascade that turns on bone specific genes in the nucleus via the Smad proteins (Fig. 5). Examples of genes that are upregulated

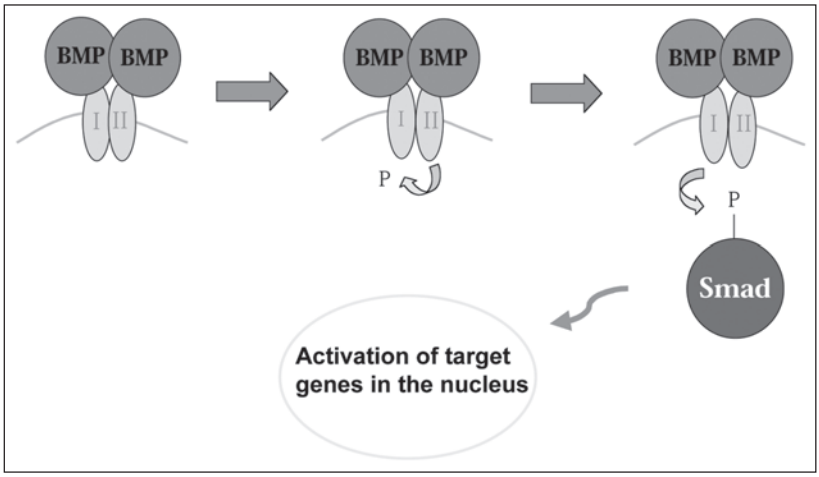

Figure 5. Mechanism of action for bone morphogenetic proteins. Upon secretion, two BMP molecules form a dimer and bind to a complex of two transmembrane receptors, Type I and Type I. Type II receptor phosphorylates Type I receptor, which then phosphorylates the Smad family of proteins. There proteins then activate target genes in the nucleus including RunX2, a bone-specific transcription factor. BMPs function as secreted signaling molecules that upon binding to a complex of transmembrane receptors, triggers a phosphorylation cascade to activate bone specific genes in the nucleus to promote osteogenesis.

during osteogensis include osteocalcin, osteonectin, bone sialoprotein and the transcription factor Runx2. Evidence that BMPs contribute to successful spinal fusions has been shown in rabbits, dogs, mice and nonhuman primates.[141,180-182] However, BMPs have a short biological half-life. Thus, coupling this treatment with the use of a biodegradable scaffold is often used. Scaffolds used include poly-lactic acid, hydroxyapatite, de-mineralized bone matrix, and hydroxyapatite calcium triphosphate.[183,184] All of these examples possess the limitations outlined in Table 1. Secondly, large quantities of BMP must be delivered in an active form to the site for effective participation in bone healing. Finally, BMPs lack tissue specificity and thus it is difficult to control the duration and localization of their activity using the traditional carrier matrices mentioned above. Gene therapy to deliver BMP to the site of desired spinal fusion can overcome many of the obstacles faced by the traditional methods described. Introducing BMP into cells at the fusion site will make it possible to achieve long-term controlled expression that is tissue specific.

In experimental spinal fusions in animals, an adenoviral vector carrying rhBMP-2 is often used. It was shown that mesenchymal stem cells transduced with the rhBMP-2 gene produced rhBMP-2 protein in vitro and could transform into an osteoprogenitor line that can produce bone in vivo.[185] This refers to an ex vivo method for growth factor delivery since rhBMP-2 delivery to the stem cell was performed in culture. Further, such cells were injected percutaneously and paraspinally at the lumbosacral junction that resulted in endochondral bone formation.[186] Proteins other than BMPs that are known to be osteoinductive such as LIM-1 (LIM mineralization protein-1) can also be delivered to spinal fusion sites to promote osteogenesis.[159]

A successful spine fusion was demonstrated; showing fusion in 100% of the sites that received bone marrow cells that were transfected with active cDNA encoding LIM-1.[187] LIM-1 is a novel secreted protein, known to act on surrounding cells to induce bone formation. In a rat model, posterior lateral spinal fusion was achieved following implantation of bone marrow cells transfected with LIM-1 cDNA.[187] (Fig. 6) Therefore, genes encoding secreted osteogenic factors are good choices for gene therapy for bone due to their ability to act on surrounding

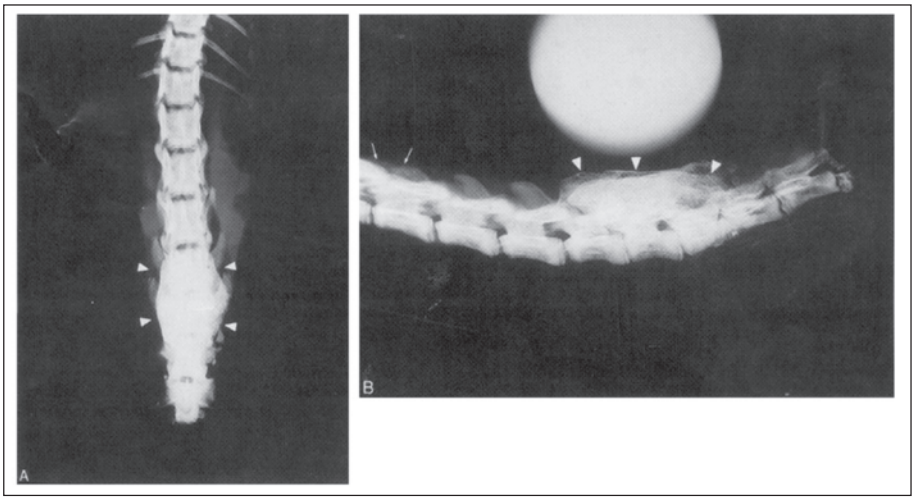

Figure 6. Spinal fusion is achieved in a rat model when devitalized bone matrix is loaded with bone marrow cells over-expressing LIM-1 cDNA. Anteroposterior (A) and lateral (B) radiographs of a rat spine 4 weeks post-surgery. Complete spinal fusion where bone marrow cells expressed LIM-1 cDNA is shown (arrowheads) and no fusion where cells contained an inactive form of LIM-1 cDNA (arrows).

host cells, rather than transfected cells only. These studies show promising results in small animals and strongly support an advance towards clinical use for the treatment of human spinal fusions. Although animal studies indicate gene therapy as an attractive treatment for spinal fusion, problems arise when translating such a therapy to humans.

For example, gene dosage is a consideration for any gene therapy application. The use of BMPs for spinal fusion is no exception. Rhesus monkeys needed eight times as much bovine-derived osteoinductive factor to achieve spinal fusion compared to the rabbit model.[180] Other issues of concern include duration of expression and localization of osteoinductive protein expression. Ossification of the ligamentum flavum and secondary spinal cord compression in mice when BMP-2 had been delivered to the lumbar extradural space has also been reported.[188] Localization and duration of BMP activity can be controlled by incorporating tissue specific, inducible promoters. For example osteocalcin is an osteoblast specific protein and the osteocalcin promoter has been used to drive tissue specific expression of growth factors for gene therapy.[188] Further, using an inducible system such as the tet on/off system, allowing for controlled expression of the transgene.[189] In the presence of tetracycline or its derivative doxycycline, BMP is not expressed. In their absence, exogenous BMP expression is turned on. A tetracycline-regulated system was able to control rhBMP-2 expression in vivo and promote bone healing in a mouse radial critical-sized defect model (Fig. 7).[189] Further, when rhBMP-2 expression was turned on, bone specific genes were up-regulated.[189] The function of BMP endogenously is another factor to consider when designing a gene therapy for bone using BMP. Bone inductive proteins of BMP vary across species.[180] Therefore, the functions of BMP must be characterized specifically in each animal to be treated by gene therapy using BMP DNA. This holds true for any gene therapy application to bone and will be echoed in gene therapy applications to other bone defects. In conclusion, osteoinductive factors that are delivered to the wound site via gene therapy were shown to promote successful spinal fusion in animal models. Other genes of interest must also be investigated. With these come additional advantages and limitations that must be considered when evaluating the potentials of such treatments for humans.

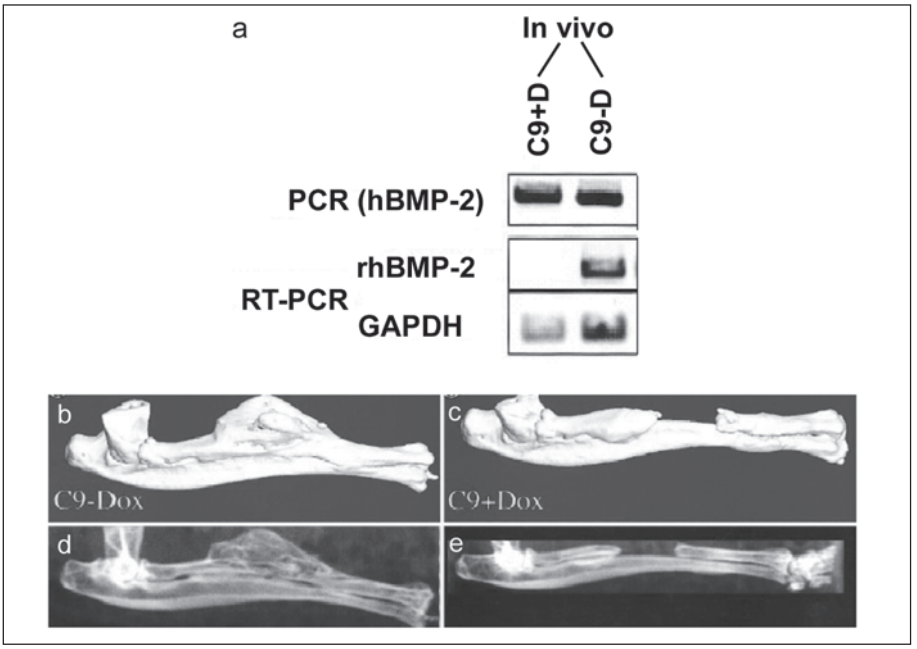

Figure 7. Doxycycline can be used to regulate exogenous rhBMP-2 expression in vivo and promote bone healing a) In the prescence of Dox, no rhBMP-2 is produced, whereas, no Dox promotes rhBMP-2 expression in C9 cells. b-d) MicroCT was used to scan mouse forelimbs with bones and muscles intact. Three-dimensional reconstructions (b and c) and simulated x-ray projection images (d and e) show that in the absence of doxycycline, new bone is formed at the defect site.

Considerations When Devising a Gene Therapy Strategy for Bone

To treat any bone defect, the physician must consider the many factors: the nature of the defect, the condition of the soft tissue surrounding the wound site, and the overall health of the individual. Gene therapy may be a good choice for wound repair if the host tissue surrounding the wound is viable and healthy. When designing a gene therapy strategy, one must consider the target tissue or cells, the duration of gene expression required (Fig. 8), and the protein being expressed.

To achieve optimal bone healing, a clearer understanding of basic bone biology must be reached. Although, we know of many osteoinductive factors that can enhance the new bone formation, we most certainly do not have the entire bone microenvironmental network characterized. We must strive to identify signaling molecules other than the BMPs and TGF-β that contribute to osteogenesis. Additional in vitro and in vivo animal studies must be conducted to further identify gaps in basic knowledge before clinical trials in humans can be conducted. As we gain specific knowledge of the bone microenvironment and understand how the signaling molecules cooperate functionally to promote osteogenesis, gene therapy strategies can coevolve to derive the ideal bone tissue engineering therapy.

With continuing advances in gene technology, gene therapy will likely become increasingly important in healing both acute and chronic wounds/pathologies. As our understanding of the physiology of bone fracture repair and the role of the various repair regulators at the molecular level increases, this will ultimately accelerate the progress of gene therapy. In addition, the transfection efficiency and the safety of the delivery systems is expected to improve, providing a clinically feasible therapy.

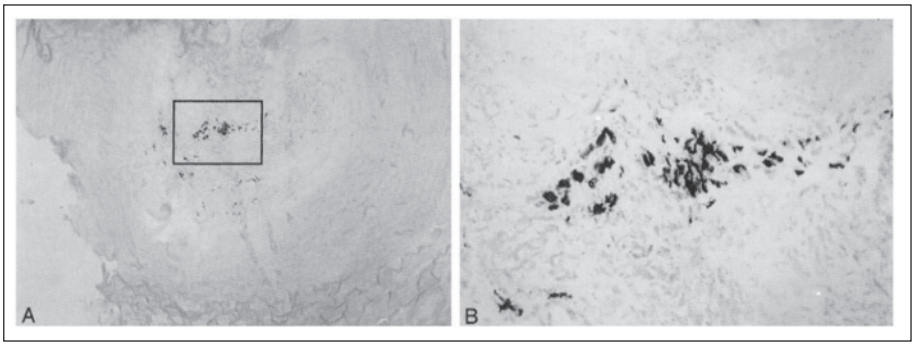

Figure 8. LacZ marker gene expression persists 12 weeks following delivery to intervertebral disc cells via adenoviral vector. A) 20x magnification of X-gal staining of intervertebral disc cells. B) 100x magnification of box denoted in (A).

In summary, newly developed comprehensive therapies based on biological understanding, using either recombinant proteins or their genes will enhance bone regeneration. The challenging task of tissue engineering bone is being tackled by many multidisciplinary research groups involving engineers, biologists, polymer chemists, and clinicians. This effort will yield to optimization of current therapies or the development of therapies that will enhance clinical treatment outcomes.

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Chapter 11

Cell Therapy for the Enhancement of Bone Repair

Hervé Petite and Rodolfo Quarto

Abstract

At variance with cartilage, bone displays an important potential of self- repair and regeneration. In vivo and in physiological conditions, this process occurs through strictly regulated sequences of cellular events. Successful replacement of bone through tissue engineering likely will be dependent on the recapitulation of this cascade of events. Bone regeneration in fact requires the cross-talk between microenvironmental factors and cells, where mesenchymal progenitor cells are recruited and properly guided by soluble signalling molecules and extracellular matrix. Tissue engineered bone repair have attempted to mimic this natural setting by delivering a source of cells capable of differentiating into osteoblasts, inductive growth factors, and bioresorbable scaffolding matrices to support cellular attachment, migration, proliferation and fate. Engineered tissue repair may in fact be viewed as the restoration of form and function to injured bone as well as to other tissues. The manipulation of these elements for therapeutic purposes might become common clinical practice.

The development of cell therapy approaches has dramatically improved in recent years as the understanding of musculoskeletal stem cell biology advances. Synthetic biomaterials developed by the industry are used to exploit skeleton's capacity to regenerate and repair. Sophisticated experimental designs have tried to combine two or more of these elements.

Many issues in this innovative field remain to be solved of course, but cell based therapies for the repair of relevant bone defects are not far from routine clinical feasibility. This chapter will attempt to highlight the development of cell based approaches for bone tissue engineering.

Introduction

Bone repair is possibly one of, if not the oldest challenge that the medical culture has been facing since the dawn of the history of medicine.[1,2] In particular, the reconstruction of large bone segments has always represented a major clinical issue, both for frequency and for difficulty of reconstruction. With the exception of the Ilizarov technique, all methods proposed for bone repair are based on a concept of "part substitution". The substituting "spare part" has to become vascularized and vital in order to be integrated in and substituted by the host tissue in order to heal the lesion. This is a difficult task to achieve when cortical bone grafts from tissue banks are used,[3-5] and represents a major problem even with autologous vascularized grafts.[6-8] Failure and complications are indeed very frequent,[9] besides the complexity of the surgery and the donor site morbidity in the case of autografts.[10-12] The use of bone allografts, which could solve a number of problems, raises further concerns about the possible transmission of infectious diseases and of immune responses by the host organism.[9,13,14] Because of all these concerns, orthopedic surgeons often adopt solutions that do not allow a complete functional recovery and in some cases they have to take drastic measures such as arthrodesis or limb amputation.

Engineered Bone, edited by Hervé Petite and Rodolfo Quarto.

Many of these issues could be solved by a Tissue Engineering approach to bone repair.[15,16] Stem cell science is advancing vigorously as well as biomaterial science.[17,18] The knowledge of soluble signalling molecules and transcription factors regulating the cellular events leading to osteogenic differentiation and finally to bone repair will soon lead to the understanding of the key mechanism involved.[19-21] Already experimental studies both preclinical and clinical shade light on the enormous potential of this new discipline in regenerative medicine.[22-25]

Here we review the impact of recent work in this interdisciplinary field on the treatment of skeletal lesions. We will present and discuss the cell based strategy for bone reconstruction. After introducing the "clinical scenario", we will present the major players in the game: osteoprogenitor cells and their potential sources, scaffolds and strategies for cell delivery as well as preclinical experimental models and clinical pilot studies.

Clinical Scenario

Bone lesions/defects occur in a wide variety of clinical situations, and their reconstruction to provide mechanical and functional integrity is a necessary step in the patient's rehabilitation. Moreover, a bone graft or a bone substitute is often required in maxillo-facial and orthopedic surgery to assist healing in the repair of osseous congenital deformities, or in the repair of defects due to trauma or to surgical excision after elimination of osseous disease processes exceeding a certain size.

Modern therapeutic approaches to repair large bone defects can be divided into two main groups: the first excluding (Ilizarov and bone transport) and the second including graft transplant (auto-, allo- and xeno- grafts; different biomaterial implants).

Osteotomy followed by bone distraction, the Ilizarov technique, lays on the bone regeneration potential. This apporach circumvents all problems related to bone grafts, but it is highly inconvenient to the patient. The relatively high rate of success obtained with this technique is counterbalanced by the long recovery time and the high rate of complications.[26,27]

Bone auto-grafts, both nonvascularized and vascularized, are considered at the moment the optimal choice. Presently vascularized grafts are the most widely used, being considered as the most successful. The success rate is high indeed, but complications, such as infections, non unions etc. are very frequent especially in large shaft reconstructions.[6-8] Furthermore, large reconstructions with autologous bone require large harvests of healthy tissue with important donor site morbidity.[10-12] Therefore this approach is limited by definition.

Many of the problems presented by the autografts, in particular the immediate availability of tissue for transplant, could be solved by allo- and possibly xeno-grafts. Host immune responses to and potential risk of infectious diseases transmission by bone allo- and xeno -grafts raise several concerns limiting their applications.

Novel materials, cellular transplantation and bioactive molecule delivery are being explored alone and in various combinations to address the problem of bone deficiency. The aim of these strategies is to exploit the body's natural ability to repair injured bone with new bone tissue, and later to remodel the newly formed bone in response to the local stresses it experiences. In general, the strategies discussed in this paper attempt to provide the reconstructed region with appropriate initial mechanical properties, encourage new bone to form in the region, and then gradually degrade to allow the newly formed bone to remodel and acquire the proper mechanical support function. Several of the concepts presented below are already finding initial clinical applications in early patient trials.

Sources of Osteoprogenitor Cells

Osteoprogenitor cells have been isolated from a variety of tissues, including periostium, bone marrow, spleen, thymus, skeletal muscle, adipose tissue, skin and retina.[28-39] Recently osteoprogenitors have also been reported to be isolated from peripheral blood, although with very low efficiency.[40] These findings shade new light into stem cell biology and cellular differentiation. However, their use for tissue engineering is not always straightforward. For this

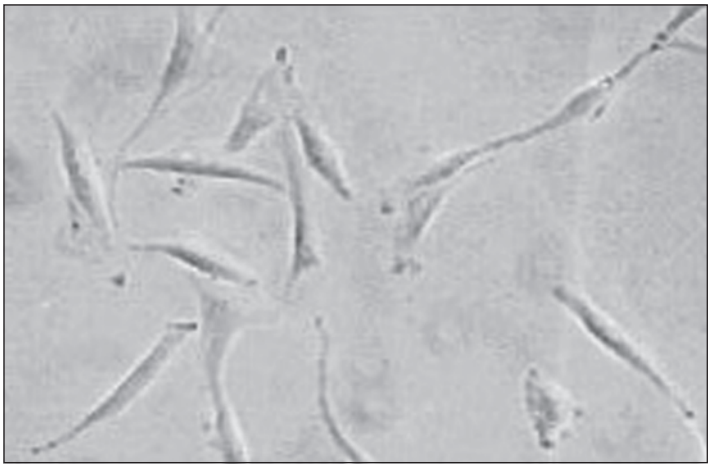

Figure 1. Phenotype of cultured human BMSC. BMSC cultured in the presence of FGF-2 maintain a stable fibroblastic phenotype.

purpose indeed a number of factors has to be considered: minimal invasivity, miminal donor site morbidity, easily accessible tissue source, high frequency of osteoprogenitors etc.

Marrow derived stromal fibroblasts (BMSC) (Fig. 1) can be isolated, expanded in culture, and stimulated to differentiate into bone, cartilage, muscle, marrow stroma, tendon, fat and a variety of other connective tissues[41] (Fig. 2). The first evidence for BMSC differentiation potential derives from transplantation studies in animal models performed by Friedenstein and coworkers.[39] The harvest of a limited bone marrow sample is a relatively easy and safe procedure. Very large numbers of BMSCs can be generated in culture from limited marrow samples, making possible to engineer constructs composed of these cells together with appropriate scaffolds which could be reintroduced into the in vivo setting. In order to obtain large number of osteoprogenitors for cell transplantation, culture conditions and the effects of growth factors on proliferation and differentiation of BMSC are of great interest and have been investigated by several groups.[42-49] BMSC represent, at the moment of writing this chapter, the most interesting and widely accepted experimental model for cell therapy. Furthermore, BMSC can be transduced with various viral vectors and are, thus, interesting potential candidates also for somatic gene therapy in local or systemic pathologies.[50-54] Very recently, a cell population with totipotent features has been isolated from the bone marrow.[55,56] These cells, termed MAPC (Marrow Adult Progenitor Cells) are able, even at clonal level, to generate meso-, endo- and ecto-dermal derived tissues when introduced into a murine blastocyste. They can be expanded for a very significant number of cell doublings without any sign of cell senescence.

The presence of osteo-chondrogenic progenitors in human skeletal muscle is suggested by the formation of ectopic bone in clinical and experimental conditions.[30,32,57-59] Recently the isolation and characterization of skeletal muscle derived cells with osteo-chondrogenic potential has been described.[30] These cells, in the early stages of culture, are highly positive for both osteoprogenitor cell and pericyte markers. Their putative identification as pericytes, perivascular cells with established osteogenic potential, suggests a cellular link between angiogenesis and bone formation in skeletal muscle and, perhaps, in embryo development. Muscle derived pericytes are easily cultured and expanded in vitro by routine techniques, they can therefore represent an alternative source of osteogenic progenitor cells for possible cell-based therapeutic use in certain conditions.[38] Still little is known on their general characteristics in vitro as well as their behavior in vivo, but for sure their source is not the easiest, their isolation is laborious and their frequency in skeletal muscle and in other tissues seems to be rather low.

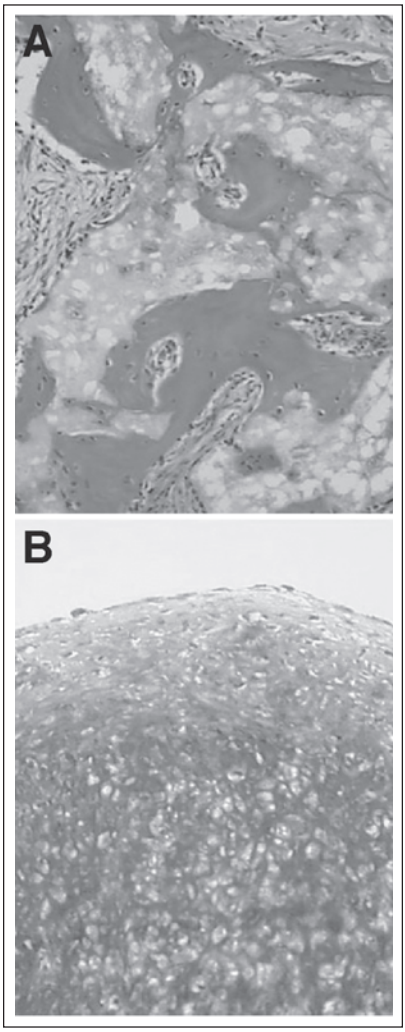

Figure 2. Cartilage and bone formation by human BMSC. The "in vivo" osteogenic potential (A) of BMSC and their "in vitro" chondrogenic potential (B) is evident in representative fields of histological sections.

An interesting potential source of osteoprogenitor cells is achievable in large quantities, under local anesthesia, with minimal discomfort.[29,31] Such a population of progenitor cells can be apparently isolated from human adipose tissue harvested by suction-assisted lipectomy (liposuction).[60] From this adipocyte-rich fraction, a fibroblast-like population of cells can be isolated, which can be maintained in vitro for extended periods with stable population doubling and, apparently, low levels of senescence. Immunofluorescence and flow cytometry show that the majority of these cells are of mesodermal or mesenchymal origin with low levels of contaminating pericytes, endothelial cells, and smooth muscle cells. Finally, they can differentiate in vitro into adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of lineage-specific induction factors.[31]

Some, if not all, of the problems raised by solid tissue osteoprogenitor cells could be solved harvesting cells with similar characteristics from peripheral blood. This of course would be the

easiest source of cells. Few reports, starting from the historical data by Luria and coworkers,[61] suggest that it is possible to isolate from peripheral blood a population of fibroblasts.[40,62] These peripheral blood fibrocytes would be in principle the population of cells reaching sites of tissue injury and contributing to connective scar tissue formation. They display a distinct cell surface phenotype (CD34-/CD45-/collagen I+/b1 integrin subunit), and are an abundant source of cytokines and growth factors that function to attract and activate inflammatory and connective tissue cells.[40]

In conclusion, cells potentially interesting for bone tissue engineering can be isolated from a variety of tissues. Still, the only cell system widely studied and used for both preclinical and clinical applications remains the one originally described by Friedenstein and will be therefore discussed in the following sections.[39] It would be intriguing to speculate that, given proper selection of subpopulations and specific culture conditions, either any tissue contains a totipotent stem cell compartment or committed cells can be induced to "dedifferentiate" up to the level of stem cell.

Scaffolds and the Design of the Delivery Vehicle

Scaffolds are the key factors required to start and direct the cascade of cellular events leading to bone repair. Delivering osteoprogenitor cells within a suitable tridimensional matrix is thus of critical importance for engineering bone tissue. Scaffolds in bone tissue engineering have to be considered as a crucial component of the experimental design; the scaffold is the element mimicking the extracellular matrix in a regenerating bone microenvironment. This concept implies that the scaffold is not only a simple inert delivery vehicle, but it has to be "informative" to the cells

The primary role of biomaterials in orthopaedic applications rests upon their osteoconductive properties coupled to the ability to integrate effectively with bone tissue.[63-69] Thus a proper biomaterial is supposed to easily integrate with the surrounding tissue and to allow new bone tissue ingrowth. But biomaterials have also to serve as delivery vehicle of cells and signaling molecules.[70,71] Their architecture has to be permissive for blood vessels to colonize even larger structures. Finally they should be biocompatible and resorbable. From this point of view, the new generation of bioceramics are indeed a undoubted candidate.[72,73] Bioceramics in fact mimics a preexisting bone surface and bone cells deposit new bone matrix on the ceramics surface.[66,74] Porous bioceramics (hydroxyapatite (HA), and tricalcium phosphate (TCP)) are osteoconductive, have a favorable bone affinity,[66,69,75-79] and are free from risks of rejection or infection.[66,67,75,78,80] Synthetic bioceramics are readily available, and can be used to custom design implants suited to individual applications. However, they do not represent per se a mechanically sound material,[79,81-83] and the performance of a theoretical ceramic implants depends on the local mechanical demands of the injuried bone, and on the efficient deposition and integration of new bone into the implant.

An important improvement in this field is represented by synthetic porous scaffolds (Fig. 3). In this case in fact the internal architecture can be intelligently designed and the density and the biomechanical properties of the material can be predetermined. The result is that the surface available for tissue regeneration as well as for cell delivery can be extremely high. The increase in surface can also have positive effects on scaffold resorbability. The alternative approach to synthetic ceramics is to use natural scaffolds which have an intrinsic structure highly compatible with tissue ingrowth. One such scaffolds is the coral exoskeleton which present a completely interconnected porous structure and good biomechanical and architectural properties.[84-87]

Because of their osteoconductive properties and their ability to "integrate" with bone tissue, HA and TCP bioceramics are perhaps the biomaterials most widely studied and used. The osteoconductivity and the ability to be invaded by blood vessels of porous bioceramics can be improved by manipulating the structural characteristics of the finished implant device.

As outlined already, bone tissue engineering strategies attempt to provide the injured segment with scaffolds of initially poor mechanical properties, highly permissive to new bone

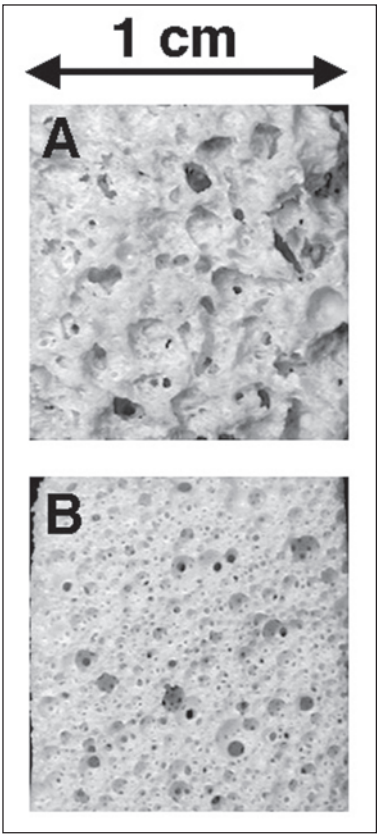

Figure 3. Bioceramics with different internal architecture. Different bioceramics with the same composition (100% HA) produced with different technologies display significant differnces in terms of density, average pore size, pore size distribution and level of interconnection.

ingrowth and blood vessels invasion. Scaffolds have eventually to be resorbed to allow the new bone to gradually remodel acquiring the required mechanical properties.

A significant improvement in this field has been represented by the association between porous bioceramics and BMSC, where the intrinsic properties of the bioceramics are integrated with the properties of the osteoprogenitor cells which allow the formation of vital, vascularised and biomechanically performing bone tissue.[22-24,88]

Experimental Studies

BMSC ability to form bone when locally transplanted in small animals was originally demonstrated in an ectopic model of bone formation by Friedenstein and coworkers.[39] Only several years later this ability of BMSC has been used to repair experimentally induced bone defects in small animals.[89,90]

Preclinical models have been developed in large size animals to verify the feasibility of this cell therapy approach in larger scale lesions.[22-24,88] Interestingly in all these studies the basic conceptual approach was similar, although the animal model was different. Marrow samples were harvested from iliac crests of each animal and the osteoprogenitors were isolated and expanded in culture. After an appropriate time interval and a number of controls on the cell cultures, the surgical procedure was initiated. A large segmental defect was surgically created in

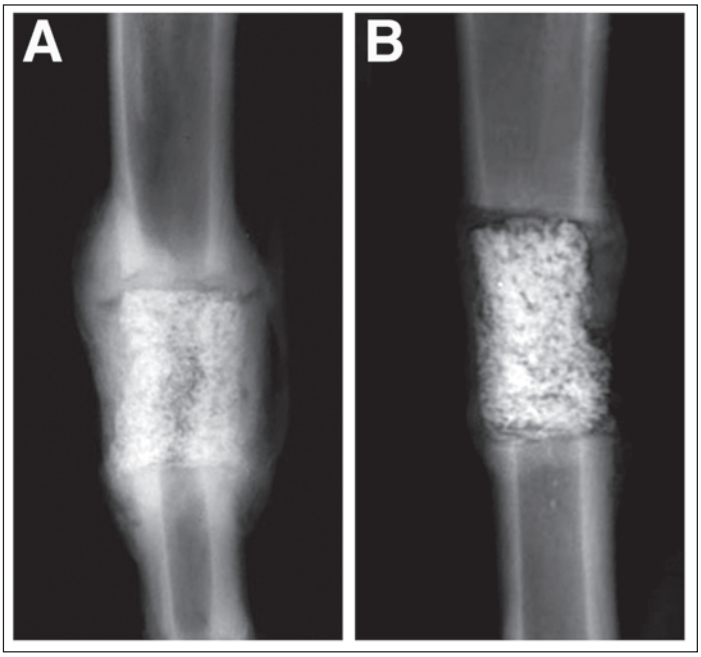

Figure 4. Radiograms of cell loaded bioceramic cylinders and control implanted in the animal tibia. A) BMSC loaded porous HA; B) cell free control HA. Radiograms were taken 2 month after transplantation in the sheep tibia model. Union was established rapidly at the bone/HA interface. Callus formation was more extensive in BMSC loaded samples.

a long bone. The surgical defect was always of critical size (i.e., it would not heal by itself). The removed bone segment was substituted with a cylinder of porous bioceramic loaded with autologous "in vitro" expanded osteogenic progenitors. A fixation device, either internal or external, was used to stabilize the bone segment and discharge the mechanical load. Animals were monitored by non invasive analysis at regular time intervals. In genera, lesion repair proceeded quite rapidly. After sacrifice, specimens were retrieved and histological analysis performed. Integration host bone/implant was excellent. The amount of bone observed was significantly higher in the scaffolds loaded with osteoprogenitors than in controls where only the bioceramic scaffold had been implanted. Both woven and lamellar bone had filled most of the pores of the implants loaded with osteogenic cells. As tested in an indentation assay, the stiffness of the composite bioceramic-bone was found to be higher in cell-loaded implants compared to bioceramic only control implants[23] (Fig. 4).

It is worth mentioning that these studies differed significantly for what was concerning the animal model (dog, sheep), the anatomical segment (femur, tibia), the nature (synthetic, natural) the chemical composition, the geometry and the resorbability of the biomaterial used.[22-24,88] Still the results were in good agreement suggesting an important advantage in bone formation and therefore in the healing of the segmental defect when marrow stroma derived osteoprogenitors were delivered together with the bioceramic scaffolds.

Clinical Studies

It is surprising that after the initial enthusiasm demonstrated by the flourishing of large animal studies, whose results were all substantially in good agreement and very encouraging, only one clinical pilot study has been performed.[25] Recently, Quarto and coworkers have transposed the cell-based tissue engineering approach verified in large animals, in the clinical setting

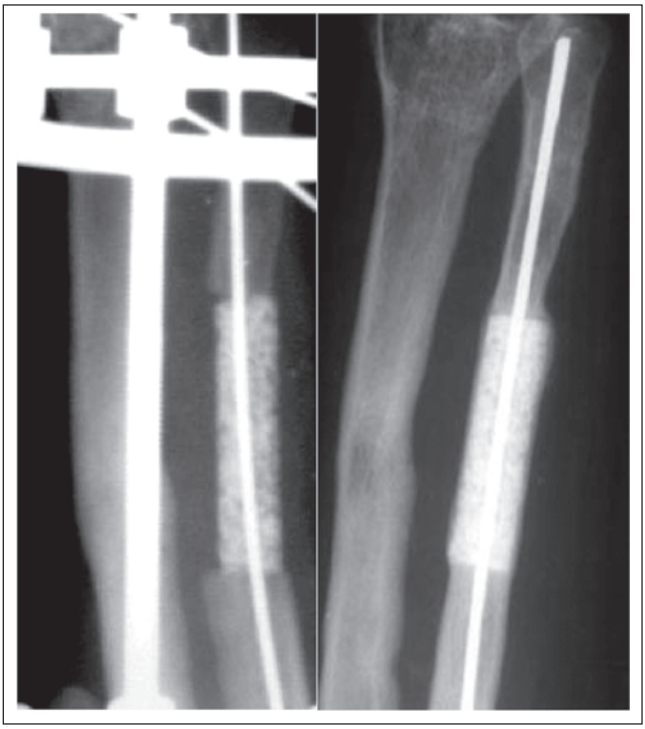

Figure 5. Radiograms of ulna treated with autologous BMSC loaded bioceramic cylinder. Bioceramic loaded with autologous BMSC were used to treat a 16 year-old girl presenting a 4.0 cm traumatic bone loss at the distal diaphysis of the ulna. Radiograms were taken 8 months after treatment. Radiogram reveals a good callus formation along the implant and a good integration at the interface with the host bone.

to treat patients with substantial (4 to 7 cm) long bone defects.[25] In principle the study was organized to directly transfer the information gained from the animal study in the clinics. The patients selected for this clinical study had experienced previous traditional surgical treaments with negative results and no therapeutic alternatives were envisaged. Autologous osteoprogenitors were isolated from the iliac crest bone marrow aspirates of each individual patient and expanded "ex vivo". As soon as the appropriate number of cells was obtained in culture, osteprogenitor cells were harvested and delivered in vivo vehicled by highly porous hydroxylapatite scaffolds. External fixation was initially provided for mechanical stability. An abundant callus formation along the entire implant and a good integration at the interface with the host bone was observed as soon as by the second month after implantation. All patients recovered limb function between 6 and 15 months (Fig. 5). Using a traditional approach, the expected recovery time would have been much longer, under the most favorable conditions and in the absence of complications.

Conclusions

In this chapter we have presented an overview of the cell based strategy for the treatment of skeletal lesions. We have seen the potential sources of osteoprogenitor cells and the basic elements required from the scaffolds for cell delivery. Experimental preclinical models and clinical pilot studies using this approach are providing results of high interest and very promising. Still the results are far from being considered optimal. Several problems have to be solved before considering cell therapy a real alternative in the clinical setting.

Cell cultures should be highly standardized and culture media and each component used should be certified for this kind of use. Which cell population will perform better and which culture technology (traditional plastic, bioreactors etc.) will provide us with the best cell preparation at the lowest cost and at the highest qualitative standards are the points that cell biologists and biotechnologists will have to solve in the next future. Convenient quality criteria and controls will have to be established. Scaffold also will have to evolve to meet the requirements of the clinics; future biomaterials will have possibly to have geometry specifically designed for hosting cell and allow their survival and a better vascularisation once delivered at the implant site in vivo. Scaffolds will have to improve in order to have resorption rates adequate to the specific application and possibly to became at least at some extent more workable and allow some movement (flexion, torsion, compression etc.).

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Chapter 12

Repair of Osteochondral Lesions

Ivan Martin, Dirk Schaefer and Beatrice Dozin

Abstract

Trauma and disease of joints frequently involve structural damage to the articular cartilage surface and the underlying subchondral bone. These pathologies result in severe pain and disability for millions of people world-wide and represent a major challenge for the orthopaedic community. Albeit a series of therapeutic approaches has been developed to treat osteochondral defects, none of them has proved yet to ensure long-lasting regeneration. The difficulties encountered mostly rely on the intrinsic biological, biochemical and biomechanical properties of the articular cartilage. An accurate knowledge of these properties is mandatory to improve current therapies and/or establish innovative procedures. This chapter will (i) describe some of the structural and functional properties of articular cartilage; (ii) critically present the current procedures to repair cartilage; (iii) introduce a variety of novel strategies currently under investigation for cartilage repair and (iv) highlight some of the main challenges to be addressed in order to improve the clinical treatment of osteochondral lesions.

Introduction

Structure and Function of Articular Cartilage

The structural organisation of articular cartilage in adult organisms reveals a rather simple architecture, being composed of one single type of highly specialised cell, the chondrocyte, embedded within a dense extracellular matrix (for a review, see ref. 1). The chondrocytes contribute little to the volume of the tissue, 1-2% in human articular cartilage. In adulthood, chondrocytes generally do not divide any longer and are aimed at maintaining the integrity of the articular surface through balanced synthetic and catabolic activities. The matrix itself is composed of (i) fluid, mostly water (75-80% of the wet weight), gases, metabolites and cations, and (ii) a framework of macromolecules that include mainly type II collagen (50-73% of the dry weight), proteoglycans (15-30% of the dry weight), other fibrillar (IX and XI) and nonfibrillar (type VI and X) collagens and additional noncollagenous molecules. A variety of interactions bridge the individual macromolecules, in particular noncovalent associations between proteoglycans and collagens and covalent bonds between different collagen species.

The framework of macromolecules mostly gives the tissue its form and stability. Water and proteoglycans are dispersed through the collagen framework as a soluble gel, making the matrix biphasic. Due to their hydrophilic nature, proteoglycans mainly confer resistance to compression, by attracting a large amount of water into the intramolecular and intermolecular space. Collagen fibrils withstand the swelling forces of the water-proteoglycan phase and provide the matrix with high tensile strength. Thus articular cartilage, although it is only a few millimeters thick, ensures to the joint smooth gliding motion, load transmission, force distribution and minimised peak focal stress on the subchondral bone.[2] The functional role of noncollagenous proteins, comprising anchorinCII, fibronectin, tenascin, fibromodulin and cartilage oligomeric protein, remains as of yet largely unknown.

Engineered Bone, edited by Hervé Petite and Rodolfo Quarto.

Throughout the tissue, both cells and matrix present a highly organised distribution within successive layers known as the superficial, transitional, radial and calcified zones. Chondrocytes within each layer are characterised by distinct size, shape and space orientation. Also collagen fibers follow a rigorous structural pattern, forming a network of parallel, arcade-like and perpendicular fibers from superficial to deeper zones. Such structural organisation both at the cellular and molecular levels is indicative of specialised functions.

Focal Lesions and Natural Repair of Articular Cartilage

Traumatic cartilage lesions affect mostly young subjects often involved in sustained sport activities. During these traumas, shear forces lead to a separation of cartilage between the radial and the calcified layers.[3] Focal defects are classified as either chondral or osteochondral. *Chondral lesions* lie entirely within the cartilage and do not penetrate beyond the calcified zone into the subchondral bone. The vasculature being unreached, these lesions do not heal spontaneously and are analogous to those observed during the early phases of osteoarthritis.[4,5] Osteochondral defects penetrate the articular surface in depth through the vascularised subchondral bone. Some spontaneous attempts of repair can be observed as mesenchymal chondroprogenitor stem cells invade the lesion, differentiate into chondrocytes and form cartilage.[6] However, repair of full-thickness defects ends up to be only transient: the repair tissue is fibrous in nature, does not have the functional properties of native hyaline cartilage, and thus results more vulnerable to the action of free radicals, metalloproteinases and catabolic cytokines including IL-1 and TNF-α.[7,8] Matrix breakdown often leads to evident clefts and fissures on the articular surface and subsequently to the onset of degenerative joint diseases such as osteoarthritis.[9]

It is thus clear that articular cartilage has a limited capacity for self-regeneration after injury. Intrinsic biological features of the tissue itself are the limiting factors:

1. the mature chondrocytes have a relatively low metabolic activity and proliferation capacity and the abundant extracellular matrix may provide a barrier for their migration to the defect;
2. articular cartilage being neither vascularised nor penetrated by lymphatic vessels, there is no direct access to progenitor cells; furthermore, the supply of growth and differentiation promoting factors is ensured only by diffusion from the synovial fluid;
3. some of the proteoglycans of the matrix have anti-adherent properties and may prevent cell adhesion, further undermining any tentative repair process;[10] herein may also lie the difficulty of achieving a good integration between repair tissue and native cartilage;
4. finally, articular cartilage is not innervated and thus insensitive to early injuries that can readily progress to degeneration much before evident clinical symptoms emerge.

Total joint replacement using artificial prostheses generally eliminates pain and partially restores functionality, but is limited by the durability of the device. Thus, particularly for young individuals, it is mandatory to identify alternative procedures to permanently repair cartilage injuries or at least to delay the implantation of an artificial joint replacement. The complexity of articular cartilage structure and the peculiarity of its biological/biophysical features explain the large repertoire of most diversified therapeutic approaches that have been developed and are being investigated to regenerate osteochondral lesions and restore joint functionality. In the following sections, the reader is provided with an overview of these techniques.

Current Clinical Treatments of Osteochondral Lesions

Microfracturing

Microfracturing is the most widely used cartilage repair technique, with more than 76,000 procedures reported in 1999 in the U.S. only.[11] The procedure, which is generally performed arthroscopically, consists in perforating the subchondral plate of (osteo)chondral defects by multiple holes, thus allowing marrow-derived mesenchymal progenitor cells to reach the lesion and form new cartilaginous matrix[12] (Fig. 1A). The popularity of the technique derives from the low morbidity and an overall clinical improvement of symptoms. However, the tissue generated after microfracturing was shown to lack the structure, composition, mechanical

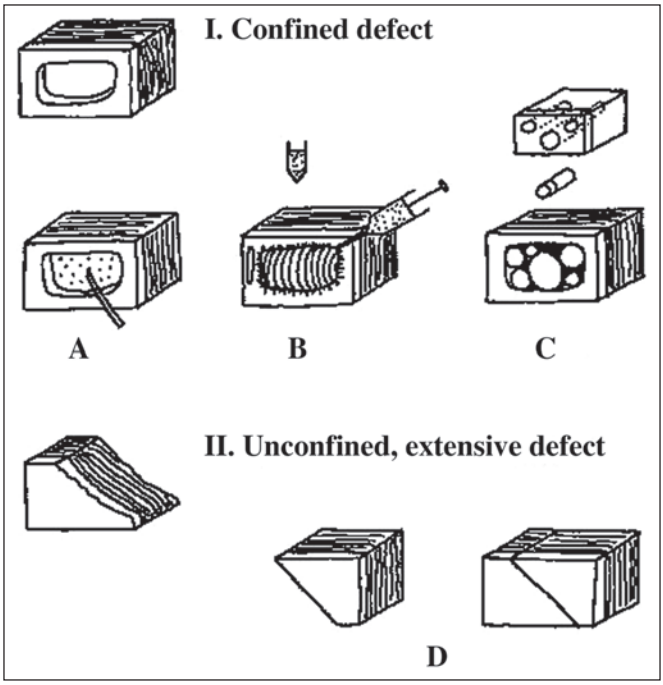

Figure 1. Current cartilage repair techniques for the treatment of a confined cartilage defect include perforation of the subchondral bone (A, microfracturing), injection of autologous chondrocytes under a periosteal flap (B, autologous chondrocyte implantation) or transfer of osteochondral cylinders from a minor weight bearing area of the joint (C, mosaicplasty). Extensive, unconfined joint defects may be treated by transfer of fresh osteochondral allografts (D).

properties, and in most instances the durability of articular cartilage.[13] Thus, despite evidence that microfracturing stimulates the formation of fibrocartilaginous repair tissue, the clinical value of this approach remains uncertain and the indications for its use are not objectively identified.

Autografts and Allografts

Autologous Periosteal Transfer

The technique consists in harvesting autologous periosteal tissue from the medial proximal tibia and suturing it into the defect, thereby introducing a new cell population along with an organic matrix (Fig. 1B).[14] Animal experiments and clinical experience have shown that periosteal grafts placed in articular cartilage defects can produce new cartilaginous tissue. However, increasing patient age adversely affected the results[13] and some groups reported not totally satisfactory outcomes.[15] The lack of consensus on the optimal orientation of the periosteum (e.g., with the cambium layer facing the bone[14] or the joint[16]) is an indication of uncertain clinical performance.

Autologous Osteochondral Transfer

The technique, also called mosaicplasty, consists in harvesting osteochondral cylinders from a joint area of minor load and press-fitting them into predrilled holes at the defect site[17] (Fig. 1C). Based on clinical improvement and, in some cases, graft integration, it was concluded that small symptomatic osteochondral or cystic lesions are appropriate indications for this

technique.[18] However, due to the limited availability of donor tissue and the induced morbidity at the donor site, the recommended size limit of the damaged area is only about 2 cm^2.

Fresh Osteochondral Allograft

The technique, used in cases of unconfined, extensive cartilage defects, consists in transplantation of osteochondral tissue from compatible donors (Fig. 1D). Clinical results indicate successful treatment of large unipolar osteochondral defects, although an unloading period of 1 year after surgery is required.[19] The procedure strongly depends on the access to osteochondral grafts from a sufficient number of donors, in order to minimise size mismatch between donor and recipient, and is therefore limited to few specialised centers.

Autologous Chondrocyte Implantation

Autologous chondrocyte implantation (ACI) consists in harvesting healthy articular cartilage from a minor weight bearing area of the knee joint. Chondrocytes are enzymatically isolated, expanded in monolayer culture and then reinjected under an autologous periosteal flap, which covers the cartilage defect (Fig. 1B). The results of a pilot clinical study[20] were judged as good to excellent in 14/16 patients with isolated femoral condyle lesions (up to 6.5 cm^2 in size). As a consequence, patients began to request this new technique and several companies started offering the service of in vitro expansion of autologous chondrocytes. Peterson et al[21] reported the clinical, arthroscopic and histological results obtained for the first 101 patients that were treated with ACI. Reported outcomes suggest some level of repair and improved symptoms (good to excellent results for 92% of isolated lesions and for 67% of multiple lesions), although the lack of comparative groups and of validated assessment methods is a noted weakness.

The accumulation of clinical experience is in contrast with the understanding of the principles of the repair process. Only recently, biopsy samples from the graft site, harvested up to 30 months after surgery, indicated an initial formation of fibrocartilaginous tissue, which progressively remodelled and where newly synthesised type II collagen accumulated.[22] However, biopsies were only taken in the center of the repaired defect, and therefore critical evaluation of graft integration could not be made.

From a surgical standpoint, the main limitation of ACI is related to the mechanism of graft fixation. It was shown that the periosteal flap delaminates regardless of the postoperative rehabilitation protocol, and therefore the control over the fate of the injected cells is poor.[23] This problem becomes even more relevant when large osteochondral defects are to be treated. In order to improve ACI, some industrial groups have introduced cell delivery systems based on membranes (e.g., Verigen) or three-dimensional scaffolds (e.g., Fidia Advanced Biopolymer). The surgical procedure results easier and provides a higher level of control over graft fixation; however, the clinical outcome of such procedures has yet to be properly assessed.

Novel Tissue Engineering Strategies

Stimulation of Repair by Bioactive Factors

The integrity of articular cartilage relies on a fine-tuned local release of hormones, growth factors and cytokines produced by chondrocytes themselves. By interacting with each other, these factors control important chondrocyte functions, including cell division, matrix synthesis and degradation. Since cartilage is not vascularised, the autocrine/paracrine network is the main, if not the only, warrant of the tissue homeostasis. When articular cartilage is injured, this equilibrium becomes unbalanced. Chondrocytes respond to injury by proliferating and increasing the synthesis of matrix molecules at the edge of the lesion. However, this response is transient and very soon ceases possibly due to a lack of sustained supply of factors.[9]

Molecular therapy, by contrast to the cell-based therapy already used in clinic, is a new emerging field to repair cartilage damage. The introduction of functional proteins (e.g., growth factors, signalling molecules, transcription factors and other effectors) at the site of the defect

for a defined period of time may provide a critical initial input to expedite regeneration processes. Success of the approach will depend on the choice both of the factor(s) to administer and the way of release and delivery.

Selection of Factors

Coordinated in vivo and in vitro experiments may guide the selection of the effector(s) that should induce cell proliferation and/or differentiation. Those retaining major attention are listed here below.

Growth factors of the TGF-β superfamily are known to participate in endochondral bone formation and fracture callus healing processes. BMPs are important signalling molecules inducing limb bud development during embryogenesis,[24] ectopical bone formation[25] and differentiation of mesenchymal progenitor cells into osteoblasts and chondrocytes.[26-29] TGF-β1 has emerged as one of the key factors promoting synthesis of type II collagen and aggrecan core protein and down-regulating metalloproteinases.[30-32]

FGF-2 is regarded to date as the most potent mitogen for chondrocytes. By contrast, this factor may have opposite effects on matrix deposition and accumulation, since it promotes both anabolic and catabolic activities.[33,34] Therefore, FGF-2 could be delivered only in early phases of tissue repair.

Insulin and insulin-like growth factors (IGF-I and IGF-II) all modulate cartilage matrix production. Insulin alone is much less potent than IGF-I in stimulating collagen synthesis but strongly enhances proteoglycan deposition. IGF-I maintains chondrocyte metabolism in cartilage homeostasis, stimulates chondrocyte synthetic and mitotic activities and inhibits chondrocyte-mediated matrix catabolism. Also IGF-II can stimulate proteoglycan synthesis but, like insulin, is much less effective than IGF-I.[35,36]

EGF alone has no effect on chondrocyte proliferation. Together with insulin, EGF synergistically stimulates proteoglycan synthesis and induces chondrocyte proliferation.[37] PDGF also enhances proteoglycan deposition but to a lower extent as compared to the other described factors.[38]

Direct or Matrix-Based Delivery of Factors

Attempts of local molecular treatment have been performed in rodent models using TGF-β1 and FGF-2, administered either by sequential injections or constant release through osmotic pumps. Both delivery systems resulted in successful regeneration of articular cartilage and subchondral bone.[39,40] However, other authors reported adverse effects secondary to local administration, including inflammatory joint response, synovial hyperplasia, proteoglycan loss, cartilage destruction and osteophyte formation.[41,42]

In the past few years, several matrix-based delivery systems have been developed and tested for cartilage repair. The use of a matrix would help not only stabilising the factors and controlling their delivery, but also providing a structural template filling the osteochondral lesion. The ideal matrix should meet essential requirements as being nonimmunogenic, nontoxic, biocompatible, biodegradable and easily manufactured. Issues related to biocompatibility, biological response and immunotoxicity evaluation of release systems have been reviewed by Anderson and Langone.[43] Here we describe some examples of delivery systems based on matrices made of naturally occurring or synthetic polymers.

Use of Matrices Made of Naturally Occurring Polymers

Collagen is a physiological substance, which can be prepared in solution or shaped in membrane film, thread or sponge. Although derived from xenogeneic sources (mostly porcine skin), purification techniques can now eliminate the immunogenic telopeptides that used to prompt foreign body responses to implantation. In such, collagen sponges impregnated with osteogenic proteins from demineralized bone matrix[44] or BMP-2[45] were prone to induce ectopic cartilage formation or improve healing of full-thickness articular defects.

Fibrin, currently utilised in clinic as fibrin glue, is a natural polymer that forms during blood coagulation and plays an important role in hemostasis and wound healing. Heparin-containing fibrin hydrogels have been reported to slowly and regularly deliver growth factors presenting heparin binding affinity, including FGF-2.[46]

Alginates are negatively charged unsulfated copolymers of L-glucuronic and D- mannuronic acids extracted from brown algae. They cross-link in the presence of multivalent cations (mostly Ca^{++}) to form an open-lattice hydrogel. The large average pore size of the mesh allows easy diffusion of macromolecules. The relatively mild gelation process of cross-linking has enabled not only biological proteins but also cells and DNA to be incorporated into alginate matrices without significant loss of full biological activity.[47]

Hyaluronic acid has unique physicochemical properties and distinctive biological functions. It binds specifically to proteins in the extracellular matrix and on the cell surface, thereby playing a role in cartilage matrix stabilisation[48] and cell mobility.[49] Partially esterified hyaluronic acid has been recognised as particularly suitable for controlled peptide release or protein delivery.[50] For instance, when loaded with BMP-2 in synergy with IGF-I, cross-linked high molecular weight hyaluronic acid was reported to promote excellent cell infiltration and cartilage formation.[51]

Use of Matrices Made of Synthetic Polymers

The use of synthetic polymers introduces high flexibility in the modulation of matrix physicochemical properties, and thus of their delivery kinetics for specific bioactive factors. Novel polymer processing techniques also allow enhanced maintenance of the biological functionality of the incorporated factors.[52,53]

Most synthetic matrices currently used for delivery of bioactive factors are made of polylactic acid or polyglycolic acid homo- or heteropolymers. An example of such a synthetic matrix used for delivery of factors in cartilage repair is a 50:50 poly-DL-lactide-coglycolide scaffold loaded with TGFβ1 and implanted in 7-mm osteochondral defects in goats. The study showed the potential for TGFβ1 delivered by biodegradable scaffolds to improve the quality of cartilage repair.[54]

Since most proteins have free aminoacid groups in their sequences, they can be covalently linked to a polyethylene glycol (PEG) hydrogel network. This covalent attachment provides a means to achieve long-term controlled release of bioactive molecules. Upon hydrolysis, PEG hydrogels degrade into low molecular weight PEG derivatives, which can be easily cleared by the body.[55,56] Alternatively, the PEG network can be used to encapsulate microspheres containing bioactive factors. Indeed, microspheres containing IGF-I and TGFβ1 photoencapsulated with bovine chondrocytes in PEG-based hydrogels promoted increased production of glycosaminoglycans (GAG).[57]

Advances in polymer science have recently led to the development of vehicles capable of delivering multiple factors with distinct kinetics.[58] The method, although currently limited to the delivery of only two factors and not specifically designed for the repair of cartilage lesions, addresses the important issue of releasing different factors at different temporal stages, which is a key for a successful enhancement of tissue regeneration.

Delivery of Factors by Gene Transfer

Bioactive molecules usually have a relatively short half-life. When they are administered directly in the joint, the maintenance of effective concentrations necessitates either very high initial doses or repeated treatment. The approach of gene transfer may circumvent these limitations, providing an enhanced and long-lasting production of bio-effectors. Thus, the introduction of appropriate genes directly at the site of injury could possibly initiate the healing response, accelerate the healing process and improve the quality of the repair tissue.

The feasibility of gene transfer in cartilage repair has been so far investigated mostly in the context of rheumatoid arthritis and osteoarthritis. In a number of animal models of inflammatory arthritis, the delivery of anti-inflammatory agents has shown efficacy in ameliorating various joint pathological symptoms.[59-61]

The delivery of the transgenes, reviewed by Evans,[62] can be either systemic or local. Systemic delivery is performed by direct intraperitoneal or intravenous injection. However, systemic gene transfer with viral vectors often results in high but transient serum levels of the expressed proteins, an approach that is not likely to produce significant clinical benefit. Moreover, host response to viral components and diffuse expression of immuno-regulatory molecules can lead to tissue inflammation, widespread immunosuppression and toxicity when high vector concentrations are used.[63,64] By contrast, the alternative local delivery within the synovial lining of the joint would lead to the accumulation of the factor(s) in and around articular tissues where they are needed the most. Genes can be locally transferred by two means: either they are delivered directly to the target site (in vivo approach) or selected cells are harvested from the joint, expanded, genetically manipulated and reimplanted at the injury site (ex vivo approach). Although both protocols have given encouraging results,[65-68] some concerns cannot be denied: when using viral vectors, acute inflammation can be revealed at the site of injection, in particular with adenoviruses that are highly immunogenic; moreover, inadvertent spread of vector into nonarticular tissues like lung, liver and spleen has been reported.[69] Ex vivo gene therapy has the advantage that a defined population of cells is genetically modulated and that the effects and dosage for optimal proliferation and/or differentiation can be controlled. However, the approach, while rather effective, is laborious and expensive, and thus less appealing for a widespread scale application. Repair strategies aiming at inducing osteochondral lesion repair by delivery of molecules have a great commercial potential, since they could lead to the development of off-the-shelf products (e.g., "intelligent" matrices) readily available for clinical use. However, in order to reach this goal in a safe way, further knowledge in the long-term properties, mode of action and interactions of growth/differentiation factors, as well as the development of safe DNA vectors, is required. Therefore, in the short-medium term we foresee that the cellular approach will meet broader consent for clinical use. In the following paragraphs, we review some of the issues that are likely to enhance osteochondral repair by cell-based approaches.

Identification of Appropriate Cell Types

For all cartilage repair methods based on autologous cells, a critical issue is related to the procurement of chondrogenic cells. In fact, a cartilage biopsy in the joint, necessary for autologous chondrocyte preparation, represents an additional injury to the cartilage surface, and has been reported to be detrimental to the surrounding healthy articular cartilage.[70] In addition, this approach is limited by the availability of cells, particularly in elderly individuals, and by their capacity to proliferate and to differentiate after expansion in monolayers. Several alternatives have been therefore proposed, either to use more efficiently a biopsy of articular cartilage or to use different cell sources, including chondrocytes from nonarticular cartilages or mesenchymal progenitor cells.

Mature Chondrocytes

It is long known that human articular chondrocytes can undergo only a limited number of cell divisions in vitro and that their proliferative potential decreases with age.[71] In addition, the redifferentiation of expanded human chondrocytes was reported to be negligible,[72] to decrease with serial passaging,[73] to fully develop only in long-term cultures,[74,75] and to be highly dependent on the lot of serum used.[76] The use of specific growth factors (e.g., FGF-2, TGFβ1, PDGFbb) during articular chondrocyte expansion was recently shown not only to increase the cell proliferation rate, but also to enhance the ability of the cells to redifferentiate upon transfer into a 3D environment (Fig. 2) and to respond to differentiating agents.[77,78] Human articular chondrocytes were also seen to maintain a higher chondrogenic commitment, as indicated by the expression of the transcription factor SOX-9, by using a defined serum-free medium during cell expansion.[79] At present, however, there is no comparative animal or clinical study reported in the literature concerning the use of articular chondrocytes expanded under conditions favouring cell proliferation and maintenance of chondrogenic ability.

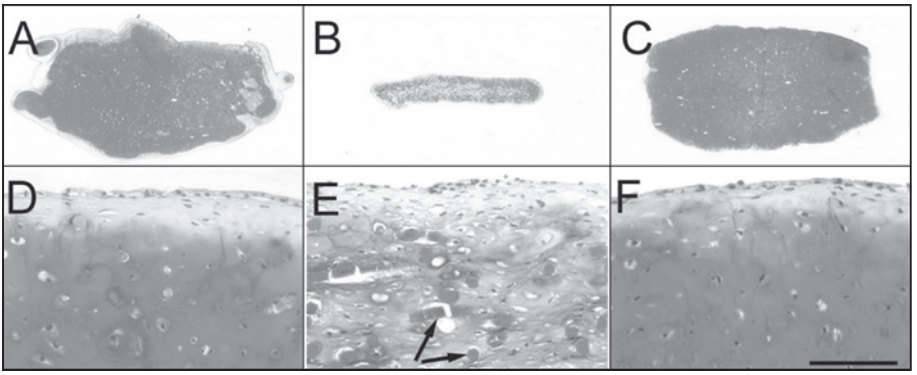

Figure 2. Cartilaginous tissues engineered using polyglycolic acid nonwoven meshes and bovine chondrocytes, either freshly harvested (A, D) or expanded in monolayers without (B, E) or with (C, F) FGF-2. Medium supplementation with FGF-2 not only increased chondrocyte proliferation rate, but also maintained in chondrocytes a higher capacity to generate cartilaginous tissues, similar to that of freshly harvested cells.[77] We recently demonstrated that also human chondrocytes may be expanded while maintaining their ability to redifferentiate, although in this case multiple growth factors are necessary.[78,79] Glycosaminoglycans are stained red by Safranin-O. Arrows indicate undegraded polymer fibers after 4 week culture. Scale bar = 2 mm (A-C) or 0.2 mm (D-F).

Differentiated chondrocytes can also be obtained by enzymatic digestion of nonarticular, hyaline cartilage tissues. For example, biopsies of nasal or rib cartilage can be removed under local anaesthetic and by a less invasive procedure than removing tissue from specific areas of the joint. Furthermore, since the donor site is not subjected to compressive forces, there is a minimal risk of morbidity at the biopsy site. It was recently shown that as compared to articular chondrocytes, human chondrocytes from the nasal septum proliferate approximately 4 times faster and have a higher capacity to generate a cartilaginous tissue after monolayer expansion.[80] However, extensive data from in vivo experimental studies will be needed to demonstrate the efficacy of nasal chondrocytes at articular sites.

Mesenchymal Progenitor Cells

An alternative to the use of differentiated chondrocytes is the use of cells with chondrogenic differentiation capacity. Mesenchymal progenitor cells with a chondrogenic potential have been isolated from a variety of tissues, including periosteum,[81] bone marrow[82] and synovial membrane.[83] Potential advantages of using mesenchymal progenitor cells as compared to differentiated chondrocytes include: (i) higher proliferative capacity, even from older individuals; (ii) higher responsiveness to growth factors and signalling molecules; and (iii) ability to differentiate into chondrocytes or osteoblasts according to the local environment. This latter possibility would allow the use of the same cell graft to repair both the articular cartilage and the subchondral bone.

There have been a few experimental studies on the repair of focal articular lesions using mesenchymal progenitor cells from bone marrow[84-86] and from periosteal/perichondrial tissues.[87,88] One of the earliest study[84] highlighted the possibility that the cartilage regenerated in osteochondral defects by marrow-derived mesenchymal progenitor cells could get thinner and fibrillated after prolonged time, consistent with the known characteristic of these cells to terminally differentiate in vitro into hyperthrophic chondrocytes. In general, the data reported are qualitative and no study has shown yet either consistent results or the generation of a functional tissue comparable to native hyaline cartilage. In addition, no report has been published so far regarding the clinical use of mesenchymal progenitor cells from any tissue source for articular cartilage repair. Thus, to date these cells have been reported to remain an unrealised promise for effective regeneration of articular cartilage lesions.[89]

Engineering Functional Cartilage Grafts

As compared to cells injected under a periosteal flap or immobilised in a hydrated gel, preformed and functional cartilaginous constructs may result less vulnerable to environmental factors and mechanical forces upon implantation.[90] A mechanically functional cartilage graft would reduce mechanical discontinuities on the articular surface and allow early post-operative function under physiological loading conditions, a key factor for a successful rehabilitation. In this regard, the use of appropriate scaffolds and of tissue culture bioreactors is central to guide chondrogenic cells at forming functional cartilage-like tissues of predefined size and shape and with biomechanical characteristics approaching those of native cartilage.

Three-Dimensional Scaffolds

Scaffolds used in tissue engineering of articular cartilage should display biological and physical properties matching both the needs of differentiating chondrocytes in vitro and of regenerating cartilage in vivo.[91] Thus, the porosity of the scaffold should allow the exchange of nutrients and waste products, while providing a sufficient physical support for cells and extracellular matrix. The degradation rate of the scaffold should be tuned on the rate of production of extracellular matrix by chondrocytes, considering that the physical environment to which the scaffold is exposed in vivo may have an effect on its degradation. In addition, the mechanical characteristics of the scaffold should be such that at the time of implantation the cell-scaffold construct will display biomechanical properties compatible with those of the joint.

Different research groups have used a wide variety of scaffolds in the attempt to generate cartilaginous tissues in vitro. The form and composition of these scaffolds range from non-woven meshes and foams of alpha-hydroxypolyesters,[52,92-95] polyglactin[96] or hyaluronan alkyl esters[97] to photo-crosslinked hydrogels[57,98] and sponges based on different types of collagen and glycosaminoglycans.[99,100] Composites consisting of a 3D scaffold filled with cells embedded in a fibrin or alginate gel have also been explored, in order to combine the advantages of a predefined shape support and of media favouring maintenance of a rounded cell morphology.[96,101]

Overall, the developed scaffolds have been tested with chondrogenic cells (mostly with nonexpanded animal chondrocytes) and reported to support the formation of cartilage-like structures in vitro. However, fundamental questions related to understanding (i) the interactions between cells and specific substrates, (ii) the influence of the pore size distribution on cell behaviour, and (iii) the effect of scaffold geometry (i.e., in the form of a foam, mesh or gel) on the induction/maintenance of the chondrocytic phenotype are yet to be answered. Efforts in this direction will be needed to identify objective criteria for the selection of scaffolds for the regeneration of osteochondral lesions.

Tissue Culture Bioreactors

Engineering functional cartilage tissue may be enhanced by the use of bioreactors that provide a controlled in vitro environment over specific biochemical and physical signals known to regulate chondrogenesis. In particular, bioreactors could improve the formation of cartilage tissues by providing: (i) mixing patterns that result in efficient and spatially uniform seeding of three-dimensional scaffolds; (ii) enhanced mass transport of chemical species to the cells cultured into the scaffold; and (iii) physical (e.g., hydrodynamic, mechanical) stimulation of the cell-scaffold constructs during their development.[102]

Uniform and efficient seeding of cells on porous scaffolds is an important prerequisite for the development of a homogeneous tissue from the lowest possible cell number. As compared to static loading of cell suspensions into scaffolds, seeding into orbitally mixed Petri dishes yielded thicker constructs with more spatially uniform distribution of cells and extracellular matrix.[103] The concept of maintaining a uniform suspension of isolated cells and providing a relative velocity between cells and the scaffold during seeding was extended by seeding in rotating vessels[104] or mixed flasks.[105] Dynamic seeding using mixed flasks was shown to achieve seeding efficiencies approaching 100%, but led to cell densities higher at the scaffold periphery. In

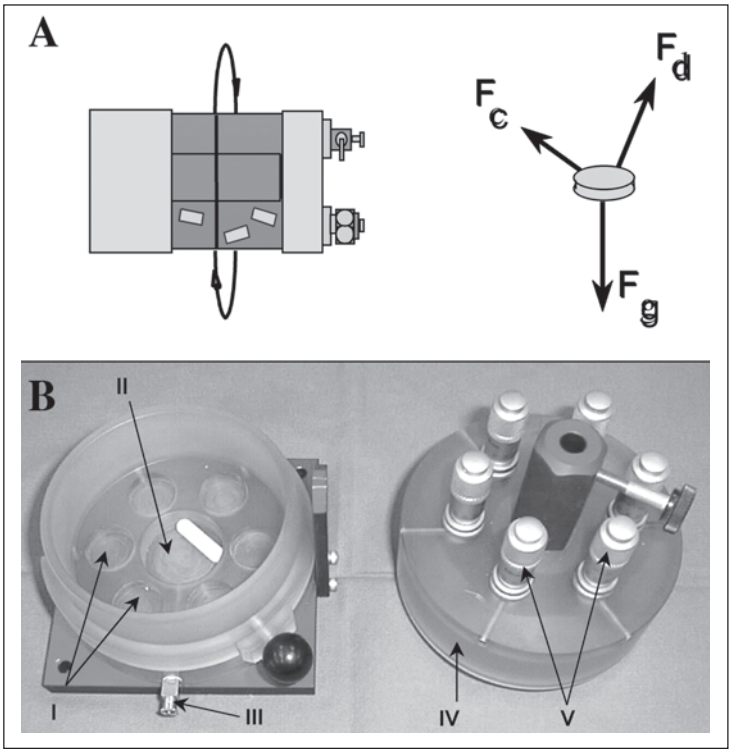

Figure 3. Bioreactors for the application of physical forces to engineered cartilage tissues. In the rotating wall vessel system (A), the rotational speed is adjusted so that the drag force of the medium (Fd) is balanced by the centrifugal (Fc) and gravitational (Fg) forces. The constructs are thus maintained in a tumble-slide regime and the resulting dynamic laminar flow enhances the production and accumulation of cartilaginous extracellular matrix.[107] Specific culture chambers (B) have been developed for the application of direct deformation to engineered constructs.[123] Chambers include wells to allocate tissue constructs (I), a magnetic bar for medium stirring (II), an inlet/outlet port for medium change (III), a cover lid to maintain sterility (IV), and micrometer screws to accurately establish the contact position between the plungers and each specimen (V).

addition, the method is mostly suited to fiber meshes as opposed to porous foams. The design of bioreactors providing the possibility of scaffold perfusion with a cell suspension in alternate directions has the potential to yield a more homogeneous seeding on a variety of scaffolds.[106]

Once the cells are associated with the scaffold, cell-polymer constructs can be cultured in bioreactors applying specific regimes of fluid flow. It was previously shown that dynamic (rather than steady) and laminar (rather than turbulent) flow patterns were associated with rapid in vitro assembly of functional cartilaginous matrix containing glycosaminoglycans and type II collagen by articular chondrocytes cultured on biodegradable scaffolds[107] (Fig. 3A). Such findings could be explained by the fact that dynamic laminar flow generated by rotating wall vessels provided efficient mass transfer at limited shear stress levels, which was essential to promote/maintain cell differentiation, synthesis and retention of extracellular matrix macromolecules.

Tissue engineering bioreactors can also expose developing cartilage constructs to specific physical stimuli that may be beneficial for tissue growth and maturation (Fig. 3B). Enhanced chondrogenesis and cartilage formation were reported upon stimulation of (i) cartilaginous nodules by intermittent motion of medium in roller bottles,[108] (ii) gel-immobilised chondrocytes

by dynamic compression,[109,110] and (iii) cell-polymer constructs by intermittent hydrostatic pressure,[111] hydrodynamic shear,[107] and cyclic loading.[112]

Despite the great efforts currently dedicated to the development and use of bioreactors for the engineering of functional cartilage tissue, it remains unclear which specific physical stimulation regimen is required to induce a specific effect on cultured chondrocytes. The scenario is further complicated by the fact that different scaffolds may transduce similar physical signals in different ways to cells, making it difficult to compare results obtained by different groups.

Engineering Osteochondral Composite Tissues

As previously described in the section "Autografts and Allografts", a promising approach for the correction of large defects involves transplantation of osteochondral units consisting of an articular cartilage layer and the underlying subchondral bone. In vitro engineering of an osteochondral composite of predefined size and shape starting from autologous cells would eliminate the main limitations of this technique, namely (i) the amount of material available, (ii) the donor site morbidity, and (iii) the difficulty to match the topology of the grafts with the injured site. In addition, since ingrowth of trabecular bone into an osteoconductive material or a preformed bone-like tissue is generally highly efficient, the approach would allow easier fixation and anchorage of the engineered graft into the joint lesion, in a way bypassing the well-known problem of cartilage-cartilage integration.[113]

Osteochondral composite tissues were first generated in vitro by culturing differentiated chondrocytes onto PGA meshes and periosteal-derived cells onto foams made of a blend of poly-lactic-coglycolic acid and polyethylene glycol.[113] The two tissue parts were cultured first separately and then sutured together for different periods of time. The study demonstrated the feasibility of generating composites of cartilaginous and bone-like tissues in vitro, and pointed out that the maturation and integration of the two parts can be modulated by the cultivation time.

Osteochondral composites were also generated using mesenchymal progenitor cells, exploiting their capacity to differentiate along different mesenchymal lineages.[114] Rat marrow-derived cells were first "committed" in monolayers with chondrogenic or osteogenic culture supplements and then seeded respectively on hyaluronic acid-based sponges or porous ceramic. The two parts were sealed together using fibrin glue and implanted subcutaneously into nude mice. The two compartments supported a selective differentiation of the mesenchymal progenitor cells and did not separate even when the fibrin sealant was totally degraded. Lamellar bone was abundant in the ceramic part, but only fibrocartilage and not hyaline cartilage filled the voids of the hyaluronic acid sponge. Implantation in a site better resembling the mechanical and biological environment of a joint was therefore concluded to be essential.

A recent study reported the implantation of engineered osteochondral composites in rabbit joints.[116] A layer of functional cartilage, generated by bioreactor culture of chondrocytes seeded onto PGA meshes, was combined with an osteoconductive support made of a ceramic/collagen sponge (Collagraft). The composites were press-fit in the femoropatellar groove of adult rabbits, in the largest ever created defects (7 x 5 x 5 mm) (Fig. 4). The engineered cartilage, which was 5 fold thicker than the native rabbit cartilage, withstood physiological loading immediately after implantation and remodelled within 6 months into osteochondral tissue consisting of a cartilaginous surface and new trabecular bone that was well anchored to the host bone. The cartilage surface contained evenly distributed GAG and type II collagen and displayed characteristic architectural features, uniform thickness as well as Young's moduli almost normal. Control defects left empty or treated with cell-free scaffolds healed with irregularly shaped fibrocartilaginous tissue. The study left many open questions, including the mechanism by which the cartilage was remodelled, the origin of the cells forming the repaired cartilage surface (from the implant or from the host), and the long-term stability of the engineered cartilage. However, it demonstrated for the first time that functional engineered cartilage grafts, combined with an osteoconductive support, might provide a template permitting the orderly repair of very large

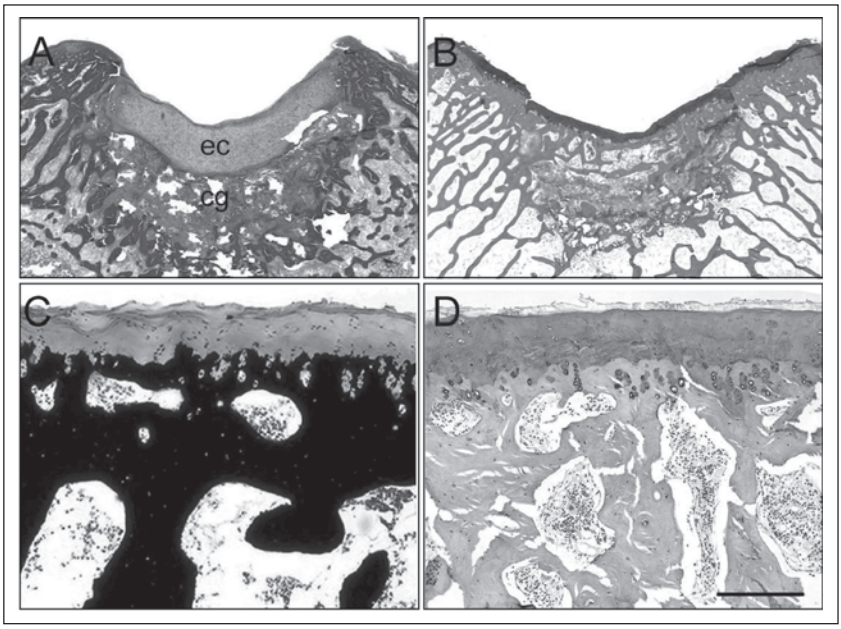

Figure 4. Repair of large osteochondral defects in adult rabbits by implantation of an osteochondral composite, consisting of a layer of engineered cartilage combined with an osteoconductive support (Collagraft).[116] After 6 weeks (A), the original shape of the engineered cartilage (ec) was still preserved, on top of the Collagraft material (cg). After 6 months (B), the engineered cartilage remodelled into a cartilaginous surface, matching the thickness of native rabbit cartilage, and new trabecular bone. A clear tidemark was separating the trabecular bone (C, von Kossa stain) and the cartilage surface (D, type II collagen immunostain). Scale bar = 2 mm (A, B) or 0.2 mm (C, D).

osteochondral defects in adult rabbits. The result could be explained by the fact that the functional extracellular matrix of the engineered cartilage layer not only provided a protective milieu for the embedded chondrocytes, but also served as a mechanical and electrochemical signal transducer that conducted various joint stimuli to the chondrocytes. This approach therefore deserves further extension to a human cell source.

Future Directions

In this chapter, we have reviewed some of the several approaches currently used or under investigation to repair cartilage injuries. The last section describes what we believe to be key challenges for the development of new procedures, which may predictably restore large osteochondral lesions.

Generation of Cartilage Grafts Using Human Cells

As compared to the current procedures, implantation of structurally and mechanically functional cartilage tissues would allow easier graft fixation, early recovering of joint functionality and reduced mechanical discontinuity, which in turn might account for improved durability of the repair (see section "Engineering Functional Cartilage Grafts"). Preformed cartilage could ultimately be combined with osteoconductive materials in order to generate osteochondral grafts (see section "Engineering Osteochondral Composite Tissues"). Despite all the recent progresses made in ex vivo synthesis of cartilage, a reproducible generation of uniform cartilaginous tissues of predefined size starting from adult human cells has not yet been achieved.

We believe that this goal could be reached only by coordinated efforts in different areas, in order to identify (i) an appropriate source of *chondrogenic cells;* (ii) the bioactive factors required by these cells; (iii) the specific features of *3D scaffolds* in which cells can be grown; and (iv) the regime of physical stimulation that a bioreactor should apply to enhance cartilage development and maturation in a controlled environment. Advances in the generation of functional cartilage grafts thus prompt for tight interactions between scientists with different backgrounds, including medical doctors, cellular and molecular biologists, engineers, and physicists.

Enhancement of the Integrative Capacity of Engineered Cartilage

Engineered cartilage constructs implanted in osteochondral defects have been reported to remodel and fuse with the host subchondral bone,[84,115,116] possibly indicating that cartilage-bone integration is not a major challenge. By contrast, the cartilaginous portion of these grafts often does not integrate with the host cartilage to form a continuous, mechanically stable attachment. Such lack of repair could lead to abnormal stress distribution during physiological activity and degeneration in the long term.[117] Elucidation of the relationships between adhesive biomechanical properties and underlying cellular and molecular processes is necessary to design new integrative cartilage repair procedures.[118]

In vitro model systems have been recently developed to study factors affecting cartilage-cartilage integration without systemic effects and variability that are inherent to in vivo studies. Bioreactor cultivation of engineered cartilage discs press-fit into native cartilage rings indicated that an important factor for integrative cartilage repair is the presence of biosynthetically active cells capable of proliferating, filling the gaps at the tissue interface, and progressively forming cartilaginous tissue.[119] In addition, proteoglycan removal from native cartilage tissue by proteolytic enzymes was shown to increase the integrative properties of engineered cartilage grafts but only if cells were present at the periphery of the construct. Therefore, a partially developed-engineered cartilage, where cells are not yet totally "embedded" within a dense extracellular matrix, could display larger integrative capacity. Using a partial apposition configuration model, it was further demonstrated that integrative repair at the cartilage-cartilage interface is mediated by deposition of newly synthesised collagen.[117] Based on all these findings, one could envision strategies for enhanced integrative cartilage repair, whereby additional chondrogenic cells or factors increasing collagen production are delivered at the time of graft implantation, possibly after treatment of the chondral surface with proteolytic enzymes.

Identification of Appropriate Animal Models

The intrinsic repair capacity of articular cartilage significantly varies according to the species, especially when comparing animals and humans. Therefore, animal models should not be selected to demonstrate the success or failure of a certain cartilage repair procedure, but rather to test selective variables of cartilage repair procedures and to understand general paradigms of repair. The intrinsic chondrogenic capacity of human cells and their interactions with polymeric scaffolds have been studied subcutaneously in nude mice, although the absence of the joint biochemical and biomechanical milieu could markedly bias the results. The feasibility of new concepts in cartilage repair has been explored in rabbits, although no statement on the efficacy of the treatments in humans may be derived. Questions related to graft survival and patterns of remodelling under loading should be addressed in larger size animal models (e.g., sheep, goat, dog, and horse), although large differences in spontaneous healing and biological behaviour of chondrocytes have been documented. A number of general requirements have been described to make an animal model more suitable.[120] In particular, (i) the size of the defect must be critical so that spontaneous healing does not occur without treatment; (ii) the animals must be in the adult age in order to reduce the extent of spontaneous healing; (iii) the evaluation must be performed also at a late time point (i.e., at least 1 year) in order to assess possible long-term degenerative changes.

Assessment of Clinical Outcome

To validate the efficacy of cartilage repair procedures in clinical settings and compare results from different methods, it will be necessary to perform rigorous controlled and randomised trials with stringent evaluation of the outcomes. Assessments should include (i) parameters related to the clinical status of the patient, (ii) radiographic findings and histological analyses of biopsies at the site of repair, (iii) biochemical assays to quantify the fractions of different collagen types in a small biopsy,[22] and (iv) measurement of cartilage physical properties, possibly using minimally invasive techniques.[121,122] The International Cartilage Repair Society (ICRS) has proposed a standard Cartilage Injury Evaluation Package (www.cartilage.org/ICRS_Evaluation.pdf), consisting of a self-assessment questionnaire for the patient, clinical evaluation forms and a histological grading system for cartilage biopsies. Standardised compilation of data at defined times after surgery will allow more objective patient evaluation, even if gathered by different surgeons.

The increasing number of groups/companies offering a cell-based cartilage repair product introduces the need to assess clinical outcome in patients grouped not only according to repair procedure, but also to the specific product used within a certain treatment concept. Objective comparison of different repair methods can be facilitated by the introduction of databases where standardised evaluation of patients treated with a specific product can be recorded.

Considering that the "success" of any procedure aimed at repairing cartilage injuries can only be quantified as the durability of a pain-free and functional joint, observation time in clinical trials should be extended for several years before any conclusion can be drawn. Since this fact represents a major obstacle in comparing different treatments within a reasonable time frame, strong emphasis should be put in the identification of "early predictors" of long-term outcomes, in order to allow establishment of shorter but equally relevant clinical trials.

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Chapter 13

Maxillofacial Bone Regeneration Using Tissue Engineering Concepts

Minoru Ueda

Introduction

The bone defect in the maxillofacial region generally can be categorized to three types according to the size of defects. The first category is the cavitary bone defect which is less than 3 cm in length and need from 5 to 10 ml of bone tissue for reconstruction. For such defects, the particular cancellous bone marrow grafting harvested from iliac crest is performed. The second category is the segmental bone defect which is from 3 cms to 6 cms in length and the bone tissue is from 10 to 15 ml. Such defects are often caused by the resection of mandible due to the benign tumor. The interpositional grafting of iliac bone is performed. The third category is the largest bone defect with a free end which is more than 6 cm in length. It needs approximately 20 ml of bone tissue for reconstruction. Such large bone defect is usually caused by the hemimandibulectomy due to the oral cancer. The free vascularized osteocutaneous flap is commonly applied for the reconstruction (Fig. 1).

Autogenous bone grafting is a well established and reliable procedure for reconstruction of the maxillofacial bone. However, it is associated with substantial morbidity in the donor site that includes infection, malformation, pain, and loss of function.[1] The complications resulting from graft harvest have inspired the development of alternative strategies for the repair of clinically significant bone defects. A previous approach to this problem focused on the development of various synthetic materials that might be developed instead of the autogenous bone. However, most of these synthetic materials were not osteogenic or osteoinductive. Therefore they can not replace the entire function of living bone. On the other hand, allogenic and heterogenic materials has been used but they have a risk of disease transmission.

The limitations of previous graft materials have led to the development of tissue engineering which is, defined as the interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, and improve the function of damaged tissue and organs.[2] Recently these tissue engineering concept has been developed by using the following factors such as signaling molecule, stem cell and scaffold (Fig. 2).

In this article, the strategy for maxillofacial bone regeneration using tissue engineering concept is introduced.

Signaling Molecule

The first approach for bone regeneration is to deliver specific signaling molecules. If a safe, recombinant growth factor could be applied to a biocompatible, resorble carrier and the correct shape of bone implant could be implanted directly out of a package, all of the previously stated shortcomings of autogenerate bone grafting would be overcome. The host would generate its own bone in response to the growth factor.

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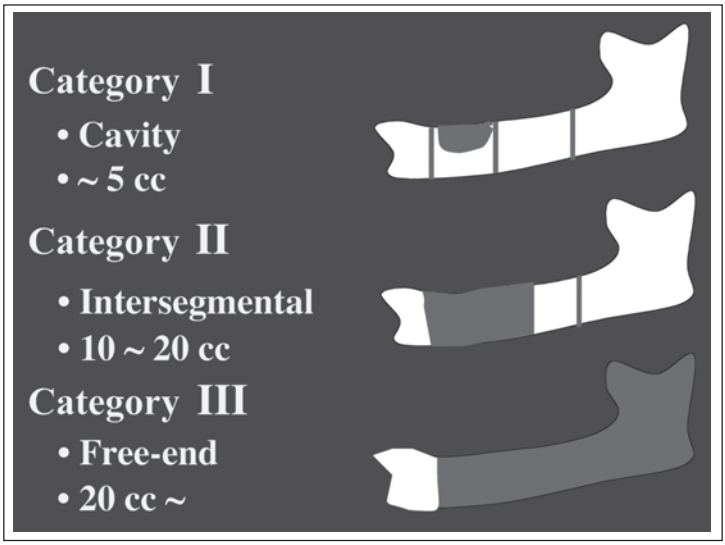

Figure 1. Three categories of maxillofacial bone defects.

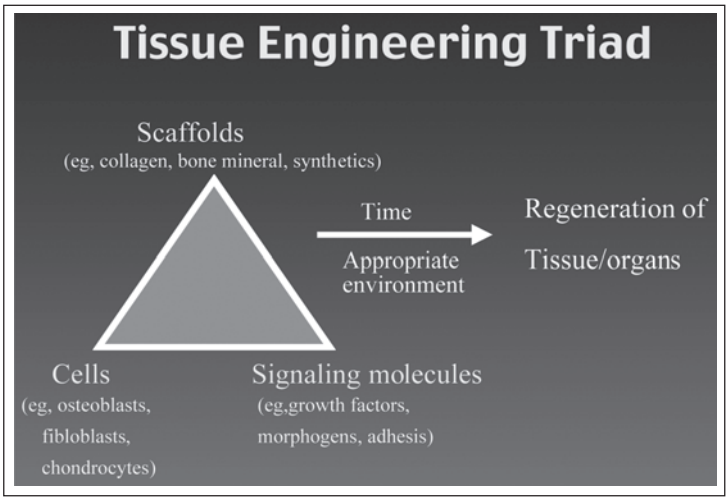

Figure 2. Tissue engineering concepts with three elements, stem cell scaffold and signal molecule.

Bone Morphogenetic Protein

The most widely studied molecules are members of the bone morphogenetic protein (BMP) family. In 1965, Dr. Urist[3] purified BMP as a substance to induce the local bone formation. Since then, at least 15 BMPs, have been cloned and their osteogenic activities have been characterized. Of these recombinant human BMPs, BMP-2 is one of the most osteoinductive agents. However, it's a just single growth factor that promote only a single regeneration pathway. So, it is suggested that the potential for bone regeneration is limited. Therefore, large amounts of rh-BMP-2 would be required for adequate bone formation and so its clinical application for a massive bone defect would not be realistic.

Tranforming Growth Factor-β

The transforming growth factor-β (TGF-β) gene superfamily is one of the largest and most diverse groups of related peptide growth factors that are important in tissue generation and wound healing. TGF-β1, the first member of this superfamily to be well studied originally purified from human platelets and subsequently found in multiple normal tissues, including cartilage and bone.[4-6] TGF-β may play a major role in bone generation, repair, and remodeling. An interesting offshoot of this line of research is the tissue engineering implications: the ability of various growth factors to induce bone formation in vivo for reconstruction of bone defects.

Transforming growth factor-β1 induces woven bone formation in vivo when injected onto the periosteum of rat and mouse cranium and onto the perichondrium of rabbit ears. In addition, when combined with a demineralized bone matrix (DBM) carrier, both TGF-β1 and TGF-βZ (recombinant hybrid of TGF-β1 / TGF-β2) accelerate osteoinduction in a craniofacial onlay mode in rabbits.

Platelet-Rich Plasma

Platelet rich plasma (PRP) contains a high concerntration of growth factors, which are universal initiators for almost all pathway of wound healing.[7] By using these identified growth factors and nonidentified growth factors in platelets, we tried to perform the bone regeneration.

Animal Experiment

Our study was designed to examine whether PRP with β-tricalcium phosphate (β-TCP) would improve ossification of the graft using rabbit. PRP is prepared from the peripheral blood by the manner of Figure 3, and the remaining blood components are delivered back into the rabbit. All animals received human care in compliance with the "Guidelines for the Care and Use of Laboratory Animals"published by the National Institutes of Health (NIH Publication no. 85-23, revised 1985). The maxillary sinus floors in 4 adult rabbits were augmented with β-TCP combined with PRP on the test side and β-TCP on the control side (Fig. 4). They were sacrificed, respectively, at 2 and 4 weeks after surgery to evaluate bone formation.

Histologic evaluation documented a significant amount of newly formed bone along the β-TCP at 4 weeks on both sides, indicationg that β-TCP used in this study had good osteoconductivity. The bone volume fraction at the augmented area on the test sites was higher

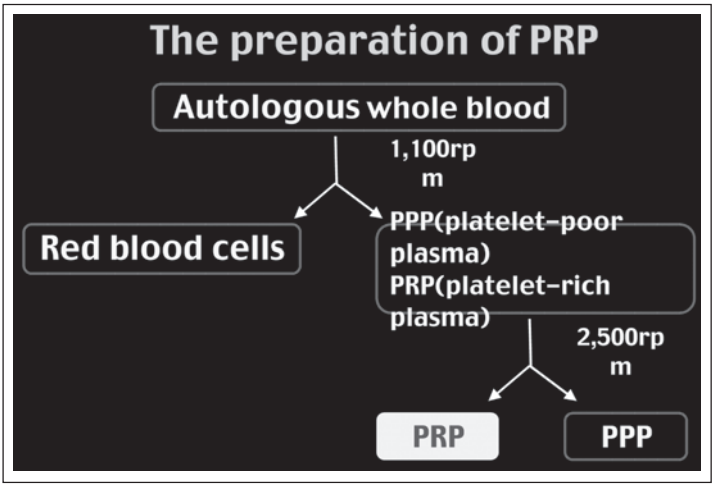

Figure 3. Preparation method of PRP by two-time revolutions.

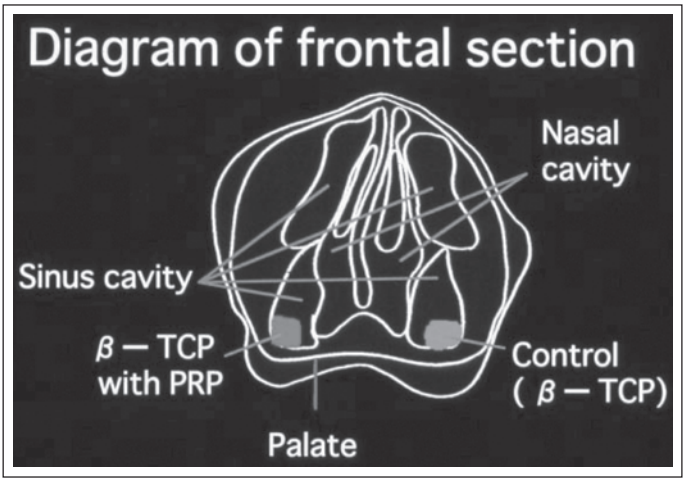

Figure 4. Experimental design of β-TCP and PRP for osteogenic capacity.

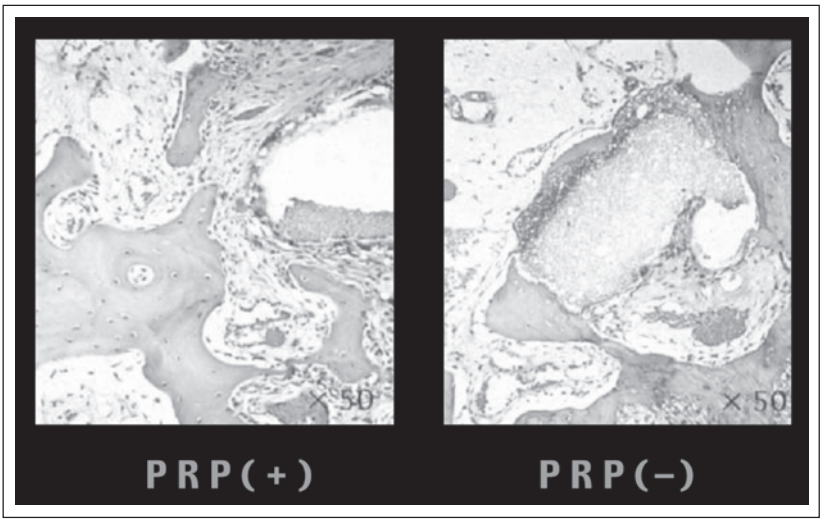

Figure 5. Histological findings of β-TCP and PRP.

than that of control sites at 2 and 4 weeks (Fig. 5). However, on the control sites, the bone volume fraction was lower than that of β-TCP at 2 and 4 weeks.

These results suggest that the application of PRP causes a more rapid resorption of β-TCP in a maxillary sinus. The change of morphology of the graft was observed in the early stage of healing in both groups, indicating that the histologic differences were seen clearly especially in the angiogenesis and absorption of β-TCP. And also PRP would have a potential to enhance bone formation and improve the healing of the graft in a maxillary sinus. Taken together, the present findings suggested that β-TCP loaded with PRP had excellent osteogenic characteristics and supported its potential in tissue engineering to repair bone defects.

Stem Cell

Stem cells create or recreate functional structures using information and signaling which have been preprogrammed. As we understand these programs and behaviors, we can use them to our advantage in the rational design of living tissues. The source of cells for creation of these structures is a major challenge. Much is known about stem cells and cell lineages in the bone marrow and blood. Recently, mesenchymal stem cells (MSCs) have received widespread attention because of their potential utility in tissue engineering applications.[8] After expansion to the appropriate cell number, the cells can then be transferred to templates where further remodeling can occur. It was reported that MSCs derived from bone marrow have osteochondral differentiation potential in vitro[9,10] and in vivo,[11,12] and therefore, may play an important role in the repair of bone defects.

Mesenchymal Stem Cells and Biodegradable Ceramics

We prepared to apply a biodegradable scaffold, pure β-TCP, especially synthesized by a wet milling method (mechanochemical method), which was an excellent material that induced bone formation, while gradually being degraded and was finally replaced by new bone. It was reported that β-TCP with a three-dimensional structure provided an excellent environment for bone regeneration and its surface facilitated cell attachment, proliferation, and osteogenic differentiation. We investigated whether β-TCP loaded with MSCs had an osteogenic potential at an ectopic site.[13]

Animal Experiments

The β-TCP was supplied by NGK SPARK PLUG CO., LTD., Nagoya, Japan. The solid and porous components of the microstructure were completely interconnected. The pore average was 200-400 μm in diameter, the interconnection average was 60 μm and the average void volume was 90%. These ceramics were a disc-shaped, 5 mm in diameter and 4 mm in thickness (Fig. 6).

For MSCs isolation and culture expansion male 7-week-old Fisher F344 rats were sacrificed by pentobarbital overdose. Both ends of the femors of a rat were cut away from the epiphysis,

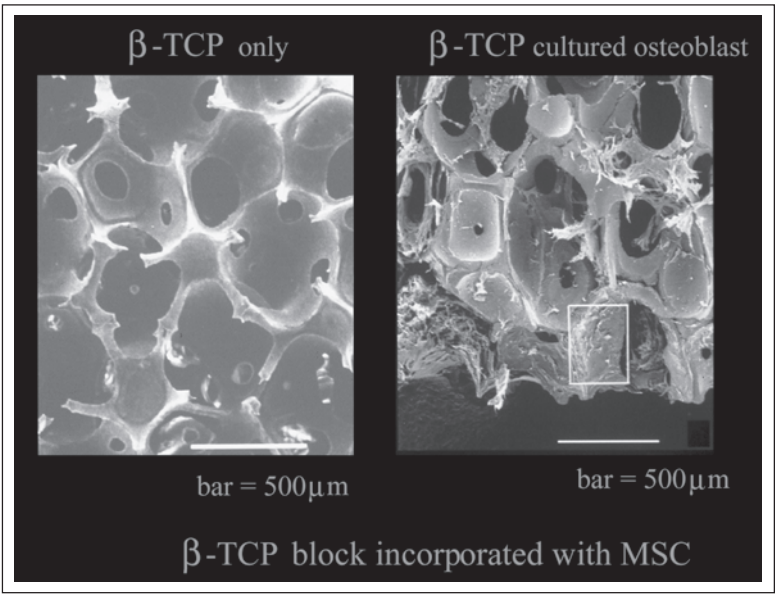

Figure 6. Scanning electric microscopic view of ceramics (β-TCP).

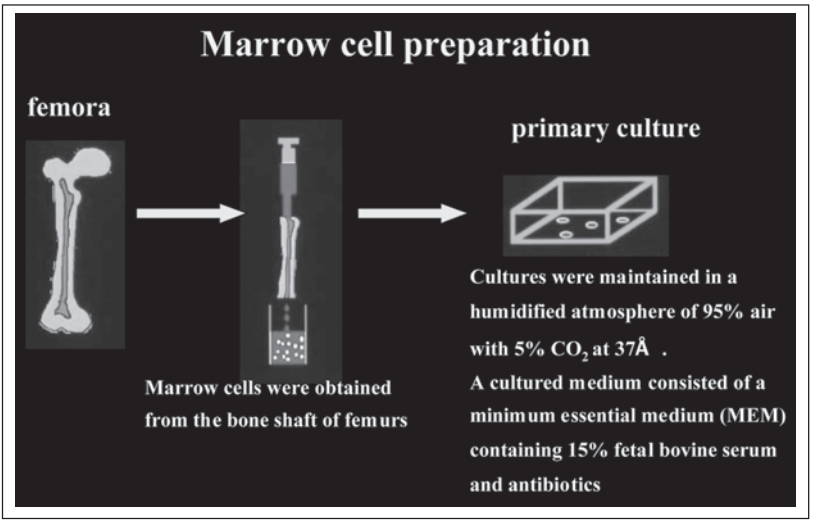

Figure 7. Schematic drawing of marrow cell preparation.

and the marrow plugs were flushed out using 10 ml of cultured medium expelled from a syringe through a 23-gauge needle (Fig. 7). The released cells were collected in two TC flasks (8×10 cm^2) containing 15 ml medium. The medium was changed after the first 24 h to remove nonadherent cells. Subsequently, the medium was removed 3 times a week. Cultures were maintained in a humidified atmosphere of 95% air with 5% CO_2 at 37°. A culture medium consisted of a minimum essential medium (MEM) containing 15% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μm/ml streptomycin, and 0.25 μg/ml amphotericine B, Sigma-Aldrich, Poole, UK). After 10 days in primary culture, bone marrow-derived MSCs were released from their culture substratum using 0.05% trypsin-EDTA. The cells were concentrated by a centrifuge at 900 rpm for 5 min at room temperature, resulting in 10×10^6 cells/ml. The β-TCP blocks were soaked in cell suspension (10×10^6 cells/ml) for 2 h in a CO_2 incubator. After 2 h of incubation, each block was transferred into a 24-well plate for subcultures. One block was subcultured in one well with 1 ml of the culture medium suplemented with 10 mM Naβ-glycerophosphate, 80 μg/ml vitamin C phosphate (L-ascorbic acid phosphate magnesium salt n-hydrate, $C_6H_6O_6$) and 10^{-8}M dexamethasone (Sigma-Aldrich, Poole, UK). The subcultures were maintained for 20 days.

Syngenic 7-week-old male Fischer rats were anesthetized by intramascular injection of pentobarbital (nemubtal 3.5 mg/100 g body weight) following light ether inhalation. The β-TCP loaded with MSCs after 20 days of subculture and each empty ceramic were implanted subcutaneously in the back of syngenic rats under sterile conditions. For each animal, empty β-TCP, and cell-loaded β-TCP, were randomly assigned to and implanted at one of four sites, not in contact with any osseous tissue.

Each implantation was harvested 1, 2, 4 and 8 weeks after implantation. The specimens were fixed in 10% buffered formalin, decalcified (K-CX, falma Co., Tokyo), and stained with hematoxylin and eosin. These specimens were examined under light microscopy. At 2 weeks after implantation, a few mature bone together with cuboidal active osteoblasts was observed when alkaline phosphatase activity achieved a peak. The osteoblast layer was in close contact with the vasculature. At 4 weeks after implantation, consistent bone formation was observed in more pores of β-TCP. At 8 weeks after implantation when osteocalcin content was significantly higher than before, bone formation was still progressing and an increase in the mature lamellar bone areas could be observed (Fig. 8). We confirmed that the bone areas were increased

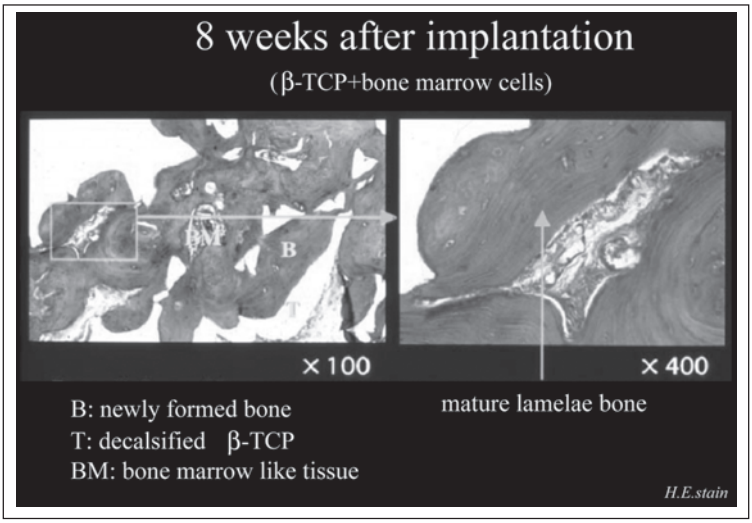

Figure 8. Histological findings of newly formed bone by MSCs and ceramics.

time-dependently. Despite of excellent bone formation in vivo, no cartilage was observed in the porous areas at any time and, thus, the process was the so-called intramembranous bone formation. In the control groups, although the vascularization was observed, the subcutaneous implantation of empty β-TCP blocks alone did not show any bone formation in the pore area at 1, 2, 4 and 8 weeks after implantation and we observed only fibrous tissues.

From this study, we confirmed that there was a osteogenic potential in β-TCP as a biodegradable scaffold.

Injectable Bone

The transplantation of bone requires an invasive operation with skin on mucosal incision and exfoliation of the periosteum. A reliable method of delivering additional autogenous bone to the maxillofacial skelton would minimize surgical trauma. If one could transplant by means of injection by syringe and cause to engraft large numbers of isolated cells, one could augment the maxillofacial osseocartilaginous skeleton with autogenous tissue but without extensive surgery. Unlike the porous ceramics used to create a cell-ceramics constructs, a liquid support matrix that polymerises to a gel would be more easily shaped and molded for custom reconstruction or augmentation. Additionally, a liquid polymer system holds the potential for injectable delivery, which would be much less invasive than open implantation.

An important property of these materials for injectable bone is its plasticity in site and stability. Fibrin glue,[14] a composite of fibrinogen and thrombin, is a physiologically relevant matrix whose principal component, fibrin, has fundamental roles in the process of blood clotting and wound healing and is a potentially suitable biological vehicle for cell transplantation since it has proven biocompatibility, biodegradability and binding capacity to cells. We performed the animal experiment to determine whether a combination of fibrin glue-β-TCP and mesenchymal stem cells (MSCs) will result in new bone formation and can result in an injectable osteogenic bone substitute with a plasticity of the matrix[15] (Fig. 9).

Animal Experiments

The animals were divided into one experimental group (injected with MSCs/fibrin glue-β-TCP admixtures) and one control group (injected with fibrin glue-β-TCP admixture) and analyzed at 2 (n=5), 4 (n=5), and 8 (n=5) weeks after injection.

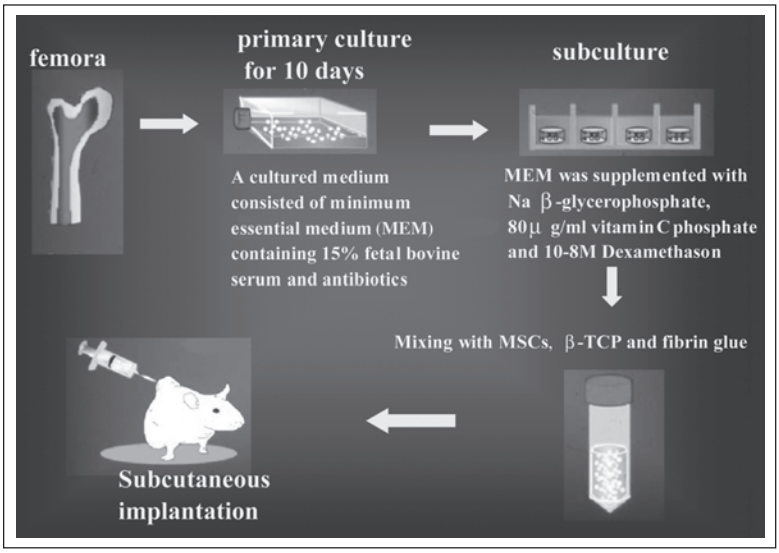

Figure 9. Schema of experimental protocol by using MSCs/fibrin glue-β-TCP admixtures.

MSCs isolation and culture expansion was performed according to previously described methods.[16] Pasteurized fibrin glue (Bolheal, the ChemoSero-Therapeutic Reseach Institute, Kumamoto, Japan) was formed by mixing two separate solutions, A and B. Solution A consisted of fibrinogen (80 mg/ml) and fibrin-stabilizing factor XIII (75 units/ml) dissolved in 1 ml of plasmin inhibitor aprotinin (1000 kIE/ml).[17] Solution B contained thrombin (250 units) dissolved in 1 ml of 40É M $Cacl_2$. Solution A and B were mixed in a 1:1 (volume/volume) ratio. The clotting reaction between A and B produced a semirigid three-dimensional network at room temparature. The β-TCP loaded with MSCs after 20 days of subculture (MSCs/β-TCP composite) was reduced to small lumps in sterilized 5ml syringe and was resuspended in a solution A. Solution A containing MSCs/β-TCP composite and solution B [1:1 (volume/volume) ratio] were placed in the barrel of the sterilized 5ml syringe and mixed by inverting the syringe repeatedly. The MSCs concentration in the admixture prepared was approximately 10 x 10^6 cells/ml. Syngenic 7-week-old male Fischer rats were anesthetized by intramascular injection of pentobarbital (nembutal 3.5 mg/100 g body weight) following light ether inhalation. The skin was prepared with povidone-iodine. An 18 gauge needle was inserted into the loose connective space between the carnosum layer and muscle fascia. 0.5 ml of the MSCs/fibrin glue-β-TCP admixture was injected in the subcutaneous space (Fig. 10A).

After injection of MSCs/fibrin glue-β-TCP admixtures into rat, subcutaneous nodules formed by 8 weeks and these were hard, resisted compression and had well-defined margins upon dissection from subcutaneous tissue (Fig. 10B). Injection of fibrin glue-β-TCP alone failed to form nodules. Vascularization had increased throughout the implants at 8 weeks. At no time point was evidence of malignant growth found in any of the specimens from the MSCs/fibrin glue-β-TCP admixture groups. On the other hand, control implants of injected fibrin glue-β-TCP admixtures had only a shiny appearance and an elastic consistency at 2 weeks, which persisted until 8 weeks. The implant margin was not detectable at 8 weeks and its dimensions remained flattened.

Histologic examination at 2, 4, and 8 weeks showed calcified bone matrix with occasional small remnants of biodegraded fibrin glue-β-TCP in the implants. At 2 weeks after implantation, we could observe the osteoblast lining and at 4 weeks, a few mature bone together with cuboidal active osteoblasts was observed. At 8 weeks after implantation when osteocalcin content was significantly higher than before, bone formation was still progressing and an increase in the mature lamellar bone areas could be observed (Fig. 11). We confirmed that the bone

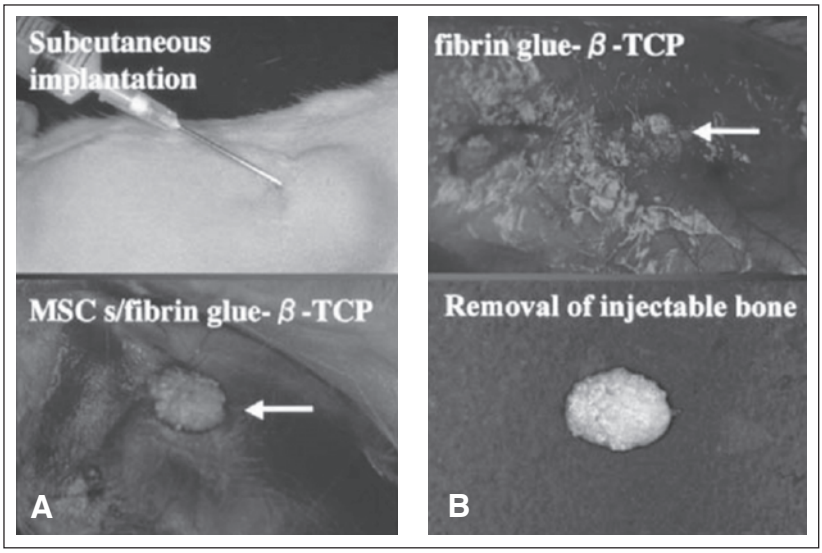

Figure 10. A) The MSCs /fibrin glue-β-TCP admixture as injected from an 18 gauge needle. B) A macroscopic view of the appearance of an implant 8 weeks after injection of the MSCs /fibrin glue-β-TCP admixture. A well-defined nodule is present in the subcutaneous space of the animal.

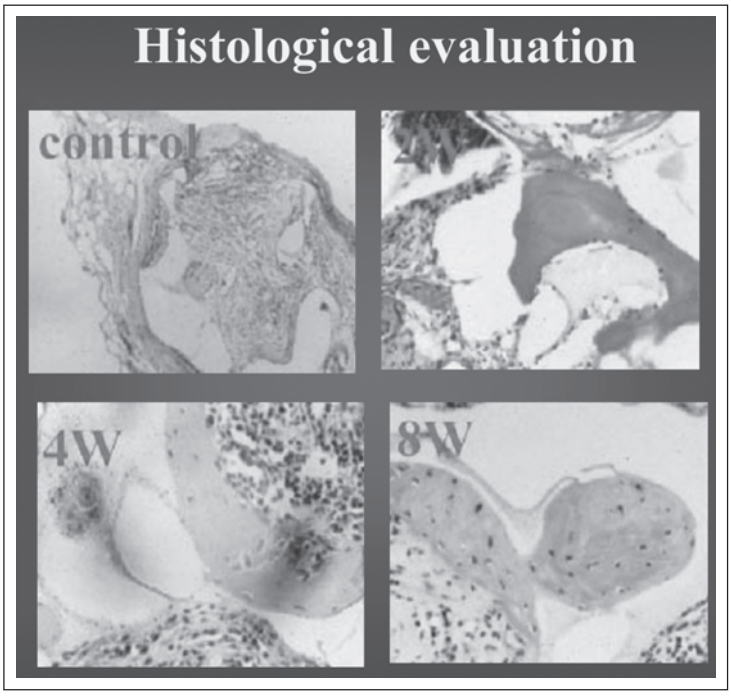

Figure 11. Histologic comparison of the implants developed from MSCs /fibrin glue-β-TCP admixtures or fibrin glue-β-TCP admixture.

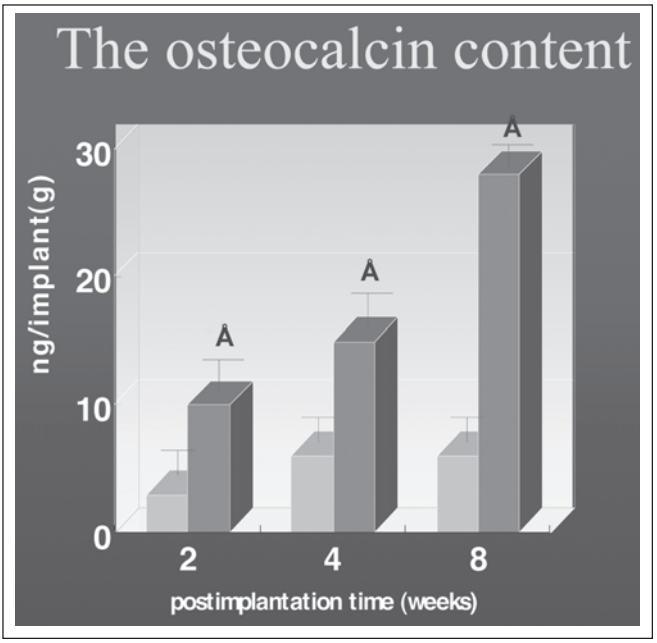

Figure 12. The osteocalcin content for mineral detection. The experimental samples (MSCs /fibrin glue-β-TCP admixture) and control (fibrin glue-β-TCP admixture) harvested at 2, 4, and 8 weeks after injection. Osteocalcin content increased over time in the tissue implants developed from the MSCs /fibrin glue-β-TCP admixture. Each point represents the mean value of osteocalcin content±SE (n=5 at each point). Asterisks indicate significant differences in osteocalcin at $p<0.05$.

areas were increased time-dependently. Despite of bone formation in vivo, no cartilage was observed in the porous areas at any time.

Control implants with fibrin glue-β-TCP admixtures alone exhibited none of these histologic features and did not show any bone formation in the area at 2, 4 and 8 weeks after implantation and we observed only fibrous tissues. With time, the fibrin glue-β-TCP was gradually resorbed, a result producing implants smaller and flatter and containing numerous pores and fibrous tissues caused by the biodegradation of the fibrin-β-TCP admixture (Fig. 12). These changes were correlated with those found by x-ray and osteocalcin content for mineral detection in the implants developed from MSCs/fibrin glue-β-TCP admixtures or fibrin glue-β-TCP admixture.

Osteopontin, a protein important in bone development, was identified with experimental groups, but it could not detect in the control groups. The osteocytes was positive with the antibody. These results were consistent with the osteocalcin content, x-ray findings, and histologic evaluations. In this regard, this study demonstrates that MSCs/β-TCP matrix composites can be transferred with fibrin glue to recipient sites in animal models without loss or viability of cultured tissue and fibrin glue allows MSCs proliferation without deforming cell structure and is an appropriate delivery substance.

Discussion

In the context of minimally invasive surgery, the next logical step is to provide a biological replacement for missing tissue without the need for a harvesting operation. Tissue engineering is defined as the fabrication of living parts for the body from cells in the laboratory. Donor cells such as stem cells or cultivated, differentiated cells are seeded on an appropriately configured scaffold

replicating extracellular matrix. Growth factors are added to the in vitro system to encourage stem cell proliferation. The engineered structure is then transplanted to the recipient.

For bone formation induced by marrow-derived MSCs has long been recognized to be useful.[18,19] Recently, a method has been developed involving enzymatic liberation of marrow cells from explants and their introduction into cell culture with subsequent expansion in cell number. These cultured cells eventually differentiated into mature osteoblasts.[20,21] Therefore, on this basis, it was considered advantageous here to transplant cultured MSC as a source of osteoblasts, but the cellular implantation procedure was complicated by problems associated with delivery vehicles. Optimally, delivery substances to be used for bone or cartilage replacement or repair through tissue engineering would provide the same environment as bone matrix in vivo and, additionally, would combine an appropriate rate of biodegradability with the capacity of the respective cells to multiply.

Vacanti[22] and his group have engineered structures such as ear cartilage and a composite distal interphalangeal joint among other structures using collagen gel. In our laboratory induction of bone tissue in athymic mice was attempted with MSCs and fibrin glue. Yamada[15] has cultivated mesenchymal stem cells in vitro and seeded them in an injectable scaffold and fibrin. After 16 weeks, the construct closely resembles the bone. The engineered structure is hard and bone is evident histologically when the specimen is sectioned. Various growth factors in fibrin glue related to wound healing have been found recently to support the growth, adhesion, migration, differentiation, and vascularization.[23] There may be an advantage, then, to using a cell/fibrin composite in that it has been suggested as more osteoinductive than cell suspension, although both the composite and cell suspensions sequester and retain osteoinductive proteins required to trigger molecular signals for local morphodifferentiation to promote calcification. In the presence of fibrin glue, the results here support the greater osteoinductive capacity of cell/extracellular matrix composites.

From these series of experiments, I can conclude that the cell and fibrin mixture can be a interesting alternative to the conventional autogenous cancellous bone grafting especially in category I bone defect. However, in categories II or II, the regenerated bone cell/fibrin mixture have not enough mechanical strength. Tissue engineering is just at the beginning and many problems remain to be solved before its clinical use.

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Chapter 14

Clinical Evaluation of BMPs and Bone Marrow Stromal Cells in Orthopedic Surgery

Didier Hannouche and Yang Yu Chien

Abstract

The development of alternative techniques to treat long bone nonunions or for spinal fusion purposes offers outstanding perspectives for a large number of patients. Among the different approaches available, the use of bone morphogenetic proteins (BMPs) and osteocompetent cells are certainly attractive approaches. Both have been extensively evaluated in pre-clinical models, and have clearly demonstrated efficacy in a number of studies. Although very promising, these techniques are invasive and expensive, and several issues should be overcome before they can be adapted for widespread clinical use. Critical are the need for the use of a high-dose of BMPs to achieve efficacy in human, the choice of suitable drug delivery systems, the difficulties encountered to design reliable clinical trials to compare these therapeutics to the standard of care. The aim of this study is to provide an update on the clinical application of these new modalities.

Introduction

Fracture repair involves a sequence of dynamic events that aim at complete restoration of both skeletal anatomy and biomechanical properties of the involved bone.[1] In some cases though (5-10%),[2] the repair potential of human bone is overtaken and further surgical procedures are required to achieve bone bridging. It is estimated that 500,000 bone graft procedures are performed every year in the US alone, half of which for bone healing purposes.[3] In most severe cases, often due to comminuted open fractures or tumor excision, autologous bone grafting procedures are routinely indicated to transplant at the target site living osteogenic cells and bone inductive proteins that stimulate cell proliferation and differentiation. However, this technique carries with it numerous problems, including a limited supply of suitable bone, and an additional surgery that may be responsible for a significant morbidity to the donor site, with pain, hematoma, or infection.[4,5] For the past 30 years, many alternative therapeutics have been considered and tested,[6] including physical methods (electromagnetic fields, ultra-sound…) and bone graft substitutes (allograft, osteoconductive materials…), but none of these have been able to give satisfactory results. Optimism has greatly increased since the discovery by Urist in 1965[7] of a family of osteoinductive proteins that are capable to elicit new bone formation by the recruitment of non-committed stem cells. These proteins, named bone morphogenetic proteins (BMPs), are members of the transforming growth factor β (TGFβ) superfamily. Subsequently, most of the proteins were individually identified and some of them were cloned and are now produced by genetic engineering techniques (rh-BMP2 and rh-BMP7). The isolation of these unique osteoinductive proteins has literally set off an intense research in the field of fracture repair and bone regeneration. Therefore, a number of investigators have proposed to supplement a variety of biomaterials with BMPs in order to speed up and improve the repair

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process and have shown overwhelming results in pre-clinical models. The BMPs have demonstrated their ability to promote bone healing in large defects in rabbits, sheep, and dogs,[8,9] and in spinal fusion models in dogs and monkeys,[10,11] and to accelerate bone healing in sheep.[12] Despite extensive evaluation of BMPs in preclinical models, confirming their substantial efficacy in the treatment of bone defects in animals, some important issues, regarding efficacy in human and safety, needed to be addressed before these proteins could be considered for routine medical use. It took almost 15 years before the Food and Drug Administration (FDA) in the US and the European Agency for the Evaluation of Medicinal Products (EAEMP) approved rh-BMP2 and OP-1 (rh-BMP7) for spinal fusions and the treatment of nonunion of long bones.

An alternative strategy to the use of BMPs is the combination of osteocompetent cells such as bone marrow stromal cells (BMSCs) and different scaffolding materials.[13] These osteogenic materials are currently under evaluation for the treatment of large bone defects in human in several countries.

The purpose of this study was to review the clinical data available on the use of BMPs and BMSCs, and to discuss the critical concerns that remain.

Clinical Experience with BMPs

Bone Morphogenetic Proteins can be used in clinical practice to accelerate the natural process of bone healing, to reduce the incidence of nonunions (higher in the case of comminuted open fractures), or to replace the current standard of care, namely bone autografting, for the treatment of several diseases: nonunion of long bones, bone defects, spinal disorders requiring spinal fusion. The treatment of these disorders is usually difficult and time-consuming and the use of BMPs is expected to reduce significantly both the failure rate and the morbidity of bone grafting.

Segmental Bone Loss and Nonunions

Most of the clinical studies reporting on the use of BMPs have focused on the treatment of nonunions and segmental bone defects. Johnson and Urist described their experience with the use of h-BMP, extracted from human bone, on five occasions.[14-18] In their latest report, 30 patients were treated with a composite made of an allograft, and a high concentration of h-BMP. When the femoral defect was greater than 2 cm, it was supplemented with bone autograft. Healing was achieved in 24 patients at an average of 6 months after BMP implantation. Although effective, the use of allogeneic h-BMP does not appear a suitable option for medical purposes: hundreds of kilograms of bone would be needed each time to obtain few milligrams of h-BMP (40 kg for 1 mg); and the implantation of an allogeneic product would involve potential risks of disease transmission and immunological reactions from host. The fabrication of recombinant forms of naturally occurring BMPs (BMP 2 and 7) in the '90s has hastened the clinical research in the field of bone delayed unions and nonunions. The application of OP-1 to heal clinical bone defects was first reported by Geesink in 1999 in a prospective randomized double-blind study.[19] This study evaluated the use of OP-1 (3.5 mg) delivered on bovine type 1 collagen implants to treat 15mm-length fibular defects performed during an opening wedge high tibial osteotomy. Control groups were filled with the matrix alone. Five of the six patients treated with OP-1 healed compared to none of the control group. Consequently, OP-1 was compared to autologous iliac crest bone grafting in the treatment of tibial nonunions. This multicenter prospective randomized trial started in 1992 and involved 17 medical centers.[20] Under a US FDA approved Investigational Device Exemption, the study enrolled 122 patients (124 tibial nonunions) with an established nonunion at least 9 months after injury. Additional inclusion criteria included the need for an internal fixation and bone autografting (in the judgement of their surgeon), and no surgery performed within 3 months prior to implantation. Half of the patients were treated with OP-1 (3.5 mg/bovine type 1 collagen) and half of them with autologous iliac crest bone grafting. A new rod was inserted in 90% of the cases. The

demographics were comparable in the 2 groups. At 9 months, given clinical and radiological evaluation criteria (bridging on at least 3 views), healing was achieved in 62% of the OP-1 treated group and 74% of the bone autograft group (p=0.158). No serious adverse effects were observed. While the outcome of both groups was comparable regarding bone healing, 20% of the autograft patients group had pain at the donor site at 6 months, and bleeding was significantly higher (345cc vs 245cc) in this group. Interestingly, the authors reported significantly less infection at the fracture site (3% vs 21%) when OP-1 was implanted compared to bone autograft. In a study performed in rats, Chen[21] confirmed the capacity of OP-1 implants to form new bone in an infected segmental defect in rats, while the implantation of bone autograft led to osteolysis in most of the cases. More study of this important question, and the potential mechanisms through which OP-1 would reduce the rate of infection is clearly warranted in the future. This first multicenter trial has confirmed the findings in earlier studies[22] of a significant efficacy of OP-1 in the treatment of nonunions, and today more 2500 implantations have been performed worldwide. Based on this study, the US FDA and the EAEMP have approved the use of OP-1 in the treatment of tibial nonunions.

In severe cases, sometimes affecting other bones than the tibia, and when conventional treatment has failed, an individual patient usage approval can be obtained to treat separately a patient. This was performed in 163 consecutive salvage cases between 1997 and 1999 in Australia[23]. The indications for OP-1 implantation included 113 nonunions, 18 revision hip surgeries, 16 failed arthrodeses, 9 bone defects, 3 peri-prosthetic fractures, 1 elective osteotomy, 1 congenital pseudarthrosis, and 2 osteochondral defects. 35% of the patients had prior autograft procedures, and 28% had pre-existing pathology such as infection, and rheumatoid arthritis…OP-1 was usually combined with autograft or other osteoconductive material (allograft, hydroxyapatite). At an average follow-up of 15 months, 95% of the patients were available for clinical and radiological assessment. Bone repair was achieved in 70% of the cases, and 25% were considered as failures.

Another recent report displayed promising results in the treatment of bone losses around loosened prostheses in combination with bulk or morcellized allograft.[24] Placed at the host-bone allograft junction either in the femur or the acetabulum, the OP-1 implant elicited extensive new bone formation, but no heterotopic bone in surrounding tissues. Although these preliminary studies suggest some positive effects of the BMPs in other skeletal sites and indications, these results must be assessed in larger controlled clinical trials comparing this treatment to the standard of care in each indication.

Acute Treatment of Tibial Fractures

Fractures of the tibia are known to heal twice as slowly than other location. When the fracture of the tibia is open, it is estimated that 47% of the patients will require further interventions to achieve bone healing. The use of BMPs is expected here to reduce the rate of bone delayed union and nonunion. In a pilot study[25] on the use of rh-BMP2 in the healing of 12 open tibial fractures with a Gustilo classification of II or higher, bone healing was achieved in 9 cases without further intervention. These preliminary results on the benefit of rh-BMP2 on the time course of open fracture healing were recently confirmed in a prospective randomized evaluation of 450 tibial fractures stabilized with an internal rod.[26] The rh-BMP2 was delivered on a bovine type 1 collagen sponge at two different doses, 6 and 12 mg of BMP. The control group was composed of patients treated with internal fixation and an absorbable sponge alone. The patients were stratified by the severity of the open wound. The period required to achieve clinical and radiological healing was significantly reduced in the 12mg-rh-BMP2 group (44% reduction in the risk of failure) and treatment with rh-BMP2 was associated with fewer secondary invasive interventions, fewer infections, faster wound healing. Minor adverse reactions were reported including a significant higher rate of headaches in the BMP treated group (11 vs 7%). Despite some interesting results, the use of rh-BMP2 in the treatment of acute tibial fractures is still under evaluation by the US FDA for pre-market approval. The main issue that

precludes the BMPs from being used routinely in acute fractures is that the panel of available treatments would be then significantly reduced in case of failure. Only one injection of BMP is recommended by the manufacturers in order to avoid allergic and immunological responses.

Spinal Disorders

Autologous bone grafting is routinely indicated in many spinal disorders in order to reduce pain and stabilize the spine (lumbar stenosis, degenerative disc disease, scoliosis, fractures…). It is estimated that half of the 200,000 spine fusion procedures performed every year in the US will fail to achieve fusion.[27] Based on the results of pre-clinical models,[11,28] Burkus et al[29] have recently reported the results of a multi-center prospective clinical trial on the use of rh-BMP2 delivered on an absorbable collagen sponge (InFUSE Bone Graft®, Wyeth-Ayerst Research) inserted in a hollow threaded titanium cage (LT device®, Medtronic Sofamor Danek) for lumbar spine anterior fusion. This study enrolled 279 degenerative disc disease patients that randomly received either the InFUSE Bone Graft or autologous iliac crest bone grafting. Blood loss was significantly lower in the rh-BMP2 treated patients (109,8cc vs 153,8cc). At 2 years follow-up, 32% of the autograft bone patients still experienced pain at the donor site. Fusion rates were similar in both groups, with a fusion rate as high as 94.5% in the BMP treated group. This high rate of fusion was consistent with a significant improvement of function and reduction of pain as assessed by a standardized low back pain questionnaire completed by the patients. The InFUSE-LT® device has received approval from the US FDA for anterior lumbar spine fusion.

The efficacy of BMPs has also been tested in posterolateral fusion.[30] In a prospective trial, 25 patients suffering from single-level disc degeneration were randomized to receive either autologous bone grafting and the Texas Scottish Rite Hospital (TSRH) pedicle screw instrumentation, or 20 mg of rh-BMP2 per side delivered on a calcium phosphate carrier (60% hydroxyapatite-40% tricalcium phosphate) with or without the TSRH instrumentation. At an average follow-up of 17 months (range 12-27 months), posterolateral fusion was achieved in 100% of the rh-BMP2 treated patients (20/20), as compared to the bone autograft group (40%). These two preliminary studies on anterior and posterolateral spine fusion demonstrated that rh-BMP2 was as efficient as autologous bone grafting to stimulate bone growth along spinal vertebrae. Although very encouraging, these results need to be validated on a larger number of patients and care should be taken to confirm the safety of BMPs implanted for spinal purposes before widespread use. Heterotopic ossification may lead to dramatic restenosis of the lumbar canal, with potential ossification of an exposed dura, and adjacent level stenosis.[31]

Although there has been some overwhelming evidence of the clinical efficacy of the BMPs in numerous indications, several issues persist and are of concern. One issue is the dose of BMP implanted in each trial and the optimal delivery system that remains to be found. The dose of BMP implanted is excessively high (3.5 to 40 mg[30]) when compared to the quantity present in bone, which could set off unpredictable local or systemic reactions: the BMPs are expressed in many tissues (kidney, peripheral and central nervous system, heart, lung…), and have potent effects on morphogenesis and growth in tissues other than bone.[32] Recent studies have concentrated on delivery systems in order to achieve a slow release of BMPs and thus reduce the dose of BMP implanted. Several investigators have focused on the use of bovine type1 collagen gel,[33] but this material carries the risk for disease transmission. One attractive alternative is the use of biodegradable polymer foams that are bioresorbable and biocompatible, and which porosity can be controlled accurately during processing. The development of a suitable drug delivery system would retain the BMP for a longer period of time, and limit the risk of bone overgrowth, immunogenicity, and potential effects on distal organs. With regards to potential systemic effects, one issue that needs to be addressed is how to secure the BMP-carrier implant at the target site. To date, the BMPs are delivered on collagen gel carriers that are not viscous enough and it is hard to ensure they will remain at the bone fracture site when the tourniquet

will be deflated. Although no major systemic effects were reported in the different clinical trials, an immune response was observed in 38% of the patients treated with OP-1 thus preventing from the implantation of another dose of OP-1 in the future.[20]

Another reason for the need in human of high dose of BMPs is probably related to the under-performance of BMPs in higher species as opposed to rodents,[34] and the fact that BMP2 and BMP7 are physiologically part of a complex cascade involving a number of molecules to achieve bone repair. Such a mixture of osteoinductive proteins can be found in the demineralized bone matrix (DBM) that is currently in use in clinical practice. It is noteworthy that the inductive potential of rh-BMP2 or OP-1 is 1 million times that of the DBM, while DBM has given similar results than the application of a high-dose of a single BMP.[19] The use of a high dose of a recombinant molecule raises also the acute problem of the cost of this new technology. This cost, although excessive (approximately 4500 euros per dose), should be balanced by the potential reduction of failure rate of currently available techniques, their morbidity, and the resulting disability. As for any new therapeutic procedure, the cost-benefit of BMPs should be carefully evaluated before they can be routinely applied in clinical practice. The comparison of BMPs to the standard of care in prospective clinical trials is of paramount importance, but the design of the study itself, the choice of outcome parameters, the analysis and interpretation of the data are critical to provide clear and non-ambiguous information to the medical community.[35] In this respect, several parameters might have been considered in previous reported trials:

1. stating that a fracture is healed should be based on both clinical parameters and several x-rays views taken at different time points, given the fact that interpretation of x-rays is very subjective and cannot be blind, as bone grafting is easily recognizable;
2. The effect of BMPs should be easily quantified and distinguished from the action of other potential procedures: tibial reaming and nailing, autologous bone grafting are by themselves effective treatments of bone nonunions and should ideally not be combined with the use of BMPs in randomized trials, because they make the interpretation more difficult;
3. With regards to long bone nonunions, the patients should be at least stratified by the severity of open wound, the presence of infection or not, the length of the bone defect, as the results might considerably vary from one group to another.

Clinical Experience with Bone Marrow Stromal Cells

The efficiency of osteoinductive biomaterials relies on the recruitment of osteocompetent cells from the surrounding tissues. Therefore, their use will be limited to clinical cases where the wound bed can provide these cells, excluding clinical situations involving necrotic areas and large bone defects. To overcome this problem, the use of osteogenic material composed of a scaffold loaded with osteocompetent cells has been proposed.[13]

There are a number of possible sources of bone progenitor cells, each with its own advantages and drawbacks. The osteogenic potential of fresh bone marrow has long been recognised and a number of investigators have experienced some success using autologous fresh bone marrow to augment bone formation in experimental studies. Connoly,[36] Healey[37] and Garg[38] have documented the use of fresh bone marrow in the treatment of ununited fractures. However, no prospective randomized clinical trial has been carried out to definitely demonstrate the efficacy of this practice. In addition, the quantity of newly formed bone is directly dependent on the number of osteocompetent cells implanted, prompting the use of techniques allowing the isolation and amplification of the osteocompetent cell pool. Once the expansion of BMSCs is achieved, the next step in the process of building an osteogenic material is to load BMSCs onto a scaffold. There is general agreement that the scaffold should favor BMSCs adhesion, proliferation and differentiation as well as encourage rapid vascular invasion. Ideally, the new extra-cellular matrix should take on the mechanical function of the template as bone forms, implying that the scaffold should disappear at a rate commensurate with new bone formation.[13] A number of carriers have been evaluated for delivering BMSCs including collagen,

alginate, polylactic polyglycolic acid polymers or calcium phosphate ceramics. However, the ideal scaffold remains to be found.

To the authors' knowledge, there are only two reported studies on the use of osteoprogenitor cells for the repair of bone defects in human. Both are short case reports, respectively on one and three patients.

In the first report, Quarto et al displayed promising results, with the use of a hBMSFs loaded on hydroxyapatite scaffolds to treat large bone defects.[39] There were 3 mid-diaphyseal bone losses, ranging 4-7 cm, in-patients aged 16 to 41 years old. Bone healing was achieved in 2 months, with a good integration at the interfaces with the host bone, and without any adverse reaction. However, owing to the nature of the article itself, which was a short correspondence, it is hard to conclude whether the technology applied here was a clinical success and did improve the outcome of the patients compared to a standard bone autografting. No mention was made on the previous treatments applied, neither on the length and the conditions of cell culture. Furthermore, careful examination of provided radiographs 8 to 18 months after surgery did not show any biodegradation of the scaffold compared to the postoperative x-rays. Resorption rates of calcium phosphate ceramics vary inversely with the calcium phosphate ratio, and hydroxyapatite is known to be almost undegradable when implanted in vivo. It is likely that the choice of the scaffold was here non-optimal.

More recently, Vacanti et al reported the case of a 36-year old man who had a traumatic avulsion of the distal phalanx of the thumb.[40] Reconstruction of this bone was performed using culture-expanded osteoprogenitor cells isolated from the periosteum and seeded on a pre-shaped coralline scaffold. At 28 months follow-up, the thumb had normal length, but strength was reduced compared to the opposite side (25%), and no active motion was observed at the interphalangeal joint. A biopsy of the implant performed 10 months after surgery showed only 5% of newly formed bone within the pores.

Although non-optimal, these studies definitely show that the use of local cell therapy for the treatment of bone defects is feasible, and these modalities will probably supersede the need of bone grafting in the near future. However, to clearly demonstrate the benefits and the superiority of these expensive technologies, they should be objectively compared to established treatments in prospective randomised trials.

Conclusion

Based on their outstanding osteoinductive capacity, the BMPs offer promising perspectives for a large number of patients suffering from long bones nonunions or spinal disorders. The BMPs have proven to offer beneficial effects in these indications and this might obviate the need for bone autograft harvest procedures in the near future. From our point of view, as for any new therapeutic approach, the application of this expensive technology must be limited until clear data on efficacy and safety are available for widespread use in clinical practice.

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Part IV
From the Bench to the Bedside

Chapter 15

Regulatory Issues:
Down to the Bare Bones

Paolo Giannoni and Ranieri Cancedda

Abstract

In this chapter we propose a brief description of the relevant issues that tissue-engineering researchers must consider when planning new therapeutic approaches, especially in the bone and cartilage repair field. An overview of the current regulatory authorities is presented to evidence their role as responsible rule-makers. The influence of the market and of the private enterprises, as well as that of the academic world, is also taken into consideration when defining rules to follow.

We will discuss existing guidelines and legislation, giving an overview of the requirements to be considered when projecting a possible product application in the field of tissue engineering. The reader's attention will be primarily focused on the cell-based composites, being these products the most challenging in terms of application of this approach to bone repair. A general outlook of the requisites for the manufacturing of tissue-engineered products will be discussed, spanning from the origin of the cellular component of the composite to the release criteria of the final product.

The nature of the products or of the therapeutic strategies will not be dealt with, unless they represent useful examples to evidence the related regulatory issues.

Introduction

Progress in cell biology and biotechnology and improvements in therapeutic treatments have made available to clinicians tissues to be used in reconstructive surgery for the repair of extensive lesions. In particular, a role for several growth factors controlling cell proliferation and differentiation has been established and cell culture techniques have been improved to allow the in vitro selective expansion of restricted cell populations. Tissue engineering, i.e., the association of ex vivo grown cells and/or biologically active molecules with different materials, is furthermore driving the production, the testing and the marketing of new generations of transplantable biomaterials. These can now be used in unlimited quantities. Ideally the new approaches based on cell-biomaterial associated products will substitute the previously used devices which do not represent sufficient or satisfactory solutions. Repair of soft tissues—previously hardly feasible—may now be envisioned and performed by transplanting the engineered tissue obtained from the expansion of cells of the patient in association with resorbable materials (either synthetic or of natural origin).

The first "manipulated" cells intended for structural repair or reconstruction were autologous keratinocytes expanded ex vivo for treatment of burn wounds and leg ulcers. Additional examples were chondrocytes cultured and implanted in focal cartilage defects, allogeneic dermal fibroblasts for dermal wound healing and the transplant of specific endocrine cells. Other applications of the tissue engineering approaches presently at a preclinical or very early clinical

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stage are the repair of skeletal and cardiac muscle fibers, the implant of fat cells for cosmetic augmentation, the realization of vascular prosthesis and the selection of cells with a neuroregenerative potential. Each of these applications may also take advantage of a possible genetic modification of the patient's or of the donor's cells by the insertion of a modified, or repaired or new gene.

Commercial distribution of such products could provide a relatively simple solution to a common type of injuries, satisfying the needs of a large number of patients. When commercial establishments began to provide these manipulated cells to surgeons within the United States it became clear that such a class of products had not been explicitly considered by the FDA. Moreover the manipulation required was much more than that needed for autologous bone marrow transplant, where the source tissue is harvested but hardly "processed" at all.

Tissue Engineering of Bone

Particularly, in the repair of bone and cartilage, the tissue engineering approaches are seeking to address the needs by the realization of viable substitutes that restore and maintain the function of the two types of tissues. With respect to standard drug therapy or permanent implants, the tissue-engineered bone becomes integrated within the patient, affording a potentially permanent and specific cure of the disease state. Any approach involves one or more of the following key ingredients: harvested cells, recombinant signaling molecules and three-dimensional matrices. Ideally the cells would attach to the scaffold and proliferate and differentiate under the control of endogenous and exogenous growth factors to ultimately organize into normal healthy bone or cartilage as the scaffold matrix degrades.

Orthopedic surgeons had previously used autologous tissues (for ex. rib cartilage) without regulatory board oversight. It was immediately obvious that different rules should apply to tissue engineered products. For example, chondrocytes dissociation from healthy tissue and their ex vivo expansion (to reach the proper number for reimplantation) would fall under the somatic cell therapy product definition. Nonetheless, since chondrocytes were implanted in defined lesion sites, contrary to systemic therapies, this treatment showed similarities with some device products.

In defining rules, one must take into account the following: (i) the need of unconventional culturing techniques. Cells do not organize into tissue or organs simply by culturing them in standard media, nor are they able to acquire the proper phenotype without specific signaling/differentiating factors; (ii) the need of appropriate matrices and/or biomaterials that provide the required structural scaffold properties at the lesion site and that allow to be remodeled while the new tissue is being formed. Combination products, derived from somatic cell and/or gene therapy products grown on different scaffolds may evidence different biological properties depending on the scaffold itself; (iii) accessibility of the lesion site by the specific surgical techniques needed to implant the cell/biomaterial construct.

Since the chemical/physical properties of many synthetic matrices can be easily defined and controlled and the surgical procedures needed for the new therapeutic applications can be easily overviewed, the real new regulatory challenge is posed by the combination products and the absence of rules for the manufacturing of their cellular components.

With respect to their mode of action, combination products can be divided into two main categories: the metabolic support systems and the tissue repair/replacement products. Products for bone and cartilage reconstructive therapies normally fall under the latter category. Cells are either autologous or allogeneic multi or pluripotent cells—stem cells included—all of which could also be genetically modified. The device part is generally identified as "natural biomaterials" (collagen sponges, amniotic membranes, demineralized bone) or as "synthetic polymers" (PLGA-derivatives, ceramics). They can be used either for temporary or permanent implants in a wide range of applications.

Tissue-Engineered Products: Drugs, Biologics or Devices?

Cell and gene therapies represent new approaches not feasible only a few years ago. They are focused on the patient, in many cases the human subject being the source of his/her own means of cure (autologous cell therapies). The first worldwide authority organization to recognize the need of appropriate guidelines for this type of therapies was the American Food and Drug Administration. It is of relevance that the US attention to the issue came as a direct consequence of the vital framework that interconnects the industry and the academic world in the North American scientific community. Presently several national and international public offices deputed to drug regulation, among which the Center for Biologics Evaluation and Research (CBER) of the american Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA), are developing rules and guideline for the manufacturing and use of tissue engineering products.

Initial regulations in the field were derived from the interim rule for banked human tissue promulgated in the American federal register on December 14th, 1993, entitled "Human tissue intended for transplantation".[1] In this document banked human tissue products are defined as "…derived from a human body which: (1) is intended for administration to another human for the diagnosis, cure, mitigation, treatment or prevention of any condition or disease; (2) is recovered, processed, stored, or distributed by methods not intended to change tissue function or characteristics; (3) is not currently regulated as a human drug, biological product, or medical device; (4) excludes kidney, liver, heart, lungs, pancreas, or any other vascularized human organs; (5) excludes semen or other reproductive human tissues, human milk and bone marrow…". In addition, recognizing that the sponsors of the developing tissue and cell based therapies were willing to render their products commercially available, another note was issued on the federal register within the same year, defining the somatic cell therapy products as "…autologous (self), allogenic (intra-species), or xenogenic (inter-species) cells that have been propagated expanded, selected, pharmacologically treated, or otherwise altered in biological characteristics ex vivo to be administered to humans and applicable to the prevention, treatment, cure, diagnosis, or mitigation of diseases or injuries…".[2] In this document a relevant statement was promulgated by the FDA, and widely accepted by the international scientific community: the definition of "manipulation of cells" was described as the ex vivo propagation, expansion, selection, or pharmacological treatment of cells or other alteration of their biological characteristics.[2] Clear emphasis was posed on the need to precisely frame a "biological characteristic" (unique and identified by any specific technical means) and its "normal" or "altered" condition.

Nonetheless these regulations were insufficient for a higher scale exploitation of the technology and for its transfer to the market, mainly for the temporary absence of reference standards, of product quality assessments and of lot release controls. Instead, the required screening of the starting material was already partially regulated, taking advantage of the issues dedicated to the transplantation of human tissues.[1]

Thus the industrial and the private investments world were looked upon as proponent counterparts by the regulatory boards. On the contrary the academic world hardly interfaces with the legislator, traditionally focusing on basic research as a primary goal. Academic scientists may often be applying an innovative approach that clinically works but that was never screened nor approved by any official board and/or national commission.[3] It should be stressed that, as a consequence, these therapeutic approaches have grown to the status of "possible marketable products" either from only partially controlled clinical trials or directly off the bench of academic institutions. In this respect, therapies that take advantage of autologous cell culturing often derive the culture conditions directly from the optimizations run on the laboratory benches. Usually several media are tested, with different self-made modifications, indeed needed for specific cell types or for particular growth conditions. Nonetheless, ancillary products, such as growth factors, cytokines or chemicals in the culture media, not intended to be part of the final product, might affect its quality, effectiveness, or safety.

Unfortunately innovative therapies were already shown to carry potential unwanted effects, besides being sometimes unclear regarding the benefits gained by the patients as already tragically experienced in the case of gene therapy.[4-7] Furthermore, few preclinical data have been published and toxicological evaluations are seldom found in the literature for the ancillary substances that may still contaminate the final product. Nontissue components, even though to a lesser extent, could also represent means of unwanted effects. It should be stressed that tissue-based products that are intended for therapeutic use, that contain synthetic or mechanical components and that achieve their primary mode of action by means other than metabolic or systemic action are regulated as devices, including combination products. In the United States, their evaluation should therefore be carried out by the Center for Device and Radiological Health (CDRH) of the FDA.[8] Premarket investigations and clinical trials would be required under investigational new drugs (IND) or investigation new device exemptions (IDE), as appropriate.

Composite products for tissue-engineering of skeletal tissues are comprised of two or more regulated components, i.e., drug/device, biologic/device, drug/biologic or drug/device/biologic that are physically, chemically or otherwise combined or mixed and produced as a single entity.[9] Composites such as a biomaterial associated to bioactive molecules (growth factors, cytokines, etc.) may ultimately be considered as devices exerting their action as a drug, and therefore be regulated by the Center for Drug Evaluation and Research (CDER) of the FDA. Other composites, such as a porous ceramic associated to autologous osteogenic cells, would obviously fall under two categories. Wisely the FDA has recognized the possible overlaps of competence in the evaluation process of these new therapeutic agents. In the same document, therefore, a clear statement was made to sustain the setting of a Tissue Reference Group, composed of six members, three of the CBER and three of the CDRH, to assist in making jurisdictional decisions and applying consistent policy to these products.[8] The bottom line is that the mode of action of the combination product, once defined, dictates which FDA branch will review applications and draw the rules for the therapeutic use.

In the "Proposed approach to regulation of cellular and tissue-based products" issued by the FDA it is also stated that "...for combination products with synthetic or mechanical components (which comprise the largest class of combination products), clinical trials and marketing applications must address the clinical safety and effectivness of the overall product, as well as the function and compatibility of the synthetic or mechanical components. The agency principal concerns are that they function correctly, that they last a predictable and adequate length of time and that they are compatible with surrounding tissues...".[8]

Thus the real challenge for national and international regulatory boards is how to step into a guideline and law proposal strategy that grants efficiency and at the same time sufficient flexibility to meet the patients' needs and rights, the scientific innovations and the market-oriented enterprises.

The Backbone of the Current Regulatory Frameworks for Cell Therapy Products

This section is a summary of most recent and updated guideline for design/manufacturing of a tissue engineered product to be used for cartilage/bone repair. It should be noted that any nation may have considered or may be considering to introduce, apply and enforce additional regulatory requirements.

In this overview, we will not encompass the current issues that rule the procedures used to manipulate whole organs or minimally-manipulated bone marrow (both of which are under the Health Resources and Service Administration in the US), or the transfusable blood products (i.e., whole blood, red blood cells, platelets and plasma) which are ruled separately. We will not deal also with the current framework for tissue-related products, such as those derived from animals, with products used in the propagation of cells or tissues, or products that are secreted from cells or tissues, (such as human milk, collagen or growth factors).

Cell Therapy Products

In the USA, the Center for Drug Evaluation and Research (CDER) has been assigned as the FDA component that has the primary jurisdiction for the regulation and the premarket approval of the products derived from autologous cells manipulated ex vivo (MAS cells). Clinical investigations of MAS cell products should be performed in accordance with the requirements for investigation of new drugs and they are subject to licensure as biological products. The current Good Manufacturing Practice regulations (GMPs) will apply, although FDA intends to consider on a case-to case basis alternative approaches to specific regulations, when these may be impractical or unnecessary to assure safety, purity and efficacy of the product.[10]

Establishments engaged in the manufacture of the MAS cell products should register as a drug manufacturer, and should be subjected to inspections on a biannual basis by a representative of the CBER and by an investigator from the closest FDA District Office.

European or Asian nations will refer to the European Agency for the Evaluation of Medicinal Products (EMEA) or to national authorities for regulatory jurisdiction and similar controlling actions. It should be stressed that US officials will not allow marketing within the USA of MAS cell products manufactured in a plant outside USA, unless directly approved by the FDA or unless evaluated through a FDA-approved review process.

Whether the MAS cell product will be applied by public institutions or sponsored by private companies, investigative clinical studies will be required. The FDA guidance on "Applications for products comprised of living autologous cells" also state that "...Prospective randomized clinical studies traditionally have been the best way to demonstrate safety and efficacy. However, where studies of MAS cells without internal patient controls provide evidence of effective structural repair which substantially and clearly represents improvements in outcomes compared to patients in an appropriate historical databases, this may be sufficient to demonstrate efficacy...".[10]

To follow the proper guidance for clinical trials, early contacts of the proponents with the relevant authorities are strongly recommended. Previous to any activity, the structures where the tissue engineered products will be manufactured and clinical experimentations will be carried out need to be credited and certified by the competent authorities.[11,12] Within the term "structure" several items are included, such as room and building requirements, hardware requirements, as well as legal and clinical/scientific responsible officers.

In several European countries, Ethical Committees within hospitals have the power to approve or refuse clinical trial proposals on the basis of the risk assessment for the patient. They also are the proper administrative body to overview adherence to international and national law requirements including the existence of adequate manufacturing structures. If the use of a new drug/device/biologic is suggested and the risk evaluation is undetermined or unclear, as in most cases of tissue engineered products, the appointed committee may directly request the Ministry of Health and/or national health institutions approval. In the case of Italy, for example, this is the proper role of the Istituto Superiore di Sanita'.[13] The Istituto Superiore di Sanita' would require, in turn, immediate interaction with the proponents to establish a controlled phase I clinical study, according to all the GMPs, law and guidance recommendations.

Manufacturing Facilities

Following are some specific points to be considered before proposing a clinical study to prove safety and efficacy of a tissue engineered product.

General Room/Building and Hardware Requirements

- all cultures for tissue engineering purposes will have to be performed aseptically and by using sterile instruments;
- source tissue harvesting and cell culturing must be performed in GMP approved rooms. Whenever necessary, as for gene therapy applications or as decided on a case-to-case basis by the appropriate authority, in biosafety containment level 3 rooms (BL3; the rooms must

be provided of controlled access, air locks, biosafety cabinets where no work is allowed on open benches; all surfaces must be sealed, all penetrations—telephone, lights, gas, vacuum, electrical line, water—are to be caulked, collared or sealed to prevent leaks, and negative airflow pressure, doublebacked ventilation system and absolute filtration units must be available, working and properly maintained);

- rooms where cell cultures are performed for clinical use must be used for that purpose only; in the case of research institutions performing a few tissue engineering cultures in a limited period of time, dedication of the same cell culturing rooms to other activities can be accepted by the competent authority upon validation of the cleaning and sterilization procedures prior to cell culturing for clinical applications;
- rooms must be scheduled for routine cleaning procedures and sterilization; additionally BL3 rooms must have independent accesses; operational procedures in the cell culture rooms must avoid cross contaminations between cells derived from different donors/patients (spatial of temporal separation are strongly suggested);
- all the hardware used in cell culturing for clinical applications should be provided of specific sensors and visual/acoustic malfunction alarms (i.e., freezers, incubators, laminar flow hoods, cryogenic hardware); backup apparatuses must be available, working and properly maintained);
- certified procedures must ensure sterility of the outgoing products, either from viruses, mycoplasma or bacteria;
- certified procedures must ensure the tracking down of any used material in any produced lot of final products;
- for each manufacturing location a floor diagram should be included that indicates facilities layout. The diagram does not need to be a detailed engineering blueprint, but rather a simple schematic that depicts the relationships between the different rooms of the manufacturing areas and that indicates the use of adjacent ones. The diagram must report the flow of production of the biological substance and any other specific activity in dedicated rooms (for example storage of raw materials, quarantine, etc.)
- in the absence of any specific regulation, Good Laboratory Practices (GLPs), GMPs and ISO 9002 certifications must be considered as the normal working standard.

For clear economical and technical reasons, controlled areas and rooms are normally included and surrounded by lower-requirement areas (i.e., where the control parameters, such as air purity, sterility, safety containment are less strict). This disposition reduces costs to maintain elevated safety levels. The interfaces between areas of different levels are critical: while planning the production process, fluxes of materials/operations from the interior to the exterior of the different areas must be considered. Segregation of the different areas can be obtained by dislocation (low pressure, high flux) or differential (high pressure, low flux) air pressure between the areas. This concept applies to both sterile and nonsterile productions and is summarized in Figure 1.

Personnel Requirements

- a legal representative, distinct from the scientific/clinic responsible officer, must be identified in each structure;
- the scientific/clinic responsible officer must have a proven expertise in the biomedical field of interest;
- a quality control responsible officer must also be identified, distinct from the scientific officer. Only in the case the structure performs a few cell cultures in a limited amount of time the scientific/clinic responsible officer can serve also as quality control responsible;
- personnel must be aware of the standard working operational procedures as well as of the safety and emergency procedures; in this respect the European Countries are also committed to follow international guidance on the safety issue, as indicated by the european directives on safety measures.[14]

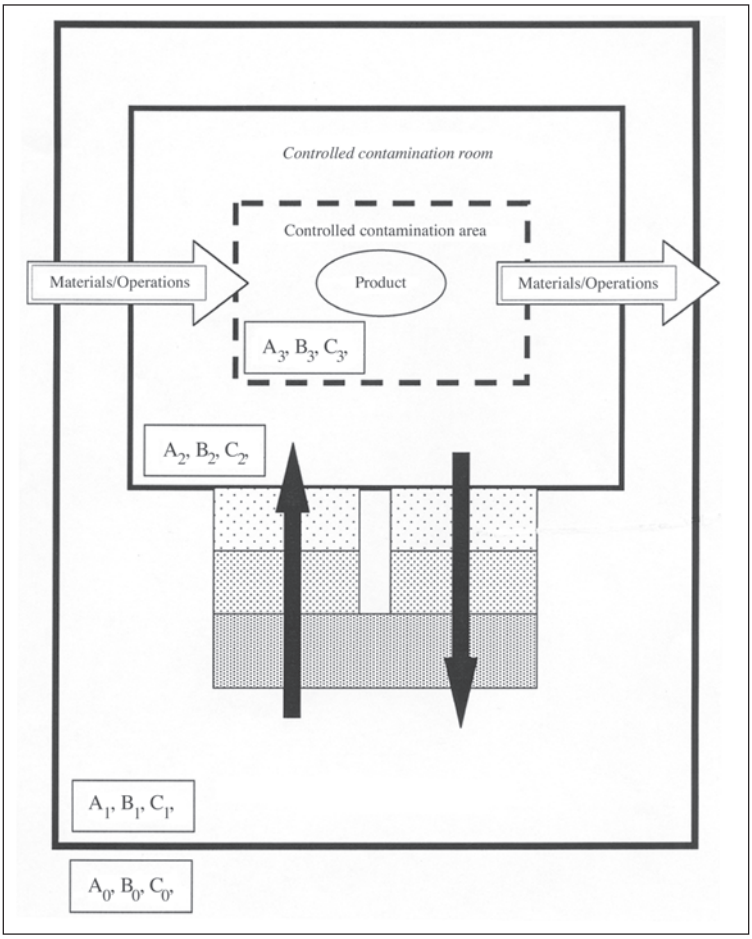

Figure 1. Schematic of the fluxes of materials/operations/personnel through controlled contamination areas. Continuous lines represent means of physical separation (exterior walls, interior walls, transfer compartments) or of contamination containment (laminar flux protected areas). Dashed lines borders the cleanest area. The different areas are monitored for the same parameters (boxed letters, A, B, C) which have to meet the specifications required for each area (A_0, A_1, A_2, A_3,...A_N). Fluxes of materials and operations are depicted as white arrows, whereas black arrows identify personnel'ins and outs. Access and exit are allowed through confinement rooms, from the dirtiest (dotted) to the cleanest zone (white), and viceversa. Note that separate entrance and exit are recommended whenever possible.

Product and/or Process Quality Assessment

In a final rule issued in 1996[15] the FDA has amended the definition of "manufacturer" to include an applicant for a license. The definition broadly encompasses academic laboratories and clinics which would assume responsibility for the safety, purity and potency of the final tissue engineering products even if not directly involved in significant manufacturing steps. By converse, "manufacturing" becomes broadened to include the more-than-minimal manipulations occurring in autologous cell culturing, as already described.

Whether it comes from the academic or the private enterprise world, the proper documentation must be submitted to the relevant authorities for the peer review and approval

procedure. So far all official authorities of countries where a tissue engineering activity is being performed have substantially agreed to request similar detailed information for the evaluation process of those products that include cells, either for autologous or allogeneic use. In particular they all request:

- scientific rationale and objectives of the clinical experimentation;
- characterization of the starting cell population and of the auxiliary components;
- procedures used to manipulate the cells and the auxiliary components;
- description of the cell banks (where needed);
- characterization of the final cell and/or tissue engineered product;
- procedures for quality control of lots of the final products;
- preclinical documentation on the toxicity of the final product;
- preclinical documentation of the final product efficacy.

To solve the lack of standards, new techniques must be set on a case-to-case basis. Typically the active principle in the cell therapy based products is poorely durable. The available cells are usually scarce and their number not sufficient for extensive quality control tests. In the case of severely aggressive diseases, the use of autologous cells imposes defined temporal limitations: a prolonged expectance before the implant may hinder the patient's condition or alter his/her morbidity to the treatment. In addition cell products normally need to be used few hours to a few days from the time of the product preparation.

Since autologous cells are to be used in the same donor/patient, and given the above mentioned limitations in lot quantities and timing, the number of applicable tests can be reduced. For example the official sterility test of the European Pharmacopea takes no less than fourteen days to be performed. Clearly, the short half-life of cell therapy products may require their use in advance to the availability of test results; nonetheless"...the manufacturer must still employ appropriate controls to provide assurance of safety, purity and potency of MAS cell products...".[10]

The requested documentation is necessary not only for the donor/patient's risk assessment, but also for the analysis of the therapy efficacy. For the FDA, in order to use and market a MAS product, there is no need to demonstrate its superiority with respect to other existing drugs/therapies.[10] On the contrary, the Committee for Proprietary Medicinal Products of the EMEA (CPMP), and therefore the national regulatory boards of the European Community countries, require a clear evidence of its effectiveness. In fact the European Committee follows a more restrictive rule, probably considering a higher intrinsic risk as associated to the autologous cell therapy with respect to other approaches.

Risk Assessment Analysis

A proper application and risk management/assessment analysis should take into account all the following factors:[8-12,16-17]

Origin of Materials to Be Used in the Composite Product Manufacturing

All culturing media, either for cells or tissue cultures, should be certified for their composition, purity, salt and organic component content and indicate a defined expiry date. Similarly all the auxiliary materials which will be part of the final product (i.e., scaffold matrices, biopolymers, ceramic components) or that are used in the manufacturing processes (i.e., plasticware, disposable materials, common reagents) should be certified by their respective producers through identified quality assessments. Lot numbers must be registered. For each chemical used, in addition to the lot number, one must register manufacturer, production date, purchase date, expiry date, composition, purity grade, biological activity and known contaminants.

All material used in the manipulations of the cellular component of the composite should be certified for clinical usage. Unfortunately for many of the culturing media components (cytokines, growth factors, antibodies, etc.) this standard is not available; however, for the ancillary products whose function is necessary only during the manufacturing procedures, a proper assay that determines their absence in the final product would be sufficient to grant their use.

All materials must be checked in terms of sterility, apirogenicity and absence of contaminating agents.

Use of any substance that may induce immune response (animal serum, serum proteins, antibiotics, antimicotic agents) should be avoided or limited to the very first manipulation procedures; indeed these substances must be absent in the final product.

Specific attention should be posed to the use of fetal bovine serum, due to the serum-associated risk of transmission of unknown pathogens. Ultimately the use of human serum, if necessary, must be properly justified.

Donor/Recipient Selection

Donors can be living or deceased. Obviously, for composites that include autologous cells, only living donors are considered. Donor files should include the following:

- identity code for each donor;
- name, age and gender; age should be limited, according to the most recent data available, to ensure proper cell response;
- health status;
- date and time of the biopsy;
- biopsy characteristics (procedures applied, size, purpose, conservation means). Logistic of biopsy shipment (sending and receiving institutions, way of travel, etc) should also be recorded.

Donor/Recipient Exclusion Criteria

Donor's cells must be preventively addressed as for autologous or allogeneic use. Allogeneic therapies are associated to a high potential risk of transmission of pathogens. Donors should be excluded if:

- affected with extended infections or septicemia;
- positive to syphilis, type B hepatitis, type C hepatitis, AIDS (or if included in AIDS-risk associated categories), HIV-I and/or HIV-II, Creutzfeldt-Jacob syndrome (CJD);
- affected with neurological diseases of viral or unknown origin;
- previously treated with the human growth hormone and/or drugs derived from the human hypophysis;
- previously treated with dura mater;
- previously treated with demineralized bone; demineralized bone falls under a different regulatory panel, as described in a following paragraph;
- previously treated with bovine pericardium;
- affected with malignant tumor(s);
- affected with genetic diseases that may compromise the recipient tissue response to the composite application;

All the donors/patients will repeat testing 6 months after inclusion in the protocols for those pathologies for which a positive test response is not immediately detectable after infection. For a second screening, alternative tests may also be applied, such as the polymerase chain reaction (PCR), if of proven validity.

Cells or tissues of donors found positive to exclusion criteria should not be acquired. If acquired they should be destroyed. This rule may not apply to autologous transplantation procedures or in rare cases of allogeneic therapies for which either the syndrome gravity or the donor's genotype rarity make other therapeutic approaches unfeasible. In theses cases the competent authority must also certify the conservation procedures of biological material explicitely identified as "biohazardous"; all the procedures for the collection, disposal and conservation of potentially biohazardous material must be described in detail prior to approval.

Prerequisites for Cell Banks (If the Cell/Biological Component of a Composite Is Retrieved from or Storaged Into)

The current guideline suggests to preserve any specific and characterized cell population in a cell bank to be able to recover the cell population used for somatic therapies. This may not be feasible for therapeutical applications, such as in the case of cartilage reconstruction where the autologous chondrocytes are few and can be expanded for a limited amount of cycles.[18] The "bank" itself should be composed of the "main storage cell bank" (MCB) and of a "working cell bank" (WCB).[11] The MCB is defined as the collection of aliquots of homogeneous cell populations that have been previously characterized in terms of genotype, phenotype, biological functions and purity and that, when feasable, were obtained by clonal expansion. The MCB must be preserved in at least three different locations in appropriate liquid nitrogen containers.

The WCB is the collection of the aliquots of an homogeneous cell population obtained from a single aliquot of the MCB via in vitro amplification for a defined amount of time and/or cell cycles. Cell culturing conditions must be described in detail. Aliquots, obtained either from the MCB or the WBC cannot be reinserted in the bank once used. The cellular component of the composite originates from one WCB aliquot.

Procedural operations performed to constitute an MCB or a WCB must be described in detail. Issues related to the cryopreservation of the cells are critical. The specific protocols must indicate:

- the composition of the preserving solution. Several solutions are commercially available. It is mandatory that cryopreservants are eliminated from the cell suspension before use. A low toxicity at the concentrations used and a total absence of mutagenicity effects are also mandatory;
- the precise temperature at which cells must be preserved; alarmed and backed-up systems are required for -80°C freezers and liquid nitrogen containers to prevent power and/or temperature failures. Upper and lower limits for working temperatures must be determined;
- the maximal duration of the preservation time for every manufactured product (to be estimated with proper experiments if not available);
- operational procedures and timings for freezing and thawing passages (in most cases, cytotoxicity of the cryopreservants at standard room temperature imposes an extremely rapid thawing procedure);
- the detailed description of all the parameters to assess the cell viability in the in the expanded cell population on a short and long term (colony forming efficiency and life span, respectively). Procedure must grant a defined percentage of viable cells;
- the precise location of each container. Aliquot amount and identity of each cell/tissue component should be recorded on a specific register.

A proper risk assessment/management should anyhow follow the general scheme proposed in Figure 2 (adapted from EN-ISO 14971).[17]

Procedure (Checking in Vitro Expansion and/or Engineering of the Cell Components)

Cell components are potentially subjected to several biological risks during the expansion and/or storage phases; to ensure the manufacturing quality, intermediate and final products should be tested for the following:

Cell Identity

The identity and composition of the cell population through the different manufacturing phases must be determined. A set of genotypical, phenotypical and functional markers must be available to follow and unequivocally identify the proper cell type.[18]

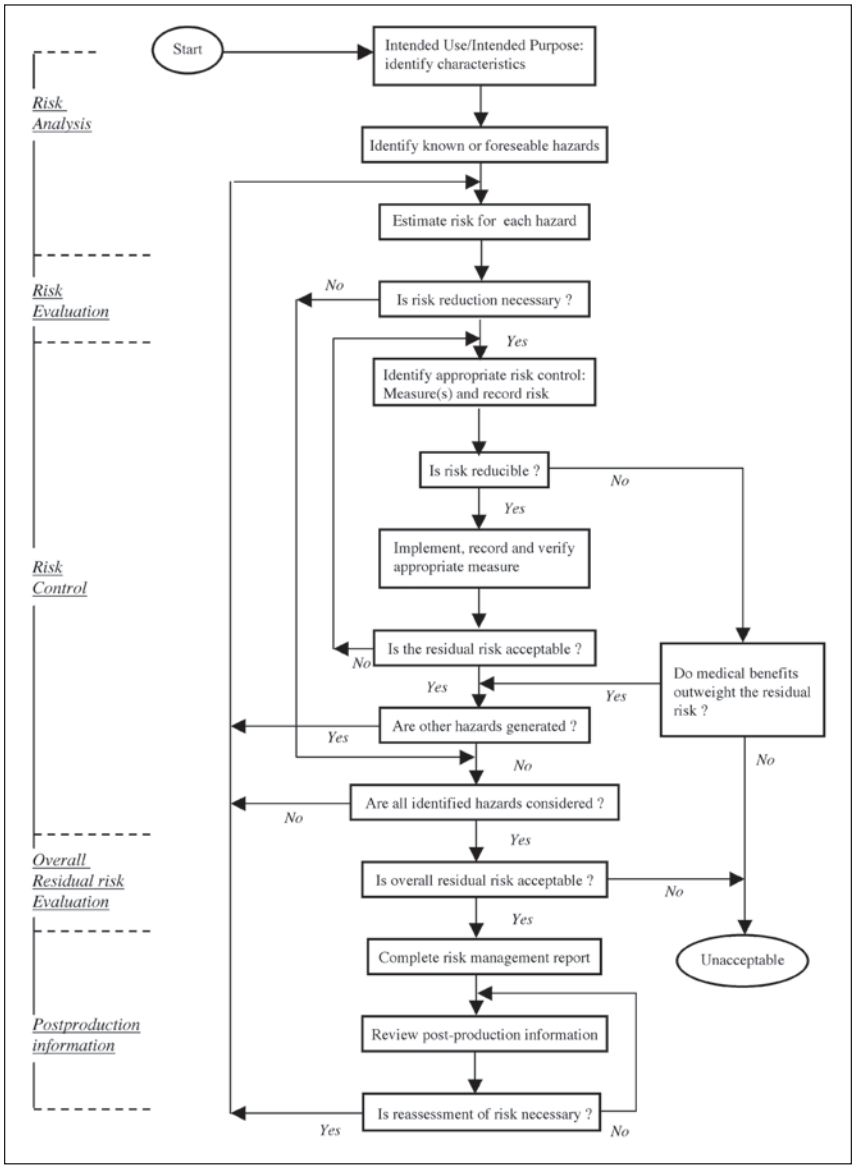

Figure 2. Schematic summarizing the risk management/assessment for a GMP/GLP and/or certified procedure.

Contaminating Agents

Risk of bacterial, viral, micoplasmic, fungal or yeast contamination during the manufacturing process and in the intermediate/final products must be reasonably excluded. It is responsibility of the manufacturer to grant the purity and safety of the final product.

In the U.S.A. the FDA requires testing of blood samples from allogeneic donors of hematopoietic stem cells in order to prevent the transmission of communicable diseases. The implementation of regulatory procedures, paragraph A-2, states: "...For peripheral blood

stem cell donors, the donor's blood, and for umbilical cord blood donors, the mother's blood, would be required to be tested for HIV, cytomegalovirus, HTLV, syphilis and hepatitis infection....physical examination of perspective donors would include screening for high risk for HIV, hepatitis, CJD and tuberculosis".[8] The same paragraph also states that "..the agency intends to recommend, but not require, that testing be performed when stem cells will be used in the person from whom they were obtained. In such a case the agency would recommend only thed following tests: HBsAg, anti-HCV, anti-HIV-1, anti-HIV-2, HIV-1-Ag and HTLV-I/II. The agency also would recommend that the history and physical examination of the donor include screening for high risk HIV and hepatitis. The agency would require that record-keeping and labeling reveal which of the recommended tests were performed and their results, as well as which of the recommended tests were not performed".

Nonetheless, for tissue engineering purposes, autologous cell preparations can also be obtained from donors resulting positive to some of the exclusion criteria. As for the circumstances justifying the use and storage of such cells or tissues, the agency requires " (1) that the cells be labeled as "biohazard", (2) that autologous products also be labeled as "autologous use only"; (3) written advanced informed consent of the recipient be documented; (4) there be documented concurrence of the recipient's physician before the cells could be released from the cell bank."[8]

Aging

Primary human cell divide for a limited number of cycles, undergoing a senescence degenerative phase thereafter. A senescent cell may also have lost the biological properties of the young differentiated phenotype that physiologically constitutes the tissue of origin. Senescent cells can be useless for tissue engineering purposes. It is therefore necessary to record number of doublings of any cell population used for tissue engineering purpose, starting with the biopsy and including procedural steps before and after the cryopreservation passage, if performed. The number of the in vitro doubling must not hinder the proliferation capacity of the expanded cells.

Chromosomal Alterations

Prolonged culturing of mammalian cells increases the risk of quali-quantitative chromosomal alterations in the cell genome. Standard karyotyping techniques should be applied to evaluate the stability of the expanded cell population karyotype.

Differentiated Phenotype Loss

During the in vitro culturing cell may loose, partially or totally, the expression of specific markers of their differentiated state. By converse they may acquire the expression of new markers that are not typical of their physiological phenotype. Both of these events may compromise the cells future reinsertion in the tissue of origin. Such an eventuality must be verified by means of immunohystochemical analysis, possibly targeting specific gene products of the tissue under investigation (for example keratins for the epithelia). The possible existence of a contaminant cell population harvested at the time of the biopsy—able to propagate and progressively substitute the cell population of interest—must also be exploited. Immunocytochemistry and PCR techniques are the suggested approach to tag and check this eventuality.[18]

Neoplastic Phenotype

The neoplastic transformation is a rare phenomenon in mammalian and human cells. Nonetheless the exposure to several ancillary products (cytokines, hormones, growth factors, viral vectors) may alter the cell predisposition and increase the risk of neoplastic occurrences. Absence of risk must be assessed before the use of the cell/composite product in humans either by in vitro culturing techniques or by transplantation in immunodeficient animals. Assays should be planned for the process quality assessment if not feasible for each different human autologous culture.

Quality Controls for the Final Product[19]

Purity Quantity and Biological Activiy

It is necessary to define the required dose of the manufactured product to obtain the biological/therapeutic effects. Doses (for example the average cell concentration in a given composite product) may be derived from preclinical experiments or may vary according to each donor's cell responsiveness to culturing conditions (typically cell response depends on the donor/patient's age/health condition).

Culture conditions may also influence the cell(s) properties or responsivness to the ex-vivo expansion; it is therefore necessary to assess the quality of the cell product by means of appropriate markers. In the case of cultured chondrocytes, for example, a certified counting method would ensure proper delivery of a correct amount of expanded cells for reimplantation purposes. At the same time, as already mentionned for the control of the cell identity of possible phenotype loss, culturing conditions must ensure the maintenance of a collagen type II producing phenotype in the manufactured chondrocytes.[18] The determination of this typical cartilage marker can be assumed as one of the possible standard assessments to be included for the validation of the manufacturing procedures. Clearly it would not be wise to consume the final product (i.e., manufactured chondrocytes) to demonstrate their collagen type II expression, leaving none to reimplant in the donor/patient. Therefore down-scaled tests should be demonstrated as proper and applied when feasible. Alternatively, the quality assessment procedure must be validated. Obviously proper markers must be identified for each specific product.

The biological activity of the final product can also depend upon the site of the therapeutic application with respect to the location of the tissue of origin of the cell component. In this respect the FDA operates a distinction between homologous and nonhomologous function whether or not the product is a structural tissue.[8] A structural tissue engineered product is exerting an homologous function when used to replace an analogous tissue that has been damaged: examples include bone allograft obtained from long bones but used in a vertebra; skin allograft from the arm but used on the face and so on.

An example of nonhomologous use is cartilage placed under the sub-mucosa layer of the urinary bladder to change the angle of the uretra and thus preventing the backflow of urine: the cartilage would be acting as a structural support in a district where that support does not normally exist. Another example of nonhomologous use of a cellular product would be the treatment of metabolic disfunctions with stem cells, intended to perform a different function other than the hematopoietic reconstitution. It is evident the need of increased quality controls either for the manufacturing process or for the final products in case of tissue-based products that are used for nonhomologous functions.

Toxicity Studies[11,20]

Toxicity studies must be performed, as for pharmaceutical drugs. In certain cases the use of human cells in living experimental animals may induce toxic/immuno response due to the inter-specie differences. Therefore proper toxicity studies on the final product must be conducted in immunodeficient animals. Proper species must be selected to give an experimental answer as close as possible to that of humans. For example if the new therapy is based on the effects of a cytokine secreted by transplanted cells, tests should be performed with animal cells that possess receptors for that molecule and where that molecule induces the same biological effects. Undesired toxic effects in body districts other than the ones subjected to the therapy must be clearly reported.

Biological Product Stability

A detailed description should be provided of the storage conditions, along with study protocols and results supporting the stability of the lot/batch of the final product. Stability data must be submitted for the final product as packaged in the container in which it is to be shipped/marketed. If the product is to be shipped under conditions in which cell viability is to

be preserved, data must be given to confirm the final product stability under the scheduled conditions. Detailed information must be provided concerning the supplier, address, compatibility results and biological tests.

In the receiving center, an analysis of the container, its closure system, its compatibility with the biological product together with the expiration date should be performed. For sterile products evidence of container and closure integrity is mandatory.

Release Criteria

To evaluate manufacturing consistency and standard adequacy of the products, at least three set of results of quality control tests, performed on independently manufactured complete products, must be submitted under the supervision of the officer responsible for the clinical application.

The documentation should include release criteria, i.e., all procedures and parameters needed to evaluate product transportation condition and its integrity and sterility. In this respect temperature and pH indicators and means of visual inspection of the final product security are recommended. Procedures for release refusal must also be planned.

Demineralized Bone

A separate evaluation is given for the demineralized bone. Decalcified freeze dried bone allografts are considered as unclassified devices, rather than tissues. This view is due to the more-than-minimal manipulations needed to obtain the final manufactured product. The device definition only applies if the allograft is processed to demineralize and preserve the bone to be used as a bone filler in orthopaedic and/or dental applications. In this case, as a Class I medical device, bone allograft would be exempted from premarket notifications and GMP requirements. In contrast, powdered bone (freeze dried bone allografts) falls under a different legislation[21] being defined as a minimally-manipulated tissue since the process does not change the integral structure of the tissue.

Conclusions

The emerging field of tissue engineering is rich of activity and promises. In the bone and cartilage repair area many of the tissue-engineered products are based on the use of a cell component associated to a natural or synthetic biomaterial. As for classical pharmaceutical drugs, the criteria for their manufacturing need to be refined and updated in the light of the achievement of the safest and most efficient product. It must be considered, though, that the cell components of the composites are extremely complex open systems,[22] on which our present knowledge and control are much more limited than the ones needed to perform a rather simple chemical synthesis. A great deal of research, data and understanding are therefore still needed to improve the state of the art in the field. This involves the public institutions and the private enterprises, but it also calls in for public discussions. New therapeutic approaches pose several ethical questions along with technical doubts, as in germline therapy for example, but they also demand solutions to widespread debates before society comes to their acceptance.[23] In this respect the function of the regulatory boards in the different nations becomes extended not only to define the rules, the standards and the manufacturing requirements but also to inform the public opinion and represent it.

In the light of the new role that the patient will have in the future, possibly being the means of his/her own cure, regulatory authorities have to take into account all the legal issues to protect the patient/donor's rights. In spite of the differences of the legal, medical and political issues in the different countries a wider distribution of the same standard techniques and references will make possible for experts to compare different protocols and/or products, allowing a better evaluation of the therapeutic advantages for the patients.

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Index

D

E

F

G

H

I

K

L

M

N

O

P

Q

R

S

T

V

W

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