Arthur J. Sytkowski

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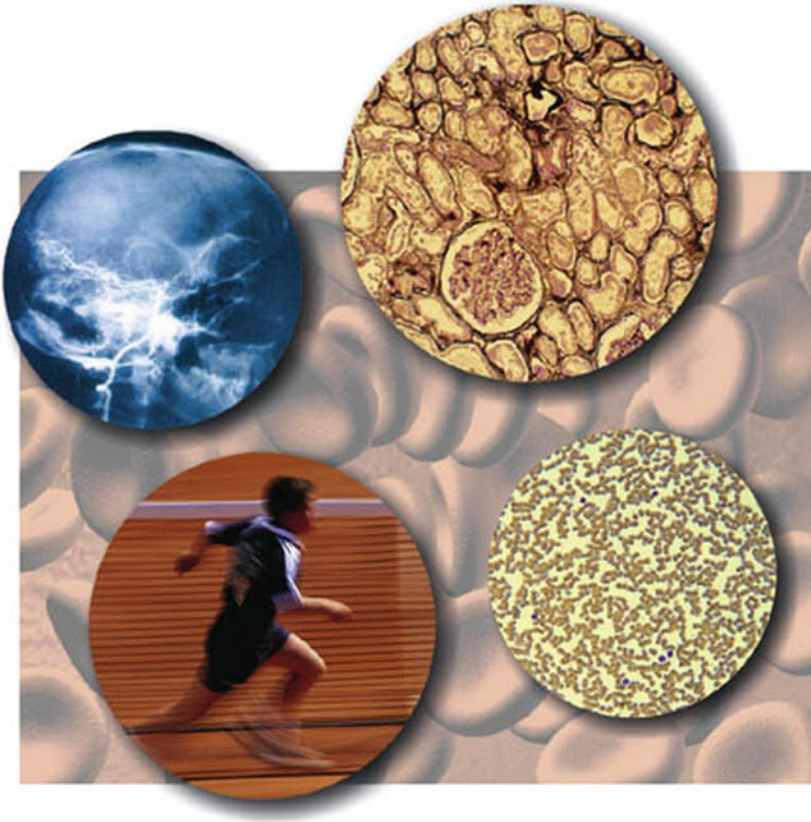
Erythropoietin

Blood, Brain and Beyond



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Blood, Brain and Beyond

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Preface

This year, 2003, marks twenty-six years since the purification of human erythropoietin, or "Epo" as it is commonly known, and twenty years since the cloning of the human Epo gene. Sadly, this year has also witnessed the passing of Allan J. Erslev, one of the giants in the field that I had the privilege to know. In the past twenty-six years since I began my own scientific work on Epo with the encouragement and support of David G. Nathan, Epo has grown from a curious glycoprotein hormone studied by a small collection of scientists and physicians to an important therapeutic agent and one of the darling pharmaceuticals of the biotechnology industry, reaching multibillion-dollar sales year after year.

This book is an attempt to bring together in a single, manageable volume the wide array of information and subject matter relating to Epo, its action and its use. The idea for this volume was conceived in October 2000 while having dinner in a small restaurant in South Boston with Karin Dembowsky, then with the Life Sciences Division of WILEY-VCH, and her husband, Klaus Dembowsky, of Bayer AG. Klaus had recently co-edited a volume entitled "Novel Therapeutic Proteins" and I had co-authored the chapter on Epo. Karin suggested that I could edit a new work devoted entirely to Epo comprising chapters written by numerous experts in the field, or consider making it a solo piece. Years earlier I had spoken with Sanford B. Krantz, who in 1970 co-authored the seminal "Erythropoietin and the Regulation of Erythropoiesis" with Leon O. Jacobson (University of Chicago Press), and I remember Sandy's telling me that the job took the better part of a year with time for little else. (Somehow, I later forgot this conversation when I began missing deadlines.) Because I admired how Krantz and Jacobson were able to integrate the chapters of their book, presumably due in part to the consistency of writing style and perspective, I chose to make this new book a single author work, hoping to achieve at least some measure of their excellence. It must be said that what the reader finds laudable between the covers of this volume is the product of numerous scientists, physicians and their

students, hopefully cited correctly. What the reader finds lacking or erroneous can only be attributed to myself.

The work comprises eleven chapters covering 1) History; 2) Development Biology; 3) Gene Regulation; 4) Physiology and Metabolism; 5) Biochemistry and Protein Structure; 6) Receptor Biology and Signal Transduction; 7) Clinical Disorders of Epo and its Receptor; 8) Epo and the Treatment of Anemia; 9) Functions of Epo Outside the Hematopoietic System; 10) Epo and Sport; 11) The Future of Epo. Each chapter stands on its own as, I hope, a thorough and accurate discussion of the topic with citations through late 2003. Indeed, with modern computer searching capabilities, especially the CiteTrack service of Science magazine, it has been possible and, indeed, became necessary to revised "finished" chapters as new papers appeared. At the same time, since the author of each chapter is myself, interchapter relationships were more easily made. At first, I hoped to be encyclopedic, but as the writing progressed it seemed that what might be more useful to potential readers – students and more experienced professionals from several disciplines alike – would be a more portable work, a type of handy reference, *vade* mecum, which one can use both as a full explication of subject matter as well as a springboard to further investigation.

It is hoped that many readers will find several of the chapters useful. I suggest that particular attention be paid to Chapters 9, 10 and 11, since many current and, in some cases, unexpected discoveries are being made in these areas. Now that we are becoming aware of Epo's actions in the brain, endothelium, heart, reproductive tract and other tissues (Chapter 9), scientists and physicians from many fields should find this volume useful. Chapter 10 describes the use and abuse of Epo in athletics, but also reviews "altitude training" and other mechanisms to upregulated endogenous Epo to enhance endurance. Analytic methods are also reviewed. Chapter 11 focuses on new therapeutic forms of Epo, Epo-like agents and other approaches to substitute for and compete with the recombinant hormone pharmaceutical.

I must acknowledge others who have helped make this book a reality. I thank my wife, Pamela, for her patience, her wonderful proof-reading and stylistic suggestions, my son, Nick, for understanding my need for private time in my home office and keeping me updated on Boston sports, and my daughter, Alexandra, for several excellent cover design ideas. I thank my laboratory administrator, Rosemary Brady Panza, who skillfully transcribed my tape dictations that I resorted to after three chapters of my own typing purgatory. The professionals at WILEY-VCH have been extremely helpful and patient, which was especially necessary given my penchant for delay. I must acknowledge the guidance of Andrea Pill-

mann, Publishing Editor, Life and Analytical Sciences, Prisca-Maryla Henheik, Project Editor, Life and Analytical Sciences, and Hans-Jochen Schmitt, Production. Finally, I thank those numerous scientific friends and colleagues who have provided ideas, encouragement and inspiration to me in my endeavors.

Arthur J. Sytkowski Boston, December 2003

Contents

1
Introduction and History

1.1
Early Observations

The discovery of erythropoietin (Epo) was not a single event that can be credited to one individual or even a group of investigators in a particular year. Rather, it was a process of slow, deliberate unmasking of the molecule that began in the nineteenth century. Many of the details of this story have appeared elsewhere. The reader is referred to the landmark monograph by Krantz and Jacobson [1] and the excellent reviews by Jelkmann [2] and Fisher [3, 4] and references therein, all of which served as background for this chapter.

Perhaps the earliest recorded observation relating to Epo was that of Jourdanet who observed in 1863 that persons living at high altitude had more viscous blood [5]. Later in that century, Viault reported that his own red blood cell count increased significantly while traveling from a lower to a higher altitude in Peru [6, 7], a report that was confirmed by Muntz [8]. A similar observation in the Alps [9, 10] led Friedrich Miescher to propose that hypoxia was the direct stimulus for red blood cell production (erythropoiesis) by the bone marrow. Attempts to treat anemia by inducing hypoxemia were made [11].

1.2
Carnot and Deflandre: A Humoral Factor that Stimulates Erythropoiesis

The first experimental evidence for a humoral factor involved in the regulation of erythropoiesis is found in the famous studies of Carnot and Deflandre [12, 13]. They made rabbits anemic by bleeding them, prepared serum from them and then injected this serum into non-anemic rabbits. After two days, the red blood cell counts of the recipient animals had increased sub-

stantially. Carnot and Deflandre wrote *"Nous avons précédemment constanté, dans le sérum des animaux préalablement saignés et en pleine crise de rénovation sanguine, la présence d'une substance capable d'activer l'hemopoïese (hémopoïétine) et de provoquer, chez les animaux neufs, une hyperglobulie rapide, considérable et constante"* [12]. (Previously, we have demonstrated, in the serum of animals that were in the active state of blood regeneration after bleeding, the presence of a substance, hemopoietine, capable of activating hemopoiesis, and of rapidly provoking, in normal animals, a high, constant hyperglobulism. – Translation from [2].) Jelkmann points out that the experiment itself is open to question, since the donor rabbits were bled only 30 mL and were probably not very anemic. Moreover, the recipient animals received only 5–9 mL of donor serum. Therefore, not all investigators succeeded in reproducing Carnot and Deflandre's observation.

Several investigators did succeed in confirming the existence of a humoral factor involved in the regulation of erythropoiesis using a variety of methods and species. For example, Gibelli made rabbits, guinea pigs and dogs anemic by bleeding or treatment with phenylhydrazine, a chemical that causes hemolysis, and showed that their sera increased the red blood cell counts of recipient animals [14]. Müller showed that the decrease in red blood cell counts in mice after bleeding could be prevented by administration of serum from anemic guinea pigs [15]. These observations were supported by Förster who showed the same effect using serum from rabbits exposed to hypobaric hypoxia [16]. Sandor demonstrated that rabbits injected with serum from donor animals exposed to hypoxia exhibited an increase in reticulocytes, indicative of active erythropoiesis [17]. Similarly, Hjort showed that serum from rabbits made anemic by bleeding could induce a reticulocytosis in normal recipients [18]. Tei reported that he detected a humoral substance capable of stimulating erythropoiesis in the serum of bled rabbits and in rabbits made anemic by treatment with phenylhydrazine [19, 20], findings also reported by Krumdieck [21]. Finally, Bonsdorff and Jalavisto used the term "erythropoietin", instead of Carnot and Deflandre's "hemopoietine", to describe the humoral factor that stimulates erythropoiesis [22].

1.3
Reissmann, Erslev, Jacobson and Stohlman: The Kidney as the Likely Source

An experiment critical to the demonstration of humoral regulation of erythropoiesis after an hypoxic stimulus was provided by the parabiotic rat experiment of Reissmann [23]. Reissmann's experiment is a true classic of

physiology characterized by simplicity of design and unambiguous interpretation. Of the two animals with a shared circulation, one breathed normal air while the other breathed hypoxic air. However, both animals exhibited increases in peripheral blood hemoglobin and red cell numbers and erythroid hyperplasia of the bone marrow. The obvious conclusion was that a humoral substance capable of stimulating erythropoiesis was produced in the hypoxic animal that could circulate to the normoxic animal, increasing erythropoietic activity in both animals. In a less well known study, Ruhensroth-Bauer used parabiotic rabbits and showed that the bleeding of one induced a reticulocytosis in both [24]. Reissmann's study reinvigorated research on Epo. Erslev showed that serum from rabbits made anemic by bleeding could stimulate erythropoiesis in normal recipient animals [25]. In order to determine the site of Epo production, Jacobson *et al*. removed various organs from rats to determine which needed to remain in place to allow the increase in Epo levels seen after bleeding. Only removal of the kidneys prevent the Epo response to bleeding, thus demonstrating indirectly the likely site of Epo biosynthesis [26]. Interestingly, support for these studies came from an experiment of nature. Stohlman *et al*. reported on a patient with a cardiovascular malformation that resulted in normally oxygenated blood flowing above the diaphragm but hypoxemic blood below the diaphragm. The patient was polycythemic. Importantly, examination of the bone marrow obtained from the sternum, which was well-oxygenated, revealed erythroid hyperplasia, suggesting that a humoral factor produced in response to hypoxia by an organ below the diaphragm (the kidney) stimulated erythropoiesis in all regions of the bone marrow [27].

1.4 More Evidence for the Kidney… Erythrogenin?

More than twenty years before Reissmann's experiment, Waltner and Waltner discovered that oral or parenteral administration of cobalt to rats resulted in polycythemia [28]. Two decades later, Weissbecker demonstrated erythroid hyperplasia in the bone marrow of humans who had received cobalt [29]. In fact, as noted by Fisher, a pharmaceutical cobalt preparation known as Roncovite® was used to treat anemia in human patients during the 1950s [3]. Knowing that, Fisher perfused dog kidneys with blood containing cobalt and demonstrated an elevation in Epo levels [30]. Shortly thereafter, a similar observation was made using kidneys perfused with hypoxemic blood [31]. Still, there was doubt that the kidney ac-

tually produced the biologically-active hormone. Gordon *et al.* hypothesized that the kidney produced an enzyme, designated "erythrogenin", that acted on a plasma protein resulting in active erythropoietin [32]. Indeed, there was significant evidence to support this hypothesis [33–41]. However, Erslev demonstrated the production of Epo by kidneys perfused with a serum-free solution [42]. Later, Schuster *et al.* used a northern blot analysis to demonstrate Epo mRNA in kidneys of hypoxic rats.

1.5 Early Assays for Erythropoietin

As related by Krantz and Jacobson [1] and Fisher [3], the earliest assays for Epo were qualitative rather than quantitative and consisted of measuring the increase in red blood cell numbers, reticulocyte numbers or hematocrit. In 1955, Plzak *et al.* published an innovative method to measure Epo consisting of the incorporation of ^{59}Fe into the circulating red blood cells of normal rats [43]. Subsequently, it was learned that both hypophysectomized rats and fasted rats exhibited reduced endogenous Epo levels and erythropoiesis, making these animals more sensitive to the action of exogenous Epo [44]. However, although these assays gained acceptance, it became clear that they were sensitive to other substances present in the test samples, such as hormones and protein [45]. In order to suppress endogenous Epo using a more specific mechanism, hypertransfusion was employed, leading to the development of the hypertransfused mouse assay, which proved to be the most sensitive and specific assay [46]. In addition to hypertransfusion, exposure to hypoxia was employed to induce plethora and thereby markedly reduce endogenous Epo levels in mice. Hypoxia has the effect of stimulating a marked increase of endogenous Epo levels and resulting in brisk erythropoiesis and resultant plethora. After two to six weeks, the animals are removed and placed in a normal atmosphere. This eliminates the hypoxic stimulus to endogenous Epo production. Endogenous Epo production is now virtually zero due to the increase of hemoglobin levels in the animals. The now plethoric animals have a highly erythroid bone marrow that is exquisitely sensitive to exogenous Epo. Several means to generate hypoxia have been employed including a gas mixture of 10% O_2 [47], a hypobaric chamber [48], exposure to carbon monoxide [49], and housing the mice in a silicone rubber membrane enclosure [50]. The first reliable *in vitro* bioassay for Epo employed ^{59}Fe incorporation into heme in cultured rat bone marrow cells [51].

1.6
Standardization: What is a "Unit" of Erythropoietin?

While these assays were being developed, it became clear that there was a need for standardization. Pure Epo was not yet available, so White *et al.* suggested that a "Unit" of Epo be defined in terms of the effect of cobalt in stimulating ^{59}Fe incorporation into the circulating red blood cells of fasted rats [52]. So the "cobalt unit" of Epo was defined as the amount of Epo that stimulated erythropoiesis in the fasted rat assay equal to that of 5 μmol of cobalt. Since not all rat strains responded to cobalt to the same degree, the Sprague-Dawley strain was specified. It became clear that crude preparations of Epo partially purified from the plasma of anemic sheep were relatively stable, so a standard of lyophilized Step IV sheep Epo (23 U/mg) (Standard A) was established [53]. This became the First International Reference Preparation (I.R.P.) [54]. Later, a second standard (Standard B) was developed that consisted of lyophilized crude human urinary Epo. It defined 1 Unit of Epo as that contained in 1.48 mg of the Standard protein 10 International Units (10 IU) per ampoule. This is also known as the Second International Reference Preparation [55] and became available from the National Institute for Biological Standards and Control in the United Kingdom in 1971. In 1990, a First International Standard consisting of pure recombinant Epo was developed (86 IU/ampoule) [56]. This is now being replaced by the Second International Standard 2003, which also is composed of pure recombinant Epo (120 IU/ampoule) [57].

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2
Developmental Biology of Erythropoiesis and Erythropoietin Production

2.1
Introduction

The developmental biology of erythropoiesis and of Epo production is a complex process that is inextricably linked to ontogeny. In recent years it has become evident that Epo is produced by numerous cell types and that the EpoR is expressed on a wide variety of cells, both hematopoietic and nonhematopoietic. This Chapter focuses on the developmental biology of hematopoiesis and erythropoiesis and on the appearance of those Epo producing cells that are relevant to erythropoiesis. The important topic of nonhematopoietic expression of the Epo receptor and Epo is discussed in detail in Chapter 9. Furthermore, this Chapter focuses on mammalian erythropoiesis, especially mouse and human. For an overview of hematopoietic development in other species as well, the reader is referred to the excellent review by Zon [1].

2.2
Yolk Sac Hematopoiesis

The first blood cells arise along with endothelial cells in the yolk sac [2–5]. This is an organ comprising two layers, extra-embryonic mesodermal cells and visceral endodermal cells. This association of germ layers is designated the splanchnopleure. The endodermal layer is in direct contact with macromolecules derived from the maternal plasma, and these molecules along with others synthesized by the endoderm itself influence development in the mesodermal layer within these so called “blood islands” [6]. As pointed out by Palis and Yoder [4], the visceral endoderm will provide molecular signals that induce formation of blood cells and endothelial structures [7–9]. Blood islands developing in the murine embryonic

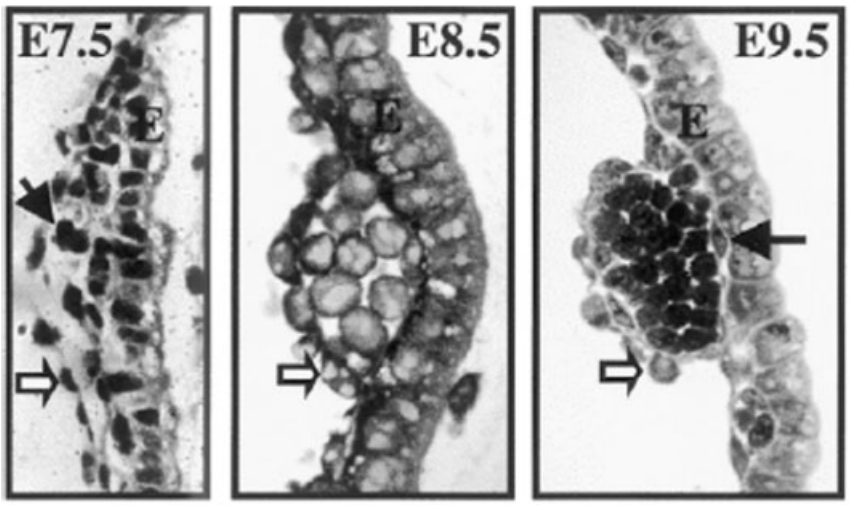

Fig. 2.1 Blood island developing in the E7.5–E9.5 mouse embryo yolk sac. Undifferentiated mesoderm cells (E7.5, closed arrow) give rise to clusters of hematopoietic cells that lie between mesothelial cells (open arrow) and a single cell layer of endoderm (E). At E9.5, inner blood island is visible surrounded by endothelium (E9.5, closed arrow). From reference 4.

yolk sac between the E7.5 and E9.5 stages are shown in Fig. 2.1. The corresponding human embryonic stages leading to blood cell and endothelium formation are shown diagrammatically in Fig. 2.2. All lineages of blood cells as well as endothelial cells appear to be derived from a multipotent progenitor designated the "hemangioblast". Support for this concept comes from the study of chick embryos and embryonic stem cells (ES) and the observation of the simultaneous development of endothelial structures and hematopoietic cells [10–13].

The erythropoiesis occurring during this process in the yolk sac is designated "primitive erythropoiesis" and is distinguished from that which is called "definitive erythropoiesis" occurring within the embryo. Yolk sac primitive erythropoiesis occurs extra-embryonically as can be appreciated from Fig. 2.2. Primitive erythropoietic cells are characterized by relatively large size, expression of embryonic hemoglobins and the morphologic property of nuclear retention, in contradistinction to the loss of nuclei that occurs during the terminal differentiation process of definitive erythropoiesis. In the embryo, these large nucleated erythroid cells are designated "megaloblasts". The megaloblasts enter the primitive blood stream where they differentiate further resulting in nuclear condensation and final enucleation by mouse embryonic stage E13.

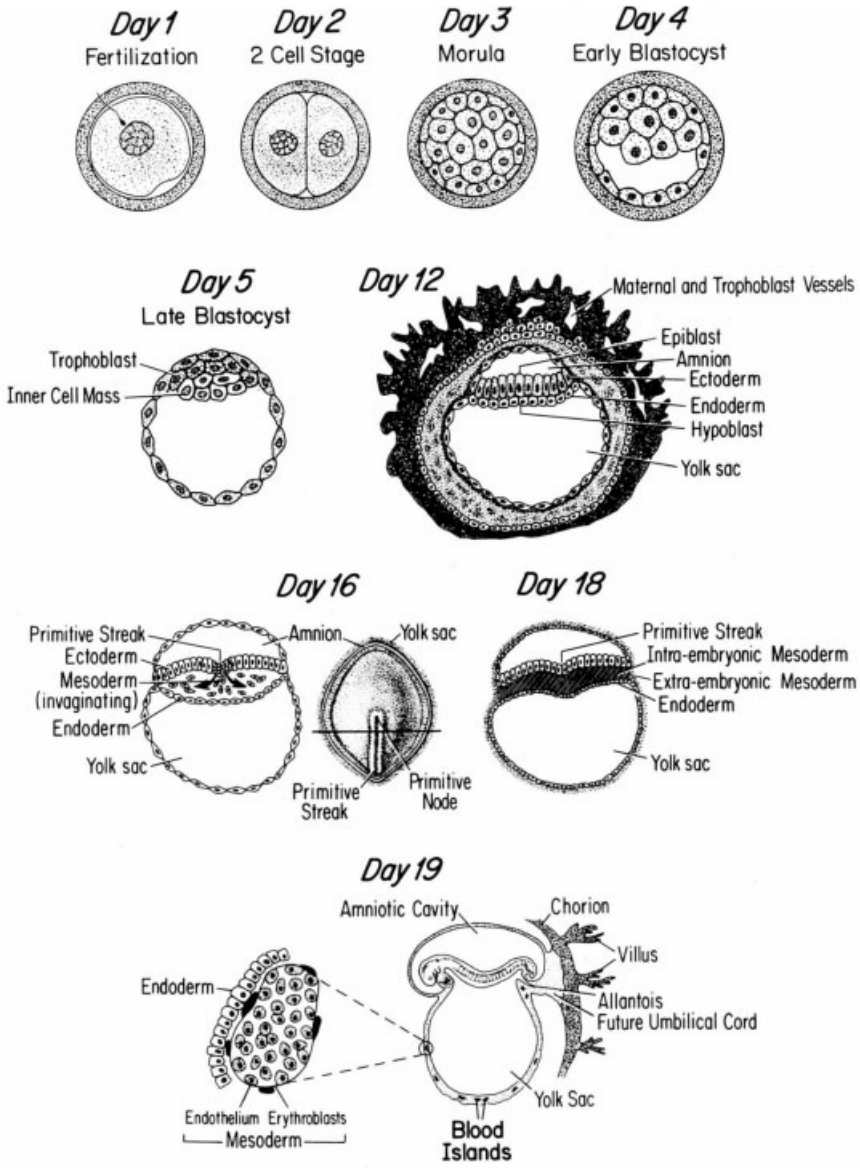

Fig. 2.2 Development of hematopoiesis in the human embryo. Epiblast cells begin to invaginate at day 16 to form the mesodermal layer. At day 18, both intra-embryonic and extra-embryonic mesoderm is present. At day 19, blood islands are present in the yolk sac. From reference 1.

2.2.1 The Role of Erythropoietin in Yolk Sac Erythropoiesis

The role of Epo in primitive erythropoiesis remains controversial. An early study by Cole and Paul on cultured mouse embryos revealed no effective sheep Epo on heme synthesis by yolk sac cells of the date 8–9 post-ovulatory embryo [14]. In contrast fetal liver responded to Epo with a marked increase in heme synthesis in the day 11 mouse embryo. In a later study, however, Epo did stimulate heme synthesis in yolk sac cells [15]. Epo binding sites or receptors were identified on hamster yolk sac erythroid cells throughout hamster gestational days 8–13 [16]. It was reported that both binding affinity and the number of receptors per cell varied during ontogeny. The functionality of these receptors, however, was not reported. McGann *et al*. used *in situ* hybridization to identify Epo receptor mRNA in mesodermal cell masses of the developing yolk sac of the mouse embryo stage E7.5 even before recognizable erythroblasts were found [17]. Epo receptor mRNA was also identified in yolk sac blood islands in stage E8.5. Using a yolk sac explant system, the investigators demonstrated both an increase in erythroblast numbers and in betaH1 globin accumulation after exposure to exogenous Epo. Furthermore, antisense oligodeoxynucleotides to EpoR reduced the numbers of differentiating primitive erythroblasts, globin accumulation and erythroid progenitor numbers. Further complicating this picture are the results from targeted disruption of the EpoR gene in mice [18, 19]. In these animals, there is a substantial reduction in circulating primitive erythroblasts by E11.5 leading to death of the embryos with severe anemia by E13.5. Nevertheless some hemoglobinized primitive erythroblasts do survive knockout of the Epo or EpoR genes, in marked contrast to the complete absence of terminally differentiating definitive erythroid cells in the fetal liver of these mutant animals.

2.2.2 Genes Essential to Primitive Erythropoiesis

Targeted disruption of several genes in the mouse has demonstrated their essentiality to hematopoiesis and/or erythropoiesis. For the most part, these essential genes encode transcription factors [20, 21].

GATA-1 was one of the first transcription factors shown to be essential to erythropoiesis. GATA-1 is a zinc finger protein that was initially discov-

ered to bind to regulatory sequences in globin genes [22, 23]. However, GATA-1 binding sites are ubiquitous in both promoters and enhancers of erythroid (and megakaryocytic) specific genes. GATA-1 expression is restricted to the erythroid, megakaryocytic, eosinophilic mast cell and multipotent progenitor cell lineages of the hematopoietic system [22, 23] and to Sertoli cells in the testis [24]. GATA-1 knockout mice died at E10.5 with severe anemia characterized by maturation arrest [25]. GATA-1 apparently plays a role in survival as well as differentiation, since GATA-1 ES cells undergo rapid apoptosis [26]. A recent study by Gregory *et al.* found that GATA-1 and Epo promote erythroid cell survival by cooperating in the induction of the anti-apoptotic gene Bcl-xL [27]. Furthermore, a study by Kapur *et al.* indicates that GATA-1-enhanced Bcl-xL expression is mediated by GATA-1's effect on EpoR and STAT5 expression [28].

FOG-1 (friend of GATA) is expressed along with GATA-1 in erythroid and megakaryocytic cells [29]. FOG-1 is a GATA-1 interacting partner and it, along with other members of the FOG family, helps direct GATA-1's interaction with DNA target sequences. FOG-1 knockout mice die at E10.5–11.5 with severe anemia and erythroid maturation similar to that seen in GATA-1 knockouts [30]. FOG knockout mice exhibit failure of megakaryopoiesis as well. **GATA-2** is expressed in hematopoietic cells along with GATA-1, but its expression is less restricted and includes endothelial cells and undifferentiated ES cells [31, 32]. Thus, the GATA-1 knockout mouse exhibits a severe hematologic defect, dying after E10–11 with profound anemia [33].

Tal-1 (SCL) is a basic-helix-loop-helix (bHLH) transcription factor [34–36]. It forms heterodimers with E proteins and binds to E-box DNA sequences that are located in erythroid-specific genes. Tal-1/SCL knockout

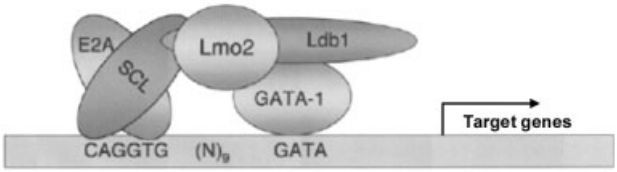

Fig. 2.3 Proposed complex of SCL, E2A Lmo2, GATA-1 and Ldb1 interacting with an erythroid-specific target gene. Note binding to E-box motif (CAGGTG) and GATA-1 binding motif. From references 20 and 40.

mice die at an early embryonic stage due to the complete absence of blood, either in the yolk sac or in the embryo itself [37, 38]. **LMO2** (RBTN2) interacts physically with Tal-1/SCL [39, 40]. Thus, it is not surprising that LMO2 knockout mice also die bloodless at an early embryonic stage [41]. Interestingly, there is evidence for the formation of a multimeric complex of Tal-1/SCL · E2A · GATA-1 · LMO2 and Ldb1 that may direct transcription of erythroid genes (Fig. 2.3) [42].

2.3 Fetal Liver and Bone Marrow (Definitive) Erythropoiesis

Definitive erythropoiesis is characterized by the formation of small, enucleate erythrocytes that result from the replication and ultimate terminal differentiation of the progeny of "committed" erythroid progenitors, that is, cells with substantial self-replicating potential but whose progeny will ultimately differentiate only in the erythroid lineage. These erythroid progenitors are derived from multipotent progenitors and, ultimately, from pluripotent hematopoietic stem cells [43–45]. When injected intravenously into irradiated mice, these cells give rise to multilineage hematopoietic colonies in the spleen [46], thus resulting in the name "colony forming unit-spleen" or "CFU-S". Whether stem cells make the decision to "commit" to a particular lineage (or lineages) by a stochastic process [47] or whether they are induced by one or more environmental stimuli has been the subject of much investigation and debate for over four decades.

The very existence of committed erythroid progenitors was deduced from the appearance of colonies of differentiating, hemoglobinized erythroid cells when bone marrow cells were grown *in vitro* in semi-solid medium, for example, plasma clot or methylcellulose. In such medium, if the daughter cells of a dividing progenitor are motile, they can move to another location to continue dividing. Once motility is lost, a characteristic of more mature cells, daughter cells of the last few divisions remain adjacent to each other, resulting in a tightly-packed, three-dimensional colony. The more mature erythroid progenitor, which gives rise to a single 8 to 64–128 cell colony, has been designated the "colony forming unit-erythroid" or "CFU-E". In turn, numerous motile CFU-E are derived from a less mature progenitor, designated the "burst forming unit-erythroid" or "BFU-E", an appellation that refers to the microscopic appearance of scattered clusters of small to medium sized colonies that loosely resembles a fireworks display.

It would appear that definitive erythropoiesis is initiated in the yolk sac, since culture studies have shown that both BFU-E and CFU-E are present

there [48–50]. However, these progenitors do not differentiate fully in the yolk sac, since maturing erythroid cells characteristic of definitive erythropoiesis do not appear. Both Epo and EpoR are essential for definitive erythropoiesis, as shown from studies of the respective knockout mice. However, both BFU-E and CFU-E were identified in the livers of the mutant fetuses, indicating that neither Epo nor the EpoR are required for development of these committed progenitors [18]. In addition to EpoR, stem cell factor, SCF, and its cognate receptor, Kit, play a role in erythropoiesis. In studies of the Epo-dependent cell line HCD-57, which expresses Kit, it was shown that SCF could replace Epo to support growth and survival [51]. Also, SCF treatment induced phosphorylation of the EpoR and Kit and associated physically with EpoR via the Box2 domain of the EpoR cytoplasmic portion. By infecting erythroid progenitors from the fetal livers of EpoR knockout mice with a retrovirus expressing wildtype EpoR, it was shown that SCF was required for development of CFU-E-derived colonies *in vitro* [52].

The source of stem cells for definitive erythropoiesis, which takes place in the fetal liver and in the adult bone marrow (and spleen in mice), had long been thought to be the yolk sac [53]. However, in the chick, fetal erythropoiesis depends upon clusters of hematopoietic cells located in intra-aortic and later para-aortic sites, suggesting the possibility of a dual origin of stem cells [54]. As noted by Palis and Segal [3], human, mouse, pig and gerbil mid-gestational embryos exhibit intra-aortic blood islands, and the aorta-gonad-mesonephros (AGM) region of the embryo may be the site of origin of definitive erythropoiesis [55–58].

Recent evidence has begun to challenge the conventional wisdom regarding the strictness of the yolk sac → fetal liver → bone marrow paradigm of mammalian hematopoiesis. Jay *et al*. showed that treatment with the proper combination of cytokines induced the growth and differentiation of hematopoietic cells from 16- to 27-week human fetal brain and muscle tissue [59]. Curiously, the cells were uniformly $CD34^-/CD45^-$ and expressed the prominin AC133. Sequeira Lopez *et al*. obtained striking histologic evidence that in the mouse embryo, erythroid progenitors arise in every tissue simultaneously with blood vessel development [60]. These investigators used transgenic mice expressing LacZ driven either by the *Tie2* promoter to mark endothelial cells or by the *β-globin* LCR-promoter combination to mark erythroid cells. Microscopic examination of embryos from E8.5 through E11.5 demonstrated $LacZ^+$ erythroid cells budding intralumenally from the endothelial walls in virtually every region of the embryo (Fig. 2.4). Further evidence for an endothelial origin of erythroid cells in the mouse embryo was provided by Sugiyama *et al*. [61]. These investigators tagged mouse embryo endothelial cells by intracardiac injec-

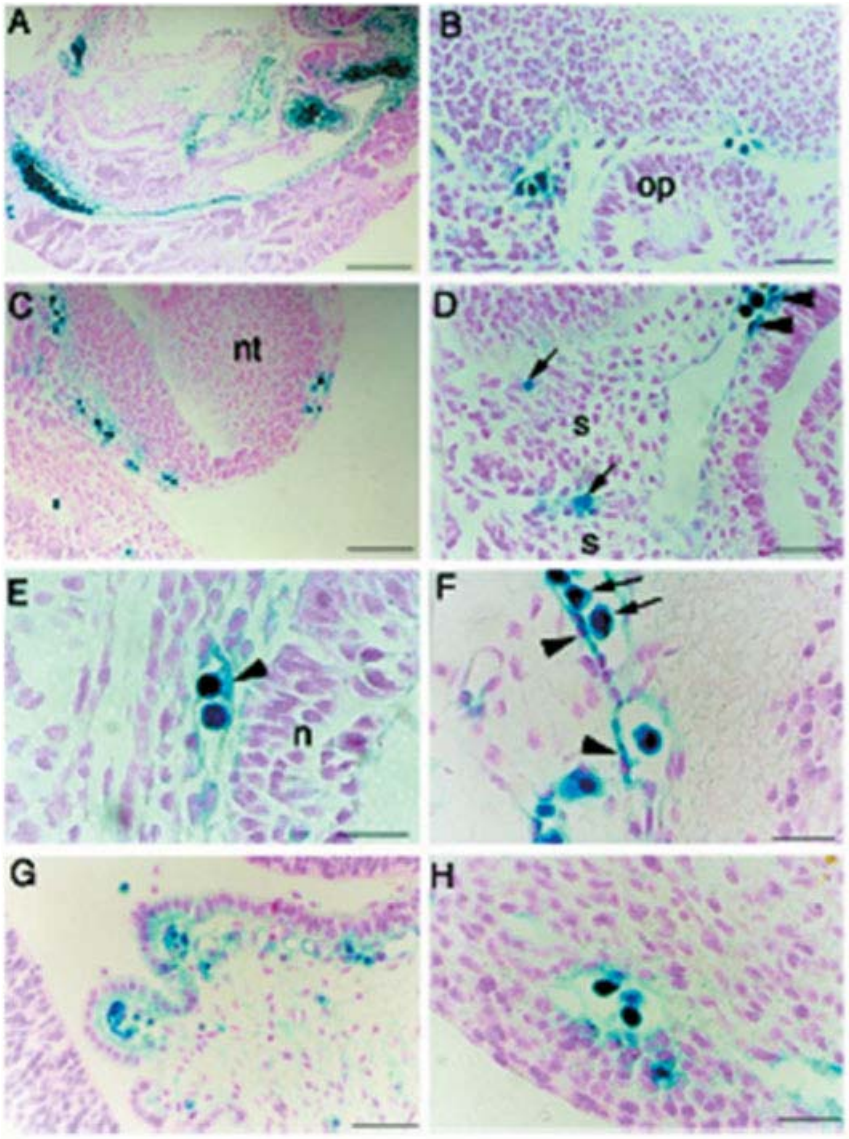

Fig. 2.4 Distribution of *β-globin* expression in mouse embryos assayed using the X-Gal reaction in *globin/LacZ* embryos at E8.5 (A to E) and at E11.5 (F to H). *LacZ* expression in the whole embryo (*A*), optic pit (op; *B*), neural tube (nt; *C*), in between developing somites (s) (arrows) (*D*), and lining the dorsal aorta (arrowheads). *E*: LacZ expression is evident in "budding" erythroid cells and in endothelial cells (arrowhead) adjacent to the neuroepithelium (n). *F*: LacZ expression in "budding" cells (arrows) and in endothelial cells (arrowheads) in the cephalic mesenchyme, in the developing coroid plexus (*G*), and in a marginal vein in the left hindlimb bud (*H*). Scale bars: 200 μm (*A*); 100 μm (*C*); 50 μm (*B*, *D*, and *G*); 25 μm (*E*, *F*, and *H*). From reference 60.

tion of the fluorescent compound 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indo-carbocyanineperchlorate-Ac-LDL (DiI-Ac-LDL). After *in vitro* culture of the embryos, fluorescent erythroid cells derived from DiI^+ endothelial cells were clearly identified.

2.4 Genes Essential for Definitive Erythropoiesis

Among those genes essential for definitive but not primitive erythropoiesis, ***c-myb***, which is widely expressed in hematopoietic cells, plays an especially complex role, for it is also a participant in the development of other lineages. Studies of Friend erythroleukemia cells, which differentiate in response to chemical inducers like dimethyl sulfoxide (DMSO), showed that DMSO induced a rapid downregulation of both c-*myb* and c-*myc* and that this pattern was required for differentiation to occur [62–67]. Todokoro *et al*. used the Epo-responsive murine erythroleukemia cell line SKT6 and demonstrated that Epo also downregulated c-*myb*. Forced overexpression of the gene blocked Epo-induced differentiation [68]. However, Gewirtz and Calabretta showed that artificial downregulation of c-*myb* with antisense oligodeoxynucleotides was shown to block normal hematopoiesis *in vitro* [69], suggesting a more global role in hematopoiesis as well as a more specific one in erythroid differentiation. That the downregulation of c-*myb* may be an important differentiation-initiating event was supported by the experiments of Chern *et al*. [70, 71]. These workers used the Rauscher murine erythroleukemia cell line, a line that grows independently of Epo but that differentiates in response to it. They showed that either Epo or DMSO downregulated c-*myb*. Importantly, when treated with antisense oligodeoxynucleotides to c-*myb*, they demonstrated the induction of differentiation along with downregulation of c-*myb* transcript and protein. As shown from the c-*myb* knockout mouse, a functional c-*myb* is required for normal fetal liver hematopoiesis [72]. Whereas yolk sac hematopoiesis appears normal, fetal liver hematopoiesis is markedly impaired, and the animals die at E15. The numbers of erythroid and other hematopoietic progenitors are markedly reduced, although megakaryopoiesis is unaffected.

Like c-*myb*, **AML-1**, the α subunit of the core binding factor (CBF), is essential to definitive but not primitive hematopoiesis. AML-1 knockout mice die at E12.5 with a block in all definitive hematopoietic lineages [73]. **EKLF** (erythroid Kruppel-like factor) is important to the regulation of the β-globin gene [74–77]. Its DNA consensus sequence can be found in

the adult β-globin promoter, and EKLF knockout mice die at the fetal liver stage due to lack of adult β-globin gene activation. EKLF may act, at least in part, by physically associating with GATA-1, along with Sp1.

2.5 Erythropoietin Production

Yolk Sac The question of whether Epo is produced extra-embryonically in the yolk sac has not yet been settled definitively. Lee *et al.* examined mouse embryos for Epo and EpoR expression using *in situ* hybridization [78]. They found EpoR expression in the yolk sac at stage E8.0 and in the yolk sac vasculature at E9.0. However, Epo transcript was detected at E9.0 in the vitelline of the embryo and not in the yolk sac itself. These results are in contrast to those of Yasuda *et al.* who carried out a very similar study on gestational day 10 embryos using *in situ* hybridization and immunohistochemistry [79]. They detected Epo mRNA in 57.6% of endodermal epithelial cells by *in situ* hybridization and Epo protein in 52.8% by immunohistochemistry.

Fetal Liver There is less controversy about Epo production by the liver. Koury *et al.* first studied adult mice using *in situ* hybridization and demonstrated Epo mRNA in isolated hepatocytes [80]. Additional, a small population of nonepithelial appearing cells in or near the sinusoids also contained Epo mRNA. Wintour *et al.* examined the 41 day ovine fetus [81]. They identified Epo mRNA in both the mesonephros and the metanephros as well as in the liver by RT-PCR. *In situ* hybridization of the mesonephros demonstrated that the Epo mRNA was present in interstitial cells lying between the proximal but not the distal tubules, similar to those cells identified in the anemic adult mouse kidney [80, 82]. Juul *et al.* used RT-PCR and immunohistochemistry in a study of various organs from human fetuses [83]. As expected, the fetal liver appeared to be the most robust producer of Epo. However, Epo mRNA and protein were identified in several other organs. A detailed discussion of this nonhematopoietic role of Epo is found in Chapter 9.

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3
Regulation of the Erythropoietin Gene: A Paradigm for Hypoxia-dependent Genes

3.1
The Structure of the Erythropoietin Gene

The human Epo gene was cloned by two groups within just a few weeks of each other, although the publications from each group were several months apart. Both groups obtained amino acid sequence from human urinary erythropoietin and screened libraries using mixed oligonucleotides. Jacobs *et al.* reported the isolation of both the gene and a human cDNA [1], whereas Lin *et al.* reported isolation of the gene only [2].

The human Epo gene spans approximately 2.2 kb from the ATG codon to the stop codon (Fig. 3.1). It encodes a protein of 193 amino acids with a calculated M_r of 18 399 in its nonglycosylated form. The gene comprises 5 exons. No promoter-like sequences were originally identified. In the intron between exons 3 and 4, there is a member of the *Alu* family of repeated sequences. It is flanked by an imperfect direct repeat.

A somewhat later report by Powell *et al.* also described cloning the human Epo gene as well as using high resolution dual laser sorting of human chromosomes to identify the gene's location on human chromosome 7 [3]. An earlier report of the cloning of the human Epo gene did not provide any sequence information and was never confirmed [4].

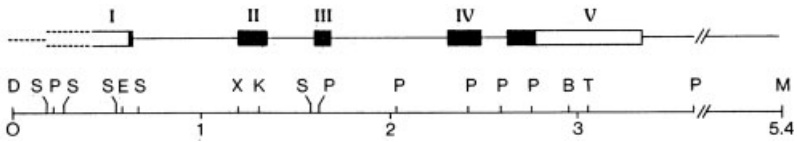

Fig. 3.1 Early structure of the human erythropoietin gene with restriction map. The boxes show exons I–V and the solid boxes show regions that are translated. The restriction endonuclease sites are: B, *Bgl* II; D, *Hin*dIII; E, *Bst*EII; K, *Kpn* I; M, *Bam*HI; P, *Pst* I; S, *Sma* I; T, *Sst* I; X, *Xba* I. From reference [2].

3.2 Tissue-specific Expression

In the mammalian fetus, Epo is expressed principally in the liver. As ontogeny continues, liver Epo production is suppressed and kidney production becomes predominant. In a series of transgenic mouse experiments, Semenza and co-workers helped define those regions of the Epo gene that are necessary for tissue-specific expression. In the first experiment, Semenza *et al*. prepared an Epo gene comprising 0.4 kb of 5′ flanking sequence and 0.7 kb of 3′ flanking sequence as well as the intact gene [5]. They microinjected this 4 kb gene into fertilized mouse eggs. The resulting transgenic mice were polycythemic, exhibiting increased red cell numbers in the blood, increased erythroid precursor numbers in the bone marrow and spleen, and increased serum Epo levels. Interestingly, homozygotes had a greater degree of polycythemia with marked extramedullary erythropoiesis. The investigators identified human Epo mRNA in the fetal liver, adult liver, kidney and in all other transgenic tissues analyzed. Some hypoxic regulation of the gene was seen; anemia raised Epo mRNA levels in the liver but not in the kidney, thus showing that at least some of the hypoxic regulatory sequences were found within the 4 kb transgene but that sequences necessary for tissue-specific expression were not.

In the group's second transgenic experiment, they compared the 4 kb transgenic mouse, designated tgEPO4, with mice obtained using a 10 kb transgene that contained 6 kb of 5′ flanking sequence and 0.7 kb of 3′ flanking sequence, designated tgEPO10 [6]. The tgEPO10 animals were characterized by the expression of Epo mRNA only in the liver that was inducible either with anemia or by cobalt injection. Like the tgEPO4 animals, the tgEPO10 mice were polycythemic. This study demonstrated that the sequences required for liver-specific expression were located within the 6 kb 5′ flanking sequence and that sequences required for kidney expression of the Epo gene were located outside of the 10 kb construct used.

In a third series of experiments, these investigators generated transgenic mice expressing an Epo gene containing 14 kb of 5′ flanking sequence [7]. This resulted in hypoxia-inducible expression of Epo in the kidneys of the transgenic animals. They also showed that this inducible human Epo expression was found in the peritubular interstitial cells in the renal cortex that also expressed endogenous mouse Epo mRNA.

3.3
Mechanism of Hypoxic Regulation – Identification of Transcription Factors and Other Regulatory Proteins

3.3.1
Is the Oxygen Sensor a Heme Protein?

In 1987, Goldberg *et al.* made an important breakthrough in the study of hypoxic regulation of Epo production [8]. They screened a large number of kidney and liver cell lines examining them for expression of Epo, either constitutive or regulated, using a radioimmunoassay and an *in vitro* bioassay. Among the 14+ cell lines examined, they found that the human hepatoma cell lines designated Hep3B and HepG2 produced Epo constitutively and, of special importance, this expression was upregulated markedly upon treatment with 50 μM cobalt chloride. Thus, since these cell lines were generally available, investigators now had an *in vitro* cellular system with which to begin to elucidate the mechanism of hypoxic regulation.

The next year these investigators provided evidence that the oxygen sensor responsible for the regulation of the Epo gene was a heme protein [9]. They used the human hepatoma cell line Hep3B and showed that either hypoxia or cobalt chloride increased expression of Epo mRNA as well as biologically active and immunologically reactive Epo protein. They found that new protein synthesis was required for this induction and that hypoxia, cobalt and nickel all appeared to operate by a common pathway. The fact that carbon monoxide blocked hypoxic induction of Epo production led the authors to conclude that "a heme protein is integrally involved in the oxygen sensing mechanism." The fact that cobalt can substitute for iron in the heme moiety and alter its oxygen affinity gave further credence to this conclusion. Goldberg *et al.* proposed a model in which a conformational change in the heme protein could explain the common mechanism of hypoxia, cobalt and nickel stimulation of Epo production.

In a study extending the use of Hep3B cells to investigate Epo production, Imagawa *et al.* investigated the Epo gene regulatory elements [10]. Using both 5′ and 3′ flanking sequence in a series of reporter gene experiments, the authors showed that regulation by hypoxia or cobalt operated through both promoter and enhancer elements through the human Epo gene.

3.3.2 The 3′ Flanking Region of the Erythropoietin Gene Contains Important Regulatory Sequences

In 1991, Semenza *et al.* identified DNase 1 hypersensitive sites 3′ to the human Epo gene in liver nuclei [11]. Using DNase 1 protection and electrophoretic mobility shift assays, they showed that a 256 bp region of the 3′ flanking sequence bound at least 4 different nuclear factors and that two of these factors were induced by anemia in both liver and kidney. Furthermore, they demonstrated in transient expression assays that this region functioned as a hypoxia-inducible enhancer. At the same time, Pugh *et al.* were working with HepG2 cells [12]. They used transient transfection assays and identified a DNA sequence 120 bp 3′ to the poly-A site of the mouse Epo gene that conferred oxygen-regulated expression using a variety of heterologous promoters. They noted that the sequence had features typical of an eukaryotic enhancer. Full activity was realized when 70 bp of the sequence was employed. In addition, they showed that the enhancer operated in HepG2 and Hep3B cells but not in Chinese hamster ovary cells or mouse erythroleukemia cells and that it responded to cobalt but not to cyanide or to 2-deoxyglucose. Therefore, this region was an accurate representation of normal Epo physiology.

Identification of proteins that interacted with the Epo gene followed rapidly. In 1992, Semenza and Wang identified a 50 bp enhancer from the human Epo gene 3′ flanking sequence that mediated a response to hypoxia in Hep3B cells [13]. Using DNase footprinting, they further delineated the binding of a nuclear factor to nucleotides 26–48. Using mutagenesis, they showed that nt 4–12 and nt 19–23 were essential for hypoxic induction. Binding of this nuclear factor was induced by hypoxia, and its induction required *de novo* protein synthesis. Although the 50 nt hypoxia-induced enhancer bound several nuclear factors, only one of these was induced by hypoxia.

Studies of Blanchard *et al.* emphasized the cooperation between promoter and enhancer elements in the hypoxic response [14]. Using transient transfection of Hep3B cells, these investigators identified a 53 bp promoter region and a 43 bp enhancer region that conferred hypoxia and cobalt inducibility. Although each alone resulted in 6–10-fold induction, together they produced a 50-fold induction after stimulation. Using electrophoretic mobility shift assays, they showed that the enhancer region exhibited specific DNA-protein interactions and that the promoter element competed with these interactions. Further studies provided even greater detail about these sequences.

3.3.3
Identification of the Hypoxia-inducible Factor – Hypoxia Regulates More Than the Erythropoietin Gene

In 1993, Wang and Semenza described **hypoxia-inducible factor 1** (**HIF-1**) [15, 16]. They identified it as a nuclear factor from Hep3B cells that could be detected when cells were cultured in 1% oxygen but not in 20% oxygen. Hypoxia also induced it in several cell lines that did not express Epo. They showed that induction of HIF-1 was inhibited by cycloheximide. They showed further that the protein kinase inhibitor 2-aminopurine inhibited induction of both HIF-1 and Epo mRNA. HIF-1 DNA binding activity was found to rapidly disappear when hypoxic cells were exposed to increased oxygen. The kinetics of HIF-1 association and dissociation from its binding site *in vitro* were extremely rapid, exhibiting a $T_{½}$ of less than one minute for both processes. In another study, they showed that the iron chelator desferrioxamine induced both Epo gene expression and HIF-1 binding activity [17]. That desferrioxamine induces HIF-1 by chelating iron in the cell was supported by the fact that coadministration of ferrous ammonium sulfate inhibited the induction. Desferrioxamine also induced Epo in the kidneys of mice *in vivo*. Importantly, the desferrioxamine induced HIF activity in non-Epo producing cells, suggesting to the authors "a common hypoxia signal transduction pathway leading to HIF-1 induction in different cell types."

HIF-1 activates the transcription of genes other than erythropoietin. Based upon their observation that HIF-1 activity was induced by hypoxia in non-Epo producing cells, Semenza *et al*. discovered that mRNAs encoding the glycolytic enzymes aldolase A, phosphoglycerate kinase 1 and pyruvate kinase M were induced in Hep3B cells or HeLa cells by exposure to HIF inducers (1% oxygen, cobalt chloride or desferrioxamine) [18]. As with Epo, cycloheximide had blocked HIF-1 activity and induction of these glycolytic mRNAs. Semenza *et al*. showed that oligonucleotides from these enzymes contained sequences related to the HIF-1 binding site of the Epo enhancer and bound HIF-1 specifically both from nuclear extracts and affinity-purified protein. Furthermore, they showed in transient expression assays that these glycolytic enzyme gene sequences containing HIF-1 binding sites mediated hypoxia-inducible transcription. These results significantly broadened the role of HIF-1 in hypoxia-induced gene regulation as well as further demonstrating the importance of hypoxic regulation outside of the erythropoietin system.

HIF-1 is a heterodimer. Wang *et al*. showed that both subunits of HIF-1 (α and β) are basic helix-loop-helix (bHLH) proteins containing a PAS domain. The PAS domain is common to the *Drosophila* PER and SIM pro-

teins and the mammalian ARNT (**a**ryl hydrocarbon **r**eceptor **n**uclear **t**ranslocator) and AHR proteins. Wang *et al.* went on to show that HIF-1 α was most closely related to SIM and that HIF-1 β was one of a series of ARNT gene products. Thus, the designations "HIF-1 β" and "ARNT" are often used interchangeably throughout the literature.

The breadth of HIF-1 action and the increasing importance of hypoxic regulation of gene expression were further emphasized by the observation that vascular endothelial growth factor (VEGF) gene transcription was activated by HIF-1. Forsythe *et al.* observed that VEGF stimulated neovascularization under several clinically important situations including ischemia of the myocardium, retinal disease and tumor growth [19]. They showed that VEGF 5′ flanking sequences were responsible for transcriptional activation of the reporter gene in hypoxic Hep3B cells. Furthermore, artificial overexpression of HIF-1 α and HIF-1 β increased both hypoxic and nonhypoxic reporter expression markedly. Also, cotransfection with a dominant negative form of HIF-1 α inhibited reporter gene activation in hypoxic cells. Separately, Levy *et al.* demonstrated that a 500 nt region of the 3′ UTR of VEGF mRNA was critical for stabilization of VEGF mRNA [20, 21]. Interestingly, they observed that this protein mRNA complex was constitutively elevated in cells lacking the von Hippel-Lindau tumor suppression gene. This observation was to have great importance in the elucidation of the oxygen sensor (see Section 3.3.5 below).

3.3.4
Other Interacting Proteins and the Regulation of HIF-1

The regulation of Epo gene expression as well as other hypoxia-inducible genes requires other regulatory proteins in addition to HIF-1 α and β. It was noted by Galson *et al.* that both the promoter and enhancer elements of the Epo gene contain consensus hexanucleotide hormone receptor response elements [22]. These investigators transcribed and translated 11 different orphan nuclear receptors *in vitro* and screened their ability to bind to elements in the Epo promoter and enhancer by electrophoretic mobility shift assay. They observed that four of these receptors bound specifically to the response elements in the Epo promoter and enhancer, namely, hepatic nuclear factor 4 (HNF-4), TR2-11, ROR α1 and EAR3/COUP-TF1. Except for ROR α1, the other three formed DNA protein complexes that had mobilities like those observed in nuclear extracts of Hep3B cells. Additionally, antibodies to HNF-4 and COUP induced super shifts in the Hep3B nuclear complexes. HNF-4 was observed not to be expressed in HeLa cells. By ectopically expressing HNF-4 in these cells, Gal-

son *et al.* observed an 8-fold increase in hypoxic induction of a reporter gene construct containing the minimal Epo enhancer and promoter. This increase in hypoxic induction by HNF-4 in HeLa cells was prevented by cotransfection with HNF-4 delta C, which lacks the C terminal activation domain. In contrast to these results using HNF-4, cotransfection of EAR3/COUP-TF1 into HeLa cells along with HNF4 or alone in Hep3B cells suppressed hypoxic induction of the Epo reporter. These studies demonstrated that HNF-4 was an important positive regulator in tissue-specific and hypoxia-inducible expression of the Epo gene and that the COUP family had a negative role.

Further demonstration of the role of ARNT/HIF-1β in the hypoxic response was provided through the study of ARNT-deficient cells. Wood *et al.* used these mutant cells, derived originally from a mouse hepatoma line HepA1C1C7 [23]. Using two stimuli, hypoxia and desferrioxamine, which had been shown to activate HIF-1, Wood *et al.* demonstrated that ARNT-deficient cells lacked hypoxic induction of DNA binding and transcriptional activity of HIF-1. Through a series of transfection experiments of ARNT/HIF-1β deletion mutants, they showed that the bHLH and PAS domains were necessary for function and response to hypoxia but that the amino terminal and carboxy terminal domains were not. This role of ARNT/HIF-1β was shown in the hypoxic induction of other genes including LDH-A, PGK-1, VEGF and GLUT-1. The essential role of ARNT/HIF-1β in the hypoxic response was confirmed by Salsada *et al.* [24].

Also participating in hypoxic regulation are the homologous transcription adaptors p300 and CBP, which are targeted by the E1A oncoprotein. Arany *et al.* searched for specific p300 binding proteins and found that HIF-1α interacted with p300 [25]. Importantly, they showed that hypoxia induced the formation of a DNA binding complex containing both HIF-1α and p300/CBP. Ectopic expression of p300 increased hypoxia induced transcription from the Epo promoter, and E1A binding to p300 inhibited it. The authors further showed that hypoxia-induced VEGF and Epo mRNA synthesis were inhibited by E1A, demonstrating that p300/CBP-HIF complexes are important in the regulation of hypoxia induced genes.

Work to define the regulatory domains within HIF-1α was carried out by Pugh *et al.* [26]. They constructed chimeric genes containing portions of HIF-1 coding sequence linked either to a heterologous binding domain or encoded between such a domain and the constitutive activation domain. They found that sequences from HIF-1α were responsible for oxygen-regulated activity of HIF-1 but those from HIF-1β were not. Amino acids 549–582 and 775–826 formed two minimal domains which independently could convey inducible responses. Further data led the authors to propose a dual mechanism activation in which the inducible activation do-

main was further amplified by regulation of transcription factor abundance.

An important insight into the complex role played by p300/CRB in hypoxic regulation was provided by Ebert and Bunn [27]. They characterized the multiprotein complex that binds to the lactate dehydrogenase A (LDH-A) promoter and demonstrated the involvement of HIF-1, p300/CRB and CREB-1/ATF-1. Further work demonstrated the formation of multiprotein complexes in both the LDH-A promoter and 3′ enhancer region of Epo. Next, Bunn and his colleagues demonstrated the mechanism of regulation of HIF-1α. Huang *et al.* identified an **oxygen-dependent degradation domain** (**ODDD**) within HIF-1α that controlled degradation by the ubiquitin-proteasome pathway [28]. They showed that the ODDD comprised approximately 200 amino acid residues in the central region of HIF-1α. Deletional mutagenesis of the entire domain resulted in a HIF-1α that was stable and active in the absence of hypoxic induction. Furthermore, the ODDD conferred oxygen-dependent instability when fused to GAL4. In a follow up study, Huang *et al.* provided further, albeit, indirect,

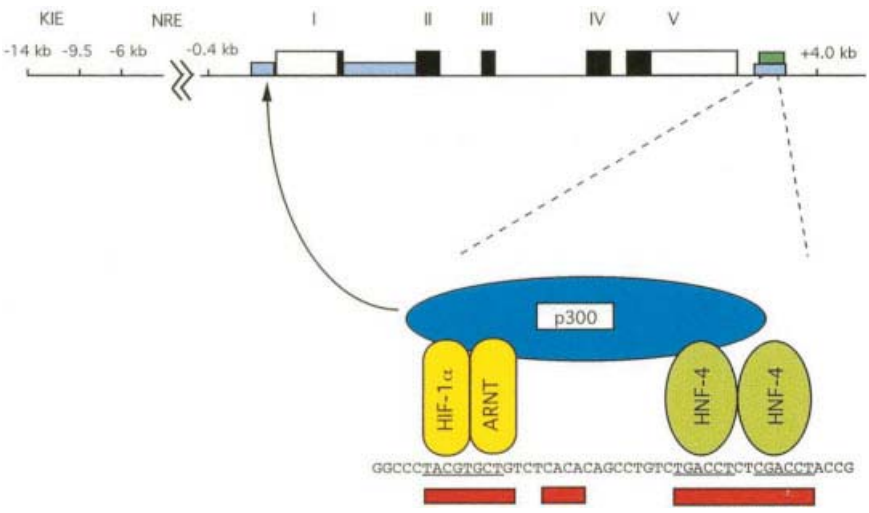

Fig. 3.2 A more detailed structure of the human erythropoietin gene. Exons are again depicted by solid black boxes; 5′ and 3′ untranslated regions are depicted by open boxes. Blue rectangles depict the areas of homology between human and mouse noncoding sequences, and the region of liver-specific DNase I hypersensitivity is shown with a green rectangle. Sites in the 3′ enhancer that are important for hypoxic induction are underlined in red. Binding of HIF-1α, ARNT/HIF-1β, HNF-4, and p300 is shown. The arrow indicates that p300 can interact with the basal transcriptional machinery in the promoter. From reference [30].

evidence that the oxygen sensor is a heme protein [29]. They studied the mechanism by which carbon monoxide and nitric oxide, two heme binding ligands, affect oxygen sensing and signaling. They showed that carbon monoxide suppressed activation of HIF-1 and induction of Epo mRNA by hypoxia but had no effect on HIF-1 induction by cobalt chloride or desferrioxamine. Using sodium nitroprusside, a nitric oxide donor, they found that both nitric oxide and carbon monoxide prevented accumulation of HIF-1α protein, thereby blocking hypoxia-induced HIF-1 DNA binding. In 1999, Ebert and Bunn presented the evidence for a heme protein as oxygen sensor and the mechanism of Epo gene regulation known at that time [30] (Fig. 3.2).

3.3.5
The von Hippel-Lindau Protein, Proline Hydroxylation and the Oxygen Sensor

The seminal clue that finally led to elucidation of the oxygen sensor was the von Hippel-Lindau protein. Von Hippel-Lindau (VHL) disease is an inherited cancer syndrome in which individuals have a predisposition to develop clear cell renal carcinoma, pheochromocytoma, spinal cord cerebellar and retinal hemangioblastoma [31–36]. Hemangiomas of other organs (adrenals, lungs, liver) and multiple pancreatic and renal cysts occur as well. There is a clear pro-angiogenic phenotype. Indeed, Levy *et al*. observed the association of VEGF expression and VHL protein (pVHL) in 1996 [21], and Iliopoulus *et al*. showed that VHL-deficient cells lacked hypoxic regulation that could be restored by ectopic expression of wild type VHL protein [37].

In 1999, Maxwell *et al*. showed that pVHL played a role in oxygen-dependent proteolysis of HIF [38]. In VHL-deficient cells, HIF-1α protein was constitutively stabilized in normoxia, and HIF-1 was activated. Ectopic expression of pVHL restored oxygen-dependent instability of HIF-1α. They found that pVHL and HIF-1α coimmunoprecipitated, indicating direct interaction, and identified pVHL in the hypoxic HIF-1 DNA binding complex. In cells treated with desferrioxamine or cobalt, pVHL and HIF-1 were dissociated. Thus, the pVHL/HIF-1 interaction was iron dependent, and it was required for oxygen-dependent degradation of HIF-1α.

It was known from the work of Huang *et al*. that HIF-1 α contained an oxygen-dependent degradation domain (ODDD) that mediated destruction of HIF-1α by the ubiquitin-proteasome pathway [28]. Cockman *et al*. demonstrated that pVHL was essential for this process [39]. They showed that HIF-1α ubiquitinylation was defective in VHL-deficient renal carcinoma cells and that exogenous expression of pVHL complemented this defect.

This effect was specific for HIF-1α subunits. They went on to demonstrate that short sequences within the internal transactivation domains of HIF-1α were sufficient for recognition by pVHL. Mutagenesis studies delineated the structural requirement for this interaction. The authors concluded that pVHL regulated HIF-1α degradation by functioning as a "recognition component" of a ubiquitin ligase complex.

In 2001, Jaakkola *et al*. demonstrated that HIF-1α targeting to the pVHL ubiquitin E3 ligase complex was dependent upon oxygen-regulated prolyl hydroxylation [40]. These and other studies of HIF-1α demonstrated that proline 564 hydroxylation was critical for this interaction [41, 42]. The authors designated the enzyme responsible for this reaction as **HIF α prolyl hydroxylase** (**HIF-PH**) and concluded that the absolute requirement for oxygen as a cosubstrate and iron as a cofactor suggested that HIF-PH functioned directly as a cellular oxygen sensor. Hydroxylation of proline 402 affords another site for pVHL binding [43].

Strong support for the role of prolyl hydroxylation came from the elegant work of Epstein *et al*. [44]. They observed that the enzymatic activity designated HIF-PH required oxygen and was inhibited by 2-oxoglutarate, suggesting that the enzyme was a member of the superfamily of 2-oxoglutarate-dependent oxygenases. All of these enzymes have an absolute requirement for oxygen (O_2; dioxygen) as cosubstrate. They identified a HIF-1α homologue as well as a pVHL analogue in *C. elegans*. In *C. elegans*, as in mammalian species, interaction of HIF-1 and pVHL was regulated by prolyl hydroxylation. Using common motifs in the 2-oxoglutarate-dependent oxygenase superfamily, they searched *C. elegans* and mammalian databases for additional candidates. Interestingly, in *C. elegans* there is a family of genes related to *C. elegans* gene *egl-9*, identified initially based upon an egg laying abnormality. The gene product of *egl-9* seemed a likely candidate for a *C. elegans* prolyl hydroxylase. Mutant worms containing defective *egl-9* showed a marked upregulation of HIF-1 under normoxic conditions and lacked hypoxic regulation. The authors showed, by expressing recombinant *egl-9*, that the interaction of *C. elegans* HIF-1 and pVHL occurred after incubation with EGL-9 protein.

Further sequence analysis identified three *egl-9* homologs in the rat genome and three in the human genome. Epstein *et al*. designated the human protein products as "prolyl hydroxylase domain containing" (PHD) 1, 2, and 3. Investigation of the interaction of human HIF-1α and pVHL *in vitro* showed that PHD-1, -2 and -3 promoted the interaction, with PHD-1 begin most effective. Also, the activity of PHD-1 was inhibited by iron chelation, cobalt, and a 2-oxoglutarate analogue. Therefore, the prolyl hydroxylases (PHDs) [45] fulfilled all criteria to qualify as oxygen sensors regulating HIF.

In addition to prolyl hydroxylation that regulates the hypoxia inducibility/stabilization of HIF-1*α*, hydroxylation at another site in HIF-1*α* plays a distinct role. Sang *et al.* demonstrated that the C-terminal activation domain (CAD) of HIF-1*α* and its regulation also involved hydroxylase activity that was not dependent upon pVHL [46]. Moreover, stimulation of CAD activity by hypoxia and desferrioxamine was blocked by oncoprotein E1A but not by a mutant E1A defective in targeting p300/CBP. They showed that a hydroxylase inhibitor, hypoxia and desferrioxamine induced the interaction between HIF-1*α* and p300/CBP *in vivo*. This CAD hydroxylation proved to be on asparagine 803 of HIF-1*α*. Indeed, Hewitson *et al.* demonstrated that a hypoxia-inducible **HIF asparagine hydroxylase** was identical to a previously identified HIF interactor designated **factor inhibiting HIF (FIH)** [47]. FIH was found to downregulate HIF-1*α* transactivation and was later shown to interact with HIF-1*α* and pVHL, thus mediating repression of HIF-1 transcriptional activity. Hewitson *et al.* showed that this asparagine hydroxylation abrogated p300 binding to HIF-1*α*. Fig. 3.3 depicts the results of HIF-1*α* proline and asparagine hydroxylation.

The crystal structure of a hydroxylated HIF-1*α* peptide containing the target prolyl residues bound to the complex of pVHL, elongin C and elongin B has been solved, demonstrating a single conserved hydroxyproline-binding pocket in pVHL [48, 49]. Similarly, the crystal structure of HIF as-

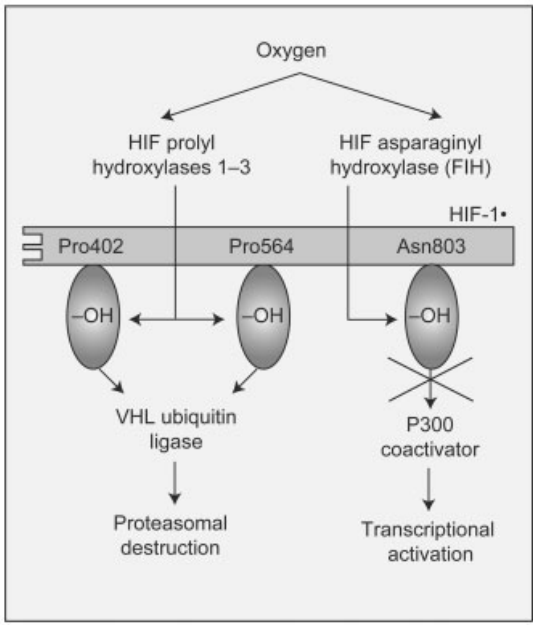

Fig. 3.3 Regulation of human HIF-1*α* by proline and asparagine hydroxylation. From reference [66].

paragine hydroxylase (FIH) complexed with iron, 2-oxoglutarate and CAD fragments reveals the structural basis of this HIF modification [50].

There is another mechanism for downregulation of HIF-1 transactivation activity. CITED2 (Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2) (also known as Mrg1 and p35srj) interacts with p300/CBP [51–55] and acts as a negative regulator of HIF-1*α*. Freedman *et al.* have shown that CITED2 binds p300/CBP and, thus, competes with HIF-1 binding [56]. They reported the high resolution solution structure of the CITED2 transactivation domain complexed to the p300 CH1 domain.

3.3.6
Modulation of HIF-1 Activity by Other Signals

Even under non-hypoxic conditions, several growth factors and cytokines can induce HIF-1*α* protein expression and induce HIF-1 target gene expression [57–60] (reviewed in [61]). Signaling by the phosphatidylinositol 3-kinase pathway appears to upregulate HIF-1*α* protein and signaling by the MAP kinase pathway increases transcriptional activity. Sang *et al.* have presented evidence that the MAP kinase signal acts on p300/CBP [62]. Therefore, the expression of Epo and other hypoxia-regulated genes is subject to a wide variety of influences besides hypoxia itself.

3.3.7
A Final Word on the Heme Protein Hypothesis

If HIF prolyl hydroxylase and arginine hydroxylase are oxygen sensors, how can we explain the data that pointed to a heme protein [30]? Some information is coming to light that provides an insight.

Both **cobalt** and **nitric oxide** can interact with heme and can mimic hypoxic induction, but each has another action. Yuan *et al.* demonstrated that **cobalt bound to HIF-2*α*** *in vitro* with high affinity and in an oxygen-dependent manner [63]. HIF-2*α* that was stabilized with a proteasome inhibitor could bind cobalt, whereas hypoxia-stabilized HIF-2*α* could not. They also made mutations within the ODDD of HIF-2*α* that prevented cobalt binding and resulted in accumulation of HIF-2*α* during normoxia. They showed further that **cobalt inhibited the interaction of pVHL and HIF-2*α*** even when HIF-2*α* was proline hydroxylation [64]. Nitric oxide mimics hypoxia by a different mechanism. Metzen *et al.* have demonstrated that **nitric oxide inhibits HIF-1*α*** ubiquitinylation and interaction with pVHL and have presented evidence that this is due to **inhibition of prolyl hydroxylases** [65].

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4
Physiology and Metabolism of Erythropoietin

4.1
The Kidney as the Site of Production

The importance of the kidney as the site of production of circulating Epo was established several decades ago (see Chapter 1). As shown in Fig. 4.1, Epo is produced in the kidney in response to the oxygen tension of the blood. Epo then circulates to the bone marrow where it stimulates the proliferation and differentiation of red blood cell progenitors, leading to more red blood cells and increased oxygen-carrying capacity. Why nature should have chosen the kidney was addressed in an intriguing article by Erslev *et al.* [1]. As the authors explain, Epo induction of red blood cell production theoretically should fail because of a positive feedback mechanism. As red cell numbers increase, whole blood viscosity also increases, eventually resulting in impaired blood flow and the induction of tissue hypoxia. This hypoxia should then stimulate increased Epo production, causing an even further increase in red blood cell numbers and viscosity. However, this does not occur. Erslev *et al.* proposed that decreased blood flow to the kidneys does not cause renal hypoxia. Rather, renal oxygen tension is determined principally by oxygen consumption used for sodium readsorption. Since sodium readsorption is proportional to glomerular filtration rate, a reduced blood flow should lead to a concomitant reduction in oxygen consumption with no net change in tissue oxygen tension. This is supported by the observation that the marked erythrocytosis seen in individuals with polycythemia vera does not, in fact, result in increased production of Epo.

The identity of the cells in the kidney responsible for Epo production has been the subject of considerable debate. Early immunohistochemistry studies by Fisher and coworkers localized Epo to the glomerulus [2]. Other groups have also identified Epo in the glomerulus and/or mesangial cells [3–5]. Using immunoelectron microscopy and highly specific antipeptide antibodies to Epo [6], Fisher and Sytkowski identified Epo in

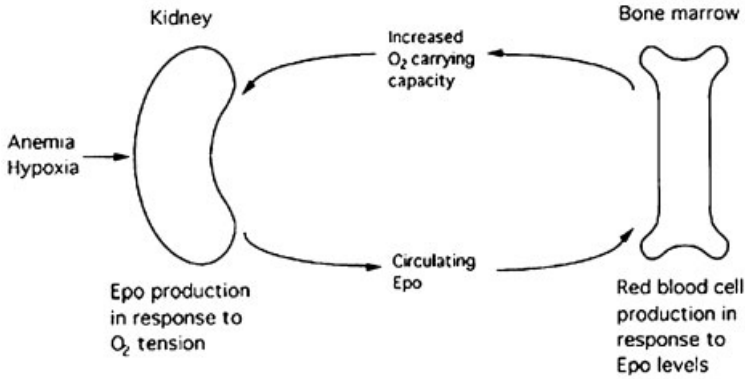

Fig. 4.1 The erythropoietin feedback loop. From reference [45].

the glomerular epithelial cells in anemic experimental animals (Fig. 4.2). However, localization of Epo protein in the glomerulus does not prove site of production.

Epo biological activity has also been associated with renal glomeruli. Jelkmann *et al.* isolated glomeruli from the kidneys of hypoxic rats [7]. The investigators prepared homogenates of these glomeruli and used an *in vitro* fetal mouse liver cell bioassay to demonstrate Epo activity that was abolished with anti-Epo antiserum. Kurtz *et al.* developed cultures of renal mesangial cells derived from the isolated glomeruli of rat kidneys and grew them as homogeneous cell lines [3, 8, 9]. Epo was detected in the culture medium, and it was increased in a lower oxygen environment or by adding cobalt chloride.

In 1988, Koury *et al.* applied *in situ* hybridization to the problem in search of cells expressing Epo mRNA [10]. These investigators isolated kidneys from anemic and non-anemic mice and hybridized them with a ^{35}S-labeled RNA probe complementary to murine Epo mRNA. They identified cells located in the peritubular areas of the anemic mouse kidney cortices that were intensely labeled. The cells were neither glomerular nor tubular. Counterstaining of the sections removed the silver grains allowing better visualization of the cells underlying the label (Fig. 4.3). Similar results were obtained by Lacombe *et al.* [11]. In the follow up study, Koury *et al.* showed that the number of cells containing Epo mRNA increased with a decreasing hematocrit in the experimental animals [12].

Other investigators have provided data indicating that the tubular cells themselves are the source of Epo production. Maxwell *et al.* used both *in situ* hybridization and immunohistochemistry and identified both Epo mRNA and Epo protein in renal tubular cells [13]. Using a different approach, Loya *et al.* studied transgenic mice carrying the Epo gene pro-

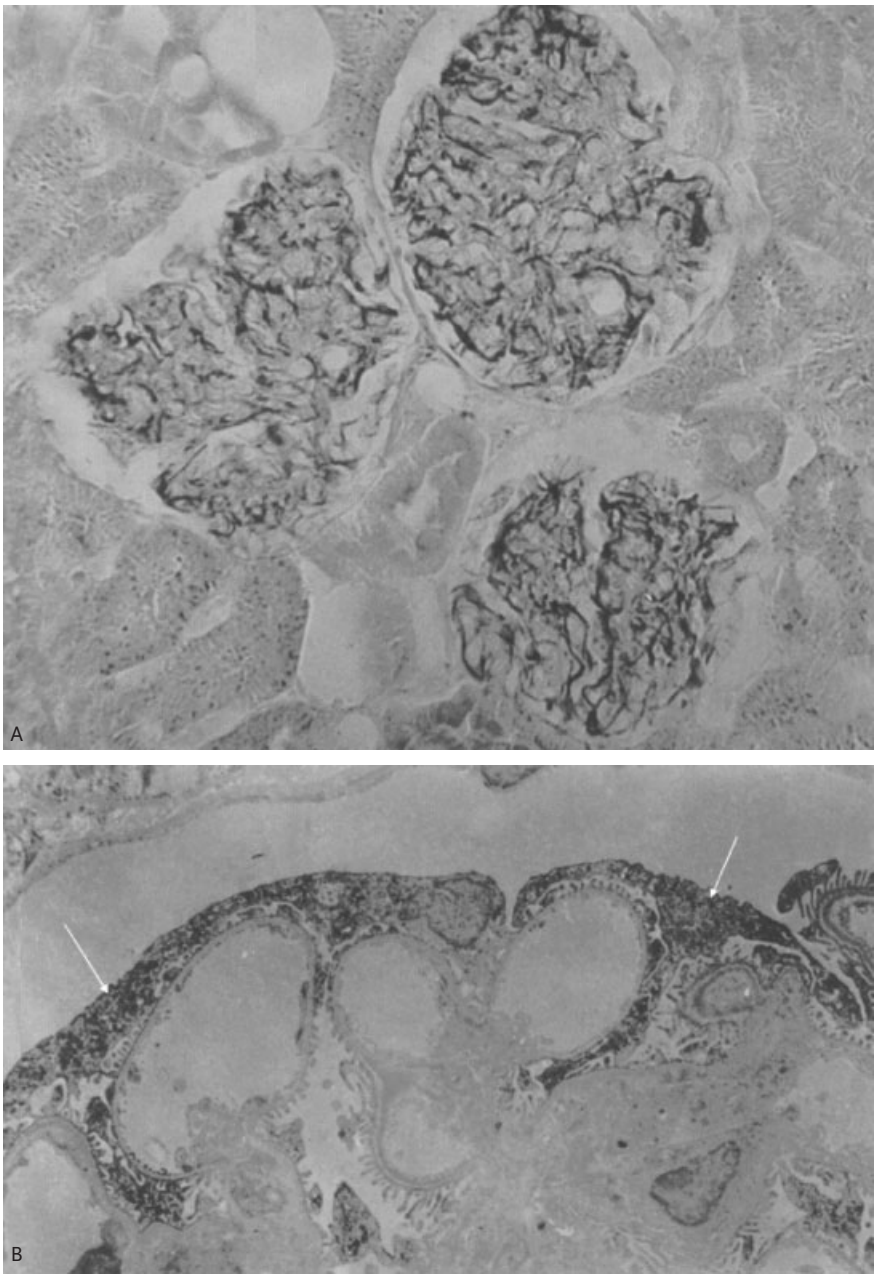

Fig. 4.2 Immunohistochemical identification of Epo protein in the renal glomeruli of anemic animals by electron microscopy. Panel A, low power view showing glomeruli containing electron dense material indicating antibody binding. Panel B, higher power view. Note electron dense material in the glomerular epithelial cells. J.W. Fisher and A.J. Sytkowski, unpublished.

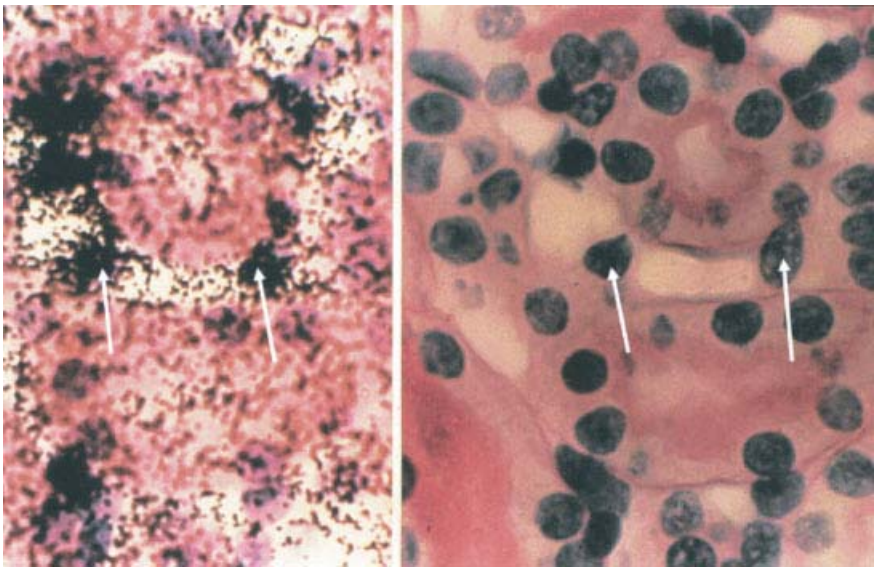

Fig. 4.3 Identification of Epo producing cells in anemic murine kidneys by *in situ* hybridization. Left panel, clusters of silver grains overlie cells containing Epo mRNA. Right panel, same view as left panel but with silver grains removed to reveal the underlying peritubular cells. Arrows point to two Epo-producing cells. Original image courtesy of Dr. M. J. Koury. Modified from reference [10].

moter linked to *lacZ* and found the reporter gene expressed in proximal convoluted tubular cells after hypoxia [14]. Da Silva *et al.* have shown that in human renal cancers expressing Epo, cells of tubular origin appear to be the site of synthesis [15]. Another *in situ* hybridization analysis of a continuous human renal carcinoma tumor line for Epo mRNA showed that expression of the Epo gene on a cell by cell basis is quite heterogeneous, with some cells expressing relatively large amounts and others little or none at all [16]. Additional support for renal tubules as a source of Epo was provided by Mujais *et al.* who detected Epo mRNA in tubular cells from microdissected kidneys using RT-PCR [17].

The peritubular interstitial cells expressing Epo mRNA identified by Koury *et al.* and Lacombe *et al.* are probably a type of fibroblast. Using a combination of *in situ* hybridization and immunohistochemistry, Bachmann *et al.* found that Epo mRNA was detected only in the peritubular interstitial cells of the kidneys of anemic rats [18]. They found that the cells were not associated with the lumina of peritubular capillaries but instead were located between adjacent tubules or between tubules and blood vessels. They designed a double labeling technique to allow both *in situ* hybridization for Epo mRNA and immunohistochemical detection of

ecto-5′-nucleotidase, which is a surface marker for peritubular interstitial fibroblasts. Indeed, Epo mRNA was localized in the central perinuclear part of the interstitial cells while the ecto-5′-nucleotidase label was present on the cell surface. A separate study also co-localized Epo mRNA with ecto-5′-nucleotidase [19]. Maxwell and coworkers derived transgenic mice using regulatory sequences from the murine Epo gene flanking the SV40 T antigen as a marker. In one of the strains, the transgene happened to integrate into the endogenous Epo locus by homologous recombination. The authors showed that, in this type of transgenic animal, renal Epo expression was restricted to cells in the interstitium of the cortex and outer medulla. Light and electron microscopic immunohistochemical studies demonstrated these cells to be fibroblast-like type 1 interstitial cells.

4.2
The Liver as a Site of Erythropoietin Production

As described in Chapter 2, the liver is the principal site of Epo production during fetal life. It seems clear from several studies that two types of cells in the liver produce Epo. Koury *et al.* applied *in situ* hybridization using antisense RNA probes to study the livers of anemic transgenic mice expressing the human Epo gene and in livers of anemic nontransgenic mice [20]. In anemic transgenic mice, they found that hepatocytes surrounding central veins contained large amounts of human Epo mRNA, whereas in the better oxygenated areas surrounding the portal triads, Epo mRNA-containing cells were very rare. In less anemic transgenic mice, the cells were less heavily labeled. In the livers of anemic nontransgenic mice, isolated cells contained murine Epo mRNA. Two types of cells were identified. Eighty percent of the Epo mRNA-containing cells were hepatocytes, and 20% were non-epithelial cells located in or adjacent to the sinusoidal spaces. Using the Epo-SV40 T antigen transgenic animals, Maxwell *et al.* also found two liver cell populations expressing the T antigen [21]. In addition to hepatocytes, they identified a second, non-parenchymal cell type. Using immunohistochemistry and electron microscopic examination, the authors identified these cells as Ito cells lying in a perisinusoidal location within the space of Disse.

4.3 Erythropoietin Produced in the Bone Marrow

There is evidence indicating that Epo is also produced in the bone marrow. Hermine *et al*. used RT-PCR and demonstrated an Epo PCR product in both human and murine bone marrow [22]. They used antisense oligodeoxynucleotides and showed that downregulating either Epo or its receptor caused a decrease in mixed erythroid/nonerythroid colony formation *in vitro*. In another study, Stopka and coworkers showed that differentiating $CD34^+$ cells expressed both Epo and Epo receptor detected by RT-PCR [23]. They further showed that cells in BFU-E-derived colonies were positive for Epo mRNA. They subjected cell lysates of BFU-E-derived colonies to SDS-PAGE and western blotting with anti-Epo antibody and detected Epo protein in the lysates. These findings suggest a potential autocrine or paracrine action of Epo within the bone marrow.

Epo and Epo receptor are also expressed by other cell types in the body. This subject is addressed in Chapter 9.

4.4 Metabolism and Clearance of Erythropoietin

The mechanisms responsible for clearance of Epo from the circulation are still under investigation. As reviewed by Jelkmann, our understanding of the "enigma of the metabolic fate of circulating Epo" must be viewed in the context of pharmacokinetic experiments carried out using urinary Epo and recombinant human Epo [24]. Glycosylation is critically important for Epo's *in vivo* half-life and action. Since both urinary Epo and various recombinant human Epo's exhibit subtle and sometimes major differences in glycosylation compared with endogenous Epo, the use of these proteins to elucidate the behavior of endogenous Epo in the circulation must, at best, give an approximation of normal physiology.

In one of the first published studies of its kind, Fu *et al*. compared the pharmacokinetics of crude human urinary Epo with that of ^{125}I-labeled, purified recombinant human Epo in dogs [25]. They showed that the plasma disappearance of both urinary Epo and recombinant Epo conformed to a biexponential equation with the central compartment being larger than the peripheral compartment. Interestingly, the mean distribution half-life for urinary Epo (75.3 ± 21.2 min) was significantly longer than that of ^{125}I-labeled recombinant Epo (23.7 ± 5.0 min). They also showed that, in anephric dogs, the clearance of recombinant Epo was significantly

slower than in intact dogs, leading the authors to conclude that the kidney contributed significantly to the elimination clearance of Epo.

Spivak and Hogans compared *in vivo* plasma clearance and organ accumulation of ^{125}I-labeled recombinant human Epo and labeled desialylated recombinant human Epo. They showed that plasma clearance of the fully sialylated molecule was multiexponential with an initial rapid distribution phase ($T_{½}$ = 53 min) and a slower elimination phase ($T_{½}$ = 180 min). As expected, 95% of the desialylated Epo was cleared with a $T_{½}$ = 2.0 min. It was found principally in the liver, since desialylation exposes the penultimate galactose residues of the oligosaccharides, allowing clearance by the asialoglycoprotein receptor in the liver. Small amounts of intact Epo accumulated in the kidneys and bone marrow.

Most studies in both healthy volunteers and in patients with uremia have revealed biexponential clearance patterns [26–31]. Brockmöller *et al.* suggested that the rapid initial phase might be due to binding of recombinant Epo to endothelial and erythroid cells [30]. As pointed out by Jelkmann [24], this nonlinear behavior of recombinant Epo suggests that its elimination involves a saturable mechanism. In another study, Kinoshita compared the pharmacokinetics and distribution of radiolabeled human urinary and recombinant Epo in rats and found no difference between the two proteins [32]. Macdougall *et al.* have reported no difference in the disappearance rate of recombinant Epo between normal volunteers and patients with chronic renal failure [33]. A comprehensive review of clinical pharmacokinetic properties of recombinant human Epo has recently appeared [34]. Considering all of the evidence, the kidney does not seem to be an important site of recombinant Epo clearance in humans.

In an interesting study, Flaharty *et al.* demonstrated different pharmacokinetic parameters based upon size of dose [35]. Using doses of 10, 50, 150, 500 and 1000 IU/kg, these authors found decreasing clearance with increasing dose with a striking three-fold difference between 10 and 50 IU/kg. Although studies in which urine samples have been collected have shown that less than 5% of dose is recovered in its intact form, the data of Flaharty *et al.* indicate a six-fold higher renal clearance at the small dose of 10 IU/kg than at 1000 IU/kg. Thus, it remains possible that the low physiologic levels of circulating endogenous Epo may clear quite rapidly and that the kidney may be a significant site of clearance of the endogenous hormone.

The liver has been considered as a possible site of Epo clearance, and it is well known that asialoglycoproteins, including desialylated Epo, are rapidly cleared by this organ. However, there is little evidence that either endogenous or recombinant Epo undergo significant desialylation while circulating *in vivo*. As has been pointed out by Macdougall, the nonphysio-

logic doses of Epo used in clinical studies make it difficult to extrapolate from the pharmacokinetic characteristics observed in these studies to those operating in normal physiology [36].

In 1959, Stohlman and Brecher hypothesized that endogenous Epo was for the most part catabolized by an Epo receptor-mediated mechanism, noting that in patients with aplastic anemia who lack erythroid progenitor cells, Epo levels are much higher than in patients with thalassemia intermedia at the same hemoglobin concentration [37]. In reflecting on the pharmacokinetic studies of recombinant human Epo and NESP, an Epo mutant with two additional N-linked glycosylation sites [38, 39], Jelkmann has pointed out that NESP may well exhibit its longer *in vivo* half-life partly due to its reduced affinity for the Epo receptor, thus emphasizing the receptor-mediated clearance hypothesis [24]. Jelkmann goes on to propose "it seems most likely that native Epo, rhEpo and NESP are degraded following Epo receptor mediated uptake, mainly in the bone marrow".

More evidence for bone marrow as a site for Epo clearance has come from the studies of Chapel *et al.* [40]. These investigators determined Epo's pharmacokinetic parameters in five adult sheep in a paired manner before and after bone marrow ablation using busulfan. They observed progressive decreases in plasma clearance, elimination half-life ($T_{½}$ beta) and the volume of distribution as ablation progressed. Similar results were obtained in animals treated with 5-fluorouracil.

Comparing the pharmacokinetic results obtained with marrow ablation in new born lambs with those seen in adult sheep, Veng-Pedersen *et al.* found a larger nonhematopoietic tissue clearance of Epo in lambs [41]. The authors stated that this much higher number of nonhematopoietic Epo receptors in the lamb suggests that they are more needed in early life, for example, in providing neuro-protection. Epo receptor numbers were not actually quantified, however. The subject of Epo and neuro-protection is addressed in Chapter 9.

4.5 Erythropoietin and the Maternal/Fetal Circulation

The question of Epo's ability to cross the placenta remains controversial. Koury *et al.* studied mice and demonstrated that significant amounts of ^{125}I-labeled Epo administered to pregnant mice was transferred to the fetuses [42]. However, Eichhorn *et al.* have shown the lack of association between fetal and maternal serum Epo levels at birth, stating that fetal blood Epo concentrations are independent of maternal levels and that Epo

does not cross the human placental barrier [43]. Malek *et al*. developed an *in vitro* profusion system of the human placenta [44]. They found that the total transfer of recombinant Epo was approximately 0.04 percent of the amount initially added to the maternal compartment, suggesting that the maternal/fetal transfer of Epo in humans is insignificant.

4.6 References

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5
Biochemistry and Protein Structure

5.1
Naturally Occurring Epo

5.1.1
Difficulties in Purifying the Hormone

The isolation and purification of naturally occurring Epo was a difficult task. As with all biochemical isolations, an abundant source of starting material and a reliable assay were required. In the case of Epo, assays were difficult to standardize and somewhat cumbersome to carry out. The source of starting material was an even greater problem. It was clear from early studies that Epo was not stored in any great quantities in any organ of the body. Unlike insulin-producing islet cells of the pancreas, there were no clusters of Epo-producing cells that could be isolated readily from which substantial amounts of hormone could be purified. Potential sources of naturally occurring Epo included the urine of anemic large animals, including humans, plasma of anemic animals, various organs such as the kidney, and cell lines derived from tumors such as renal tumors that spontaneously produced Epo.

After several years of work to purify Epo from the plasma of anemic sheep, Goldwasser and Kung reported the isolation of sub-milligram amounts of apparently pure material with a potency of 7450–8250 U/mg [1]. Because the amount available was so small, further characterization was limited. These workers later reported an apparent molecular weight of 46 000 obtained by SDS polyacrylamide gel electrophoresis and a sedimentation coefficient of 4.6 S [2].

An interesting approach to the isolation of Epo in the late 1970s was taken by Spivak *et al*. [3]. These investigators discover that wheat germ agglutinin and phytohemagglutinin had bound to Epo significantly in crude urinary preparations. Immobilizing wheat germ agglutinin on agarose allowed an 8- to 100-fold purification of urinary Epo with recoveries of greater than 40%. However, homogeneity was not achieved.

Even without pure material, some information on Epo's biochemical properties was obtained. For example, Epo was found to be sensitive to tryptic digestion. Furthermore, treatment with sialidase markedly shortened its *in vivo* half life [4], demonstrating that terminal sialic acid residues were present on the protein's oligosaccharides. Also, the presence of one or more disulfide bonds critical for biological activity was proposed [5].

5.1.2 The Purification of Human Urinary Epo

In 1977, Miyake *et al.* described a seven step procedure that yielded highly purified human Epo [6]. Remarkably, the starting material was approximately 2550 liters of urine collected in Japan from patients with aplastic anemia. The urinary protein was isolated and lyophilized. After significant trial and error, a purification scheme was developed that yielded a preparation with a potency of 70 400 U/mg with a 21% yield. Interestingly, two pure fractions were obtained that exhibited slightly different mobilities when subjected to gel electrophoresis at pH 9. An asialo form was also identified. The apparent molecular weight determined by SDS polyacrylamide gel electrophoresis for the native form was 39 000.

A second purification of human urinary Epo was reported by Sasaki and colleagues [7, 8]. These investigators had developed monoclonal antibodies to Epo [9]. They prepared an immunoaffinity column by coupling these antibodies to agarose and isolated approximately 6 mg of Epo from approximately 700 liters of human urine. They reported a specific activity of 81 600 U/mg. Some heterogeneity shown by SDS-PAGE and Western blot presumably was due to partial deglycosylation. They also reported an N-terminal amino acid sequence of 30 amino acids, which differed in three positions from the N-terminal sequence disclosed by Goldwasser [10].

5.1.3 Biochemical Properties of Human Urinary Epo

Despite the publication of two purification methods, pure human urinary Epo remained difficult to obtain, principally due to paucity of starting material. Therefore, several years passed before any significant biochemical characterization of the material was published.

An initial carbohydrate composition of human urinary Epo was reported [11]. It was shown that treatment with endoglycosidase F removed

most of the carbohydrate from the protein indicating that the oligosaccharides were attached to asparagine residues. There was an apparent lack of O-linked sugar indicated by the absence of N-galactosamine. Removal of carbohydrate resulted in complete loss of *in vivo* biological activity in mice but activity was retained when assayed *in vitro* in bone marrow cell culture.

In 1986, fully three years after the human Epo gene was sequenced (see below) [12, 13], Lai *et al.* reported the primary structure of human urinary Epo as well as the apparent glycosylation sites, disulfide bonds and a preliminary secondary structural characterization by circular dichroism [14]. The authors reported that the hormone was a polypeptide of 166 amino acids. Four cysteine residues at positions 7, 29, 33 and 161 were found with disulfide bond formation between C7 and C161 and between C29 and C33, confirming the prediction of the presence of intrachain disulfides [15]. The calculated molecular weight was reported as 18 398. Consensus sequences for N-linked glycosylation sites at positions 24, 38 and 83 were reported. The authors deduced that position 126 was a serine that was O-glycosylated, in contrast to previous work from the same group one year earlier [11]. The circular dichroism spectrum obtained indicated a helix content of 50%, antiparallel and parallel beta sheet content of 0%, and "turns and others" of 50%.

5.1.4
Some Biochemical Properties of Human Serum Epo

In 2001, Skibeli *et al.* reported the isolation of Epo from the serum of anemic human donors using an immunoaffinity method [16]. The main purpose of the study was to carry out analyses of the oligosaccharide structures and to compare them to those found in various forms of recombinant human Epo (see below). The authors used charge analysis of oligosaccharides released from Epo and found that human serum Epo contained only mono-, di-, and tri-acidic oligosaccharides, lacking the tetra-acidic oligosaccharides present in the glycans of recombinant Epo. They suggested that such sugar profiling may be useful in the diagnosis of medical conditions, in pharmaceutical quality control and in evaluating misuse of recombinant Epo in sports. Interestingly, they found that a substantial fraction of human serum Epo lacks O-glycosylation on serine 126. This may explain the apparently contradictory results reported by Dordal *et al.* and Lai *et al.* [11, 14].

5.2
Recombinant Human Epo

5.2.1
Cloning the Human Epo Gene

The human Epo gene was cloned independently by two groups within a few weeks of each other. Although second to publish, Lin and coworkers were apparently the first to isolate the human Epo gene from a genomic phage library using mixed oligonucleotide probes derived from primary amino acid sequence information [13]. Jacobs *et al.* used a similar approach and obtained a human cDNA from a human fetal liver library as well as the gene itself [12]. The gene encodes a 27 amino acid peptide followed by a 166 amino acid mature protein. The primary structure along with disulfide bonds and glycosylation sites are depicted in Fig. 5.1.

There is some debate regarding the fate of arginine 166 (R166) of the mature protein. Although Lai *et al.* demonstrated its presence in human urinary Epo by amino acid sequence determination [14], Recny and coworkers used peptide mapping and fast atom bombardment mass spectrometry (FABMS) and found that R166 was completely missing from the purified recombinant protein obtained by expressing the human Epo cDNA in Chinese hamster ovary (CHO) cells [17]. They also reported that

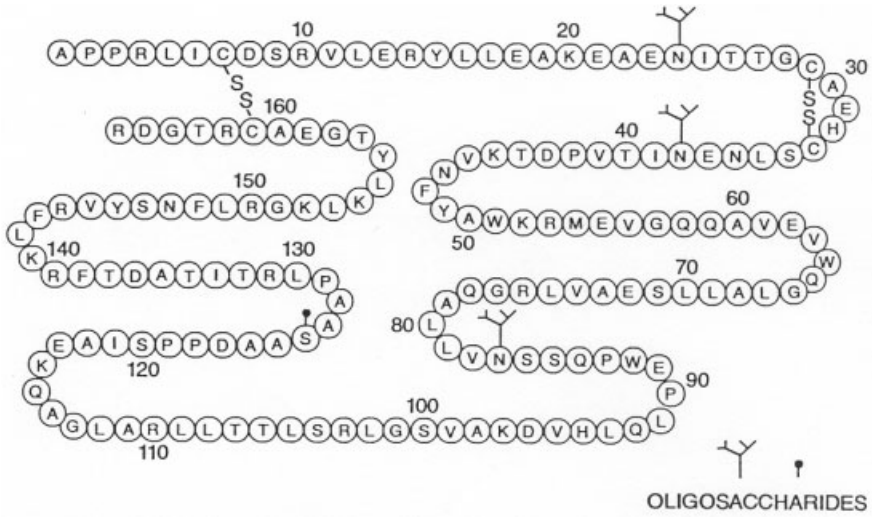

Fig. 5.1 The primary structure of human erythropoietin. The sequence of the mature 166 amino acid protein is shown in single letter code. Note the presence of two disulfide bonds (C7–C161 and C29–C33) and the four oligosaccharide chains attached to N24, N38, N83 and S126. From reference [44].

R166 was missing from purified human urinary Epo, a finding that contradicted the direct sequence analysis of Lai *et al*. Most literature states that mature human Epo is a 165 amino acid protein, based upon the Recny paper. It is possible that the absence or presence of R166 on human Epo may relate to the cells in which it is expressed, and it is also possible that Epo possessing R166 and lacking it may both exist in the circulation of humans.

There is a high degree of amino acid sequence homology among mammalian Epos. Wen *et al*. sequenced Epo cDNAs from Rhesus monkey, rat, sheep, dog, cat and pig and compared the deduced amino acid sequences with those of human and cynomolgus monkey and mouse that had already been reported [18]. They found that human Epo is 91% identical to monkey Epo, 85% to cat and dog Epo, and approximately 80–82% to pig, sheep, mouse and rat Epos. All three N-linked glycosylation sites are conserved and the long disulfide bridge linking the amino and carboxy termini (C7–C161 in humans) is conserved in all species. The short disulfide bridge (C29–C33 in humans) is not present in rodents; instead, C33 is replaced by proline. Also, the site of O-link glycosylation (S126 in humans) is preserved across all species tested except rodents. These homologies are shown diagrammatically in Fig. 5.2.

5.2.2 Glycosylation of Epo

Glycosylation and, especially, terminal sialylation of the oligosaccharides, are essential for Epo's biological activity *in vivo*. Treatment of Epo with sialidase to remove terminal sialic acids exposes the underlying galactose residue leading to rapid clearance of Epo mediated by the asialoglycoprotein receptor in the liver [4]. In contrast, increasing the number of sialic acids prolongs Epo's *in vivo* half life. NESP (**n**ovel **e**rythropoiesis **s**timulating **p**rotein) is a mutated Epo derivative in which five amino acids have been changed resulting in the addition of two additional N-linked glycosylation sites [19, 20]. This protein exhibits a plasma half life in humans that is longer than that of recombinant human Epo.

Glycosylation affects *in vitro* biological activity and affinity for the Epo receptor, although the effect on activity is opposite to that seen *in vivo*. Tsuda *et al*. subjected recombinant Epo to digestion with specific glycosidases and isolated the proteins that were deglycosylated to different extents [21]. They showed that removal of sialic acids yielded Epo with a higher affinity for the receptor and an increased *in vitro* activity. They also found that removal of the oligosaccharides decreased thermal stability of

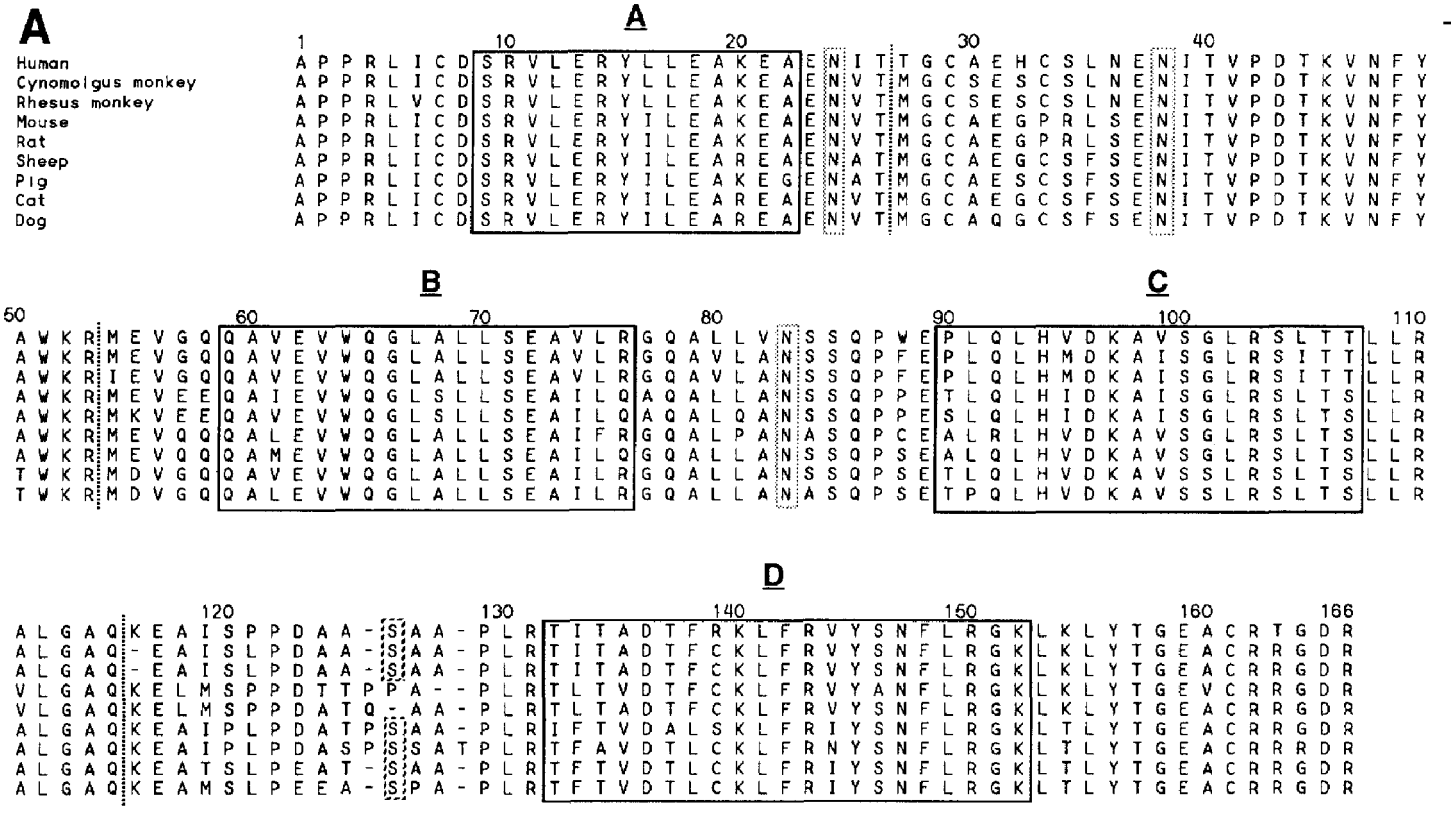

Fig. 5.2 Alignment of the primary structures of mature mammalian Epo proteins. Plain boxes indicate the positions of the predicted four α helices in the human sequence [38]. The N- and O-linked glycosylation sites are shown by dashed boxes. The vertical lines delineate the boundaries between exons. From reference [18].

the protein. Yamaguchi *et al.* prepared seven mutant genomic clones that lacked the N-glycosylation sites in all possible combinations and expressed these transiently in baby hamster kidney (BHK) cells [22]. Expression of Epo in which all three N-glycosylation sites were mutated was only 10% that of the wild type hormone. These authors confirmed that removal of N-linked glycosylation sites increased affinity for the receptor and that *in vitro* activity was retained while *in vivo* activity was low to absent. Delorme *et al.* showed that removal of any of the N-glycosylation sites reduced *in vivo* but not *in vitro* biological activity [23]. These authors specifically addressed the question of serine 126 glycosylation and found that mutation of serine 126 to valine resulted in a molecule that was only slightly less active *in vivo* than was wild type Epo. They concluded that O-linked glycosylation of serine 126 is not essential for activity. In a study of the role of N-linked sugars on Epo's activity, Higuchi *et al.* used enzymatic digestion to partially or completely remove N-linked or O-linked glycans [24]. They observed that as N-deglycosylation progressed, the *in vivo* activity decreased markedly and that this activity was correlated with the number of sialic acid residues on the molecule. In contrast, *in vitro* activity was increased by N-deglycosylation. The completely N-deglycosylated Epo had a 3-fold higher specific activity than did the fully glycosylated hormone. On the other hand, removal of the O-linked sugar on serine 126 had no effect on either *in vivo* or *in vitro* activity.

Glycosylation also affects the efficiency of protein production. Using site-directed mutagenesis, Dubé *et al.* observed that glycosylation appeared to be critical for proper biosynthesis and secretion of Epo [25]. Mutant proteins lacking glycosylation at the N-linked sites were secreted far less efficiently and remained associated intracellularly with membrane components or were degraded. Takeuchi *et al.* carried out a comparative study of the N-linked oligosaccharides of recombinant Epo prepared from CHO cells with those of human urinary Epo [26]. They found that the sugar chains were similar except for the sialyl linkage. All oligosaccharides from recombinant Epo contained only NeuAc alpha 2–3Gal linkage while those of human urinary Epo contained NeuAc alpha 2–6Gal linkage together with the NeuAc alpha 2–3Gal linkage. They reported that the major sugar chains were fucosylated tetra-antennary complex type with and without N-acetyllactosamine-repeating units in their outer chain and small amounts of 2,4- and 2,6-branched triantennary and biantennary sugar chains. Further studies have provided even greater detail about oligosaccharide structures of recombinant human Epo. For example, Nimtz *et al.* reported on the structures on N- and O-linked glycans of recombinant human Epo expressed by BHK cells [27]. As expected, some differences were observed between the N-linked structures on BHK cell-produced Epo and those of

CHO-cell produced Epo. Additionally, only 60% of the Epo protein from BHK cells was O-glycosylated at serine 126. Interestingly, this mixture of O-glycosylated and non-glycosylated Epo molecules is also seen in Epo expressed in COS cells [23] as well as in human serum Epo [16]. This may be a function of the glycosylation machinery found in kidney-derived cells. Its biological significance remains to be determined.

5.2.3 Physicochemical Properties of Epo

Davis *et al.* carried out a physicochemical characterization of recombinant human Epo produced in CHO cells and made some comparisons with human urinary Epo [28]. They used circular dichroism, UV absorbance and fluorescence spectroscopy and found no significant differences could be found between the recombinant protein and human urinary Epo. Using sedimentation equilibrium, a molecular weight of 30 400 was calculated. The authors estimated the Stokes' radius of the recombinant protein to be 32 Å – not the 20 Å calculated for a sphere of the observed molecular weight. They ascribed this difference to the extensive glycosylation. Acid titration demonstrated a conformational transition with a midpoint of pH 4.1, suggesting that "net charges on the protein moiety rather than on the whole molecule play a role in protein structural stability".

Narhi *et al.* studied protein unfolding, assessed by circular dichroism, and compared stability among fully glycosylated Epo, asialo Epo and nonglycosylated Epo [29]. They found that Epo was stabilized by glycosylation. The oligosaccharides increased Epo stability to denaturing agents such as heat and guanidinium hydrochloride. With respect to asialo Epo, it was similar to that of fully glycosylated Epo at acidic pH, suggesting that although glycosylation was important for tertiary structural stability, the sialic acid residues were not. The stability of nonglycosylated Epo was less than either of the two species.

5.3
Structure-activity Relationships

5.3.1
Antibody Studies

Once the gene was cloned and sequenced and the amino acid sequence was available, structure-activity relationship studies began in earnest. Even before the complete sequence was made public, a preliminary 28 amino acid N-terminal sequence of Epo was reported [10]. This allowed Sue and Sytkowski to prepare antipeptide antibodies directed against the first 26 amino acids of that sequence [30]. Even though the sequence ultimately proved to contain two errors, these antipeptide antibodies bound to radiolabeled human urinary Epo and immunoprecipitated it from solution. Importantly, the antibodies also failed to neutralize Epo's activity in an *in vitro* bioassay, thereby leading the authors to conclude that the amino terminus of Epo did not play an important role in receptor recognition. Similar results were obtained with a monoclonal antibody directed against this same peptide [31].

Sytkowski and Donahue subjected the amino acid sequence derived from the cDNA sequence of Epo to a hydrophilicity analysis to identify domains that have a higher probability of being on the surface of the molecules and, thus, being accessible to antibody probes [32]. Using this **epitope mapping** approach, the authors raised antibodies against synthetic peptides corresponding to six hydrophilic domains. Antibodies to five of these peptides immunoprecipitated Epo. Antibodies to the carboxy terminal peptide 147–166 failed to do so. The antibodies were affinity purified, and their capacity to inhibit Epo's activity in a bioassay was assessed. Only antipeptide 99–118 and antipeptide 111–129 antibodies blocked the hormone's action, suggesting that these domains played a functional role in Epo's receptor recognition. The importance of domain 99–118 was confirmed later by mutagenesis studies (see below) [33–35].

Using a similar epitope mapping approach, Fibi *et al.* prepared antibodies to five different hydrophilic peptides [36]. They found that antibodies to peptide 152–166, the carboxy terminus of Epo, inhibited Epo's biologic activity, leading them to suggest that the carboxy terminal region of the molecule was essential for biologic activity.

5.3.2
Mutagenesis Studies

Boissel and Bunn initially used **deletion mutagenesis** of Epo to examine a hypothetical four alpha helix bundle structure [37]. They found that mutations in any of the four proposed helical domains eliminated biological activity. In 1993, Boissel *et al.* provided more detail on the proposed structural model of Epo [38]. This model was based upon the primary amino acid sequence and on the location of the disulfide bonds of the molecule. The ribbon diagram of the predicted Epo tertiary structure that they proposed is shown in Fig. 5.3.

These investigators went on to carry out an analysis of this model by expressing in COS cells site-directed mutants of Epo and analyzing them by radioimmunoassay and bioassay. They found that deletions of 5–8 resi-

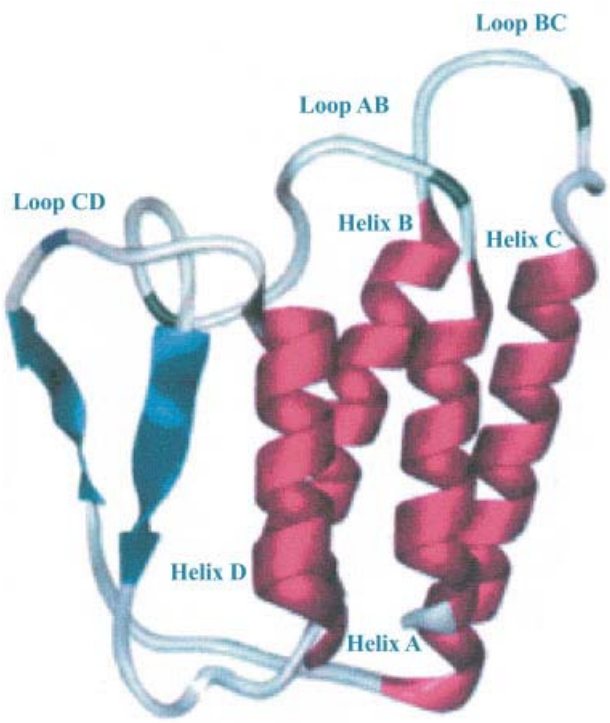

Fig. 5.3 Ribbon diagram of the predicted tertiary structure of human erythropoietin. The four α helices are labeled A–D (magenta). The interhelical loops are named for the helices they interconnect. Two regions of extended structure that could form hydrogen bonds lie between Loop AB and Loop CD (cyan). N- and O-linked glycosylation sites are shown in green and blue, respectively. Disulfide bonds are not shown. From reference [38].

dues within the predicted alpha helixes prevented export of the mutant protein from the cell. In contrast, small deletions at the amino terminus, the carboxy terminus or in the predicted intrahelical loops had no effect on Epo secretion, and these mutants were biologically active. Furthermore, they showed that replacement of C29 and C33 by tyrosine also yielded a fully active protein, indicating that the C29–C33 disulfide bond is not critical to Epo's stability or activity.

Somewhat prior to the studies of Boissel *et al.*, Chern *et al.* used **linker scanning mutagenesis** to study the 99–119 and 111–129 domains of Epo that had been suggested to be important for activity when studied by epitope mapping [32, 33]. Chern *et al.* found that mutagenesis across amino acids 111–129 had no effect on biological activity even though antipeptide antibodies directed to this region had blocked Epo's action *in vitro*. However, mutagenesis across residues 99–119 prevented secretion of the protein, indicating that amino acids 99–110 played an important structural role in Epo.

Grodberg *et al.* further refined the studies of Chern and applied **alanine scanning mutagenesis** to the region comprising amino acids 100–109, found within the proposed third alpha helix of Epo [34]. Substitution of R103 with alanine resulted in secretion of a protein with no detectable biological activity. In addition, alanine substitution of adjacent amino acids singularly also diminished Epo's activity but did not eliminate it completely. It was confirmed later that R103 and adjacent residues participate in Epo receptor binding mediated by the "low affinity site 2 domain of Epo" (see below) [39, 40]. Burns *et al.* have shown that R103A is an effective inhibitor of the action of wild type Epo [41].

Simultaneously, Wen *et al.* continued refinement of their four alpha helix model by carrying out mutagenesis at 51 conserved sites predicted to be on the surface of Epo [42]. Each of these mutant Epo cDNAs was expressed in COS cells, and the biological activity of the proteins was assessed. Wen *et al.* confirmed that the R103A mutation described by Grodberg *et al.* resulted in a protein completely lacking in biological activity and that S104A and L108A also had decreased activity, consistent with Grodberg's findings. Additionally, Wen *et al.* discovered that an R14A mutation on helix A resulted in substantial loss of biological activity and substitution by glutamic acid resulted in total loss. Other substitutions on the molecule also effected biological activity to a greater or lesser extent. From these studies, the authors proposed domains of the predicted three-dimensional structure important for biological activity.

5.4
The Tertiary Structure of Epo

In 1998, Syed *et al*. reported the X-ray crystal structure of Epo complexed to the extracellular ligand-binding domain of the Epo receptor determined at 1.9 Å resolution [39]. The extracellular domain of the Epo receptor, designated by the authors as "EPObp", was expressed in *Pichia pastoris* cells with the N-linked glycosylation site mutated (N52Q) along with two other mutations. The Epo was a soluble analog of *E. coli* expressed Epo with mutations at the three N-linked glycosylation sites (N24K, N38K, N83K).

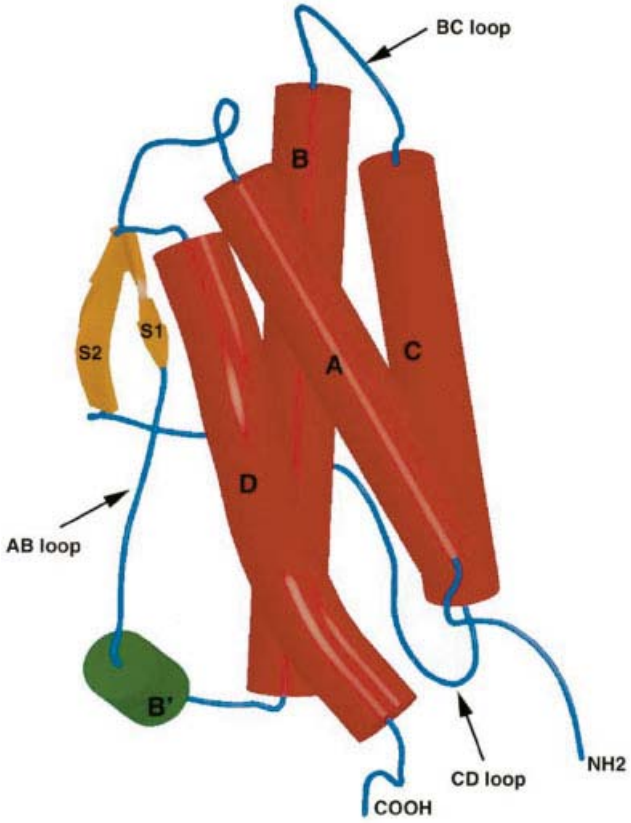

Fig. 5.4 The average minimized NMR structure of MKLysEPO. The view is perpendicular to the four-helical bundle axis, parallel to the AD plane. The four α-helices (A, S9–T26; B, G57–K83; C, L91–A111 and D, F138–A160) are shown in red and the two β-strands in the crossover loops (S1 and S2) are highlighted in brown. The short helical segment preceding the B helix (B′) is shown in green. The remainder of the Ca chain trace is colored blue. N- and C-termini are indicated for reference. From reference [40].

Using these mutant, nonglycosylated proteins, they succeeded in co-crystallizing Epo bound to its receptor. One important finding of this study was that one molecule of Epo bound two receptors, confirming solution studies carried out by Philo *et al.* [43]. Indeed, the four alpha helical bundle motif of Epo proposed by Boissel *et al.* [38] based on the structural similarities with other four alpha helical bundle proteins was confirmed. Interestingly, Epo binding to two receptors resulted in a 120° angular relationship of the two receptors that the authors concluded "is responsible for optimal signaling through intracellular kinase pathways". The model of Epo bound to two EPObp units is shown in Chapter 6.

It is interesting to view this important study in light of the epitope mapping in mutagenesis studies discussed above. As both Grodberg *et al.* and Wen *et al.* had shown by mutagenesis experiments R103 and some flanking amino acids (S100 and S104) are important in the site 2 (low affinity) interaction with the Epo receptor [34, 42]. Indeed, R103 has no fewer than six interactions with amino acids on the Epo receptor. Also, R14 discovered by Wen *et al.* to be relevant to Epo's biologic function is present in the site 2 interaction. Among the amino acids near the carboxy terminus of Epo proposed by Wen *et al.* to be important to Epo's biologic function (K140, R143, S146, N147, G151, K152), K140, R143 and N147 play direct roles in the site 1 binding domain. Thus, the epitope mapping and mutagenesis studies had quite accurately delineated those amino acids important for the interaction of Epo with its receptor.

While these X-ray crystallography studies were being carried out, the solution structures of free Epo and of Epo bound to EPObp were being solved by nuclear magnetic resonance spectroscopy (NMR) [40]. Cheetham *et al.* used an *E. coli* expressed mutant Epo to which methionine and lysine were added to the amino terminus to enhance bacterial expression and in which the N-linked oligosaccharide attachment sites were mutated to lysine (N24K, N38K and N83K). The NMR structure agreed in great detail with that determined by X-ray crystallography and with the proposed structure of Boissel *et al.* (Fig. 5.4). Furthermore, NMR structural determination of Epo bound to EPObp delineated the residues of Epo that formed the high and low affinity receptor binding sites (site 1 and site 2). For the most part, they agree with mutagenesis studies and with X-ray crystallographic analysis. The surface representations of these residues are shown in Fig. 5.5.

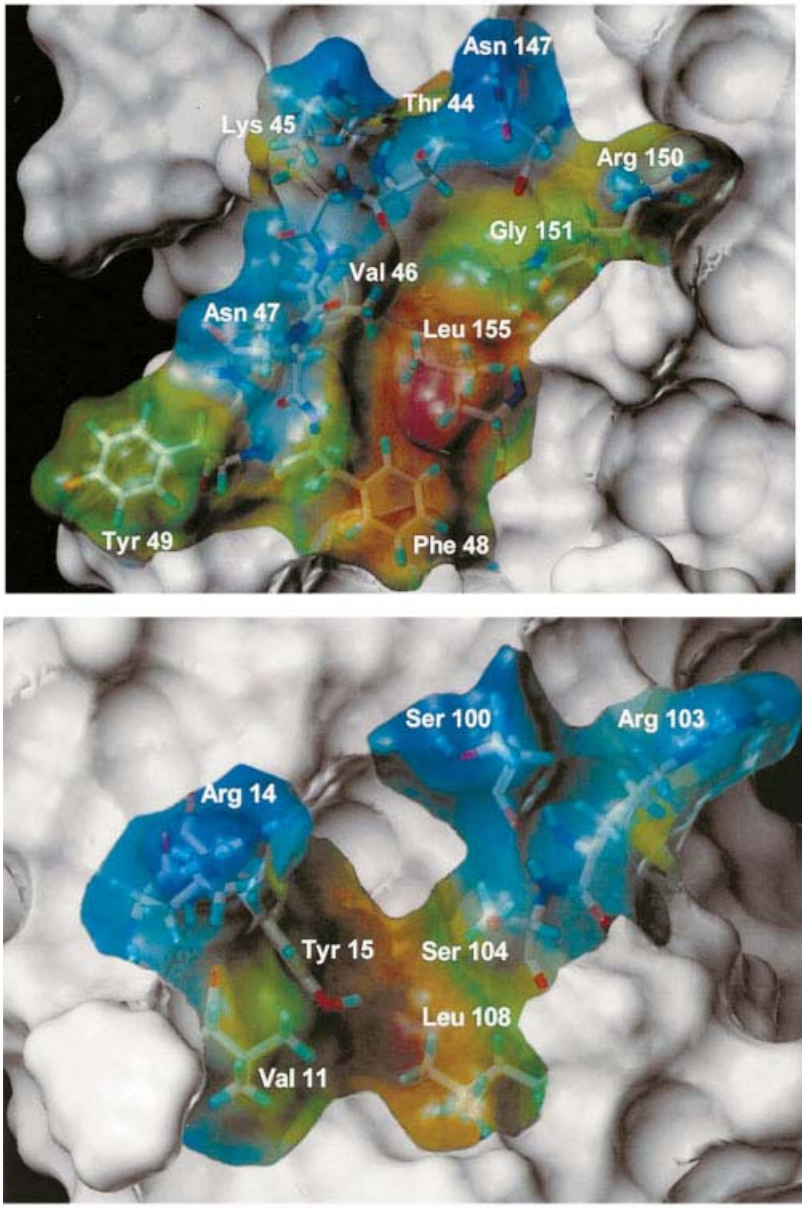

Fig. 5.5 Surface representations of the high affinity (a) and low affinity (b) receptor-binding sites of MKLysEPO. Labels indicate residues identified by mutagenesis studies as important for receptor binding. From reference [40].

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6
Receptor Biology and Signal Transduction

6.1
Receptor Biology

6.1.1
Identification of the Erythropoietin Receptor

Initially, the Epo receptor proved more elusive than might have been expected. Seven years passed between the purification of human urinary Epo [1] and the first description of "specific binding sites" for Epo on erythroid cells [2]. Several factors were responsible for this. Firstly, although the purification procedure was published in detail, the starting material – the urine from very anemic human donors – was not readily available. Secondly, attempts to radioiodinate Epo using the popular chloramine-T method employed in radioimmunoassay as well as other methods resulted in virtually complete inactivation of the hormone, rendering it useless for receptor identification. Thirdly, a source of large numbers of highly enriched erythroid progenitor cells was generally lacking. Ultimately, Epo was radiolabeled by incorporation of ^{3}H into its sialic acid residues while retaining full biologic activity. Using this reagent, Epo-specific binding sites or EpoR were demonstrated on erythroid cells from mice infected with the anemia strain of the Friend virus (FVA) [2]. Later, it was found that radioiodination in the presence of 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (IODO-GEN®) under gentle conditions resulted in the labeling of only one of the four tyrosyl residues of Epo (0.6 g-at ^{125}I/mol Epo) and yielded a product with nearly full biologic activity that proved useful in the study of EpoR binding and receptor-mediated endocytosis in the same FVA-infected erythroid cell model system [3, 4].

With the availability of recombinant Epo and a reliable radioiodination method, numerous investigators began to study the EpoR on a variety of erythroid cells, both normal and transformed, and EpoR numbers ranging from 34/cell [5] to $\approx$3000/cell [6] were described. A density of 1050

EpoR/cell was reported for highly enriched human erythroid colony-forming cells (ECFC), which are predominantly CFU-E [7]. In addition, early analyses of the thermodynamics of binding revealed the presence of two different affinity classes of receptors (binding sites) in several cases, usually by Scatchard analysis [4, 8], with the higher affinity ranging from $K_D \approx 90$–900 pM and the lower affinity from $K_D \approx 200$–9000 pM, depending on the study (Fig. 6.1). However, some studies identified only a single class [9, 10]. The molecular basis for these disparate observations has not been fully explained, but interaction with another protein on the cell surface, in addition to the EpoR, has been suggested. Nagao *et al.* expressed the wild-type murine EpoR in BHK cells and demonstrated two different affinity classes of binding sites by ^{125}I-Epo binding [11]. Treatment of the cells with tunicamycin to inhibit N-glycosylation prior to binding resulted in conversion of the biphasic Scatchard plot to one with but a single, high affinity phase. Importantly, when a glycosylation-deficient Asn51Gln mutant murine EpoR was expressed in BHK cells, biphasic binding thermodynamics were also observed, which were converted to a single phase by tunicamycin. The authors concluded that the N-linked sugar of the murine EpoR was not responsible for the biphasic binding, but rather that another glycoprotein "crucial for the ligand-saturation characteristics" of the EpoR was involved.

Prior to cloning of the murine and human EpoR, some information, sometimes contradictory, was obtained using cross-linking methods. In several studies, ^{125}I-Epo was incubated with cells to allow binding to the EpoR followed by addition of one of several homobifunctional cross-link-

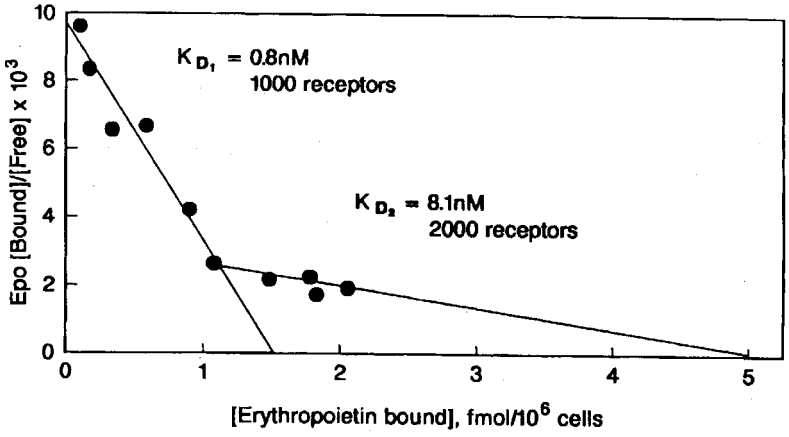

Fig. 6.1 Scatchard analysis of ^{125}I-Epo binding to EpoR on Rauscher murine erythroleukemia cells. Note biphasic curve and two affinity constants. From reference [19].

ing reagents such as disuccinimidyl suberate. The cells were then dissolved, and the proteins were subjected to SDS-PAGE and autoradiography (Fig. 6.2). Radiolabeled species of 85–150 kDa were reported, and in most reports, more than one species was visualized [3, 8, 12, 13]. It was proposed that such differences in the sizes of the ^{125}I-Epo/EpoR complexes might be due to post-translational processing of a single EpoR protein [14]. Also, the existence of a receptor heterodimer was suggested [15]. It should be noted that most of these studies used cross-linking reagents that could penetrate the plasma membrane. Therefore, it is entirely probable that some of the complexes comprised ^{125}I-Epo, EpoR and one or more intracellular proteins associated with the cytoplasmic domain of the EpoR, such as signaling molecules and adapter proteins, about which little was known at the time.

In one Epo-sensitive erythroid cell line, Rauscher murine erythroleukemia [16, 17] (clone PAN-4), pretreatment of the cells for 24 h with dimethyl sulfoxide (DMSO) resulted in a marked amplification of the biologic response to Epo characterized by 3–6-fold increase in the number of cells responding, a significant increase in the rate of response and a markedly left-shifted Epo dose-response curve, indicating greater sensitivity to the hormone [18]. Studies of the EpoR of these cells by ^{125}I-Epo binding revealed that DMSO treatment had induced the appearance of a

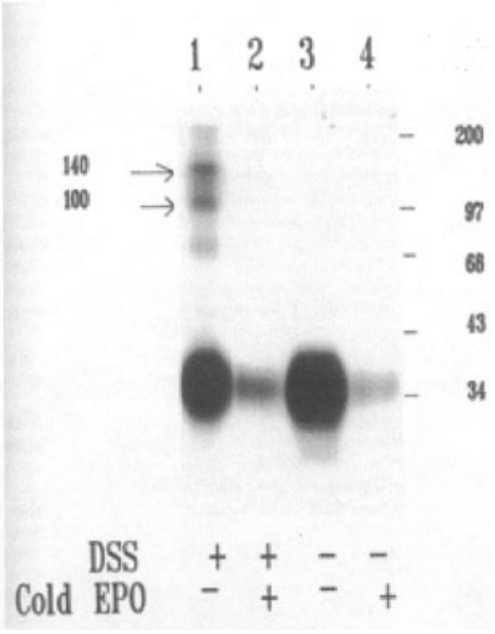

Fig. 6.2 Autoradiogram of SDS-PAGE analysis after DSS cross-linking ^{125}I-Epo to murine EpoR expressed in COS cells. Note ^{125}I-Epo/EpoR complexes of 140 and 100 kDa. From reference [20].

new population of high-density EpoRs, increasing the EpoR number from 3000/cell to over 20 000/cell [19]. This could not be accounted for by a corresponding increase in receptor mRNA. Interestingly, ^{125}I-Epo binding to these new receptors yielded a Scatchard analysis with an upwardly convex curve, indicative of positive cooperativity, and a Hill coefficient, nH, of 6.75. These results were consistent with the presence of receptor oligomers or clusters, possibly hexamers, consisting of several EpoRs and, potentially other accessory molecules.

6.1.2 The Erythropoietin Receptor Gene

A cDNA encoding a predicted 507 amino acid murine EpoR was isolated by D'Andrea *et al.* using a pXM expression library constructed from Friend murine erythroleukemia cells [20]. There was a certain risk with this approach since the cells used do not differentiate in response to Epo, potentially indicating (at the time) that the EpoR might be defective. However, this did not prove to be the case. Interestingly, whereas ^{125}I-Epo binding studies of the original Friend cells revealed a single affinity class, expression of the cDNA in receptor-deficient COS cells resulted in two affinity classes. The full-length coding sequence of the predicted 508 amino acid human EpoR was assembled from a partial cDNA obtained by screening a λgt11 human fetal liver library using synthetic oligonucleotides derived from the reported murine EpoR cDNA and exon sequence obtained from 5′ genomic DNA [21]. Independently, a cDNA encoding the human EpoR was isolated from cDNA libraries derived from OCIM1 erythroleukemia cells and from fetal liver by screening with the murine EpoR cDNA [22]. The human EpoR gene has been localized to chromosome 19pter-q12 and the murine locus was found to be tightly linked to the murine Ldlr locus near the centromere of murine chromosome 9 (9A1-A2) [23].

The EpoR gene spans $\sim$6 kb and comprises 8 exons ranging from 81 bp to 2.1 kb bp [24–26]. The extracellular (exoplasmic) domain of EpoR is encoded by exons 1–5. Exon 6 encodes the transmembrane domain, and exons 7 and 8 encode the cytoplasmic domain. The exon-intron boundaries are conserved between the mouse and human receptors. Studies in transgenic mice revealed that the human EpoR can rescue the EpoR null mouse [27].

Importantly, EpoR and EpoR mRNA and/or protein have/has been detected in several nonhematopoietic tissues [28–31] such as brain [32–34], kidney [35], and human placenta [36] and in cancers such as human breast

cancer [37], vestibular schwannomas [38], and melanoma [39]. EpoR mRNA was detected in endothelial cells, myocardiocytes, macrophages, retinal cells and cells of the adrenal cortex and medulla as early as the first two trimesters of human embryonic and fetal development [40]. The non-hematopoietic actions of Epo are discussed in detail in Chapter 9.

EpoR mRNA is transcribed continuously [41], however, anemia and hypoxia upregulate EpoR expression in hematopoietic tissue and brain [42]. EpoR mRNA is spliced to its mature form more efficiently in erythroid cells than in brain [42]. EpoR gene expression is also induced by stem cell factor [43] and interleukin-1 alpha [44], and it is downregulated by interferon-γ [45], ionomycin and the phorbol ester PMA [44]. The 5′ flanking region of the human EpoR gene contains GATA-1 [46] and Sp1 [47] regulatory elements. There is a negative CCACC motif located in the +79 to +135 fragment of the human EpoR gene promoter [48]. An upstream repetitive element in the murine EpoR gene 5′ flanking region contributes to the inhibition of transcription [49].

6.1.3 The Structure of the Erythropoietin Receptor: A Member of the Cytokine Receptor Superfamily

The human and murine EpoR preproteins comprise 508 amino and 507 amino acids, respectively, each with a predicted molecular mass of ~56 kDa. They exhibit approximately 72 percent overall sequence identity at the amino acid level. However, cross-linking and photoaffinity labeling experiments have resulted in higher molecular weight estimates of 65 kDa to 105 kDa [15, 22, 50]. Expression of the murine EpoR cDNA in COS cells followed by western blotting with antipeptide antibodies revealed multiple EpoR bands of 62, 64 and 66 kDa (Fig. 6.3) [51]. Similar results were obtained with the endogenous receptor of erythroid cells, although of somewhat higher apparent molecular weight. Potential reasons for these differences in estimated molecular weight include glycosylation, ubiquitinylation, other post-translational modifications and interaction with other proteins. Both the human and mouse EpoR preproteins contain a 24-amino acid signal peptide. The first 225 amino acids of the mature human EpoR protein form the extracellular portion. The murine EpoR protein has an extracellular portion of 224 amino acids. The transmembrane regions of both receptors contain 22 amino acids while the C-terminal 236 amino acids form the cytoplasmic portions.

EpoR is a founding member of the **cytokine receptor superfamily** [52–54], which also includes the receptors for thrombopoietin (TPO) [54],

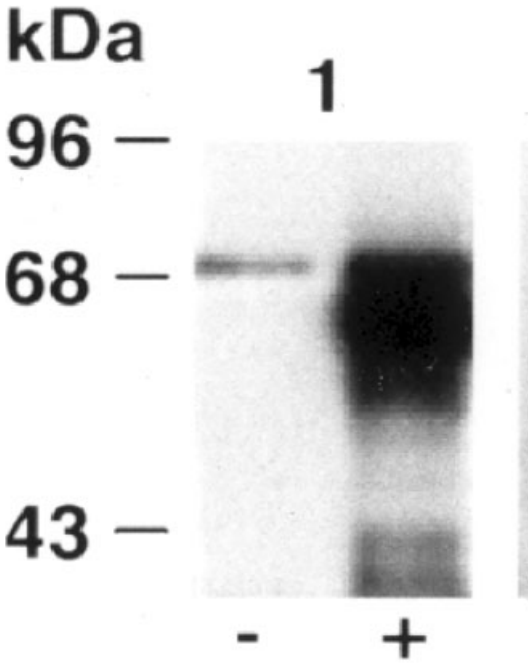

Fig. 6.3 Western blot detects 62-66 kDa EpoR protein in COS-7 cells transfected (+) with the murine EpoR cDNA. Minor band of 70 kDa in the (–) mock-transfected lane is a non-specific cross-reacting protein. From reference [51].

granulocyte colony-stimulating factor (G-CSF) [55], IL-2 [56], IL-3 [57], IL-4 [58], IL-5 [59], IL-6 [60], IL-7 [61], IL-9, IL-11, IL-12, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF) [62], leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and oncostatin M (OSM) [63]. Each receptor is characterized by a single hydrophobic transmembrane spanning region, a highly variable cytoplasmic portion and an extracellular portion with conserved sequences yielding an overall 15–35% homology (Fig. 6.4). The extracellular portion of each receptor of this superfamily contains conserved cysteine residues and a so-called WSXWS motif [53, 54, 60, 64]. This motif in the EpoR, specifically WSAWSE, is critical for ligand binding, internalization and signal transduction [65, 66]. The cytoplasmic portion of each receptor of the superfamily member lacks a kinase domain. The receptors exhibit limited homologies in the membrane proximal regions, designated the Box1/proline-rich motif and the Box2 motif [67–69], which are important for overall receptor function, particularly in supporting mitogenesis. The Box1/proline-rich motif lies within the first 20 amino acids of the cytoplasmic portion. Box2 sequences begin with a cluster of hydrophobic amino acids and end with one or two positively charged amino acids. The distal cytoplasmic regions of these receptors are necessary for differentiation or other signaling effects [70].

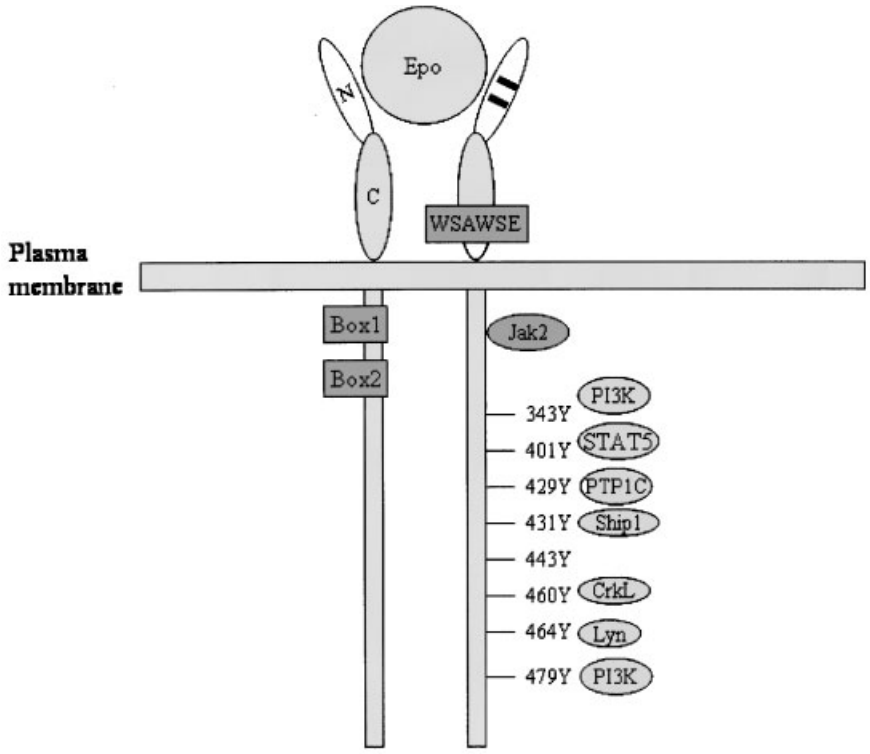

Fig. 6.4 Structure of the EpoR. One erythropoietin molecule (Epo) is shown binding to two EpoRs, resulting in receptor dimerization. The extracellular portion contains the N- and C-domains (D1 and D2) and the membrane-proximal WSAWSE motif. The dark bars in the N domain represent conserved cysteines. The cytoplasmic portion contains a Box1 and Box2 domain and eight tyrosines that can be phosphorylated. The approximate binding sites of various signal transduction molecules are also shown.

6.1.4
The Extracellular Portion of the Erythropoietin Receptor

The extracellular portion of the human EpoR comprises 225 amino acids. Based upon X-ray crystallographic analysis (Fig. 6.5) [71], the extracellular portion of the EpoR consists of two fibronectin type III (FNIII)-like domains (designated N- and C-terminal, or D1 and D2 domains) with the same topology. Each domain comprises seven beta strands, designated A, B, C, C′, E, F and G, starting from the NH_2-terminus of the domain. These seven beta strands are connected by six loops. The loops contain most of the amino acid residues involved in Epo binding. Phenylalanine 93, located on loop 3 between the beta sheets E and F of D1, and Phe

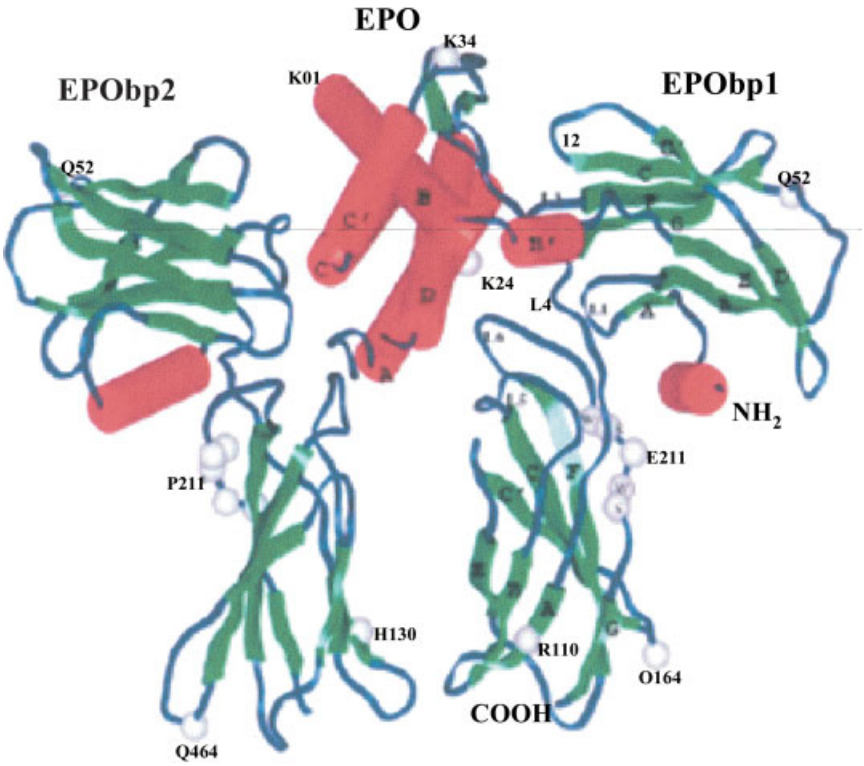

Fig. 6.5 Crystal structure of the Epo/soluble EpoR complex. Note one Epo molecule binding two receptor (EPObp="EPO binding protein") molecules. From reference [71].

205, located on loop 6 between beta sheets F and G of D2, are especially important for binding Epo. In contrast, although Met 150 is not important for Epo binding, it is critical for binding of an Epo-mimetic peptide [72, 73].

Solution studies of Epo's interaction with the extracellular portion of the EpoR (the so-called "soluble EpoR" or "sEpoR") demonstrated that one Epo molecule can bind two sEpoR molecules, albeit with markedly different affinities. These two non-identical binding sites on the Epo molecule have been designated Site 1 (high affinity)(K_d=0.2 nM) and Site 2 (low affinity)(K_d=2.1 μM), respectively [74]. Residues on the six loops of EpoR, consisting of 3 loops of D1 and 2 loops of D2 and 1 loop between the two domains, are involved in Site 1 but only five loops participate in Site 2 (without the loop between two domains participating). There is a short N-terminal helix consisting of residues 9 to 22 of D1 leading the seven beta

sheets. This helix may help stabilize the folding of the EpoR. Downstream of loop 6 of D2 and 1 is the WSAWSE motif, which is involved in the binding of Epo as well as in the folding, transport and internalization of the receptor [65].

The C-terminal domain of the EpoR extracellular region (D2) includes a region designated the "dimerization domain". Dimerization of receptors upon ligand binding is a frequently described means of initiating signal transduction in mammalian cells [65, 75–80]. The EpoR also appears to dimerize in the absence of ligand [73]. Binding of Epo to such preformed EpoR dimers results in a conformational change triggering intracellular signaling. In several members of the tyrosine kinase receptor family (e.g., platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)), two ligand molecules (L) bind to two receptors (R) forming an L2R2 tetrameric complex, resulting in activation of the receptor tyrosine kinase [75, 76, 81]. For CSF-1, the ligand is a disulfide-linked dimer, each subunit of which binds one CSF-1R. However, in the cytokine receptor superfamily system, one ligand induces dimerization of two receptor subunits, either heterologous or identical [82–84]. A principal example of dimerization in the cytokine receptor superfamily is the growth hormone receptor (GHR). The crystal structure of the ligand-bound complex reveals GH binding to two identical GHRs, resulting in GHR dimerization, apparently aided by a dimerization domain in the C-terminal beta sandwich (domain 2). This dimerization domain comprises eight residues [85], five of which were identified by a functional assay [86]. In the case of the EpoR, mutation of Arg 129 and Glu 132, located in beta sheet A of the D2, and Glu 133, located in the loop between beta sheets A and B of D2, to Cys resulted in the formation of disulfide-linked EpoR homodimers that initiated the proliferation signal spontaneously in the absence of Epo [87, 88].

6.1.5
The Cytoplasmic Portion of the Erythropoietin Receptor

The EpoR has a 236 amino acid cytoplasmic portion that, unlike growth factor receptors such as the epidermal growth factor receptor (EGFR), lacks a kinase domain. In the membrane proximal region, there are two motifs, designated Box1 and Box2, which exhibit some limited homology within the superfamily [67–69]. The Box1 motif of EpoR, IWPGIPSP, comprises amino acids 257–264 of the mature murine protein (human 258–265). The Box2 motif, PAHLEVL (murine), PASLEVL (human), comprise amino acids 303–309 of the mature murine protein (human 304–

310). The Box1 motif and its flanking regions are necessary for the binding of Jak2 kinase to the receptor and for Jak2 activation (see below) [81, 89, 90]. A carboxy-terminal truncated EpoR, with only the Box1 motif, is sufficient to induce Epo-dependent cell proliferation [91]. The membrane proximal 125 amino acid residues of the EpoR cytoplasmic portion, containing both Box1 and Box2 as well as Tyr 343 of the murine EpoR, reportedly are sufficient to induce erythroid development [91]. Other studies have demonstrated that a region between Box1 and Box2, but not Box2 itself, is required for erythropoietin-dependent cell proliferation [92]. A 9 amino acid stretch, FEGLFTTHK (reported as residues 267–276, but 268–277 from the GenBank sequence) of the murine EpoR, participates in receptor internalization [93]. A domain consisting of amino acids 352–384 is necessary for p70 S6 kinase phosphorylation and activation [94]. Interestingly, the distal portion of the EpoR cytoplasmic region can induce myeloid differentiation in 32D cells [95].

6.2 Signal Transduction Pathways

6.2.1 Phosphorylation of the Erythropoietin Receptor

The binding of Epo to the EpoR dimer (or Epo's consecutive binding to two EpoRs) triggers a conformational change in the receptor pair resulting in the activation of numerous kinases and other signaling molecules. Some kinases activated by Epo are Jak2 kinase, phosphatidylinositol 3-kinase (PI 3-kinase), protein kinase C (PKC), Lyn kinase [96] and Tec kinase [97]. Activated Jak2 and Lyn then phosphorylate the EpoR [96, 98].

Within the cytoplasmic portion of the murine EpoR, there are eight tyrosine residues at positions 343, 401, 429, 431, 443, 460, 464 and 479 of the mature protein. The human EpoR has homologous tyrosines and positions 344, 402, 430, 432, 444, 461, 465 and 480 as well as a ninth cytoplasmic tyrosine at position 285, which is not present in the murine receptor. Phosphorylation of the eight tyrosines (tyrosine 285 of the human EpoR apparently has not been studied) allows the docking of SH2 domain-containing proteins (e.g., Grb2, STAT5, Shc and CrkL), thus linking the EpoR to its signal transduction pathways. Numerous functions have been assigned to these tyrosines, both individually and collectively. Tyrosine 343 and/or Tyr 401 is involved in STAT5 activation [99–102] and is necessary for phosphorylation of the docking protein Gab2 [103]. Tyrosine

401 also plays a role in the docking of **c**ytokine-**i**nducible **S**H2 protein-**3** (CIS3), a negative regulator of fetal liver erythropoiesis, which results in the inhibition of proliferation and STAT5 activation [104]. Tyrosine 429 is involved in the association of the tyrosine phosphatase PTP1C with EpoR [105]. Tyrosines 343 and 479 are required for phosphatidylinositol 3-kinase binding to EpoR [99, 106] and support immature burst-forming unit-erythroid (BFU-E) progenitor development [107]. Tyrosines 401, 429 and 431 appear to be necessary for the docking of **SH**2 **i**nositol 5-**p**hosphatase 1 (Ship1), which is involved in the Epo-activated MAP kinase pathway [108]. Tyrosine 460 plays a critical role in Epo-dependent Ca^{2+} influx [109] and is a binding site for the CrkL adaptor protein, which is, in turn, phosphorylated by Lyn leading to activation of the MAP kinase pathway [110, 111]. Tyrosines 464 and 479 have been proposed as docking sites for Lyn [96]. Despite these assignments, however, all of these tyrosines may not be necessary for receptor function, as noted above. Additionally, in one study, human cord blood $CD34^+$ cells were infected with a retrovirus encoding a chimeric receptor consisting of the extracellular domain of the prolactin receptor and a truncated cytoplasmic domain of the murine EpoR lacking any cytoplasmic tyrosines (PRLR/EpoR). These cells were cultured in the presence of prolactin, and after 14 days, normal appearing erythroid colonies were observed, suggesting that the tyrosine residues of the EpoR may not be required for growth and terminal differentiation of human $CD34^+$ erythroid progenitors [112].

6.2.2
Kinases, Phosphatases and the Signal Transduction Cascade

Many kinases, adapter proteins and other molecules involved in signal transduction are phosphorylated in response to EpoR activation. However, it must be noted that the pattern of these phosphorylation events may be very dependent upon the cell type. Examples of proteins phosphorylated in response to Epo stimulation include Jak2 kinase, PKC, PKB (Akt), MAP kinase kinase (MEK), MAP kinase (ERK1/2), hematopoietic protein tyrosine phosphatase PTP1C, Gab1 and Gab2. Others include GSK-3b, phospholipase C-γ1 [113], GAP [114], Raf-1 [115], Ras [114], Shc [116], Vav [117], c-fps/fes [118], Lyn [119] and c-Cbl [120]. These diverse phosphoproteins function in numerous pathways, delivering the Epo signal to targets in the cytoplasm, mitochondrion and nucleus.

6.2.2.1 **Jak2-STAT5 Pathway**

Jak2 is a protein tyrosine kinase (PTK) [121] that associates with the EpoR [89]. When Epo binds to its receptor, Jak2 is tyrosine phosphorylated and activated. Jak2 then phosphorylates the EpoR and a number of other proteins, including **S**ignal **T**ransducer and **A**ctivator of **T**ranscription-1 and -5 (STAT1 and STAT5) [122, 123]. Tyrosine-phosphorylated STAT proteins homodimerize and translocate to the nucleus where they bind to specific DNA elements and direct gene transcription. STATs are known to participate in the regulation of several genes [124, 125]. However, their precise role in erythropoiesis is the subject of some debate. STAT5 was shown to be required for globin expression in both Epo-responsive SKT6 cells and ELM-I-1 cells [126, 127]. However, adult STAT5a$^{-/-}$ 5b$^{-/-}$ mice were essentially normal in the erythroid lineage and had normal steady-state hematocrits [128]. In contrast, STAT5a$^{-/-}$ 5b$^{-/-}$ **embryos** studied by other investigators exhibited severe anemia, fewer erythroid progenitors and increased apoptosis. They were also less responsive to Epo [129]. Replacement of the STAT5-binding sites (residues 343 and 401) in the EpoR cytoplasmic region with the STAT3 binding/activation motif from gp130 resulted in the activation of only STAT3 yet supported fetal liver and adult erythropoiesis [87]. Transfection of fetal liver cells from EpoR$^{-/-}$ or STAT5a$^{-/-}$ 5b$^{-/-}$ mice with the granulocyte colony-stimulating factor receptor (G-CSFR) gene and treatment with G-CSF resulted in terminal erythroid differentiation, strongly suggesting that STAT5 is not essential for erythroid cell differentiation [130]. It would appear that the Jak2-STAT5 pathway in erythropoiesis is anti-apoptotic, supporting erythroid cell proliferation. Epo induces the anti-apoptotic protein Bcl-xL gene through STAT5 [124], and STAT5 interacts with the regulatory subunit of PI 3-kinase, activation of which is required for cell cycle progression [65].

Epo also activates STAT1 in the human erythroleukemia cell line HEL [123] and in erythroid cells from the spleens of FVA mice [131], and it activates STAT1 and STAT3 in BaF3 EpoR cells [132] and in UT-7/EPO cells [133]. The importance of STAT1 and STAT3 activation in Epo signal transduction and their target genes in erythroid cells remains to be elucidated more completely.

6.2.2.2 **Ras-Raf-MAP Kinase Pathway**

The Epo induced Ras-Raf-MAP kinase pathways are complex, and there is significant disagreement, since dissimilar results have been obtained using different cell types, both normal and transformed. In the "classic" Ras-Raf-MAP kinase cascade,

Receptor → Shc → Grb2 → Sos1 → Ras → Raf-1 → MEK → MAP kinase,

Grb2, an adaptor protein with one SH2 domain and two SH3 domains [134, 135], binds to tyrosine-phosphorylated EpoR or other phosphoproteins (Shc, CrkL) [136, 137] through its SH2 domain and simultaneously to Sos1 through its SH3 domain, activating the Ras pathway [136, 138, 139]. Ras then activates Raf-1 kinase [140–142], leading to the activation of several other kinases such as the MAP kinase kinases designated MEKs (**M**APK/**E**RK **k**inases) [143, 144]. MEKs are "dual specificity kinases" that activate MAP kinases by phosphorylation on tyrosine and threonine residues [145, 146]. Activated MAP kinase then may translocate to the nucleus, phosphorylating transcription factors like Elk to modulate gene expression [147, 148]. Epo has been reported to induce activation of all of the Ras-MAP kinase pathway-signaling molecules. Epo triggers tyrosine phosphorylation of Ship1. Ship1 then binds to the EpoR and recruits Shc and Grb2 to the EpoR [108]. Apparently, Shc need not be phosphorylated for the activation of Ras-MAP kinase pathway, since Epo stimulation also triggers the phosphorylation of EpoR and other adaptor proteins that associate with Grb2 [120, 149]. Indeed, even though Epo does not activate Shc in the erythroleukemic cell line HB60-5, these cells proliferate and terminally differentiate in response to the hormone [150]. Whether other adaptor proteins such as Epo-activated SHP2 and c-Cbl are the up-stream of the Ras-MAP kinase pathway is unclear. It is not certain that Epo activates the Ras-MAP kinase pathway by following the "classic" sequence in all cells. For example, Jak2 can associate with and phosphorylate Raf-1 [151]. Studies of BaF3-EpoR cells in the author's laboratory demonstrated that activation of the EpoR resulted in phosphorylation of Raf-1, MEK and ERK1 and ERK2, but inhibition of Raf-1 activation did not alter the phosphorylation of MEK. Furthermore, ERK1 is preferentially phosphorylated over ERK2 in normal erythroid cells in contrast to essentially equal phosphorylation of ERK1 and ERK2 by other growth factors in other cell types [152]. It is possible that Raf-1 is up-stream of MEK and the MAP kinases and that ERK1 and ERK2 are regulated differently in erythroid cells than in other cell types. Although Epo activates both Raf-1 and Ras [114, 115], the role of this pathway in erythropoiesis is not fully understood. Studies of purified human erythroid colony-forming cells indicate the MAP kinase pathway is essential for erythropoiesis [153]. However, in a Friend murine erythroleukemia cell line, inhibition of MAP kinase induced erythroid differentiation [154]. Experiments using a truncated EpoR demonstrated that the activation of MAP kinases might not be required for Epo-induced proliferation [155].

6.2.2.3 **Phosphatidylinositol 3-Kinase Pathway**

The phosphatidylinositol 3-kinases (PI 3-kinases, PI3K) are a conserved family of lipid kinases comprising three classes based upon substrate specificity. Class I PI 3-Ks each consist of a catalytic subunit and a regulatory subunit. There are four different catalytic subunits in mammals: p110alpha, p110beta, p110gamma and p110delta [156]. P110alpha and p110beta are widely expressed in mammalian tissues, whereas p110gamma and p110delta are more restricted, though they are found in hematopoietic cells [156]. There are five isoforms of the regulatory subunit: p85alpha, p85beta, p55alpha, p55gamma and p50alpha. The p55alpha and p50alpha isoforms are the result of alternative splicing of p85alpha transcripts [157]. Activated PI 3-kinases phosphorylate phosphatidylinositol 4,5-bisphosphate (PI-(4,5)-P_2) at the D-3 position of the inositol ring yielding phosphatidylinositol 3,4,5-trisphosphate (PI-(3,4,5)-P_3), the activator of 3′-**p**hosphoinositide-**d**ependent protein **k**inase **1** (PDK1). PDK1 then phosphorylates Akt (protein kinase B, PKB), a serine/threonine kinase. The activated Akt kinase phosphorylates and inactivates several pro-apoptotic targets such as Bad, a member of the Bcl-2 family, forkhead transcription factor and caspase-9 [158, 159] and thereby inhibits apoptosis. Akt also phosphorylates glycogen synthase kinase 3 (GSK-3) [160], which has been implicated in the regulation of cyclin D1 [161] and in the regulation of c-*myc* by phosphorylating and destabilizing beta-catenin [162]. Akt has also been identified as a regulator of the Raf-MEK-ERK-signaling pathway by phosphorylation and inactivation of Raf at Ser 259 [163].

Stimulation of the EpoR results in its tyrosine phosphorylation and its association with the N- and C-terminal SH2 domains of the PI 3-kinase regulatory subunit p85 [99, 106, 164, 165] mediated by tyrosines 343 and 479 of EpoR. Epo also induces the tyrosine phosphorylation of insulin receptor substrate-2 (IRS-2) [166]. Tyrosine-phosphorylated IRS-2 interacts with the PI 3-kinase regulatory subunit p85. The IRS-related proteins Gab1 and Gab2 are also rapidly phosphorylated on tyrosine residues after Epo stimulation and provide sites for the docking of the p85 subunit of PI 3-kinase and the protein tyrosine phosphatase SHP2 [103]. Vav protein is tyrosine-phosphorylated after Epo stimulation and was shown to associate with the EpoR in the human Epo-responsive cell line F-36P [117]. The PI 3-kinase regulatory subunit p85 bind to phosphorylated Vav through its SH2 domains. Both anti-*vav* and anti-*p85* antisense oligodeoxynucleotides abolished Epo-induced cell proliferation and PI 3-kinase activity.

Work in the author's laboratory has demonstrated that Epo treatment of normal murine erythroid cells results in Akt and GSK-3 beta phosphorylation and in upregulation of c-*myc*. The PI 3-kinase inhibitors LY294002

and wortmannin block this Epo-dependent phosphorylation of Akt and GSK-3 beta and upregulation of c-*myc* (C Chen and AJ Sytkowski, unpublished). PI 3-kinase inhibitors also block Epo-induced initiation of c-*myc*, in contrast to the MAP kinase inhibitor PD98059, which blocks the elongation of c-*myc* [152]. GSK-3 beta is activated upon Epo stimulation, however, it appears that beta-catenin is not responsive to Epo stimulation in erythroid cells, precluding its involvement in erythroid cell c-*myc* gene regulation. Hence, the complete mechanism by which the PI 3-kinase pathway upregulates c-*myc* in erythroid cells remains to be elucidated. PI 3-kinase also appears to be involved in cell survival during erythropoiesis and in Epo's activation of p70 S6 kinase (p70S6k), which phosphorylates ribosomal protein S6 and plays an important role in cell cycle progression [167].

6.2.2.4 **Protein Kinase C Pathway**

The protein kinase C (PKC) family of serine/threonine kinases comprises 13 members [168–171]. The PKC family members are divided into several classes, based upon their structure and cofactor regulation:

1. the conventional PKCs (cPKCs), including α, β, $\beta 2$ and γ, which require Ca^{2+} and diacylglycerol (DAG) for their activation;
2. the novel PKCs (nPKCs), including δ, ε, η, θ and μ, which are Ca^{2+} independent but DAG-dependent;
3. the atypical PKCs (aPKCs), including ζ, ι and λ, which are not activated by Ca^{2+} or by DAG;
4. the recently described ν, which the discoverers proposed to be grouped with μ based upon structural homology [172].

DAG is produced by hydrolysis of the phosphodiester bond in phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) by phospholipase C, or in phosphatidylcholine by phospholipase D [168, 173]. Epo induces tyrosine phosphorylation of phospholipase C (PLC)-γ, increasing DAG and intracellular Ca^{2+} in human BFU-E-derived erythroblasts [174–176], and inducing rapid activation of PKC in Rauscher murine erythroleukemia cells and normal murine erythroid cells [177, 178]. Classical activation of PKC by DAG involves translocation of the enzyme from a cytosolic location in resting cells to a membrane-associated site after stimulation [179].

Activated PKC is a serine/threonine kinase. It can phosphorylate Raf-1 kinase [180, 181]. Thus, it is plausible that Ras localizes Raf-1 to the cell membrane where phosphorylation of Raf-1 by PKC can occur. However, data from the author's laboratory demonstrate that Epo-induced phosphorylation of MAP kinase can be blocked by PKC inhibitors but not by Raf-1

inhibitors in BaF3-EpoR cells, suggesting that PKC might not be the upstream of Raf-1 in this system [152]. Formation of CFU-E-derived colonies was blocked by the PKC inhibitor H-7, and phorbol ester and calcium ionophore increased normal CFU-E-derived colonies, consistent with the hypothesis that activation of PKC is required for erythropoiesis [182]. Furthermore, one report indicates that the PKC-α isoform plays a role in the erythroid differentiation of the $CD34^+$ progenitor cells from human bone marrow [183].

PKC is important in erythroid cell gene regulation [177, 178, 184, 185]. In Rauscher murine erythroleukemia cells, Epo-dependent up-regulation of c-*myc* requires the PKC-ε isoform, and this signal is required for Epo-induced DNA synthesis (growth signal) but not for Epo-induced globin gene expression (differentiation signal) [186]. Several other cell proliferation-related genes are regulated by Epo through one or more PKC-mediated pathways including c-*fos*, c-*jun*, *Bcl-3*, *GATA-2* and *Bcl-xL* [152, 187, 188].

6.2.2.5 **Other Epo-dependent Signaling Pathways**

The adapter protein CrkL is tyrosine phosphorylated in response to Epo leading to its transient association with Shc and c-Cbl [111, 189]. CrkL complexes with the guanine nucleotide exchange factor C3G, which activates Rap1 GTPase. In addition, Epo triggers release of the beta2 subunit of heterotrimeric GTP-binding proteins from the EpoR [190].

6.3 Summary and Conclusions

Docking of Epo with two EpoRs results in a conformational change in the receptor that triggers a signaling network resulting in the growth and differentiation of erythroid progenitors and the production of enucleate, hemoglobinized erythrocytes. The EpoR is also expressed by non-hematopoietic cells and tissues. The EpoR is a founding member of the cytokine receptor superfamily, all members of which possess an extracellular ligand-binding portion, a single hydrophobic transmembrane-spanning domain and a cytoplasmic portion that lacks a kinase domain. A WSXWS motif in the membrane-proximal region of the extracellular portion is conserved throughout this superfamily. This WSAWSE sequence in the EpoR is important for ligand binding, internalization and signal transduction. Within its cytoplasmic portion, the EpoR has a Box1/proline-rich

motif and a Box2 motif, which are important for overall receptor function, especially in supporting mitogenesis. One Epo molecule binds to two identical EpoR, resulting in receptor dimerization. Spontaneous dimerization of the EpoR in the absence of ligand also occurs. However, in the absence of ligand, these preformed dimers lack the proper conformation to initiate signaling. The ligand-induced conformational change results in the activation of several kinases and in the tyrosine-phosphorylation of the EpoR. When phosphorylated, the eight tyrosine residues of the EpoR cytoplasmic portion function as docking sites for different SH2-containing proteins, to initiate multiple signal transduction pathways that include the Jak2/STAT5, Grb2/Sos1/Ras/Raf-1/MEK/MAP kinase, PI-3 kinase/Akt/GSK-3 and PKC pathways.

Research into Epo signal transduction has yielded vast amounts of data, and many of the published studies are complicated and, sometimes, contradictory. Discrepant results can arise from the use of different cell lines and systems employed by various research groups. To cite one example, Epo induces Shc tyrosine phosphorylation by Jak2 kinase in the FDC-P1 cell line stably expressing the wild type murine EpoR [191]. In contrast, in another cell line, CTLL-EpoR, Shc is not activated by Epo, although it is activated by IL-2 or IL-15 [149]. In CTLL-EpoR cells, Epo triggers tyrosine phosphorylation of SHP2 resulting in the formation of SHP2/Grb2/cytokine receptor complexes and activation of down stream targets. Numerous signaling molecules have been shown to interact with the EpoR or to play some role in Epo signaling in one or another cell type studied. Additionally, Epo induces the expression of numerous genes, but the pathways involved are disparate and overlapping. For example, PI 3-kinase appears to be required for Epo's upregulation of c-*myc* but not of c-*fos*. However, the MAP kinase ERK1 is required for up-regulation of both genes [152]. There is substantial cross-talk among signaling pathways, and Epo-activated pathways are also modulated by other growth factors and cytokines operating on the same cell such as stem cell factor, IL-3, etc. Therefore, Epo's precise mechanism(s) of action may depend not only on the developmental stage of the erythroid (or other) cell under consideration, but also upon the relative concentrations of other ligands and their cognate receptors, each of which is subject to different regulatory influences.

6.4 References

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7
Clinical Disorders of Erythropoietin and its Receptor

The Epo gene is very tightly regulated at the molecular, cellular and physiological levels. However, there are a number of clinical conditions, due either to genetic or environmental factors, in which Epo production or signaling is disordered leading to either increased or decreased red blood cell production. Some of these conditions are due to constitutive high level expression of Epo resulting from loss of regulatory mechanisms. Others include increased production in response to exaggerated physiologic (hypoxic) stimuli and/or environmental toxins. Pathologic states resulting from reduced Epo production as well as from Epo receptor defects and mutations also exist. Conditions such as high affinity hemoglobin, methemoglobinemia and 2,3-DPG defects will not be addressed here.

7.1
Erythropoietin Production by Tumors: Paraneoplastic Syndromes

7.1.1
Von Hippel-Lindau (VHL) Disease and Acquired VHL Mutations

Von Hippel-Lindau disease is an autosomal dominant cancer syndrome resulting from a germ line mutation in the VHL gene [1–5]. This mutation or loss of the VHL gene results in a propensity to develop numerous tumors, malignant or benign, as well as cysts in several organ systems. Within the central nervous system, individuals may develop retinal hemangioblastomas, endolymphatic sac tumors and cranial spinal hemangioblastomas of the cerebellum, brain stem, spinal cord, lumbar sacral nerve roots and supratentorial structures. Disorders of the viscera include renal cell carcinoma and cysts, pheochromocytomas, pancreatic tumors or cysts, epididymal cystadenomas and broad ligament cystadenomas [1]. Tab. 7.1 (from reference [1]) shows the age of onset and frequency of VHL disease lesions.

Tab. 7.1

	Mean (range) age of onset (years)	*Frequency in patients (%)*
CNS		
Retinal haemangioblastomas	25 (1–67)	25–60
Endolymphatic sac tumours	22 (12–50)	10
Craniospinal haemangioblastomas		
Cerebellum	33 (9–78)	44–72
Brainstem	32 (12–46)	10–25
Spinal cord	33 (12–66)	13–50
Lumbosacral nerve roots	Unknown (…)	<1
Supratentorial	Unknown (…)	<1
Visceral		
Renal cell carcinoma or cysts	39 (16–67)	25–60
Phaeochromocytomas	30 (5–58)	10–20
Pancreatic tumour or cyst	36 (5–70)	35–70
Epididymal cystadenoma	Unknown (…)	25–60
Broad ligament cystadenoma	Unknown (16–46)	Unknown

Given the critical role that VHL protein plays in the regulation of the Epo gene (see Chapter 3), it is not surprising that some VHL disease tumors can produce Epo constitutively. Among the many types of tumors that VHL patients may exhibit, renal cell carcinomas or cysts and cerebellar hemangioblastoma are most frequently associated with elevated circulating Epo levels [6–14]. There is no intrinsic reason why other VHL disease lesions may not also produce Epo and, presumably, they do, but perhaps not at levels high enough to be detected clinically. There appears to have been no systematic assessment of this phenomenon among VHL disease patients or their pathological tissues.

There are also acquired VHL mutations, the most frequent of which is seen in clear cell renal cell carcinoma [15–21]. In this disease, loss of chromosome 3p or loss of heterozygosity can result in inactivation of VHL. However, the incidence of erythrocytosis associated with renal adenocarcinoma has been reported to be between 1–5% [14, 22] and has not been correlated with the frequency of VHL mutations. Besides loss or mutation of VHL, other genes have been associated with renal cell carcinoma including FHIT, FOXP1 and others [15, 21, 23–28]. This multiplicity of potential causes of renal cell carcinoma presumably accounts for the relatively low incidence of Epo production by this tumor. Furthermore, using fluorescence *in situ* hybridization, Moch *et al.* demonstrated heterogeneity of VHL gene deletions within individual renal cell carcinoma tumors [16].

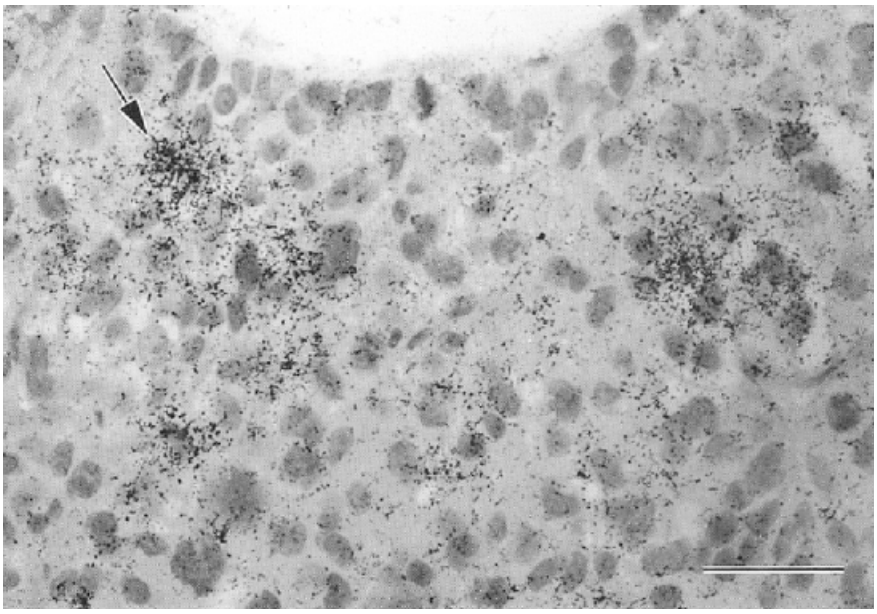

Fig. 7.1 Localization of Epo mRNA in human renal carcinoma cell tumor growing in nude mice by *in situ* hybridization. Silver grains overlie cells expressing Epo. Note heterogeneity of expression. Arrow points to highly expressing group of cells. From reference [11].

This may explain heterogeneity of Epo mRNA expression in one such Epo-producing tumor demonstrated by Shiramizu *et al.* using *in situ* hybridization [11] (Fig. 7.1).

7.1.2 Other Tumors Producing Erythropoietin

Tumors not usually associated with VHL disease or acquired VHL mutations have also been reported to produce Epo. Perhaps the most common of these is hepatocellular carcinoma [29–31]. Expression of the Epo gene by these cells may simply reflect "de-differentiation" from the adult to a more fetal phenotype, since the liver is the principal source of Epo production in the fetus. In addition to hepatocellular carcinoma, Wilms' tumors have been reported to produce Epo [32–37]. More unusual types of tumors reportedly producing Epo include pancreatic ductal carcinoma [38], renal capillary hemangioma [39–54] and uterine leiomyoma [40–54]. It must be recognized that in most of these situations, an investigation for VHL mutations was not carried out.

7.2 Inherited Defects in Oxygen Sensing: Primary Familial Congenital Polycythemia (PFCP) and the Von Hippel-Lindau Gene

Disorders known as *p*rimary *f*amilial and *c*ongenital *p*olycythemias (PFCP) or familial erythrocytoses are a heterogeneous group, all of which are characterized by an increase in circulating red blood cell mass. Some cases are primary, however, others are secondary to increased Epo levels. Causes include reduced production of 2,3-DPG. Also, PFCP can be mimicked by high affinity mutant hemoglobins or methemoglobinemia [55, 56].

Of special interest, in view of its molecular basis, is congenital Chuvash polycythemia. The Autonomous Republic of Chuvashia is located approximately 400 miles east of Moscow on the west bank of the Volga river. The population is $\sim$1.5 million, and 70% are of Chuvash ethnicity. Congenital polycythemia was first reported by Polyakova [57]. In 1997, Sergeyeva *et al.* provided a comprehensive description of the clinical and hematological findings in congenital Chuvash polycythemia [58]. Affected individuals exhibited markedly elevated hemoglobins of 22.6 ± 1.4 g/dL with normal platelet and white blood cell counts. Reported serum Epo levels were inconclusive to allow a definitive statement regarding the role of Epo in this disorder, and Sergeyeva *et al.* concluded that "Chuvash polycythemia might represent a secondary form of familial and congenital polycythemia of a yet unknown etiology".

Although Vasserman *et al.* had localized the gene responsible for this disorder to chromosome 11q23 [59], Ang *et al.* could not corroborate this finding and instead used a genome-wide screen to localize a region on chromosome 3 with an LOD score >2 [60]. They sequenced three candidate genes and identified a mutation in the VHL gene of C to T at nucleotide 598 (R200W amino acid change). Knowing that disruption of pVHL function can result in an accumulation of HIF-1α and upregulation of downstream target genes including Epo (see Chapter 3), Ang *et al.* concluded that Chuvash polycythemia was a congenital disorder of oxygen homeostasis. In a larger study, Ang *et al.* confirmed their findings of the prevalence of an R200W mutation underlying Chuvash polycythemia and demonstrated the accumulation of HIF-1α in cells from these individuals as well as an increased expression of the Epo, GLUT1, transferrin, transferrin receptor and VEGF genes, consistent with global activation of HIF-1 responsive genes.

VHL gene mutations are not restricted to congenital polycythemia in Chuvasia. Pastor *et al.* studied the VHL genes of eight children with a history of polycythemia and an elevated serum Epo level, finding three different germ line VHL mutations in four of the children [61]. Of these four,

one was homozygous for the R200W mutation, while a second was compound heterozygous for R200W and V130L. The other two children, who were siblings, were heterozygous for D126Y. Pastore *et al*. suggested that mutations of VHL might "represent an important cause of pediatric sporadic polycythemias…".

Percy *et al*. have shown that Chuvash-type polycythemia exists outside the geographical confines of Chuvashia itself [62]. They screened 78 patients with erythrocytosis and found a homozygous R200W mutation in eight patients of Pakistani and Bangladeshi origin. The ninth patient, of English ancestry, was heterozygous for the mutation. Whether this mutation, which is clearly more geographically widespread than originally thought, arose independently of the Chuvash population remains to be determined.

7.3 Elevated Erythropoietin Disorders Secondary to Extraordinary Hypoxic Stimuli

Cyanotic congenital heart disease, with its right to left heart shunting of venous blood, results in an extremely strong stimulus to renal production of Epo. Depending upon the degree of cyanosis, the hemoglobin levels can exceed 25 g/dL, resulting in marked increased viscosity of blood flow in all organs, further compromising tissue oxygenation [63–71]. An especially serious consequence of this hyperviscosity is cerebrovascular accident [67]. It has been noted that the reduction of viscosity accomplished by periodic phlebotomy and reduction of hematocrit can worsen this tendency toward vascular occlusion since the accompanying iatrogenic-induced iron deficiency leads to profound microcytosis.

A rare but intriguing disease complex associated with chronic hypoxia and excess Epo production is **chronic mountain sickness (Monge's disease)** [72–79]. This condition develops gradually as a maladaptation to chronic hypoxia in residents of high altitudes after many months or years of exposure. It is most commonly seen in residents of the high Andes in South America. Affected individuals experience dyspnea, lethargy, arthralgias and myalgias. Venous thrombosis and thromboemboli are common, and heart failure is the rule. All of this pathology can be attributed to erythrocytosis and its resulting hyperviscosity. The reason why some individuals develop this condition while others at the same attitude and even within the same family do not remains unknown, although other factors may play a role (see below).

Studies of individuals residing at high altitudes in the Andes, where this disease was first observed, in comparison to those in Tibet and Ethiopia revealed significant differences in human adaptation to high altitude hypoxia. Tibetans in the Qinghei-Tibetan plateau exhibit a significantly lower incidence of chronic mountain sickness than do residents of the Andes in South America [80]. Beall *et al.* compared the patterns of high altitude hypoxia adaptation of Andean, Tibetan and Ethiopian high altitude dwellers [81]. Each of these three groups inspired oxygen at 60–64% partial pressure of sea level. Andean dwellers exhibited both erythrocytosis and arterial hypoxemia, whereas Tibetans exhibited far less or no erythrocytosis in the presence of the same level of hypoxemia. Strikingly, Ethiopian high altitude dwellers exhibited neither erythrocytosis nor arterial hypoxemia. Nutritional and other factors may, in part, explain these differences. No doubt, further studies of these three populations at the molecular level will elucidate further aspects of the physiologic response to the hypoxic stimulus.

Based upon the observation that cobalt stimulates Epo production, presumably through its effect on HIF-1α, inhibiting the interaction of HIF-1α with pVHL (see Chapter 3), it is not surprising that **cobalt intoxication** should result in polycythemia. In the 1960s, a brewer added cobalt to beer as a foam stabilizer resulting in an epidemic of cobalt toxicity in Quebec (reviewed in [82]). Affected individuals developed severe cardiomyopathy and polycythemia. Additionally, individuals with relatively high industrial cobalt exposures have been reported to exhibit elevated packed red cell volumes [82, 83]. In an interesting twist on these observations, Jefferson *et al.* noted that there was some correlation between chronic mountain sickness observed at high altitude and mining activities carried on in or near these communities [84]. They studied males with and without excessive erythrocytosis living in Peru at an altitude of 4300 m, comparing them with controls living at sea level in Lima, Peru. Among the study participants with excessive erythrocytosis (hematocrit greater than 65%), 11 of 21 (52%) had toxic serum cobalt concentrations greater than 15 nmol/L. This surprising result suggested that, at least in some communities, cobalt exposure may contribute to the chronic mountain sickness observed. The authors noted that since cobalt is rather rapidly cleared from the circulation, their measurements would have failed to detect chronic exposure. Therefore, the incidence of cobalt intoxication may have been even higher than the 52% observed. These findings may help explain the difference in incidence of chronic mountain sickness among Andean, Tibetan and Ethiopian high altitude dwellers.

Defects in renal blood flow can also result in increased Epo production [85–96]. This is seen especially in renal artery stenosis, most often found after renal transplantation, although in some instances stenosis is not evident, despite increased Epo levels. This is usually a self-limited process.

7.4
Erythropoietin Deficiency States

Congenital absolute deficiency of Epo in humans has not been described. Studies of Epo knockout mice have shown that this is an embryonic lethal mutation. Relative deficiencies exist in abundance, most often associated with chronic renal failure, due to destruction of Epo-producing cells in the kidney but also with cancer and inflammatory diseases in which cytokines and other factors downregulate Epo production, causing it to be lower than expected for a specified hemoglobin level. These deficiency states and their treatment with recombinant Epo are addressed in Chapter 8.

A particularly interesting disease associated with Epo deficiency that sheds further light on the molecular basis of oxygen sensing is **itai-itai disease** [97–102]. This was first recognized as osteomalacia found in the Toyama prefecture of Japan. It was designated as a disease caused by cadmium in 1968. In addition to bone effects, cadmium exposure in these affected individuals causes renal tubular defects, reduces male fertility and induces anemia. In 1994, Horiguchi *et al.* carried out a clinical study on itai-itai patients and discovered low serum Epo levels despite the presence of severe anemia [103]. Further experimental studies in rats revealed that long term cadmium exposure reduced Epo production by the kidneys and resulted in anemia [104]. Though the mechanism of cadmium-induced Epo downregulation needs further study, Chung *et al.* demonstrated that cadmium inhibits the HIF-1-mediated response hypoxia by increasing degradation of HIF-1α in proteasomes [105]. While the precise mechanism of this proteasome activation by cadmium is not yet clear, it does provide an interesting insight into the multiple environmental effects that can alter oxygen sensing and its downstream signaling.

7.5
Erythropoietin Receptor Mutations and Defects

The Epo receptor cytoplasmic region contains an inhibitory domain near its carboxy terminus. Furthermore, the tyrosine phosphatase SHP-1, also designated HCP and SHPTP-1, binds at phosphotyrosine 429 and reduces receptor activity by dephosphorylating JAK-2. Clearly, mutations affecting either of these domains could result in an EpoR with increased activity, leading to a phenotype of erythrocytosis (reviewed in [106, 107]). The first such mutation was discovered in a family with idiopathic erythrocytosis [108, 109]. It was characterized by a G to A mutation at nucleotide 6002,

Tab. 7.2 Erythropoietin Receptor Mutations Causing Erythrocytosis

Mutation	*Nucleotide position*	*Effect of mutation*	*Truncation*	*Reference*
G to T	5881	Missense	110 aa	[110]
G to T	5959	Stop codon	84 aa	[111]
C to G	5964	Stop codon	82 aa	[112]
Insertion-T	5967	Frameshift	65 aa	[113]
Duplication	5968–5975	Frameshift	79 aa	[114]
Insertion-G	5974	Frameshift	64 aa	[115]
Deletion	5985–5991	Frameshift	59 aa	[113, 116]
C to T	5986	Stop codon	74 aa	[117]
G to A	6002	Stop codon	70 aa	[109, 118]
A to G	6146	Missense	Point mutation	[118, 119]
C to T	6148	Missense	Point mutation	[120]

Modified from [107]

resulting in a stop codon leading to a truncation of seventy amino acids. A number of different mutant receptors have been described. Each is characterized either by introduction of an early stop codon, a frame shift or a missense point mutation, resulting in a hyperresponsive EpoR both *in vitro* and *in vivo*. The *in vivo* phenotypes are uniformly characterized by increased red cell mass that may or may not be symptomatic. Tab. 7.2 (modified from [107]) shows these mutations and their effects on the EpoR amino acid sequence. No doubt further receptor mutations will continue to be described.

Mutant Epo receptor genes encoding EpoR proteins with reduced activity have not yet been described. A totally inactive receptor would be inconsistent with survival, as evidenced by EpoR knockout mouse data [121]. However, EpoRs with reduced activity, due either to cytoplasmic domain mutations or to extracellular region mutations that might reduce affinity for Epo, would in all likelihood be compensated for by increased Epo production leading to a normal or subtly abnormal phenotype. In a similar fashion, mutant Epo proteins may exist in nature that exhibit reduced (but not absent) affinity for the EpoR. Similarly, the physiology of the Epo feedback loop should result in increased expression of these proteins, thereby compensating for their reduced activity for the receptor. It can be anticipated that such hypoactive EpoR and Epo proteins eventually will be discovered.

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8
Recombinant Erythropoietin and the Treatment of Anemia

8.1
Therapeutic Indications

The cloning and expression of the human Epo gene permitted the development of recombinant human Epo (rhEpo) as a pharmaceutical. Since the late 1980s, rhEpo has become one of the success stories to emerge from the biotechnology industry. The availability of rhEpo has provided important new therapeutic options in a variety of disease and deficiency states.

There are numerous approved or investigational indications for the use of rhEpo [1, 2]. They include:

- Anemia of chronic renal failure
- Anemia of HIV infection on zidovudine (AZT) treatment when serum Epo level is < 500 mU/mL
- Anemia of non-myeloid malignancies (preferably if Epo level is < 200 mU/mL)
- Perioperatively, to reduce the need for allogeneic blood transfusion in non-vascular and non-cardiac surgery
- Preoperative autologous blood donation
- Anemia of prematurity
- Autologous blood donation
- Bone marrow transplantation
- Anemia of chronic inflammation
- Anemia of myelodysplastic syndromes and marrow failure states
- Anemia in hematologic malignancies
- Anemia associated with other malignancies in the absence or presence of chemotherapy
- Surgical blood loss
- Sickle cell anemia and thalassemia (to induce Hb F synthesis)

The use of rhEpo is relatively contraindicated in patients with uncontrolled hypertension and for those patients whose medical conditions suggest that rhEpo treatment would result in polycythemia.

In virtually all instances, the cause or causes of anemia should be vigorously investigated prior to institution of rhEpo therapy. The most obvious ones include deficiency states such as iron, vitamin B_{12} or folic acid deficiency. In addition, high levels of endogenous circulating Epo (>500–1000 mU/mL) ordinarily are predictive of therapeutic failure [1, 2].

8.2 Dosage and Route of Administration

The initial dose to be used varies somewhat on the condition to be treated, but an average dose of 100–150 IU/kg body weight administered subcutaneously 2–3 times per week in patients without renal failure (lower doses in the case of renal failure) represents a good starting point for induction therapy. rhEpo can be administered either intravenously or subcutaneously and, although subcutaneous administration allows for a reduction in overall dosage, intravenous administration is especially applicable to dialysis patients and is devoid of compliance concerns (see below). Unfortunately, the response to therapy can be somewhat slow, due in part to the biology of erythropoiesis in humans, but also to individual variation and the effect of disease processes on the bone marrow. Indeed, a robust response to rhEpo therapy may require 12 weeks or more, even though slight increases in hematocrit are often seen after a 4 week period. If such a response is not observed, dosing can be increased by 50 IU/kg every four weeks. Iron supplementation is virtually always advisable, ordinarily by parenteral administration, since absorption of oral iron cannot keep up with the demands of an rhEpo-stimulated bone marrow during induction.

8.3 Side Effects and Therapeutic Failure

The administration of rhEpo results in side effects only infrequently, and, for the most part, they are limited to patients with renal failure. This may be due primarily to the fact that this group of patients represents the vast majority of individuals treated thus far. The most common side effects

are hypertension and hyperviscosity, potentially leading to seizures and thromboemboli. Possible mechanisms for hypertension are discussed below. Obviously, therefore, careful monitoring of blood pressure is essential in all patients treated with rhEpo. Other reported side effects include extramedullary hematopoiesis, splenomegaly and flu-like symptoms [2–4], which can be reduced by gradual dose increases from a low starting level [5, 6]. Recently, there have been reports of pure red cell aplasia (PRCA) in patients undergoing rhEpo therapy for various forms of renal disease. The extent of this serious side effect remains to be determined as does it cause, whether related to the rhEpo itself or to other factors such as extraneous contaminants or excipients (see below).

Failure of rhEpo therapy, defined by inability to achieve the target hemoglobin or the need for extraordinary doses, is relatively infrequent. Its causes include nutrient deficiencies such as iron, folic acid or vitamin B_{12}, infection and inflammation, bone marrow failure, blood loss, hemolysis and other hematologic diseases. Obviously, the causes of therapeutic failure should be investigated vigorously.

8.4 Pharmaceutical Designations

The rhEpos used in pharmaceutical preparations have the same amino acid sequence, that is, amino acids 1–165 of the mature protein. However, they can vary in their degree of glycosylation and in their glycan (oligosaccharide) composition and/or structure due to differences in the mammalian cell lines used to express the protein, differences in downstream processing (purification) or a combination of both. Because of these issues, five International Nonproprietary Names (INNs) have been recommended by the World Health Organization for recombinant Epos each with the same amino acid sequence but with differences in their glycosylation. They also have corresponding United States Adoptive Names (USAN). These names are based upon the root *epoetin* and include *epoetin alfa, epoetin beta, epoetin gamma, epoetin epsilon* and *epoetin omega*. In the United States, only epoetin alfa is available under the trade names Epogen® (Amgen) and Procrit® (Ortho Biotech). In Europe, epoetin alpha and epoetin beta are available under the trade names Eprex® or Erypo® (Janssen-Cilag) and NeoRecormon® (Roche), respectively. In Japan, epoetin alpha and epoetin beta are marketed as ESPO® (Kirin Brewery Company, Pharmaceutical Division) and Epogin® (Chugai Pharmaceutical Company), respectively. In a small number of countries, epoetin omega

has been marketed under the trade names Hemax® or Epomax® (originally Elanex Pharmaceuticals; now owned by Baxter). There are other rhEpos available in some countries that have not utilized the INN or USAN designation. There is a sixth *epoetin* INN, *epoetin delta*, which has been recommended for a human Epo therapeutic produced by gene activation technology rather than by recombinant DNA methods, so-called "GA-EPO™", co-developed by Transkaryotic Therapies (TKT) and Aventis (trade name Dynepo™). There is a small number of reports suggesting pharmacological and/or clinical differences among these epoetins, which would not be unexpected in view of the important role of glycosylation in Epo's *in vivo* activity [7, 8]. Some studies have suggested that epoetin omega is either more potent or more efficacious than epoetin alpha or beta in reaching such endpoints as median hemoglobin attained, mean hemoglobin increment and time required to reach target hemoglobin [9, 10]. However this area has not been researched in depth.

8.5 Treatment for Specific Indications

8.5.1 The Anemia of Chronic Renal Failure

Prior to the availability of rhEpo, the anemia associated with chronic renal failure was treated principally by blood transfusion. In addition, **androgen therapy** was used with some success to at least partially correct the anemia of renal failure [11–15] and still may have utility under special circumstances [16–25]. However, androgen therapy, especially in women, can be accompanied by significant side effects. Before rhEpo became available, there was very little evidence that administration of any form of Epo would correct the anemia of renal failure. Some information was available from animal models [26, 27], and early studies in humans showed that administration of Epo-rich plasma obtained from aplastic anemia patients resulted in a transient increase in reticulocyte count, indicative of new erythropoiesis [28–30].

Progress in assessing the clinical efficacy of rhEpo in the uremic setting was remarkably fast, considering the stage of development of the biotechnology industry at the time. The first clinical trials were published less than five years after the human gene was cloned [3, 31]. Initial studies used doses ranging from 10–150 IU/kg and up to 1500 IU/kg three times per week, respectively. Both reported increases in hemoglobin and reduc-

tion in transfusion requirements. Interestingly, higher doses, which achieved a more rapid increase in hemoglobin level, also resulted in a plateauing of the response (Figs. 8.1 and 8.2). This was due to the absence of iron supplementation, which became the standard of care after more experience with rhEpo therapy had been accumulated. In these early studies, adverse effects were observed including the appearance or exacerbation of hypertension, seizures and hemodialysis access failure. It was later shown that the appearance of hypertension could be reduced or eliminated if relatively lower doses of rhEpo were used resulting in a more gradual increase in hemoglobin.

Resistance to rhEpo therapy has been well documented in patients with renal failure. Causes include malnutrition, iron deficiency, malignancies, infection, hemolysis, bleeding, aluminum intoxication (overt or subclinical), secondary hyperparathyroidism and inadequate dialysis [32–35]. Also implicated have been interactions with other agents such as cyclosporin and aminophylline [36–38]. Angiotensin converting enzyme (ACE) inhibi-

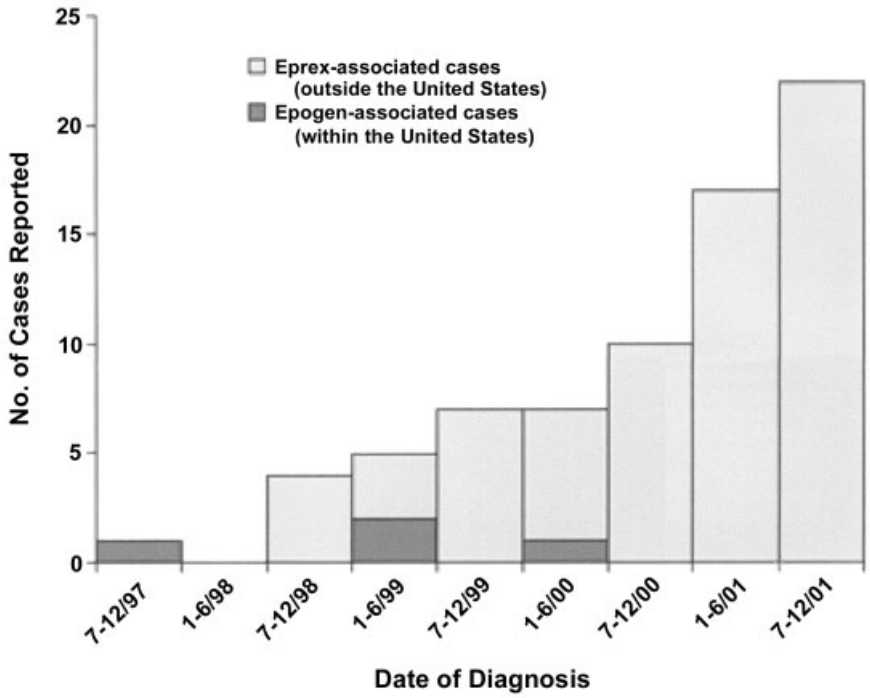

Fig. 8.1 Pure red cell aplasia among epoetin alfa recipients as reported to the United States Food and Drug Administration. The date of diagnosis was not reported for nine Eprex-associated cases. From reference [52].

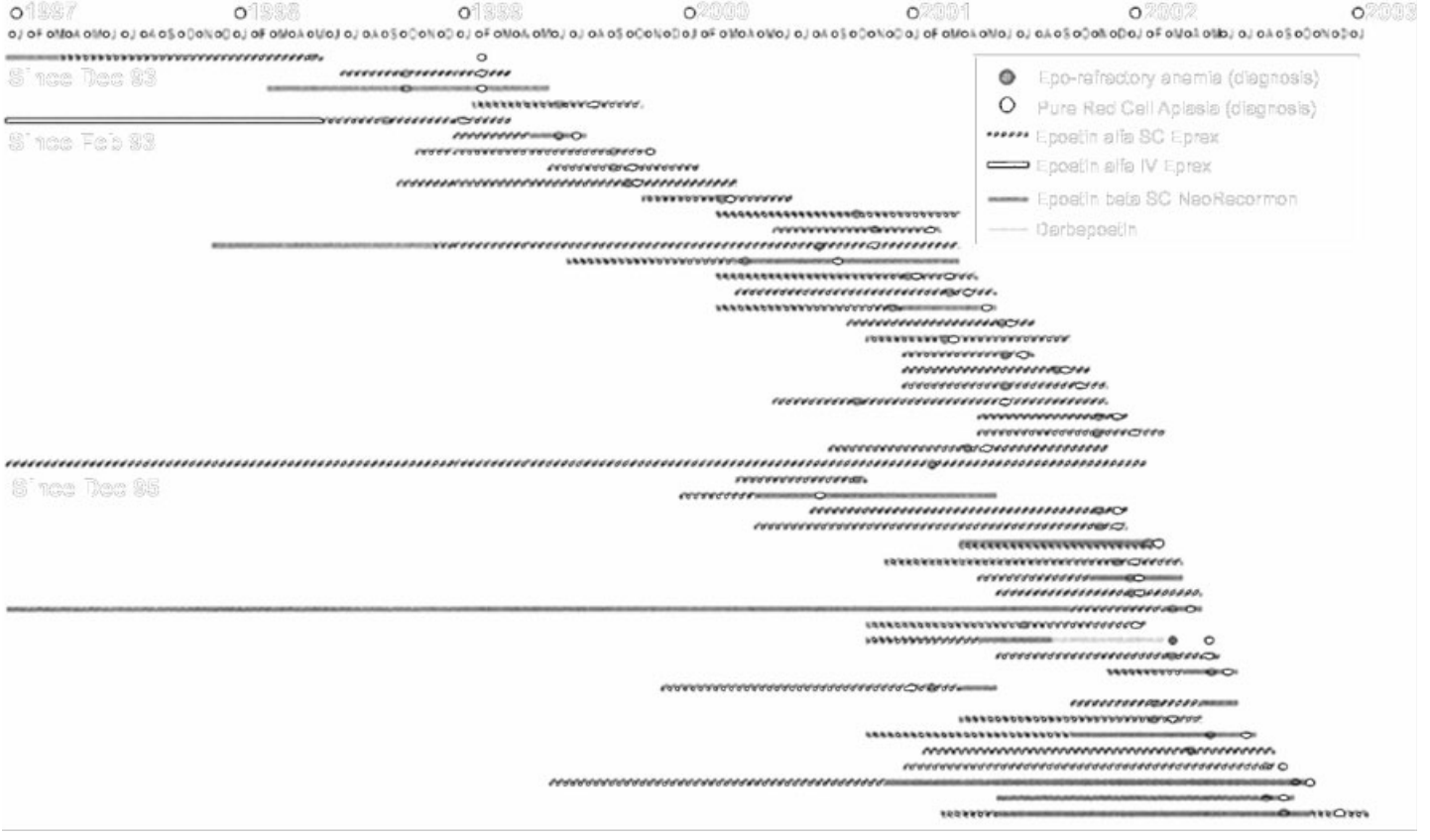

Fig. 8.2 Individual epoetin-induced pure red cell aplasia cases studied in the Department of Hematology, Hôpital Hôtel-Dieu, Paris, France. The graph depicts all cases in which anti-Epo antibodies were demonstrated by immunoprecipitation of ^{125}I-Epo from 1998 through 2002. From reference [53].

tors suppress endogenous Epo production and have been suspected to cause rhEpo resistance. The role of ACE inhibitors and rhEpo responsiveness in human dialysis patients remains controversial. Several mechanisms have been postulated through which ACE inhibitors might induce rhEpo resistance in dialysis patients [39]. They include the observation that angiotensin 2 has a mitotic action on erythroid progenitor cells [40]. Also, N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), which is an endogenous regulator of stem cell proliferation, inhibits movement of stem cells and progenitors into the S phase. ACE inhibitors increase plasma levels of this peptide significantly and, thus, could inhibit erythroid cell growth [41–43]. Also, an effect of ACE inhibitors on insulin like growth factor 1 (IGF-1) has been suggested [44]. Lastly, ACE inhibitors decrease production of interleukin-12, which is known to enhance erythroid cell growth [45]. Despite these potential mechanisms, clinical studies indicate that ACE inhibitors do not cause resistance to rhEpo [46].

8.5.1.1 **Anti-Epo Antibodies and Pure Red Cell Aplasia (PRCA)**

The report of a dialysis patient receiving rhEpo therapy who developed pure red cell aplasia (PRCA) [47] prompted Castelli *et al*. to examine the sera of 40 dialysis patients receiving rhEpo for the presence of anti-Epo antibodies [48]. They reported that a surprisingly high 67% of these patients had such antibodies, detected by an enzyme immunoassay, although none of these antibodies appeared to be neutralizing. This very high frequency has not been confirmed. Prior to 2002, only two other cases of patients treated with rhEpo who developed clinically-significant neutralizing antibodies had been reported [49, 50]. However, in 2002, Casadevall reported 13 rhEpo-treated patients in Europe who developed neutralizing anti-Epo antibodies (detected by immunoprecipitation of ^{125}I-labeled rhEpo) and PRCA [51], and more cases followed, almost exclusively in Europe [52, 53]. The large majority of these patients had been treated subcutaneously with Eprex® (Johnson and Johnson/Janssen-Cilag), a formulation of epoetin alpha manufactured in Puerto Rico and marketed outside the U.S. There has been speculation about the cause of this phenomenon, which remains under active investigation. It has been suggested that cyclosporine may reverse PRCA in some of these patients [54].

8.5.1.2 **Other Issues**

Predialysis There have been some objections to the use of rhEpo in patients with chronic renal failure but in the predialysis phase. There are

concerns that increases in blood pressure and viscosity seen with rhEpo administration, along with other mechanisms, could accelerate the loss of renal function [55]. In contrast, however, if blood pressure is controlled, the use of rhEpo in this group of patients can be recommended [56, 57].

Graft Survival There are also some interesting data suggesting that the use of rhEpo may enhance graft survival in kidney transplant patients, at least partly by avoidance of allosensitization accompanying blood transfusion [58–60].

Adjuvants There is increasing literature suggesting that the administration of other agents may enhance the action of rhEpo. These agents include ascorbic acid, folic acid, L-carnitine, vitamin D, and cytokines [61–64]. Of special interest is the role of ascorbic acid and iron utilization [65]. Even when hemodialysis patients are iron overloaded, functional iron deficiency may result from slow mobilization of iron stores. Ascorbic acid plays several roles in iron metabolism and appears to accelerate the mobilization of iron from tissue stores, thus making it an ideal adjuvant in rhEpo therapy.

Hypertension: Vascular Effects One of the most serious side effects of rhEpo treatment in renal dialysis patients is hypertension. Recently, the mechanism of rhEpo-induced hypertension has received greater attention, partially due to the observations that rhEpo has biological effects outside of the hematopoietic system (see Chapter 9). Factors potentially contributing to rhEpo-induced hypertension are increased reticulocyte mass, altered vascular reactivity to endogenous catecholamines, changes in the renin/angiotensin system, increased production of endothelin-1, effects on prostaglandin production and vascular responsiveness, resistance to the vasodilatory action of nitric oxide (NO resistance), changes in cytosolic calcium, direct vasopressive action of rhEpo, and rhEpo effect on endothelial and other vascular cell growth factors [66–72]. Studies exist to both confirm and contradict these possible actions of rhEpo in hypertension. Therefore, it is critical that further investigations be carried out to clarify this issue to allow better management of hypertension in rhEpo-treated renal dialysis patients.

8.5.2 Acquired Immunodeficiency Syndrome (AIDS)

Anemia accompanies HIV/AIDS disease very frequently and can be due to a several factors. Suppression of the bone marrow is the most common mechanism, which may be caused by infections, neoplasms, cytokines, medications and nutritional deficiencies. Zidovudine (AZT) is used to treat HIV disease and is most often associated with anemia. In early HIV infection at AZT doses of 400–800 mg, 2–20% of patients develop anemia [73, 74]. This incidence is higher in advanced AIDS cases. Although red blood cell transfusions are effective in treating this anemia, questions about potential immunosuppression secondary to these transfusions have arisen. It has been suggested that donor lymphocytes and alterations in prostaglandin E metabolism and function play a role in immunosuppression [75, 76]. An important study also demonstrated that blood transfusions represented a risk factor for progression from HIV to AIDS [77, 78]. Cytomegalovirus (CMV) is also an issue.

Recombinant Epo therapy is effective in treating anemia in AIDS patients. Endogenous Epo levels should be less than 500 mU/mL for therapy to be considered, since patients with endogenous Epo levels greater than this reportedly exhibited no improvement in any of the endpoints of rhEpo therapy [79–81]. In the absence of AZT therapy, however, rhEpo treatment is far less efficacious [82]. Whether rhEpo therapy causes a reduction in the death rate in AIDS patients is uncertain, although preliminary results are encouraging [83]. However, studies clearly show an improvement in quality of life [84]. Therefore, rhEpo treatment of anemic AIDS patients, especially with a hematocrit less than 30% and endogenous Epo levels less than 500 mU/mL can be recommended. Initial dosage may be individualized; 100–200 IU/kg weekly for 12 weeks has been used with success.

Interestingly, **autoantibodies to endogenous Epo** have been reported in anemic patients infected with HIV type 1 [85]. Forty eight of 204 patients studied had circulating autoantibodies, and these antibodies were an independent predictor of anemia. The autoantibodies were associated with higher endogenous Epo levels, and the authors suggested that these autoantibodies may have been a contributory cause to HIV-related anemia. Obviously, as with hemodialysis patients in whom rhEpo therapy is considered, all other treatable causes of anemia should be evaluated.

8.5.3 Chemotherapy-induced Anemia of Non-hematologic Malignancies

Like the anemia associated with HIV/AIDS, the anemia of cancer has multiple causes. They include increased levels of interferon-γ, tumor necrosis factor and interleukin-1 (all the result of inflammation), occult bleeding or hemolysis, tumor growth in the bone marrow (myelophthisic anemia), and the effects of radiotherapy and of chemotherapy. Both relative Epo resistance as well as reduced endogenous Epo production have been documented in cancer patients [86, 87]. Among the numerous chemotherapeutic agents, cisplatin has been especially associated with reduced production of Epo [88, 89]. This is due, at least in part, to the drug's nephrotoxicity. The efficacy of rhEpo therapy in ameliorating chemotherapy-induced anemia has been especially well-documented in randomized clinical trials [90, 91]. In patients with either a cisplatin- or a non-cisplatin based chemotherapeutic regimen, controlled studies of rhEpo therapy show a significant reduction in transfusion requirements and increase in hemoglobin/hematocrit [92]. This occurred at doses of 150 IU/kg three times weekly for 12 weeks. Among the numerous disease-specific trials published have been ovarian cancer [93] (although one investigator has recently called for a *definitive* Phase 3 randomized trial [94]), primary bone tumors [95], small cell lung cancer [96], head and neck cancer [97] and others. In addition to the hematologic endpoints, there is evidence suggesting an improvement in quality of life in cancer patients treated with rhEpo [96, 98–104]. The use of rhEpo in the setting of gynecologic malignancies has been reviewed favorably [105]. A large meta-analysis concluded that rhEpo reduced the odds of transfusion for cancer patients, but that rhEpo's effect on anemia-related symptoms had not been well addressed [106]. This study also found that data are insufficient to determine whether rhEpo therapy should be instituted before hemoglobin concentrations decline toward the 10 g/dL level.

rhEpo is effective in correcting the anemia of patients receiving **radiation therapy** even in the absence of chemotherapy and may enhance the anti-tumor effects of radiotherapy [107, 108]. This may be explained by increased oxygen delivery to the tumor cells, since radiation cytotoxicity is enhanced with tumor oxygenation. This possibility has been supported by preclinical observations in rats in which the reduction in cyclophosphamide cytotoxicity toward rat tumors that was induced by anemia was corrected upon rhEpo administration, leading to the conclusion that rhEpo enhanced tumor sensitivity to cyclophosphamide as a result of improved oxygen supply [109]. Other intriguing preclinical observations include the description of rhEpo induction of tumor regression and stimulation of

anti-tumor immune responses in murine myeloma tumor models [110], and the observation that rhEpo increased the radiosensitivity of xenografted human tumors in anemic nude mice [111]. There is substantial interest in this issue among radiation oncologists [112–115].

It should be noted that a potential exists for an adverse impact of rhEpo on tumor growth in patients, since a number of studies have demonstrated Epo receptors on human tumor cells (see Chapter 9).

8.5.4 The Perioperative Setting

Recombinant Epo Without Autologous Blood Transfusion. In cases in which patients have insufficient time to donate or will not accept autologous blood, rhEpo therapy is a useful modality. Recombinant Epo use pre- and/or post-operatively has been evaluated especially in orthopedic surgical patients. 300 IU of rhEpo/kg daily administered subcutaneously for 10 days prior to and for 4 days after operation reduced transfusion requirements in patients undergoing hip replacement surgery [116]. This study has been supported by other similar studies [117, 118]. Additionally, lower total doses or different dosing intervals have been investigated [119]. The results suggest that lower total doses or shorter preoperative periods may be equally effective if iron supplementation is employed. Since endogenous iron stores cannot be mobilized rapidly enough to accommodate the brisk erythropoiesis resulting from such therapy, iron supplementation either orally or, if necessary, intravenously is to be encouraged. Several dosing possibilities have been explored [120], and a number have been proposed, but none has proven superior [121]. Several issues exist, including time left until operation, the availability of an autologous blood donation system and convenience. In addition, cost is a factor that must be taken into consideration. The principle of acute, normovolemic hemodilution combined with rhEpo therapy remains under consideration [122, 123]. However, the data are conflicting both with respect to superiority of rhEpo over autologous blood transfusion and with respect to any potential cost savings.

Application of rhEpo therapy to problems encountered in **cardiac and vascular surgery** is still investigational. It is a concern that rhEpo may increase the incidence of thrombotic events in these patients [124, 125]. However, some studies indicate that rhEpo may be used safely in these surgical patients. There are data suggesting that rhEpo may increase extractable oxygen and decrease the tendency toward lactic acidosis through its effect on red blood cell 2,3-diphosphoglycerate (2,3-DPG) in open-heart

surgery [126]. This effect may be due to the increase in circulating young erythrocytes (neocytes) that accompanies rhEpo administration. These young red blood cells contain increased amounts of 2,3-DPG.

Recombinant Epo as an Adjunct to Autologous Blood Donation rhEpo continues to be considered for use to enhance autologous blood donation. Although allogeneic blood supplies are extremely safe, autologous transfusion can reduce the small risk even further. Additionally, it has been suggested that in cancer surgery, allogeneic blood transfusion may increase risk of recurrence, potentially by means of a mild immunosuppressive effect [127]. Preoperative autologous blood donation is a real option, but is limited by the time factor and by the donor's hematocrit. Due to the relatively slow physiologic response of endogenous Epo at restoring hemoglobin levels after donation, rhEpo has been used with some success to stimulate erythropoiesis both before and after donation [128, 129]. There are a number of reports of the successful use of rhEpo in this setting, but the benefits are somewhat limited [130–134]. Reduction of allogeneic blood transfusion was reported to be restricted to patients who were already anemic prior to operation [133–136]. Randomized trials in Europe demonstrated that 300–600 IU of rhEpo/kg twice weekly for three weeks before an orthopedic procedure was useful when combined with iron supplementation. Lower doses were not very effective, and higher doses offered no increased benefit. In almost all instances of autologous blood donation, iron supplementation should be used.

8.5.5 The Anemia of Prematurity

Premature infants usually exhibit lower hematocrits than term infants. This is at least partly due to altered Epo physiology, with respect to both endogenous Epo production by the kidney and the response of erythroid progenitors to Epo in the immature bone marrow [137, 138]. Administration of rhEpo has been employed with some success in these situations, often resulting in a reduced need for red cell transfusion [139–141]. The doses of rhEpo employed have varied widely from 75–6300 IU/kg per week. In virtually all studies, iron supplementation was used. A dose of 600–700 IU/kg weekly seems optimal, but how these doses should be divided is less certain [142]. The most benefit has been achieved in infants weighing between 1000 and 1500 g. Infants with birth weights of less than 1000 g do not respond very well to exogenous rhEpo. After the first

few weeks of life, rhEpo administration is usually no longer required, since the physiologic response of Epo production by the kidney and the capacity of the bone marrow erythroid progenitors to respond has matured by then [143]. This area has been the subject of a recent review [144].

8.5.6
The Anemia of Hematologic Malignancies

Lymphomas, leukemias, myeloma and other hematologic malignancies are usually accompanied by anemia, sometimes profound. The anemia may have multiple causes including cytokine production, bone marrow infiltration and replacement by malignant cells (myelophthisis), hemolysis and chemotherapy. Additionally, inappropriately low endogenous Epo levels may contribute [145]. Myeloma and lymphoma have been most studied.

Recombinant Epo therapy has been used in myeloma patients for a number of years. A small, early study reported that a dose of 150 IU/kg three times weekly, with the potential of dose escalation, yielded a response rate of 85% [146]. The response appeared not to be related to chemotherapy. Additional trials provided data supporting this study, applying rhEpo therapy both to myeloma and to non-Hodgkin's lymphoma (NHL) patients [147–149]. Using rhEpo doses of 5000 IU/kg subcutaneously daily for eight to 24 weeks reduced transfusion requirements and increased hemoglobin levels. Approximately 50% of patients responded. Predictors of a positive response included a pretreatment Epo level of less than 50 mU/mL and an adequate bone marrow reserve, evidenced by the pretreatment platelet count. A meta-analysis of 300 anemic myeloma patients demonstrated a response to rhEpo therapy of 64% [150]. Studies have confirmed improvements in quality of life in patients with hematologic malignancies undergoing rhEpo therapy [146, 149].

rhEpo has been used in patients with chronic lymphocytic leukemia (CLL). In these patients, hemolysis and splenic sequestration of red cells are especially important components of the anemia. Randomized trials have found that a dose of 150 IU/kg three times weekly for 12 weeks resulted in an increased hematocrit, reduced transfusion requirements and enhanced quality of life [151]. It is not known whether rhEpo therapy affects the long-term outcome or natural history of CLL.

8.5.7
Myelodysplastic Syndrome

The term "myelodysplastic syndrome" (MDS) refers to a group of hematopoietic stem cell disorders, which are clonal in nature, affecting elderly people primarily. They exhibit reduced cell numbers (cytopenias) in the erythroid, myeloid and megakaryocytic lineages. In many cases, the erythroid lineage is especially involved. Endogenous Epo levels in MDS patients vary widely, from inappropriately low to increased above normal [152, 153]. These findings may be due to the fact that most early trials of rhEpo therapy in MDS were not randomized. One analysis reported a response rate (elimination of transfusion) in 16% of patients [154]. A response rate of 15 to 20% has been found in other studies [155, 156]. Effective doses began at approximately 450 IU/kg per week for at least 8–10 weeks. Predictors of response to rhEpo therapy includes a normal karyotype, endogenous Epo levels of less than 100–200 mU/mL and the refractory anemia (RA) subtype. Patients with a lesser degree of anemia, characterized by transfusion independence, are more likely to respond. These rather disappointing response rates are due, no doubt, to the heterogeneity of diseases that the designation MDS comprises. *In vitro* data [157, 158] suggested the use of rhEpo therapy combined with granulocyte-colony stimulating factor (G-CSF) in MDS. In some studies, response rates of 40% have been achieved [159–162]. G-CSF doses of up to 5 μg/kg subcutaneously, depending upon absolute neutrophil count, have been employed. A validated decision model has identified a population of MDS patients who exhibited a 61% response rate to rhEpo + G-CSF treatment [163]. Granulocyte/macrophage-colony stimulating factor (GM-CSF) also has been used in combination with rhEpo in MDS. However, these results are less encouraging [164, 165].

8.5.8
Bone Marrow Transplantation

Bone marrow transplantation or peripheral blood stem cell rescue are strategies that are potentially curative for certain hematologic and other malignancies. However, these modalities result in suppression or elimination of erythroid, myeloid and megakaryocytic lineages of the host. Long-lasting anemia ensues, usually requiring frequent blood transfusion. Therefore, therapy with rhEpo has been investigated.

In the setting of bone marrow transplantation, alterations in the physiology of Epo and erythropoiesis have been observed. There are characteris-

tic changes unique to allogeneic and autologous transplantation, respectively [166, 167]. Shortly after autologous transplantation, there is a reduced response to Epo, although endogenous Epo is produced by the kidney in appropriately increased amounts. Later, responsiveness of the transplanted marrow to Epo returns, and transfusion requirements are decreased or eliminated completely. In the setting of allogeneic transplantation, the converse occurs. Shortly after transplantation, the transplanted bone marrow responds quickly to Epo, and hematopoiesis is more rapidly restored. Thereafter, however, inflammatory cytokines characteristic of graft *versus* host disease and immunosuppressive therapy cause a reduction of endogenous Epo production as well as some diminution of the bone marrow response. The results of clinical trials of rhEpo in the setting of bone marrow transplantation reflect these altered physiologic states [168–171]. Immediately after allogeneic transplant, rhEpo therapy has been shown to be effective in reducing blood transfusion requirements, although somewhat higher doses of 75–200 IU/kg daily intravenously have been used. As expected, immediately after autologous transplantation, studies show that rhEpo therapy is not particularly useful [172, 173].

8.5.9
Sickle Cell Anemia and Thalassemia

In both sickle cell anemia and the thalassemias, erythropoiesis usually operates at near maximum levels as a consequence of intramedullary and/or peripheral destruction of erythroid cells and increased production of endogenous Epo. Therefore, it would be expected that administration of exogenous rhEpo would be of little value. Moreover, administration of rhEpo in those cases of sickle cell anemia with relatively low endogenous Epo levels may be contraindicated, since an increase in hematocrit might only serve to increase viscosity and lead to more crises. It is well known that hemoglobin F (Hb F, fetal hemoglobin) interferes with the polymerization of hemoglobin S (Hb S) molecules and, thus, inhibits sickling of sickle cell erythrocytes [174, 175]. There are interesting data demonstrating that rhEpo therapy may result preferentially in the production of new erythrocytes containing increased levels of Hb F. The use of rhEpo in combination with hydroxyurea, another agent that increases Hb F, has been explored with some success in the treatment of sickle cell patients [176].

In a similar manner, an increase in Hb F may be beneficial to patients with thalassemia. rhEpo therapy has been shown to increase Hb F in

these patients as well as potentially correcting some imbalance between alpha and beta globin chain synthesis. Nevertheless, despite encouraging preliminary data [177], the use of rhEpo in these disorders must still be regarded as investigational.

8.5.10
The Anemia of Chronic Inflammation

The state of chronic inflammation, such as encountered in rheumatoid arthritis and inflammatory bowel disease, is characterized by reduced production of endogenous Epo as well as an inhibited response of erythroid progenitor cells to its action [178]. Recombinant Epo has been used to treat patients with rheumatoid arthritis and has been shown to restore the hematocrit towards the near normal level [179]. There is also evidence that treatment with rhEpo lessens disease activity and improves quality of life [180, 181]. In patients with inflammatory bowel disease, there may be both an inflammatory and an iron deficiency component to the anemia. Recombinant Epo has been proven effective in treating anemia in these patients when iron supplementation alone is ineffective [182]. Iron deficiency should be suspected and corrected before administration of rhEpo to these patients. Interestingly, antibodies to rhEpo have been detected in the sera of patients with systemic lupus erythematosus, the levels of which correlated positively with the degree of anemia [183].

8.6
Practice Guidelines, Patient Response and Medical Economics

Some guidance on the use of rhEpo in the anemia of renal failure has been provided by both the European Renal Association-European Dialysis and Transplant Association and the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI). Clinical practice guidelines for the use of epoetin in patients with cancer have been recommended by the American Society of Clinical Oncology (ASCO) and the American Society of Hematology (ASH).

8.6.1
European Guidelines 9–12

Guideline 9: Route of Administration of Epoetin

A. *Epoetin should normally be administered subcutaneously in predialysis and peritoneal dialysis patients since this is almost always more convenient, especially if self-administration is practised.*
B. *According to patient characteristics and preference, epoetin can be administered either subcutaneously or intravenously in patients on regular haemodialysis, but the subcutaneous route will usually lead to lower doses of epoetin being required, and, in general, this route is preferable.*
C. *When epoetin is given subcutaneously, the site of injection should be rotated with each administration.*
D. *Patients using the subcutaneous route should be encouraged to self-administer epoetin whenever possible.*
E. *In a few peritoneal dialysis patients in whom both subcutaneous and intravenous administration of epoetin is not feasible, e.g., in some paediatric patients, intraperitoneal administration may be considered.*
F. *Intraperitoneal administration must be given into a dry abdomen, which should remain dry for at least 6–8 h. Intraperitoneal epoetin dose requirements may be higher than those associated with intravenous and subcutaneous administration.*

Guideline 10: Initial Epoetin Administration

A. *The starting dose of epoetin should be 50–150 IU/kg/week (typically 4000–8000 IU/week), depending on body weight, the total epoetin requirement and the need to utilize the whole vial with some preparations.*
B. *When epoetin is administered subcutaneously (see Guideline 9), doses in the lower part of this range should be used, 2–3 times per week. For intravenous administration, the starting dose should be in the upper range (typically 6000 IU/week) 3 times per week.*
C. *Higher initial doses of epoetin may be used if the patient has either complicating disorders leading to anemia, or severe anemia (Hb concentration < 8 g/dL).*
D. *Titration of dosage: epoetin doses in the upper range can be reached progressively as the individual patient's maintenance dose is established, usually by decreasing the interval between subcutaneous injections. Intravenous injection should be continued thrice weekly with a dosage increase. If the patient requires less than the starting dose to maintain the target Hb concentration (see Guideline 5), either the intervals between each subcutaneous dosage can be extended, or thrice weekly intravenous dosage decreased.*

E. *Paediatric patients younger than 5 years of age may require greater doses of epoetin on a body weight basis (up to 300 IU/kg/week) than older paediatric patients and adults.*

Guideline 11: Monitoring of Haemoglobin Concentration During Epoetin Treatment

A. *The Hb concentration should be measured every 1–2 weeks following initiation of treatment or following a dose increase or decrease, until a stable Hb concentration and epoetin dose have been reached. The target should be to increase the Hb concentration by 1–2 g/dL per month.*
B. *Once a stable target Hb concentration and epoetin dose have been reached, the Hb concentration should be monitored every 4–6 weeks in both haemodialysis and CAPD patients and less often in pre-dialysis patients, unless intercurrent diseases occur that may influence the Hb concentration.*

Guideline 12: Titration of Epoetin Dosage

A. *If the increase in Hb concentration after initiation of epoetin therapy or after a dose increase has been < 0.7 g/dL (haematocrit < 2%) over a 2–4 week period, the dose of epoetin should be increased by 50%.*
B. *If the absolute rate of increase of Hb concentration after initiation of epoetin therapy or after a dose increase is > 2.5 g/dL (haematocrit >8%) per month, or if the Hb concentration exceeds the target Hb concentration, the weekly dose of epoetin should be reduced by 25–50%.*
C. *When the weekly epoetin dose is being increased or decreased, a change may be made in the amount administered in a given dose and/or in the frequency of dosing (if given subcutaneously). It is preferable to round off the dose to the nearest whole vial to prevent wastage.*
D. *The median maintenance dose of epoetin in a non-selected population of patients given subcutaneous epoetin will usually be < 125 IU/kg/week. The lowest effective doses are likely to be about 50 IU/kg/week, with > 90% of patients receiving < 300 IU/kg/week.*

The complete European guidelines are found in reference [184].

8.6.2 NKF Guidelines 11–16

Guideline 11: Route of Administration of Epoetin

A. *Epoetin should be administered subcutaneously (SC) in CKD and peritoneal dialysis patients.*

B. *The most effective route of epoetin administration is SC in hemodialysis patients.*
C. *When epoetin is given SC, the site of injection should be rotated with each administration.*

Guideline 12: Initial Epoetin Administration

A. *SC Administration*
   1. *When epoetin is given SC to adult patients, the dose should be 80–120 units/kg/wk (typically 6000 units/wk) in two to three doses per week.*
   2. *Pediatric patients < 5 years old frequently require higher doses (300 units/kg/wk) than older pediatric patients and adults.*
B. *IV Administration. If the initial administration of epoetin is IV for hemodialysis patients, the dose should be 120–180 units/kg/wk (typically 9000 units/wk), given in three divided doses.*

Guideline 13: Switching From Intravenous to Subcutaneous Epoetin

A. *For hemodialysis patients who are being switched from IV to SC administration of epoetin but have not yet achieved the target Hgb/Hct, the total weekly IV dose should be administered SC in two to three divided doses.*
B. *For hemodialysis patients who are being switched from IV to SC administration of epoetin after achieving the target Hgb/Hct, the initial weekly SC dose should be two-thirds the weekly IV dose. Subsequent dose adjustments should be made as recommended in Guideline 16: Titration of Epoetin Dosage.*

Guideline 14: Strategies for Initiating and Converting to Subcutaneous Epoetin Administration

- *The use of the strategies listed below is suggested to increase patient acceptance of SC administration of Epoetin.*
- *When patients begin dialysis treatments, continue Epoetin administration subcutaneously.*
- *Educate hemodialysis patients on the advantages of SC administration (improved Hgb/Hct response and economic savings).*
- *Establish a unit-wide policy under which all hemodialysis patients are started on SC ad- ministration at the same time.*
- *Use the smallest possible gauge needle for injection (e.g., 29 gauge).*
- *Use a multidose Epoetin preparation that contains benzyl alcohol.*
- *Divide the doses (a smaller volume for injection may reduce discomfort).*
- *Administer a single, weekly injection to patients receiving a small dose.*
- *Rotate injection sites between upper arm, thigh and abdominal wall areas.*
- *Encourage patients to self-administer Epoetin when possible.*

Guideline 15: Monitoring of Hemoglobin/Hematocrit During Epoetin Therapy

For purposes of monitoring response to Epoetin, Hgb/Hct should be measured every 1 to 2 weeks following initiation of treatment or following a dose increase or decrease, until a stable target Hgb/Hct and Epoetin dose have been achieved. Once a stable target Hgb/Hct and Epoetin dose have been achieved, Hgb/Hct should be monitored every 2 to 4 weeks.

Guideline 16: Titration of Epoetin Dosage

If the increase in Hct after initiation of Epoetin therapy or after a dose increase has been < 2 percentage points over a 2- to 4-week period, the dose of Epoetin should be increased by 50%.

If the absolute rate of increase of Hgb/Hct after initiation of Epoetin therapy or after a dose increase exceeds 3 g/dL (or 8 Hct percentage points) per month (e.g., an increase from a Hgb 7 to 10 g/dL or Hct change from 20% to 28%), or if the Hgb/Hct exceeds the target Hgb/Hct, reduce the weekly dose of Epoetin by 25%. When the weekly Epoetin dose is being increased or decreased, a change may be made in the amount administered in a given dose and/or the frequency of dosing (if given SC).

The complete NKF guidelines are found in reference [185].

Clinical practice guidelines for the use of epoetin in patients with cancer, recommended by the American Society of Clinical Oncology (ASCO) and the American Society of Hematology (ASH) are outlined as follows.

1. *The use of epoetin is recommended as a treatment option for patients with chemotherapy-associated anemia and a hemoglobin concentration that has declined to a level less than or equal to 10 g/dL. Red blood cell transfusion is also an option depending upon the severity of anemia or clinical circumstances.*
2. *For patients with declining hemoglobin levels but less severe anemia (those with hemoglobin concentration below 12 g/dL but who have never fallen below 10 g/dL), the decision of whether to use epoetin immediately or to wait until hemoglobin levels fall closer to 10 g/dL should be determined by clinical circumstances. Red blood cell transfusion is also a therapeutic option when warranted by severe clinical conditions.*
3. *The recommendations are based on evidence from trials in which epoetin was administered subcutaneously thrice weekly. The recommended starting dose is 150 U/kg thrice weekly for a minimum of 4 weeks, with consideration given for dose escalation to 300 U/kg thrice weekly for an additional 4–8 weeks in those who do not respond to the initial dose. Although supported by less strong evidence, an alternative weekly dosing regimen (40 000 U/wk), based on common clinical practice, can be considered. Dose escalation of*

weekly regimens should be under similar circumstances to thrice-weekly regimens.

4. *Continuing epoetin treatment beyond 6–8 weeks in the absence of response (e.g., less than 1–2 g/dL rise in hemoglobin), assuming appropriate dose increase has been attempted in non-responders, does not appear to be beneficial. Patients who do not respond should be investigated for underlying tumor progression or iron deficiency. As with other failed individual therapeutic trials, consideration should be given to discontinuing the medication.*
5. *Hemoglobin levels can be raised to (or near) a concentration of 12 g/dL, at which time the dosage of epoetin should be titrated to maintain that level or restarted when the level falls to near 10 g/dL. Insufficient evidence to date supports the "normalization" of hemoglobin levels to above 12 g/dL.*
6. *Baseline and periodic monitoring of iron, total iron-binding capacity (TIBC), transferrin saturation, or ferritin levels and instituting iron repletion when indicated may be valuable in limiting the need for epoetin, maximizing symptomatic improvement for patients, and determining the reason for failure to respond adequately to epoetin. There is inadequate evidence to specify the optimal timing, periodicity, or testing regimen for such monitoring.*
7. *There is evidence from one well-designed, placebo-controlled randomized trial that supports the use of epoetin in patients with anemia associated with low-risk myelodysplasia, but there are no published high-quality studies to support its use in anemic myeloma, non-Hodgkin lymphoma, or chronic lymphocytic leukemia patients in the absence of chemotherapy. Treatment with epoetin for myeloma, non-Hodgkin lymphoma, or chronic lymphocytic leukemia patients experiencing chemotherapy-associated anemia should follow the recommendations outlined above.*
8. *Physicians caring for patients with myeloma, non-Hodgkin lymphoma, or chronic lymphocytic leukemia are advised to begin treatment with chemotherapy and/or corticosteroids and observe the hematologic outcomes achieved solely through tumor reduction before considering epoetin. If a rise in hemoglobin is not observed following chemotherapy, epoetin should be used in accordance with the criteria outlined above for chemotherapy-associated anemia if clinically indicated. Blood transfusion is also a therapeutic option.*

The complete guidelines are found in references [102, 186].

Recombinant Epo therapy is almost universally efficacious in the correction of anemia associated with renal failure. However, outside of this setting, the probability of meaningful hematologic response is reduced and is often unpredictable on a case by case basis. Since rhEpo therapy is very expensive, it would be useful to employ a decision making model or mod-

el of response prediction, especially when applied to off-label indications. This may have special significance, since a recent evaluation of rhEpo usage in a large medical center in the United States found that only 49% of inpatients receiving rhEpo met labeled indications [187]. There have been some attempts to construct a uniform approach to decision making in rhEpo therapy. Baseline endogenous Epo levels and observed-to-predicted (O/P) rhEpo level ratios may be useful here.

The model suggested by Cazzola and co-workers, proposed before the ASCO/ASH cancer guidelines, represents a thoughtful attempt to design such an approach [1]. It is outlined below.

A. *For elective surgery patients:*

1) *If the patient is anemic (Hct < 40), time to operation is adequate (i.e. > 3 weeks), an autologous blood donation program exists and expected blood loss is significant (> 3–4 units) then rhEPO can be utilized to facilitate autologous blood collection before surgery.*
2) *If the patient is anemic, but time to operation is not adequate or an autologous blood donation program does not exist, then rhEPO can be used as an adjunctive therapy perioperatively (as specified above).*
3) *If the patient is not anemic, or the expected blood loss is not significant, then rhEPO administration can probably be avoided.*

B. *For other (non surgical) anemic patients or patients who are likely to become anemic (i.e. chemotherapy patients):*

1) *If the patient is symptomatic, significantly anemic (Hct < 24), transfusion dependent, or likely to become anemic, then evidence of blunted endogenous EPO production should be sought. This evidence can be an endogenous EPO level of less than 100 mU/mL or an observed to predicted (O/P) ratio of less than 0.9.*
2) *If blunted EPO production is confirmed and correctable causes of anemia have been ruled out, then rhEPO, usually with iron supplementation, can be started.*

8.7 Pharmacokinetics, Dosage, Routes of Administration and Effect Monitoring

The dosages of rhEpo used for therapy vary widely depending upon disease process, route of administration, convenience and individual patient response. Because of rhEpo's high cost, there is a constant pressure to reduce dosage. In this setting of uremia, doses of 50–100 IU/kg or lower if subcutaneous administration is used, two or three times weekly are ade-

quate to maintain or even increase hemoglobin levels. However, in most non-uremic conditions, dosages upwards of 150–1000 IU/kg per week may be necessary. Endogenous Epo levels prior to treatment may be used as a guide as can an assessment of bone marrow function estimated by platelet count and transfusion requirements. The above described decision making model incorporates these concepts [1].

During the last several years, the route of administration of rhEpo and frequency of dosing have come under study. Among hemodialysis patients with good vascular access, the intravenous route of administration has been used primarily in the United States, whereas many European and physicians from other countries have favored the subcutaneous route. Recent studies now show that the subcutaneous route of administration, despite reduced bioavailability of the rhEpo, results in equivalent hemoglobin levels among patients while reducing the total dose required and, hence, the cost. This is due principally to the pharmacokinetic and pharmacodynamic characteristics of rhEpo and their relationship to normal rhEpo physiology. Relatively low plasma levels of rhEpo maintained over longer time periods or, ideally constantly, are much more effective in promoting erythropoiesis than are large intravenous boluses that clear the plasma quickly. This certainly favors the subcutaneous over the intravenous route of administration. Additionally, it has been shown that reducing the frequency of administration from the original three times weekly to once weekly may be sufficient to maintain hemoglobin levels in stable hemodialysis patients, thereby reducing the number of injections markedly [188]. Also, if larger total doses are acceptable, once weekly subcutaneous administration can serve as an induction regimen as well. It should be noted that there has been a report of noncompliance with subcutaneous rhEpo administration in peritoneal dialysis patients [189]. These patients are ordinarily instructed to self-administer subcutaneous rhEpo at home. Physicians caring for such patients should be aware of this problem and consider appropriate alternatives.

The preferred route of administration is still an area of some debate among practitioners. Significant differences exist in clinical practice between intravenous or subcutaneous administration even among patients with renal failure. A recent report from the Dialysis Outcomes and Practice Patterns Study (DOPPS) on rhEpo therapy in Europe noted that the subcutaneous route is used in at least 75% of hemodialysis patients in France, Italy, Spain and the UK, whereas in Germany the value is close to 2% [190]. Subcutaneous administration results in lower peak levels but more prolonged increases, thereby more closely resembling normal rhEpo physiology. Obviously, subcutaneous administration is to be preferred when rhEpo is used on an outpatient basis, especially when self-adminis-

tration is employed. However, as stated above, compliance may be a significant issue, especially among peritoneal dialysis patients [189]. Among dialysis patients, intravenous access provides the ready means of rhEpo administration. Comparison between intravenous and subcutaneous administration has shown a reduced maintenance dose requirement in subcutaneously-treated patients in several but not all clinical studies. At present, in many Western European countries, though not all, the subcutaneous route of administration is preferred even for uremic patients. In the United States, the intravenous route is more often used in these patients. It should be noted that at the time of this writing, the epoetin alfa product of Janssen-Cilag (Eprex® or Erypo®) has been restricted to intravenous administration within the European Union due to the appearance of pure red cell aplasia in patients receiving the drug subcutaneously [51–53, 191] (see above).

Efficacy of rhEpo treatment varies widely among all patient groups, with the anemia of renal failure being most responsive. Also, lower response rates have been reported in some lymphoproliferative disorders and other malignancies, and, as noted above, relatively poor response rates have been seen in the myelodysplastic syndromes. Among any group of patients treated for the same indication, individual responses made vary widely and some objective measure of treatment efficacy is an absolute requirement for dose optimization and cost reduction. Proposed indicators of optimal response have included an increase in reticulocyte count and an increase in hemoglobin level after two or four weeks of therapy. An increase in transferrin saturation is also indicative of a response [192]. Some combination of an increase in hemoglobin of greater than 1 g/dL and/or in increase in reticulocyte count of greater than 40×10^9/L has been proposed as a reliable indicator in clinical practice [193]. Additionally, an early prediction of response after two weeks of treatment may be obtained by combining an increase in hemoglobin of at least 0.5 g/dL with a decrease in endogenous Epo to less than 100 mU/mL. Baseline values such as the observed-to-predicted endogenous serum rhEpo ratio and hemoglobin or transferrin increases at two weeks of therapy have predictive value [148, 192].

8.8 Iron Supplementation During rhEpo Treatment

Iron is essential to erythropoiesis, and during the brisk erythropoiesis that accompanies rhEpo therapy, endogenous iron stores can rapidly become rate-limiting. Even with normal iron stores in the bone marrow, maximal

erythropoiesis cannot be achieved, since the rate of iron utilization is greater than the marrow's capacity to liberate it from its storage form. This "functional iron deficiency" is often encountered during initiation of therapy and should be distinguished from true iron deficiency, which may be present initially but also may develop later in the course of rhEpo therapy. Functional iron deficiency should be considered if the serum ferritin is less than 100 ng/mL or if there is an increase in the percentage of hypochromic red blood cells [194–196]. Additionally, low reticulocyte hemoglobin content occurs several days before hypochromic red blood cells appear and can, therefore, be used as a marker of functional iron deficiency [196]. Precisely when to initiate iron therapy and the mode of administration are still somewhat controversial. Immediate supplementation has been reported as an absolute requirement only if the ferritin level is less than 100 ng/mL [1, 2]. However, other authors contend that iron should be administered routinely to all patients in the first month of rhEpo therapy except those with increased serum iron levels or transferrin saturation [1]. Although intestinal iron absorption may increase during rhEpo therapy [197], the use of oral iron supplementation is ordinarily insufficient to meet the needs of initial rhEpo treatment. Therefore, intravenous iron supplementation such as iron dextran or iron saccharate, in the US and Europe, respectively, have been employed [1, 119]. There is an approximate 0.1% incidence of anaphylactic reactions to iron dextran injections.

8.9 Future Directions

New forms of rhEpo and rhEpo-like agents are under development. One new compound, originally called **N**ovel **E**rythropoiesis **S**timulating **P**rotein (NESP) is a mutated rhEpo analog. Because of two additional N-linked glycans, NESP exhibits an *in vivo* half-life approximately twice that of rhEpo, allowing for less frequent dosing [198]. Studies in rodents showed that NESP was as effective in treating the anemia associated with chemotherapy, kidney failure and inflammatory conditions as rhEpo, and human studies supported these findings [199]. NESP was approved in Europe and the US as ARANESP (Amgen) and has the INN designation *darbepoetin*. This and other promising therapeutics are discussed in Chapter 11.

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9
Functions of Erythropoietin Outside of Hematopoiesis

9.1
Introduction

For decades, Epo was considered to be strictly a hormone that regulated red blood cell production. However, during the last several years, it has become increasingly clear that Epo exerts a wide variety of actions on numerous tissues and organ systems. These include endothelial cells, the central nervous system, the female and male reproductive organs, the heart, the gastrointestinal system, muscle cells and the kidney itself. In some cases, these actions are thought to be autocrine and/or paracrine in nature, while in others, the endocrine action of Epo may be involved. Recent studies demonstrating the presence of EpoRs and Epo itself in certain cancer cells have prompted a strong cautionary signal regarding the use of recombinant Epo as a therapeutic in cancer patients.

9.2
Erythropoietin, the Endothelium and Angiogenesis

Epo appears to play an important role in endothelial cell proliferation and angiogenesis. This is not entirely unexpected since evidence shows that erythroid progenitors and endothelial cells arise from a common ancestor, the hemangioblast (see Chapter 2). The first publication in this regard was that of Anagnostou *et al.* who observed serendipitously that addition of recombinant human Epo to human umbilical vein in endothelial cell cultures (HUVECs) triggered increased cell proliferation [1]. The authors verified this observation using recombinant Epo from three independent sources studying both HUVECs and bovine adrenal capillary endothelial cells (BACECs). They found that the growth stimulation effect was linear over a range of 1–5 IU rhEpo/mL. They also showed, using radiolabeled

Epo, that HUVECs expressed specific Epo binding sites (receptors) of approximately 12 000–27 000 per cell with a K_d of 2.4–3.8 nM. Crosslinking studies revealed a prominent 79 kDa crosslinked band as well as two less intense bands of 97 and 165 kDa. Two bands greater than 200 kDa were also identified. Anagnoustou *et al.* also demonstrated that Epo increased both HUVEC and BACEC migration. In a follow up study, Anagnoustou *et al.* demonstrated EpoR mRNA in endothelial cells using RT-PCR and identified EpoR protein by immunohistochemistry in umbilical cord vessels and in placental vascular endothelial cells [2].

Hypertension is one of the most frequent side effects of recombinant Epo therapy and patients receiving this therapy were reported to have a higher mean arterial blood pressure and plasma endothelin-1 level (ET-1) (see Chapter 8). Carlini *et al.* carried out experiments to determine whether increased serum ET-1 was a direct effect of Epo's action on endothelial cells [3]. Using bovine pulmonary artery endothelial cells (BPAECs), the authors found maximal ET-1 production after 12 hours of Epo exposure at all doses of Epo tested (0.8–6.6 IU/mL). An increase in cell proliferation was also observed. The increase in ET-1 production stimulated by Epo was blocked by the protein synthesis inhibitor cycloheximide. Carlini *et al.* went on to show that Epo stimulated ET-1 release from endothelial cells involved calcium signaling [4]. Using fura-2-loaded BPAECs, they showed that Epo treatment increased intercellular calcium nearly five-fold. Addition of nifedipine decreased both Epo-induced calcium increase and ET-1 production. They also showed that Epo upregulated preproET-1 mRNA in endothelial cells. In another study, Carlini *et al.* used an *in vitro* rat aortic ring model and demonstrated that Epo increased angiogenesis *in vitro* [5]. Addition of rhEpo to rat aortic rings embedded in a reconstituted basement membrane increased vessel outgrowth significantly concomitant with increasing supernatant ET-1 levels. Interestingly, Epo-induced angiogenesis was reduced when an anti-ET-1 antibody was added to the cultures. Carlini *et al.* also showed that Epo appeared to reduce apoptosis in endothelial cells [6]. They induced apoptosis in cultured BPAECs using lipopolysaccharide (LPS) and quantified the apoptosis by propidium iodine staining and flow cytometry. In the absence of Epo, 33 ± 8% of LPS-treated cells were apoptotic, compared to only 9 ± 3% of Epo-pretreated cells. These results are consistent with the anti-apoptotic action of Epo on other cell types.

Not all investigators have observed Epo-induced ET-1 production by endothelial cells. Banerjee *et al.* compared primary cultures of endothelial cells from human coronary arteries, pulmonary arteries and umbilical veins [7]. With either short term (4 hours) or long term (24 hours) exposure to 4 IU Epo/mL, no increase in ET-1 was observed. However, nitric

oxide synthase (NOS) activity was upregulated by Epo after 24 hours. The authors concluded that "the hypertensive effect of EPO is not likely to be caused by a direct effect on ECs [endothelial cells]".

Ribatti *et al.* determined that Epo induced a "proangiogenic phenotype" *in vitro* and simulated neovascularization *in vivo* [8]. They studied a human endothelial cell line EA · hy926 and identified EpoRs by Western blot analysis. They went on to show that Epo treatment of these cells induced phosphorylation of the EpoR and of JAK2 kinase, hallmarks of Epo signaling. Addition of Epo to serum-free medium induced cell proliferation by several fold over a dosage range of 2–5 IU Epo/mL. Also, Epo increased matrix metalloproteinase-2 (MMP-2) production by these cells. Finally, Ribatti *et al.* showed that addition of Epo stimulated angiogenesis in the chick embryo chorioallantoic membrane (CAM) model system, a commonly used assay to screen pro- and anti-angiogenic molecules. The degree of angiogenesis stimulated by Epo was equivalent to that observed using FGF-2.

Fodinger demonstrated the upregulation of immediate/early genes in human vascular endothelial cells by Epo [9]. These investigators used differential display and semi quantitative RT-PCR and showed that Epo upregulated genes involved in vascular functions (thrombospondin-1 and 20 kDa myosin regulatory light chain), gene transcription and/or translation (c-*myc* purine-binding transcription factor PuF, tryptophanyl-tRNA synthetase, S19 ribosomal protein), subunits of mitochondrial enzymes related to energy transfer (NADH dehydrogenase subunit 6, cytochrome C oxidase subunit 1) and regulators of signal transduction (protein tyrosine phosphatase G1). This study provides some insight into the gene-regulatory actions of Epo on endothelial cells.

There has been some progress in elucidating the signaling pathways triggered by Epo's action on endothelial cells. Fuste *et al.* studied HUVACs and showed that Epo triggered both the intracellular signaling pathway and increased the thrombogenicity of the extracellular matrices [10]. Treatment of HUVACs with Epo induced phosphorylation of JAK2 and STAT5 as well as increased expression of tissue factor. Chong *et al.* studied rat brain vascular endothelial cells and showed that Epo treatment protected against hypoxia-induced apoptosis by activation of AKT-1, a potent antiapoptotic regulatory factor, and by maintenance of mitochondrial membrane potential, also essential in inhibiting apoptosis [11]. In a study important to wound healing, Haroon *et al.* utilized an *in vivo* wound assay consisting of Z chambers filled with fibrin and implanted subcutaneously in rats [12]. They observed that administration of rhEpo into the fibrin matrix increased granulation tissue in a dose-dependent fashion. Injection of soluble EpoR or anti-Epo monoclonal antibodies resulted in inhibition

of granulation tissue formation. The authors demonstrated EpoR expression in macrophages. Epo also stimulates endothelial cell progenitor mobilization. Heeschen *et al.* studied the bone marrow of Epo-treated mice and found a significant increase in number and in proliferation of stem and progenitor cells as well as colony forming units [13]. Using *in vivo* models of postnatal neovascularization, they found that Epo treatment increased inflammation- and ischemia-induced neovascularization, fundamental to the healing process.

In addition to its action on endothelial cells, Ito *et al.* demonstrated that Epo exerts a mitogenic effect on vascular smooth muscle cells [14]. Using rat vascular smooth muscle cells, they demonstrated activation of the MAP kinase cascade by Epo. Additionally, DNA synthesis increased and was inhibited by forskolin or cilostazol. Increasing intracellular cyclic AMP had an antiproliferative effect on these vascular smooth muscle cells suggesting a modulatory role for cyclic AMP in Epo-induced smooth muscle cell proliferation.

9.3 Erythropoietin and the Central Nervous System

Perhaps the earliest description of Epo in the central nervous system was that of Tan *et al.* [15]. These investigators were studying renal and hepatic Epo mRNA expression as a response to anemia and hypoxia. However, they also found hypoxia-induced increases in Epo mRNA in the brain, testis and spleen that was unexpected. The anatomical localization of Epo and EpoR in the brain has been provided by several independent studies. Digicaylioglu *et al.* used radioiodinated Epo to detect binding sites on mouse brain sections [16]. Using RT-PCR, these investigators detected the expression of Epo and EpoR mRNA in mouse brain. They found that radiolabeled Epo bound principally in the hippocampus, capsula interna, cortex and midbrain. Studies on hypoxic animals showed hypoxic upregulation of Epo. The authors proposed that Epo plays a role in the brain. Marti *et al.* studied Epo gene expression in human, monkey and murine brain [17]. They examined biopsies from human hippocampus, amygdala and temporal cortex, as well as various areas of the monkey brain. Epo mRNA was found in all of these samples. Additionally, they found that hypoxia increased Epo mRNA production in the monkey brain. Furthermore, EpoR mRNA was detected in all brain biopsies from human, monkey and mouse. Juul *et al.* carried out an immunohistochemical study of Epo and EpoR in developing human brain [18]. They studied brains rang-

ing from five weeks postconception to adult. After 5–6 weeks postconception, Epo and EpoR were localized to cells in the periventricular germinal zone. At ten weeks, Epo was present throughout the cortical wall and was most intense in the ventricular and subventricular zones. EpoR was localized to the subventricular zone with little staining in the ventricular zone. In later fetal brains, EpoR was most prominent in astrocytes but also in certain neuronal populations. In contrast, Epo staining was found primarily in neurons in fetal brain although some astrocytes were also Epo positive. Epo and EpoR were identified in astrocyte and neuronal populations generally in postnatal brains. Because of these changes in distribution during development, the authors speculated that Epo is important in neural development.

Dame and coworkers also studied Epo gene expression in the developing human central nervous system [19]. Epo mRNA was abundant in the cerebellum and in two pituitary gland samples. Levels varied in the cerebral cortex and were significantly greater than that found in the amygdala and hippocampus or basal ganglia. Epo mRNA levels were very low in the spinal cord. Shinglo and colleagues studied embryonic mouse brains and found EpoRs by immunohistochemistry and RT-PCR in the embryonic germinal zone during neurogenesis and in the adult subventricular zone, the site of continued neuronal generation during adulthood [20]. Siren and colleagues used immunohistochemistry to study Epo and the EpoR in normal and ischemic/hypoxic human brain [21]. In normal brains, they found weak Epo and EpoR immunoreactivity principally in neurons. In fresh infarcts, however, Epo appeared in the vascular endothelium and EpoR in microvessels and neuronal fibers. In older infarcts, Epo and EpoR immunoreactivity were found in reactive astrocytes. Similarly, acute hypoxic brain damage resulted in vascular Epo expression whereas older hypoxic damage yielded Epo and EpoR immunoreactivity in immunoastrocytes. These results suggested to the authors that Epo and EpoR have a role as "an endogenous neuroprotective system" in the brain.

In a highly interesting study, Yu *et al.* investigated the EpoR knockout mouse and observed extensive apoptosis in the embryonic brain [22]. By embryonic day E10.5 there was a significant decrease in neuronal progenitors and increase in apoptotic cells. Cultures of these cells revealed a decrease in neuronal regeneration and an increase in hypoxic sensitivity compared to those of cells derived from normal embryos of the same stage. However, the work of Suzuki *et al.* [23] has caused a reassessment of the findings of Yu *et al.* (see below).

9.3.1 Cellular Biology of Erythropoietin in the Central Nervous System

Numerous studies have demonstrated the expression of Epo and/or EpoR by cells of the central nervous system at the cellular level and have also demonstrated effects of Epo on CNS-derived cells as well as Epo-dependent signaling. Masuda *et al.* discovered that radioiodinated Epo could bind specifically to two non-erythroid cell lines expressing neuronal properties, PC12 and SN6 [24]. In contrast to receptors on erythroid cells, PC12 cells exhibited a single class of binding sites with a relatively low affinity ($K_d = 16$ nM) compared to erythroid cells with two classes of binding sites with affinities of $K_d = 95$ pM and 1.9 nM, respectively. Crosslinking studies revealed a single 105 kDa crosslinked product for PC12 cells compared to two products of 140 and 120 kDa for erythroid cells. The authors also observed that addition of Epo to PC12 cultures caused an increase in cytosolic free calcium due to an influx from the extracellular medium. Epo also resulted in increases in intracellular monoamines in PC12 cells.

Further evidence for Epo's modulation of intracellular calcium in neuronal cells was provided by Assandri *et al.* [25]. These investigators used the human neuroblastoma cell line SK-N-MC as an *in vitro* model for undifferentiated neuronal cells. EpoR on these cells was detected by RT-PCR, western blot and immunofluorescence. Patch clamp studies demonstrated expression of T-type calcium channels. Importantly, addition of recombinant Epo resulted in increased peak macroscopic current, and confocal laser scanning microscopy demonstrated a transient increase in intracellular free calcium in response to Epo. This response was dependent on external calcium. Koshimura *et al.* studied Epo's action on PC12 in greater detail focusing especially on calcium-signaling membrane potential, dopamine release and nitric oxide production [26]. Addition of Epo to cultures of PC12 increased radiolabeled calcium uptake and intracellular calcium concentration in a dose-dependent fashion. These increases were specific to Epo since they were inhibited by anti-Epo antibody. Membrane polarization of PC12 was also observed in response to Epo treatment, and Epo increased the number of cells in a concentration- and time-dependent fashion. Finally, Epo treatment increased dopamine release and tyrosine hydroxylase activity as well as NO production. Sugawa and coworkers investigated the action of Epo on glial cell development including the maturation of late stage immature oligodendrocytes and proliferation of astrocytes [27]. They used cultures of 18 day embryonic rat cerebral hemispheres and found Epo mRNA to be much higher in oligodendrocytes than in neurons or astrocytes. In contrast, EpoR mRNA was approximately the same in these cell types. Treatment of cultures with recombi-

nant Epo increased the number of mature oligodendrocytes and treatment of mixed cultures of oligodendrocytes and astrocytes with anti-Epo antibody or soluble EpoR inhibited differentiation of oligodendrocytes. The authors suggested that stimulation of astrocytes with Epo may further accelerate maturation of oligodendrocytes.

9.3.2 Expression of Erythropoietin and the Erythropoietin Receptor by Neuronal Cells at the Cellular Level

Production of Epo by neurons *in vitro* was shown definitively by Masuda *et al.* [28]. The authors cultured cerebral cells of rat fetuses and assayed the supernatant medium for Epo using an enzyme-linked immunoabsorbant assay. They determined that Epo production was dependent upon the oxygen tension of the cell culture and that hypoxia enhanced Epo production. Furthermore, they showed that Epo purified from this supernatant medium stimulated the growth of Epo-dependent hematopoietic cells and stimulated the formation of fetal liver erythroid colonies, confirming the biological activity of the hormone. They used immunocytochemical staining of immortalized clonal cell lines and showed that astrocytes produced "brain Epo". The authors suggested that Epo acts on neurons in a paracrine fashion. Marti *et al.* analyzed primary cerebral cells from newborn mice and found that astrocytes but not microglia cells expressed Epo mRNA [17]. Moreover, expression was increased 100-fold when cells were incubated at 1% oxygen. Bernaudin and colleagues found that both astrocytes and neurons express Epo mRNA [29]. They used patch clamp and RT-PCR methods in their study. They found that both astrocytic and neuronal expression of Epo mRNA were induced by hypoxia, desferrioxamine, and cobalt chloride, similar to induction of Epo in hepatoma cells. The induction was blocked by cycloheximide, indicating *de novo* protein synthesis was required. Studer *et al.* studied embryonic day 12 rat mesencephalic precursor cells at 20% and at 3% oxygen [30]. They noted that at 3% oxygen, proliferation was increased and apoptosis was reduced, resulting in greater numbers of precursors. Moreover, differentiation of precursors into neurons was different. The number of dopaminergic phenotype neurons increased from 18% to 56% upon lowering oxygen concentration. Importantly, the authors noted that Epo supplementation of 20% oxygen cultures partially mimicked the increased dopaminergic differentiation seen at lowered oxygen tensions. Also, increased proliferation was seen with added Epo.

Chin *et al.* characterized human EpoR transcript in brain and neuronal cells [31]. They screened a human brain cDNA library and found that the

EpoR gene locus was transcriptionally active. Interestingly, in contrast to erythroid cells, which exhibited efficient splice of EpoR transcripts to mature form, brain EpoR transcripts were inefficiently or alternately processed favoring the 3′ coding region. In cultures of neuronal cells, hypoxia induced EpoR expression and increased cell sensitivity to Epo. Nagai *et al.* studied the effect of proinflammatory cytokines (IL-1β, IL-6 and TNF α) on Epo and EpoR expression in purified cultures of human neurons, astrocytes, microglia and oligodendrocytes [32]. They found Epo mRNA only in human astrocytes but detected EpoR mRNA in neurons, astrocytes and microglia. Neither transcript was found in oligodendrocytes. Interestingly, they found differential regulation of these transcripts by inflammatory cytokines. Epo mRNA and secreted Epo protein were downregulated when astrocytes were exposed to any of the three cytokines. In contrast, TNFα increased EpoR expression in human neurons. Hypoxia inducibility of Epo gene expression has been demonstrated in human neuroblastoma cells. Stolze and coworkers showed that hypoxic induction of Epo production by neuroblastoma cells was proceeded by accumulation of HIF-1, as expected [33].

9.3.3 The Neuroprotective Action of Erythropoietin

Within the last five years, it has become clear that Epo acts as a neuroprotective agent. Morishita *et al.* showed that the EpoR was expressed in both hippocampal and cerebral cortical neurons of the day 19 rat embryo [34]. He used the model of glutamate toxicity to demonstrate Epo's neuroprotectivity. As described by Sasaki *et al.*, glutamate is an important excitatory neurotransmitter in the mammalian central nervous system and can also cause pathologic neuronal injury [35]. Glutamate activates the N-methyl-d-aspartate (NMDA) receptor resulting in the opening of a channel permeable to both sodium and calcium. If this channel is open too long, neuronal death results due to excessive increases in intracellular calcium. Morishita *et al.* cultured hippocampal neurons for 7–10 days and exposed them to glutamate for 15 minutes and assayed neuronal survival 24 hours later. These investigators showed that Epo protected against neuronal death in a dose-dependent fashion. Importantly, this protection was blocked by soluble EpoR, which bound added Epo, preventing it from interacting with the neurons. Neuroprotection from glutamate toxicity required Epo-pretreatment of neurons for 8 hours before glutamate exposure for maximal protection.

Digicaylioglu and Lipton also showed that Epo has neuroprotective actions *in vitro* and that its mechanism involves crosstalk between the JAK2

and NFκB signaling pathways [36]. They induced apoptosis with IL-1β, TNFβ and IFNγ in cultures of rat cerebral cortical neurons. Pretreatment with 5 IU Epo/mL for three hours reduced the number of apoptotic neurons by greater than 50%. Concomitantly, they showed that Epo treatment of neurons induced nuclear accumulation and cytoplasmic depletion of NFκB p65 subunit and induced DNA binding activity of NFκB and transactivation in reporter gene assays. The Epo-induced nuclear translocation of NFκB was specific for neurons rather than astrocytes in mixed neuronal/glial cultures. In addition to NFκB activation, Epo treatment of neurons also activated JAK2 as evidenced by coimmunoprecipitation with anti-EpoR antibody and subsequent immunoblotting with anti-EpoR antibody and anti-JAK2 antibody. Finally, the authors showed that inhibition of JAK2 by transfection of a dominant negative kinase-deficient JAK2 mutant prevented Epo-mediated neural protection. The authors proposed that Epo's activation of NFκB results in subsequent upregulation of expression of neuroprotective genes.

Ruscher *et al.* used an *in vitro* model of cerebral ischemia, designated "oxygen glucose deprivation (OGD)", to determine whether Epo played a role in ischemic preconditioning [37]. Using rat primary cortical neurons, the authors found Epo time-dependent and dose-dependent protection against OGD. Some protection was seen in as little as 5 minutes of Epo treatment and was maximal after 48 hours. This protection was blocked by addition of soluble EpoR or anti-EpoR antibody. Interestingly, addition of medium from OGD-treated astrocytes to untreated neurons induced protection in these neurons against further OGD, and this protection was reduced by application of soluble EpoR or anti-EpoR antibody, implying that medium from OGD astrocytes contained astrocytes-produced Epo. The authors further showed that Epo treatment of neurons induced phosphorylation of the proapoptotic Bcl family member BAD. Both neuroprotection and BAD phosphorylation were reduced by the PI3 kinase inhibitor LY294002, consistent with Epo activation of the PI3 kinase/AKT pathway.

Epo has been shown to improve synaptic transmission. Weber and colleagues established cultures of rat corticohippocampal slices and studied them before and after OGD [38]. They analyzed evoked extracellular field potentials to test the effect of Epo on synaptic transmission. They found that treatment of cultured slices with 40 IU Epo/mL for 48 hours significantly increased field potentials during and following OGD compared to control tissue. The JAK2 inhibitor AG490 blocked this Epo-induced effect. The presence of both endogenous Epo and EpoR in sliced cultures was demonstrated convincingly.

Numerous studies have demonstrated a neuroprotective action of Epo *in vivo* using a variety of CNS insults. Sadamoto and coworkers studied

spontaneously hypertensive rats with permanent occlusion of the left middle cerebral artery [39]. They found that infusion of Epo into the cerebral ventricles alleviated ischemia-induced place navigation disability. They showed that the left (ischemic) to right (contralateral nonischemic) ratio of cerebral cortical area was larger in the Epo-infused animals than the control group. Epo prevented degeneration of the thalamic area and enhanced neuronal survival in the ventral posterior thalamic nucleus. Sakanaka *et al*. studied gerbils in which brain ischemia was induced by occlusion of the common carotid artery [40]. They showed that infusion of Epo into the lateral ventricles prevented ischemia-induced learning disability and blocked lethal damage of hippocampal CA1 neurons. Synapse numbers were also increased in the Epo-treated group. In contrast, infusion of soluble EpoR into animals that received only a mild ischemic treatment enhanced neural degeneration and impaired learning ability. Infusion of heat-denatured soluble EpoR had no effect, thus showing that endogenous brain Epo is important in neuronal survival. Bernaudin *et al*. used focal permanent ischemia in mice to induce cerebral infarcts and studied the temporal and spatial expression of Epo and EpoR during infarct evolution [41]. After one day of ischemia, Epo expression was found in endothelial cells. After three days, Epo was found in microglia/macrophages and after seven days in reactive astrocytes. Interestingly, EpoR expression preceded that of Epo for each cell type. The authors showed a significant reduction in infarct volume in mice treated with Epo 24 hours prior to induction of cerebral ischemia.

Studies such as these have suggested the use of recombinant Epo in clinical situations associated with CNS insult. Brines *et al*. used immunotransmission electron microscopy and showed that EpoRs were concentrated on astrocytic endfeet surrounding capillaries and on capillary endothelial cells, an anatomical relationship ideal for transcellular transport of circulating Epo to the CNS [42]. These investigators administered recombinant Epo systemically before or up to 6 hours after focal brain ischemia and found that injury was reduced by approximately 50–75%, thus confirming that Epo crosses the blood brain barrier. They administered biotinylated Epo systemically and found it localized around capillaries and in the brain parenchyma five hours after intraperitoneal injection. Other studies by these authors showed that Epo ameliorated the extent of concussive brain injury, immune damage of experimental autoimmune encephalomyelitis and kainate toxicity. Fig. 9.1 shows the effect of systemic administration of rhEpo on attenuation of injury after blunt trauma to the cerebral cortex of mice.

Siren *et al*. demonstrated that Epo prevented neuronal apoptosis after cerebral ischemia and metabolic stress *in vivo* [43]. They showed that sys-

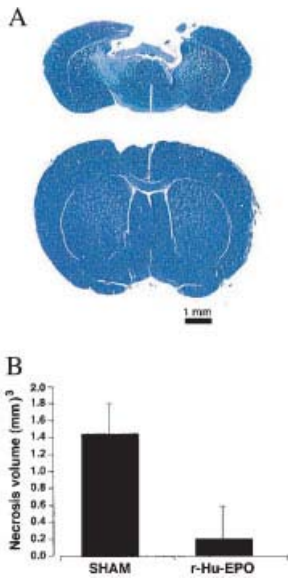

Fig. 9.1 Epo treatment attenuates brain injury after blunt trauma. Mice received a calibrated, non-penetrating blow to the frontal cortex. Panel A, *upper.* Saline-treated controls exhibited extensive cavitary necrosis 10 days after injury. Panel A, *lower.* Epo-treated mice had minimal injury. Cresyl violet stain. Panel B, results from experiment using 5000 IU recombinant Epo/kg administered 24 h before trauma. 6 Animals/group. $P < 0.05$. From reference [42].

temic administration of Epo after middle cerebral artery occlusion in rats markedly reduced infarct volume and almost completely blocked apoptosis within the ischemic border.

Epo's neuroprotective action also extends to the retina. Junk and coworkers studied acute ischemia infusion injury in experimental animals by raising intraocular pressure, a model relevant to retinal disease [44]. Systemic administration of Epo reduced damage as evidenced by histologic examination and promoted functional recovery measured by electroretinography. Reduced apoptosis of retinal neurons was noted.

Gorio *et al.* studied the action of Epo on experimental spinal cord injury [45]. They used two rodent models. The first was moderate compression at level T-3 for one minute and the second was a more severe injury consisting of a contusion at level T-9. In the first model, administration of recombinant Epo resulted in partial recovery of motor function within 12 hours after injury and nearly complete recovery after 4 weeks. In the second model, Epo administration markedly reduced secondary inflammation as well as reduced cavitation within the cord.

In a similar fashion, Epo has been shown to exert an anti-inflammatory effect in a model of experimental autoimmune encephalomyelitis [46], to prevent motor neuron apoptosis after experimental spinal cord injury [47], and to ameliorate effects of experimental subarachnoid hemorrhage in rabbits [48]. Erbayraktar and colleagues have shown that enzymatically desialylated erythropoietin retains its broad neuroprotection *in vivo* in mice

or rats without increasing the hematocrit due to the short half-life of the desialylated molecule [49]. Molecules similar to this may be useful in longer-term treatment of patients with neurological disease in whom increases in hematocrit may not be beneficial or may be deleterious.

Studies to exploit the neuroprotective action of Epo are ongoing in the clinical setting. Ehrenreich *et al.* have studied acute stroke in man [50]. Restricting themselves to acute middle cerebral artery involvement, the authors conducted a combined safety study and efficacy study. In the safety study, they treated 13 patients intravenously with 33 000 IU rhEpo once daily for the first three days after stroke. In the efficacy study, 40 patients were randomized to receive either recombinant Epo or saline. No safety issues were noted. Cerebral spinal fluid Epo concentration in treated patients was 60–100 times that of untreated patients, demonstrating that rhEpo crossed the blood brain barrier. In the efficacy portion, treatment with Epo was "associated with an improvement in follow up and outcome scales". Using magnetic resonance imaging (MRI), the authors reported a strong trend for reduction in infarct size. A larger, multicenter trial is now in progress and preliminary results are very encouraging [51].

9.4 Erythropoietin and the Reproductive System

Yasuda *et al.* demonstrated that erythropoietin is produced in the uterus in an estrogen-dependent manner and may play a role in uterine angiogenesis [52]. Studying uteri from ovariectomized mice *in vitro*, they found that Epo mRNA and protein were produced at low levels but were stimulated to significantly higher levels by added estrogen. *In vivo*, they found that administration of estrogen to ovariectomized mice induced rapid and transient Epo mRNA production in the uterus. Additionally, Epo injected directly into the uterine cavity enhanced angiogenesis in the endometrium. In contrast, injection of soluble EpoR to block Epo's action in the uterus inhibited endometrial transition to the proestrus stage in non-ovariectomized animals. These results implicate estrogen-dependent Epo production in the cyclic angiogenesis of the uterus.

Epo production in the female reproductive tract is not restricted to the uterus. Masuda *et al.* studied other murine female reproductive organs and showed that Epo mRNA was detectable in both the ovary and the oviduct [53]. Estrogen treatment upregulated Epo mRNA in the oviduct but not in the ovary. Additionally, Epo mRNA expression of the oviduct was

inducible by hypoxia either in the absence or presence of estrogen. In a later study, Yasuda *et al.* used a combination of RT-PCR, immunohistochemistry and western blotting and showed that Epo mRNA was expressed in the normal human cervix, endometrium and ovary [54]. Additionally, EpoR was detected by immunohistochemistry in the endothelium of vessels in glandular and surface epithelial cells and decidual cells of the endometrium and in the follicles of the ovary at various stages of oogenesis. This work was confirmed and extended by Yokomizo *et al.* who studied Epo and EpoR expression in the human endometrium during the menstrual cycle [55]. Using RT-PCR, they showed expression of Epo and EpoR mRNA in isolated endometrial epithelial and stromal cells. Isolated epithelial cells had higher Epo mRNA levels in the secretory phase of the cycle suggesting that Epo may be involved "in cyclic proliferation and differentiation of endometrial glandular epithelial cells, acting in an autocrine manner." Epo may be involved in the pathophysiology of endometriosis. As shown by Matsuzaki *et al.*, Epo and EpoR were localized by immunohistochemistry to glandular epithelial cells in both normal endometrium and peritoneal endometriosis [56].

The EpoR is expressed by cells in the human placenta. Interestingly, this was first noted by Sawyer *et al.* in 1989 in studies of radiolabeled Epo binding to mouse and human erythroid cells and placental membranes [57]. However, further cellular localization was not reported. Indeed, Fairchild Benyo and Conrad reported the expression of EpoR by trophoblast cells in the human placenta [58]. Using immunocytochemistry, they detected EpoR in both villous and extravillous cytotrophoblast cells in addition to syncytiotrophoblasts at all gestational ages. They reported that this EpoR was functional, since the receptor was tyrosine phosphorylated in response to Epo treatment of cultured trophoblast cells.

Epo may also play a role in the male reproductive system. Magnanti and colleagues detected Epo expression in primary rat Sertoli cells and peritubular myoid cells [59]. Using RT-PCR, they detected Epo transcripts in these cells but not in adjacent Leydig cells. Interestingly, cobalt exposure of Sertoli cells increased mRNA levels as did FSH. In contrast, testosterone reduced Epo mRNA in peritubular myoid cells. A function for Epo in the testes has been suggested by the study of Cortes *et al.* [60]. They studied young boys with cryptorchidism, three of whom had renal failure. The authors noted that in the two boys treated with rhEpo, the number of spermatogonia in testicular biopsies was unusually high compared to control material from boys not treated with Epo. The authors concluded that "erythropoietin may have a positive effect on germ cell proliferation in cryptorchidism".

9.5 Erythropoietin and the Heart

Stuckmann *et al.* discovered that both Epo and retinoic acid are secreted from the epicardium during embryogenesis and appear to be required for cardiac myocyte proliferation [61]. A search for potential epicardial growth or trophic factors found that blockade of either retinoic acid or Epo signaling from the epicardium inhibited the proliferation and survival of cardiac myocytes. Interestingly, blockade of myocyte proliferation by a retinoic acid antagonist could be rescued by exogenous Epo and, conversely, blockade of Epo signaling with anti-Epo antiserum was rescued by exogenous retinoic acid. Furthermore, Stuckmann *et al.* found that neither Epo nor retinoic acid acted directly on myocytes. Rather, the authors hypothesized that both retinoic acid and Epo stimulated the production of a yet undefined myocyte mitogen.

Epo appears to be responsible for the protection against ischemia-reperfusion injury afforded by prior intermittent hypoxia. Calvillo *et al.* reported that Epo treatment of cultured adult rat myocardiocytes subjected to 28 hours of hypoxia markedly reduced apoptosis of the cells [62]. Furthermore, parenteral administration of recombinant Epo to rats for seven days prior to coronary ischemia-reperfusion reduced cardiomyocyte loss by 50% resulting in normal hemodynamic function within one week after reperfusion. In another study on ischemia-reperfusion injury of the heart, Cai *et al.* discovered that, whereas exposure of wild type mice to intermittent hypoxia protected their isolated hearts against ischemia reperfusion injury, this protection was lost in HIF-1$a^{+/-}$ mice heterozygous for the knockout allele of HIF-1a [63]. Although Epo production was increased in kidneys in wild type mice subjected to intermittent hypoxia, no such increase in production was observed in the HIF-1$a^{+/-}$ animals. These results strongly suggest that endogenous Epo plays a role in the ischemia reperfusion protection afforded by intermittent hypoxia. In a related study, Sterin-Borda *et al.* studied the reduced cardiac contractility induced by prolonged exposure of mice to hypoxia [64]. They found that in animals treated with recombinant Epo, cardiac contractility was enhanced, whereas it was decreased upon treatment with a monoclonal anti-Epo antibody. Studies such as these raise the hope for the use of rhEpo as a therapeutic agent in ischemic heart disease.

9.6 Erythropoietin and the Gastrointestinal System

Epo has been detected in the milk of humans and other mammals, but its role in the development of the gastrointestinal tract has only begun to be explored. After identifying EpoRs in the small bowel of human fetuses, Juul *et al.* hypothesized that these receptors would also be present postnatally and that recombinant Epo would increase enterocyte migration and decrease apoptosis [65]. Using RT-PCR and immunohistochemistry, they demonstrated EpoR expressed on enterocytes in postnatal rats and humans. Studying rat intestinal epithelial cells (IEE-6), they confirmed that cells treated with 0.05 or 5.00 IU Epo/mL migrated faster than untreated cells. Furthermore, pretreatment of cells prior to exposure to TNFα and cycloheximide reduced cell death, an effect that was blocked by anti-Epo antibody.

Interestingly, in low birth weight infants treated with recombinant Epo for anemia, Ledbetter and Juul noticed a reduced incidence of necrotizing enterocolitis than in similar birth weight infants not treated with Epo, supporting the hypothesis that Epo plays a role in development of the intestinal tract [66]. Juul *et al.* showed further that Epo acts as a trophic factor in the neonatal rat intestine [67]. They dosed rat pups both enterally and parenterally with rhEpo and found no increase in hematocrit or reticulocyte count in the enterally-dosed animals. However, in both enterally- and parenterally-dosed animals, small bowel length was greater and the villous surface was increased in a dose-dependent fashion independent of route of dosing. The authors concluded that recombinant Epo "acts as a trophic factor in developing rat small bowel whether given enterally or parenterally".

The role of Epo in human milk has achieved further interest and is the subject of an excellent review by Kling [68]. The author cites a number of studies demonstrating the increased stability of Epo in milk when exposed to gastric and/or intestinal juices. In a human study, Britton and Christenson fed rhEpo to premature infants and noticed a higher plasma Epo level immediately after treatment, consistent with intestinal absorption of the hormone [69]. Whether the endogenous Epo present in human milk consumed by infants is absorbed into the infants' bloodstream in any physiologically significant amounts remains to be determined.

9.7
Erythropoietin and Other Cell Types

Westenfelder *et al.* used RT-PCR to study normal human and rat kidney tissue as well as cell lines derived from human, rat and mouse kidney for the expression of EpoR mRNA [70]. EpoR mRNA was detected in the cortex, medulla and papillae of human and rat kidney, in the human and rat mesangial cells, human and mouse proximal tubular cells and human medullary collecting duct cells. A single class of radiolabled Epo-binding sites (EpoR) was identified in each cell line with K_d ranging from 96 pM–1.4 nM. Using murine proximal tubule cell membranes, western blotting with anti-EpoR antibody revealed an EpoR protein of 68 kDa. The function of Epo within the kidney itself is not known.

Epo has also been reported to have an action on myoblasts. Ogilvie and colleagues studied primary murine satellite cells and myoblast C2C12 cells and found that both expressed EpoR [71]. Addition of Epo to cultures resulted in a proliferative response and a marked decrease in terminal differentiation (the formation of myotubes). Activation of the JAK2/STAT5-signaling pathway was observed, as was an increase in cytoplasmic calcium. Expression of GATA-1 and EpoR were upregulated by Epo in C2C12 cells as was Myf-5 and MyoD with concomitant inhibition of myogenin induction during differentiation. The authors suggest that Epo "may have a potential role in muscle development or repair".

EpoR have been demonstrated on the pancreatic islet cells of humans, nonhuman primates and rats using both western blot and immunohistochemistry [72]. *In vitro* evidence suggests that Epo may have an anti-apoptotic effect on these cells.

9.8
Are Erythropoietin Receptors Outside of the Hematopoietic System Really Necessary?

As described previously, Wu *et al.* demonstrated defects in cardiac morphogenesis in both Epo knock out and EpoR knock out mouse embryos [73]. Also, Yu *et al.* demonstrated reduction in neural progenitors and increase in apoptosis in EpoR knockout mice [22]. These studies would underline the importance of the EpoR in the development of these nonhematopoietic tissues. However, work by Suzuki *et al.* appears to contradict these findings [23]. These investigators used a GATA-1 mini-gene cassette with hematopoietic regulatory domains and established two lines of transgene-rescued

EpoR null mutant mice that expressed EpoR exclusively in the hematopoietic lineage. Surprisingly, the mice developed normally and were fertile, although EpoR expression was absent in non-hematopoietic tissues. Although a complete analysis of the non-hematopoietic tissues of these animals, such as brain, heart and endothelium, which ordinarily express EpoR, was not presented, this work clearly raises questions about our current understanding of the role of EpoR outside of the hematopoietic system.

9.9 Erythropoietin and Malignancy

The first report of EpoRs present on malignant cells is that of Okuno *et al.* who studied the human myeloma cell line (MM-S1) [74]. The authors showed that proliferation of MM-S1 cells was stimulated by Epo in a dose-dependent manner. Binding of radiolabeled Epo was saturable, and a Scatchard analysis showed an affinity constant (K_d) of 0.56 nM with 330 EpoRs per cell. Westenfelder and Baranowski showed that Epo stimulated the growth of human renal carcinoma cells, a nonhematopoietic lineage [75]. They screened human and mouse renal adenocarcinoma lines for EpoR transcripts and protein as well as for radiolabeled Epo binding and mitogenic response to Epo. EpoR transcripts and protein were found in both renal tumors and cell lines. Radiolabeled Epo binding was specific and saturable. Finally, Epo stimulated cell proliferation in a dose-dependent manner in several cell lines tested.

In an interesting case report, Bunworasate and colleagues reported the apparent Epo-dependent transformation of myelodysplastic syndrome to acute monoblastic leukemia [76]. They described a patient under therapy with rhEpo for refractory anemia with ringed sideroblasts in whom acute monoblastic leukemia type M5A with leukemia cutis developed six weeks after initiation of therapy. After discontinuation of Epo, evidence of leukemia disappeared from both bone marrow and skin. Using multiparametric flow cytometric analysis of bone marrow cells, the authors demonstrated expression of EpoR along with CD45 and CD13 on the surface of blast cells. Importantly, incubating the bone marrow cells with Epo increased [^{3}H]thymidine incorporation and bromodeoxyuridine incorporation into $CD13^+$ cells. This case raises an important concern regarding the treatment of patients with Epo whose malignant cells express the EpoR, a concern that is heightened by the studies that follow.

Yasuda *et al.* have studied the role of the Epo/EpoR axis in tumors of the female reproductive tract [77]. They studied human uterine and ovar-

ian tumors *in vivo* transplanted into nude mice and demonstrated the expression of Epo and EpoR mRNA by RT-PCR. They injected the tumors either with anti-Epo antibody or with soluble EpoR to block the action of endogenous Epo on the tumor's EpoR. Transplants were resected and examined. Importantly, among the "Epo signal deprived transplants", that is, transplants receiving either anti-Epo antibody or soluble EpoR, tumor size was reduced significantly. Immunohistochemistry showed that malignant cells and capillary endothelial cells exhibited enhanced apoptotic death. Moreover, the amount of tumor regression was correlated with the dose and frequency of signal blocking injections, control xenografts grew normally as expected. Yasuda and colleagues also carried out *in vitro* studies using cultured blocks of tumor specimens [78]. They injected either anti-Epo antibody or soluble EpoR into these blocks and compared their growth with control blocks injected with heat-denatured soluble EpoR, mouse serum or saline. Both capillaries and tumor cells were decreased significantly in a dose-dependent manner after either signal blocking injection, and an increased number of cells contained fragmented DNA and the histopathological appearance of apoptosis. Staining for phosphorylated JAK2 and phosphorylated STAT5 was intense in control tumor specimens but was markedly reduced or eliminated in treated blocks of tissue. The authors concluded from these studies that Epo signaling "contributes to the growth and/or survival of both transformed cells and capillary endothelial cells in these tumors. Thus, deprivation of erythropoietin signaling may be a useful therapy for erythropoietin producing malignant tumors".

Epo may also affect the response of some cancer cells to chemotherapeutic agents. In the first study of its kind, Solar *et al.* demonstrated that Epo treatment of human ovarian carcinoma cell *in vitro* resulted in a paclitaxel-resistant phenotype [79]. They cultured the human ovarian carcinoma cell line A2780 in the absence or presence of 5 or 35 U rhEpo/mL for 2 months and then treated the cells with specified concentrations of paclitaxel for 24–72 h. Cell survival following paclitaxel treatment was significantly greater for Epo-treated cells than for control cells (61% vs. 37%) after exposure to 1 nM paclitaxel for 72 h, and the appearance of mono- or oligonucleosomes, a measure of DNA fragmentation and apoptosis, was significantly decreased in the Epo-treated cells after exposure to 100 nM paclitaxel for 24 h. Flow cytometric analysis demonstrated an increased number of Epo-treated cells in G1/S and a reduced number in G2 following paclitaxel treatment. Levels of pro-apoptotic Bax and anti-apoptotic Bcl-X_L proteins following paclitaxel treatment were unaffected by Epo treatment. However, levels of anti-apoptotic Bcl-2 and the weakly pro-apoptotic Bcl-10 were decreased in the Epo-treated cells following paclitaxel treatment, suggesting a novel mechanism of paclitaxel resistance.

Acs and coworkers studied both human breast cancer cell lines and clinical specimens of breast carcinoma [80]. They found expression of both Epo and EpoR in human breast cancer cells and that Epo stimulated tyrosine phosphorylation and growth enhancement of these cells *in vitro*. Immunohistochemical studies of clinical specimens revealed "high levels" of both Epo and EpoR in malignant cells and tumor vasculature in contrast to normal breast tissue, benign papillomata or fibrocystic tissue. It appeared that hypoxic regions of the tumors expressed the highest levels of Epo and EpoR. In a further study, Acs and colleagues carried out a more detailed immunohistochemical study of Epo and EpoR in breast carcinoma [81]. They studied 184 invasive mammary carcinomas and 158 *in situ* carcinomas and benign mammary epithelia and analyzed the correlation of Epo and EpoR immunostaining with clinical pathologic tumor features and the patients smoking history. The authors found weak to moderate expression of Epo and EpoR in benign mammary epithelial cells and found that EpoR staining was increased in carcinomas in both nonsmokers and smokers. Epo staining was increased in carcinomas in nonsmokers only. Epo staining was especially prominent in tumor cells adjacent to necrotic areas and at the edge of tumors. Interestingly, EpoR staining was greater in tumors with a higher histologic grade, necrosis, lymphovascular invasion, lymph node metastases and loss of hormone receptor expression. Fig. 9.2 shows some of the authors' findings.

Arcasoy *et al*. have addressed the functional significance of EpoR in breast cancer [82]. They studied clinical breast cancer specimens for Epo and EpoR expression, characterized EpoR expression with relation to tumor hypoxia, analyzed breast cancer cell lines for EpoR and studied the function of EpoR expression in breast cancer cells *in vivo*. They found high levels of EpoR in cancer cells in 90% of tumors studied. Additionally, Epo expression was found in 60% of tumors, and Epo and EpoR colocalization was frequent. There was no consistent relationship between Epo expression and tumor hypoxia. They studied rat syngeneic R3230Ac mammary adenocarcinoma cells in a tumor Z-chamber model and found that a single administration of neutralizing anti-Epo antibody, soluble EpoR or JAK-2 inhibitor delayed tumor growth in a dose-dependent manner, demonstrating a function of EpoR on this breast cancer cell line *in vitro*.

Functional EpoRs have been reported on nonmalignant transformed prostatic epithelial cells and prostate cancer cells. Feldman *et al*. used RT-PCR and demonstrated EpoR mRNA in a series of transformed prostatic epithelial lines and human prostate carcinoma cell lines LNCaP and PC-3 [83, 84]. Additionally, examination of RNA prepared from primary surgical specimens of human prostate cancer and adjacent normal tissue revealed variable levels of EpoR mRNA as well. Interestingly, growth of both hu-

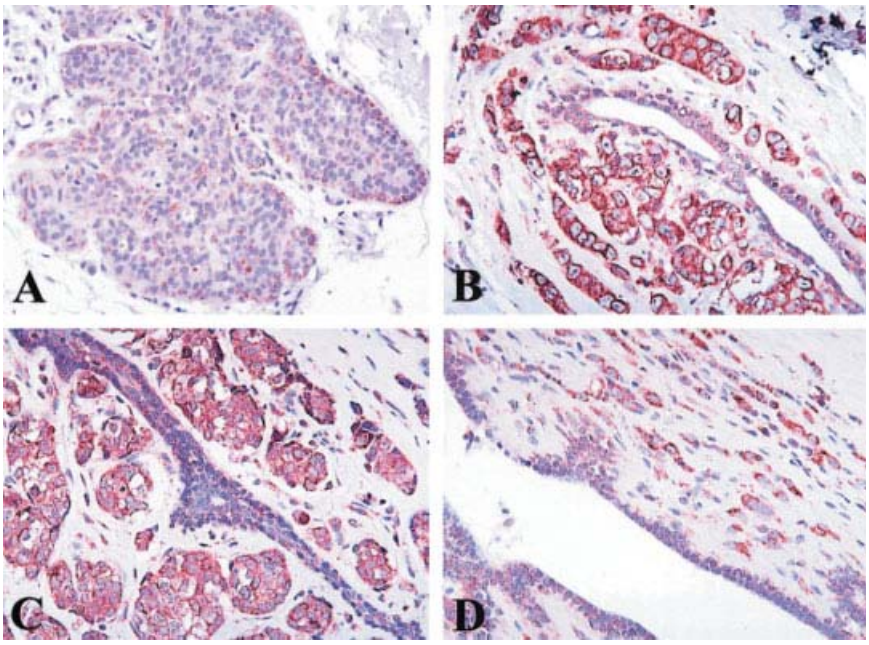

Fig. 9.2 Epo receptor (EpoR) expression in benign breast tissue and in breast carcinoma detected by immunohistochemistry. (A) Low intensity EpoR immunostaining in ductal hyperplasia without atypia. (B) Strong EpoR immunostaining in cytoplasm and on plasma membrane in invasive ductal carcinoma. (C) Strong EpoR immunostaining is seen in invasive ductal carcinoma. (D) Intermediate-intense EpoR immunostaining in invasive lobular carcinoma. Benign duct stains weakly. Hematoxylin counterstain. From reference [81].

man prostatic cell lines and transformed epithelial lines *in vitro* was enhanced by addition of rhEpo to the culture medium whether at low serum or in defined, serum-free conditions. These results heighten concern over the use of recombinant Epo in the treatment of patients with cancers expressing the EpoR.

Westphal *et al.* carried out a survey of human malignant cell lines by RT-PCR, western blot and immunofluorescence staining [85]. They found EpoR, mRNA and/or protein in almost all of the cell lines surveyed. Immunohistochemistry confirmed surface expression of EpoR in selected cell lines. Interestingly, in some cell lines, G-CSF receptor was also present. Addition of Epo to cells *in vitro* did not stimulate tyrosine kinase activity however. In a further study, Westphal *et al.* used an enzyme-linked immunoabsorbant assay and found soluble EpoR in the growth medium of a variety of tumor cell lines and in the plasma of patients [86]. This finding is especially relevant in view of the discovery by Arcasoy *et al.* of

the expression of EpoR splice variants by a variety of human cancer cells [87].

Epo may be involved with other clinically important tumors as well. Ribatti *et al.* have proposed that Epo may act as an angiogenic factor in gastric carcinoma [88]. Based upon the previous demonstration that increased vascularity is associated with enhanced metastasis and poor prognosis in gastric carcinomas, these investigators correlated vessel density and EpoR expression on endothelial cells and tumor cells with histopathologic type. They studied 40 surgical specimens and found that Stage IV had a higher degree of vascularization and that EpoR expression on endothelial and tumor cells increased in proportion to malignancy grade and was highly correlated with the degree of angiogenesis. Acs and colleagues have suggested a role for hypoxia-inducible Epo signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and in cervical carcinogenesis and tumor progression [89]. They used RT-PCR and Western blotting and found that both HeLa and SiHa cervical carcinoma cell lines as well as primary human cervical carcinomas expressed EpoR and that this expression was enhanced by hypoxia. Exogenous rhEpo upregulated tyrosine phosphorylation and reduced cytotoxic effect of cisplatin on HeLa cells. In benign epithelia, EpoR expression was confined to basal cell layers but appeared in more superficial layers in dysplasia. The level of expression was correlated significantly with severity of dysplasia. Diffuse expression of EpoR was found in all interstitial cell carcinomas. Finally, Batra *et al.* surveyed a wide variety of pediatric tumors and found EpoR expression to be widespread [90]. Interestingly, they found that addition of Epo to the cell lines HT-29 and TC-32 stimulated production of VEGF by the cells, suggesting that Epo promotes angiogenesis and tumor cell survival in these tumors.

The presence of functional EpoR on non-hematopoietic tumor cells requires that a careful reevaluation of the generalized use of recombinant Epo for the treatment of anemia in cancer patients with active disease be made. A recent study by Henke *et al.* serves to emphasize this point [91]. Based upon the general principle that an increase in tumor oxygenation improves the tumor's response to radiotherapy, Henke and coworkers set out to investigate whether anemia correction with rhEpo could improve outcome of curative radiotherapy in head and neck cancer patients. They carried out a multi-center, double-blind, randomized, placebo-controlled trial in 351 patients with carcinoma of the oral cavity, oral pharynx, hypopharynx or larynx. All received curative radiotherapy for either completely and histologically incomplete resected disease or macroscopically incompletely resected advance disease. All patients received subcutaneous placebo or rhEpo 300 IU/kg three times weekly from 10–14 days **before** radio-

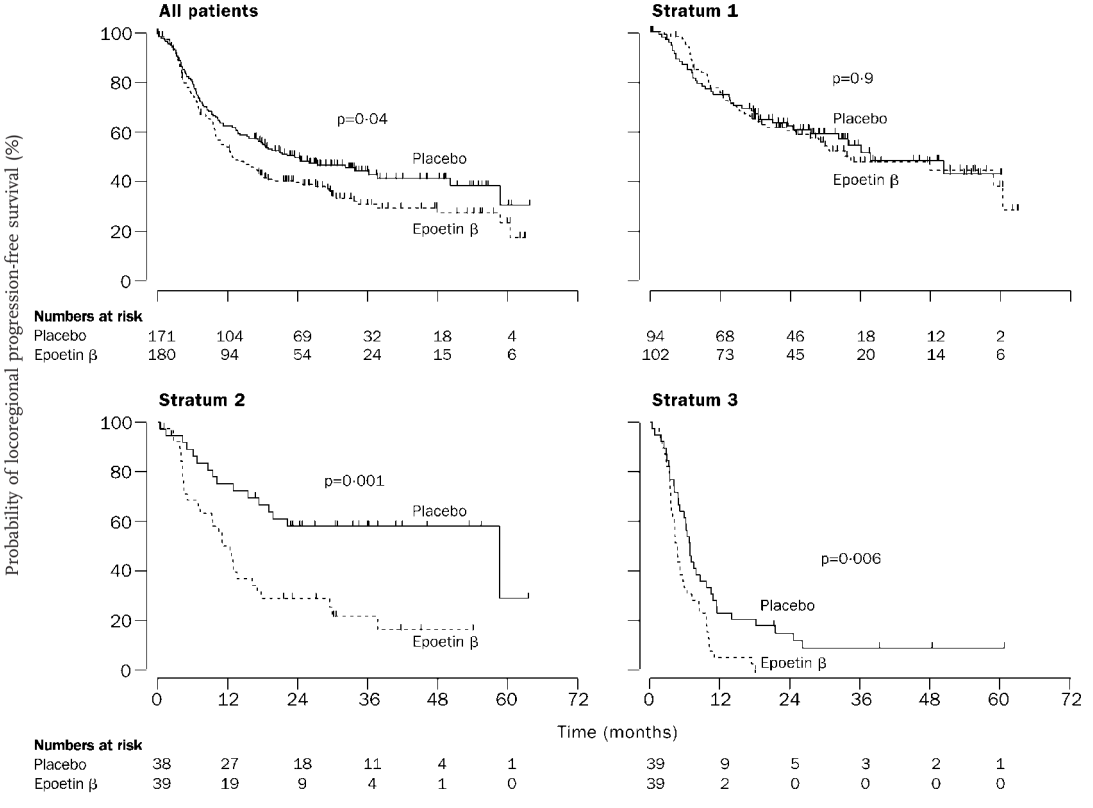

Fig. 9.3 Locoregional progression-free survival of head and neck cancer patients on randomized rhEpo treatment prior to and during radiotherapy; all patients and three treatment groups (Stratum 1, 2 and 3) from reference [91]. Stratum 1, post-operative radiation of complete resection. Stratum 2, post-operative radiation of incompletely resected disease. Stratum 3, primary definitive radiotherapy.

therapy and continuing throughout therapy. As expected, 82% of patients receiving rhEpo raised their hemoglobin concentrations. Importantly, however, progression-free survival was better for the placebo group with an adjusted relative risk of 1.62 (95% CI 1.22–2.14; P = 0.0008). The relative risk for locoregional progression was 1.69 (95% CI 1.16–2.47, P = 0.007), and the relative risk of death was 1.39 (95% CI 1.05–1.84, P = 0.02). Fig. 9.3 shows the locoregional progression-free survival curves.

Although evaluation of EpoR expression by the tumors of the patients under study was not a part of the protocol, these results clearly show that "patients receiving curative cancer treatment and given erythropoietin should be studied in carefully controlled trials", as stated by the authors, and that the role of Epo treatment in the progression and survival of patients with other neoplasms be investigated thoroughly.

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10
Erythropoietin and Sport

10.1
The Hypoxic Stimulus to Erythropoietin Production

The primary stimulus for Epo production is the quantity of oxygen in the blood. As this is reduced, HIF-1α is stabilized and hypoxia-inducible genes, including Epo, are upregulated (see Chapter 3). Epo then enters the circulation and reaches the bone marrow where it stimulates the production of increased numbers of red blood cells, resulting in increased oxygen-carrying capacity. As reviewed in Chapter 7, this mechanism can result in clinical disorders if oxygen levels are too low. However, under normal circumstances, this is an excellent mechanism to compensate for changes in ambient oxygen, such as that experienced by humans when living in altitudes significantly above sea level.

10.2
Altitude and Erythropoietin in the World of Elite Athletics

The effects of altitude and Epo entered the world of elite athletics in force at the 1968 Mexico City Olympics. Mexico City lies at an altitude of approximately 2240 m (7349 feet), which presents a moderate hypoxic stimulus to individuals acclimated to life at or near sea level. Interestingly, although 34 world records were set during these Olympics, no world records were set for any event longer than 400 m or approximately one minute in duration. Especially surprising to the world of elite runners was the outstanding showing by athletes from Kenya and Ethiopia who had apparently trained at altitudes similar to that of Mexico City, as was their custom.

Events at the 1968 Mexico City Olympics may be considered to have initiated an era of "altitude training" for elite athletes, especially those in-

volved in endurance sports, and also to have initiated the study of ways to maximize the benefits of altitude training. This topic remains of constant high interest and is an area of frequent discussion in both the scientific and popular sport press [1, 2]. Indeed, for those athletes who are unable to train at high altitudes due to cost, access or other factors, artificial hypoxic sleeping devices, which allow individuals to sleep under conditions of relative hypoxia, have been developed.

One of the most important parameters used by exercise physiologists to determine the likelihood of success of endurance athletes, especially those involved in running, cycling and cross country skiing, is the volume of oxygen that can be delivered maximally to the muscles. This maximum oxygen consumption parameter – $VO_{2\,max}$ – is described by the Fick Equation:

$$VO_{2\,max} = Q \times (AVO_2)$$

where Q is cardiac output (HR×Stroke volume) and AVO_2 is the difference in oxygen content between arterial and venous blood. AVO_2 represents the amount of oxygen utilized by the skeletal muscle.

In the exercise laboratory, $VO_{2\,max}$ is determined during a maximal exercise test by open circuit calorimetry using an automated metabolic cart. These devices are available from a number of manufacturers worldwide. The $VO_{2\,max}$ provides an objective measure of the effect of training, including altitude training (and/or blood doping) on an athlete's performance.

Altitude training has taken on such importance that a "high altitude environment training system" has been developed that includes a low oxygen athletes' hotel, a low pressure mini-dome for athletic activities and a low oxygen "Alticube", a small room designed for training with treadmills or other equipment [3]. There are also societies such as The Society of Altitude Training and Hypoxic Environmental Study or the High Altitude Training Environment System Research Society that have the aim of "exchanging information concerning training using high altitude natural environment and artificial high altitude environment systems, to research and review from a sports medicine perspective the future direction of appropriate training in high altitude environments, and to return the results to society, contributing to the development of sports and sports medicine". It must be pointed out that all of these efforts to enhance performance based upon altitude training depend strictly upon the induction of increased levels of Epo and correspondingly increased red blood cell parameters (hematocrit and hemoglobin) in the participating individuals. Therefore, it should be no surprise that as recombinant Epo became available as a therapeutic, its abuse amongst athletes spread quickly.

Optimization of high level athletic performance utilizing living at high altitudes has been shown to be most effective when a "living high-training low" strategy is employed. Levine and Stray-Gundersen subjected 39 runners (27 males, 12 females) to a randomized protocol of 4 weeks of training either "high-low" (living at 2500 m and training at 1250 m), "high-high" (living and training at 2500 m), or "low-low" (both living and training at 150 m of altitude) [4]. They showed that both groups exposed to high altitude increased their $VO_{2\,max}$ approximately 5%, proportional to the increase in red cell mass volume (9%; $r = 0.37$, $p < 0.05$). There were no changes in the "low-low" group. Importantly, Levine and Stray-Gundersen showed that both running velocity at $VO_{2\,max}$ and maximal steady state ventilatory threshold improved only in the "high-low" group. The authors concluded that "living high, training low" was a superior strategy for altitude training for endurance sports.

In another study, Stray-Gundersen *et al.* studied 22 male and female elite athletes who lived for twenty-seven days at 2500 m and carried out high intensity training at 1250 m [5]. Using this protocol, they found that fully 1/3 of the individuals participating achieved personal best times in the 3000 meter test event. They experienced, on average, a 3% improvement in $VO_{2\,max}$ from 72.1 ± 1.5 to 74.4 ± 1.5 ml $\cdot$ kg^{-1} $\cdot$ min^{-1}. Within 20 h after reaching the 2500 meter altitude, circulating Epo levels were approximately twice those seen at sea level, and soluble transferin receptor levels were elevated, consistent with increased erythropoiesis (see below). As expected, hemoglobin levels increased an average of 1 g/dL [1] during the four week period. As reported by Robertson, improvements such as those demonstrated by Levine and Stray-Gundersen and Stray-Gundersen *et al.* represent the difference between the first and 25th place runners in the 2002 Sydney Olympics Men's 10 kilometer run [1].

Interestingly, there is significant individual variation amongst athletes in response to altitude training. Chapman *et al.* studied 39 collegiate runners (27 males and 12 females) and tested their sea level 5000 meter run time before and after 28 days of "living high-training low" or "living high-training high" [6]. The authors noted that the runners could be divided into a group of "responders", whose times were shortened by 20–60 s, and a group of "nonresponders", whose times were unchanged or actually increased. Responders uniformly exhibited an increase in Epo levels over there sea level control values, whereas nonresponders had increased Epo levels but somewhat less than responders. Chapman *et al.* found a significant increase in total red cell volume and $VO_{2\,max}$ among responders only. No such increase was seen among nonresponders. The physiologic/molecular basis for this differential response to altitude among healthy individual collegiate athletes remains to be elucidated.

Ge *et al.* have tried to refine our understanding of Epo release in response to short term hypobaric hypoxia [7]. They measured blood Epo, arterial O_2 saturation and urine pO_2 in 32 men and 16 women at sea level and after 6 and 24 h at simulated altitudes of 1780, 2085, 2454 and 2800 m. They found significant increases in Epo after 6 h at all altitudes and even further increases after 24 h at 2454 and 2800 m. The increases varied markedly from –41 to 400% after 24 h at 2800 m, consistent with the individual variation to altitude described earlier by Chapman *et al.* Unfortunately, the authors did not report Epo levels at later time points, which would have provided some indication of the sustainability of increased Epo production after 6 and 24 h of simulated altitude exposure. Given that erythroid progenitors respond best to a relatively constant Epo stimulus for enhanced growth and differentiation, it can be anticipated that exposure of an individual to hypoxia for periods of approximately 6 h, such as are experienced in artificial hypoxic environments used during sleep to simulate high altitude training, will result in significantly less elevation in steady state Epo levels and much less enhancement of erythropoiesis than would be observed with 24 h or greater (constant) hypoxic exposure.

10.3 Erythropoietin Use by Athletes

Information regarding the use of Epo by athletes can be derived only peripherally from standard medical and scientific literature. Indeed, "information" on this topic is found in popular magazines, reports and monographs by various athletic organizations including the International Olympic Committee (IOC), newspapers and word of mouth or rumor within the elite athletic community. For all but the medical and scientific literature information, this section of Chapter 10 has benefited greatly from the recent review by Catlin *et al.*, to which the reader is referred for an even further in depth insight into this issue [8].

As related by Catlin *et al.*, in 1983 the IOC established The Tribunal Arbitral du Sport or Court of Arbitration for Sport (TAS-CAS) to adjudicate disputes involving individual athletes, sports federations, Olympic committees, game organizers and others [1]. In 1998, as a direct result of the apparently wide spread use of recombinant Epo by cyclists, particularly in the 1998 Tour de France (see below), the IOC encouraged the develop-

1) http://www.tas-cas.org/

ment of the World Anti-Doping Agency (WADA), an independent organization that was charged with controlling the use of "prohibited substances", including Epo, by competitive athletes [2]. The WADA came into existence on November 10, 1999 in Lausanne, Switzerland several months after a meeting to discuss the Epo doping scandal at the 1998 Tour de France.

10.4 Enhancing Performance by Blood Transfusion

Even before the availability of recombinant Epo, it was well known by athletes that the increased oxygen carrying capacity of the blood that accompanies increased red cell mass enhanced endurance. Therefore, surreptitious blood transfusion was employed and may still be. As stated by Catlin *et al.* [8], expansion of blood volume by transfusion or by Epo or Epo-like proteins is designated "induced erythrocythemia", which is more commonly known as "blood doping". Both autologous and homologous blood transfusions have been employed by athletes, and the terms "blood boosting" and "blood packing" are the common names for autologous and homologous transfusion, respectively. Examples of blood doping by transfusion include the first documented case at the 1980 Moscow Olympics in which a runner was transfused with two units of blood shortly before winning medals in the 5 and 10 kilometer races [9], and the 1984 Los Angeles Olympics in which seven American cyclists were involved, including transfusion in a motel room [10]. Notably, of these seven involved cyclists, four won medals. Until these events came to light, the IOC did not specifically ban blood doping. A final documented case was that of a U.S. skier in 1987 [11].

10.5 Recombinant Erythropoietin as a Blood Doping Agent in Athletics

Catlin *et al.* report that the possibility of using recombinant Epo to enhance performance was rumored in the athletic community even before marketing approval was granted in 1987 and that "rumors of actual use circulated before the 1988 Winter Games of Calgary." After recombinant

2) http://www.wada-ama.org/

Epo became available, numerous unexplained deaths were noted among competitive cyclists in Belgium and the Netherlands, believed to involve Epo use [12, 13]. Despite widespread publicity and "an official investigation" no further information was made public. In 1990, the IOC included Epo and Epo analogs to its list of prohibited substances.

It was nearly another ten years before abuse of recombinant Epo by the elite athletic community was documented. The precipitating event was the 1998 Tour de France [3)]. The scandal began on July 8, 1998 when a masseur for the Festina cycle team (France) was stopped at the Franco-Belgian border with a car containing more than 400 doping products including recombinant Epo. Two days later, the masseur was placed under formal investigation in Lille, and on July 11th the Tour de France began. The masseur admitted that the products were for the Festina team, and the Festina director and team doctor were questioned by police. The Festina director was suspended by the International Cycling Union on July 16th, and the following day he admitted supplying drugs to the team to improve performance. The director of the Tour de France expelled the Festina team. After a series of further events, including admissions by Festina members and an investigation of the Dutch TVM team, other riders in the Tour began to protest. All in all, by July 31st 1998, only 14 teams out of the original 21 remained with fewer than 100 of the original 189 riders. No doubt some of these withdrawals were due not to abuse of Epo or other drugs by riders, but rather reflected a sense of unity amongst the elite cycling community. Nevertheless, these events served to demonstrate worldwide the potential for abuse of recombinant Epo and the possible magnitude of the problem.

Tab. 10.1 provides a chronology of blood doping among athletes as assembled by Catlin *et al.* [8].

It is difficult to know with any degree of precision how widespread blood doping actually is. However, as early as 1990, Scarpino *et al.* reported that among 1015 Italian athletes, 17% admitted "regular" use of red cell transfusions or recombinant Epo and 25% admitted "occasional" use [14]. Scarpino *et al.* reported that among professional coaches, managers and team physicians, the consensus was that approximately 7% of athletes regularly doped with transfusion or recombinant Epo. With the increasing availability of recombinant Epo from a variety of manufacturers and sources, it is not unlikely that doping with Epo has increased. Despite this relatively high prevalence, Catlin *et al.* report that the TAS-CAS had adjudicated only two cases of alleged doping with recombinant

3) http://sportsillustrated.cnn.com/cycling/1998/tourdefrance/news/1998/08/02/drug_chronology/

Tab. 10.1 Blood doping in sport: chronology of seminal events

Year	*Description of event*	*Venue*	*Place*	*Source*
1968	Rumors of whole-blood doping	Olympic Games	Mexico City	Second-hand accounts
1976	Rumors of transfusions	Olympic Games	Montreal	Second-hand accounts
1980	5-km medallist admits blood doping	Olympic Games	Moscow	Primary account, newspaper
1980	Autologous transfusion increases performance	Key finding		Scientific literature
1984	Homologous and autologous transfusions (n=7)	Olympic Games	Los Angeles	Public admissions
1987	Epoetin alfa approved by US FDA			
1988	Rumors of rHuEPO misuse	Olympic Games	Calgary	Newspaper
1989	Several unexplained cyclist deaths		Netherlands	Newspaper
1998	rHuEPO confiscated by authorities	Tour de France	France	Court proceedings, newspaper
2000	Practical test method for rHuEPO published	Key finding		Scientific literature
2000	rHuEPO test deployed	Olympic Games	Sydney	Official IOC reports
2002	IEF tests positive for rHuEPO	Cycling race		Court decision, CAS
2002	Darbepoetin alfa found in urine (n=3)	Olympic Games	Salt Lake City	Official IOC reports

CAS, Court of Arbitration for Sport; IEF, isoelectric focusing; IOC, International Olympic Committee; rHuEPO, recombinant human erythropoietin

Epo by late 2002, upholding the laboratory result in one case and finding it flawed in the second [8].

10.6 Recombinant Erythropoietin Enhances Athletic Performance

In view of the direct relationship between Epo levels, increased red cell mass, $VO_{2\ max}$ and performance enhancement seen with altitude training, it is no surprise that administration of recombinant Epo has been docu-

mented as a performance enhancer in endurance events. Birkeland *et al.* carried out a double blind placebo-controlled study in which 5000 IU of recombinant Epo or placebo was administered 3 times weekly for four weeks to male athletes [15]. They found that the hematocrit increased in the Epo group from 42.7 ± 1.6% to 50.8 ± 2.0% along with an increase in soluble transferrin receptor and transferrin receptor/ferritin ratio (see below). Importantly, the $VO_{2\,max}$ increased from 63.6 ± 4.5 $ml \cdot kg^{-1} \cdot min^{-1}$ before Epo to 68.1 ± 5.4 $ml \cdot kg^{-1} \cdot min^{-1}$, representing a 7% increase in the Epo group ($p = 0.001$). Russell *et al.* examined the effect of low doses of recombinant Epo on submaximal and maximal exercise [16]. They divided 21 volunteer recreational athletes into three treatment groups: 1) recombinant Epo plus intravenous iron; 2) recombinant Epo plus oral iron; and 3) placebo. Both Epo treatment groups exhibited significant increases in their $VO_{2\,max}$, ranging from 3.1% to 9.7% measured at 4, 8, and 12 weeks after initiation of Epo administration, which occurred for the first eight weeks only. Importantly, this study documents the prolonged effect of Epo treatment on performance lasting weeks beyond the cessation of its use. While developing markers of altered erythropoiesis to detect recombinant Epo abuse by athletes, Parisotto *et al.* treated 27 recreational athletes in three groups with Epo plus intramuscular iron, Epo plus oral iron or placebo [17]. Both Epo-treated groups showed significant increases in $VO_{2\,max}$ of 6.3 ± 1.8% and 6.9 ± 1.1% above baseline, respectively.

Despite the apparent prevalence of recombinant Epo use by athletes, Catlin *et al.* report that no surveys describing adverse effects have been carried out [8]. In fact, a single case of superior sinus thrombosis has been reported linked to recombinant Epo use [18]. However, other drugs were also involved.

10.7 Identifying Athletes Who Use Recombinant Erythropoietin

10.7.1 Indirect Tests for Erythropoietin Use

In sport, doping tests are classified as direct or indirect [8]. Whereas a direct test identifies the doping substance chemically or biochemically using various analytical methodologies, indirect tests measure biologic markers that accompany the use of the substance without necessarily directly identifying it. The first indirect test for Epo use was the hematocrit "health" test promulgated by the International Cycling Union in which

athletes with hematocrits greater than 50% for men and 47% for women were not allowed to compete [19, 20]. Similarly, the International Ski Federation based its rule on hemoglobin values of 185 g/L or 165 g/L maximal for men or women, respectively. This hematocrit test was designated a "health test" rather than an identifier of blood doping based upon the consideration that competition with these relatively high hematocrits or hemoglobins presented a danger for the athlete. Catlin *et al.* point out that such tests would fail to consider athletes who have "naturally high hematocrit values" but they also report on the small study by Saris *et al.* who did a retrospective study of 344 hematocrits determined on cyclists prior to the availability of recombinant Epo, finding that the average was 43%, with a range of 39–48%, thus supporting the hematocrit health test parameters [21].

Other markers of Epo use have been considered both alone and in combination. Of special interest have been the soluble transferrin receptor (sTfR) and serum ferritin levels. As a natural consequence of the increased erythropoiesis accompanying Epo administration, more erythroid progenitors and their progeny are formed that are actively synthesizing hemoglobin, necessitating increased iron uptake and increased cycling of the cell surface transferrin receptor. As erythropoiesis increases, a "soluble form" of the transferrin receptor is produced that is detectable in the serum, the concentration of which is proportional to the degree of active erythropoiesis. Concomitantly, serum ferritin, which represents a transport form of iron from storage sites, is decreased due to the increased utilization of iron. Thus, the soluble transferrin receptor/ferritin ratio increases with enhanced erythropoiesis and can be used as an indicator of possible recombinant Epo use. In 2001, Parisotto *et al.* chose five indirect markers of increased erythropoiesis identified previously and tested them in combination as indicators of "current or recently discontinued" recombinant Epo use [22]. They found that changes in hematocrit, reticulocyte hematocrit, percent macrocytes, serum Epo concentration and soluble transferrin receptor correlated with current or recent Epo use. Using Fisher's discriminant analysis, the authors developed statistical models for ON recombinant Epo (reflecting current use) and OFF recombinant Epo (reflecting recently discontinued use) derived approximately two weeks after the final test injection of recombinant Epo. The authors found that they could establish an indirect blood test that "offers a useful means of detecting and deterring r-HuEPO abuse." As described by Catlin *et al.*, the ON score was approved for the 2000 Sydney Olympic Games as an indicator of athletes whose urine should be subjected to a direct test for Epo (see below). It was not approved in and of itself as an index of abuse of recombinant Epo. Catlin *et al.* relate that an athlete would be declared positive

for recombinant Epo use if the ON score were greater than a specified cut off score and that the urine test performed later identified recombinant Epo. Samples were "suspicious" if only one of the blood or urine tests was positive or if the OFF score was increased. During the Sydney Games, no athletes were declared positive but 7 were "suspicious" [23]. Currently, the OFF score is used to identify athletes requiring close monitoring with other testing.

10.7.2 Direct Tests for Erythropoietin Use

A direct test of recombinant Epo must identify the substance in a sample obtained from the athlete. In 1995, Wide *et al.* showed that recombinant Epo could be detected in the blood and urine of volunteer subjects using an electrophoretic procedure [24]. The authors showed that recombinant Epo differed in its electrophoretic mobility from endogenous reproduced Epo. A seminal contribution to direct testing for Epo and Epo analogs was reported by Lasne and de Ceaurriz who developed an isoelectric focusing (IEF) technique coupled with double immunoblotting to identify multiple "glycoforms" of Epo in the urine of test subjects [25, 26], using the widely used anti-Epo monoclonal antibody AE7A5 [4]. As described in Chapter 5, Epo has three N-linked and one O-linked glycosylation sites with highly complex oligosaccharides attached, resulting in numerous molecular forms of the hormone with different isoelectric points based principally upon the number of sialic acids present per molecule. These "glycoforms" can be separated by isoelectric focusing and, importantly, are different in overall pattern from the isoelectric focusing pattern of endogenously produced human Epo. Fig. 10.1 shows the results of Lasne and de Ceaurriz demonstrating markedly different glycoform patterns in the urine of a control individual (endogenous Epo) compared with those either treated with recombinant Epo or using it to enhance performance.

This IEF and double immunoblotting method serves not only to identify recombinant Epo and discriminate it from endogenously produced Epo but has also been employed to identify at least one Epo analog. As reported in more detail in Chapter 11, darbepoetin alfa (Aranesp, Amgen; Nespo, Dompé Biotec) is a recombinant human Epo mutant protein engineered to contain two additional N-linked glycosylation sites resulting in a protein with a higher number of oligosaccharides and, hence, a higher

4) This monoclonal antibody was developed by the author of this volume in collaboration with James W. Fisher [27].

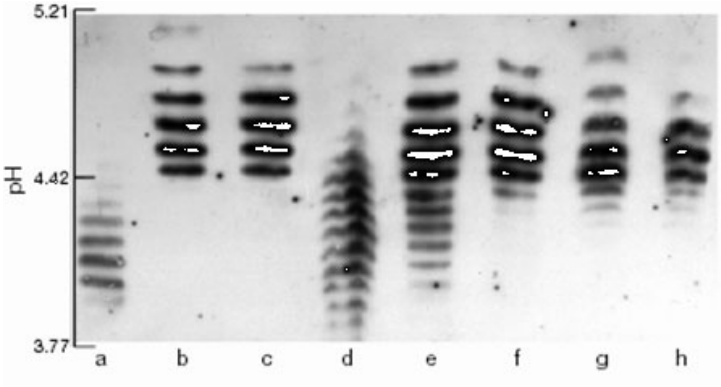

Fig. 10.1 Isoelectric focusing and double immunoblotting can discriminate between endogenous Epo and recombinant Epo in human urine. Lane a, commercially available human urinary Epo (endogenous); lane b, recombinant epoetin beta standard; lane c, recombinant epoetin alfa standard; lane d, endogenous Epo in the urine of a control subject; lanes e and f, epoetin beta in the urine of two patients treated with the drug; lanes g and h, Epo in the urine of two cyclists who competed in the 1998 Tour de France. Note how glycoform patterns of Epo in lanes g and h are virtually identical to recombinant epoetins (lanes b and c) and do not resemble endogenous Epo in lanes a and d. From reference [25].

number of sialic acids. This gives the protein a prolonged *in vivo* half-life and a distinct isoelectric focusing pattern because there are more acidic glycoforms than seen with either endogenous Epo or recombinant human Epo. Fig. 10.2 shows the results of IEF and double immunoblotting of a series of samples including recombinant Epo, endogenous Epo and several samples containing Aranesp, being either quality control samples or samples from urine obtained from potentially offending athletes, from a case adjudicated by the TAS-CAS [5)]. The darbepoetin containing samples are clearly easy to discriminate from both endogenous Epo and recombinant human Epo. At the 2002 Salt Lake City winter games, darbepoetin first appeared as an abused substance. As reported by Catlin *et al.*, the UCLA Olympic Analytical Laboratory received 77 combined blood and urine samples of which samples from 3 athletes indicated darbepoetin alfa use [8]. At least one of these cases has been adjudicated by the TAS-CAS, which found against the offending athlete [5)]. The urine specimen from the athlete who was the subject of this case is seen in Fig. 10.2, lane 20 (arrow). Recently, further study of the ON and OFF score models has been carried out and additional refinements have been made [28–30]. Cat-

5) CAS 2002/A/374 Muehlegg v/IOC http://www.tas-cas.org/en/pdf/juris2.pdf

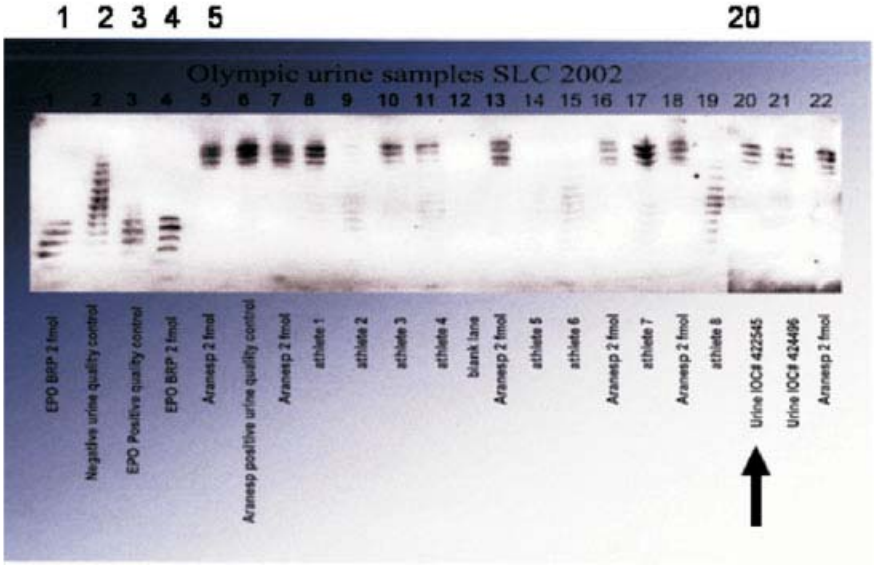

Fig. 10.2 Isoelectric focusing and double immunoblotting of various samples presented in a case before the TAS-CAS. Lane 1, recombinant Epo reference preparation BRP-2; lane 2, normal endogenous urinary Epo; lane 3, recombinant Epo in urine positive quality control; lane 4, recombinant Epo reference preparation BRP-2; lane 5, Aranesp (darbepoetin alfa) standard; lane 20, urine IOC # 422545 test sample. Note that the glycoform pattern of lane 20 and others closely match that of darbepoetin. From CAS 2002/A/374 Muehlegg v/IOC.

lin *et al*. report that darbepoetin alfa is detectable in urine for up to 12 days after the last dose.

The return to baseline hematocrit from an elevated hematocrit after cessation of recombinant Epo or Epo analog use is slower than might be anticipated intuitively. Indeed, studies show that the hematocrit remains at a relatively unchanged plateau for 12–20 days after cessation of recombinant Epo use [15, 16, 31]. The explanation for this lies in the physiology of erythropoiesis. Administration of recombinant Epo raises the hematocrit, resulting in down-regulation of endogenous Epo production by the kidney. Upon cessation of recombinant Epo administration, active erythropoiesis ceases within days since endogenous Epo production is shut off. Normal red blood cells have a life span of approximately 120 days; therefore, the population of red blood cells in the circulation of a non-Epo treated normal individual comprises cells at various points along this life span with a mean life span of approximately 60 days. If erythropoiesis is acutely switched off in a normal individual, for example, because of a viral infection or other insult to the bone marrow, the hematocrit normally

decreases at about 1% per day. However, after Epo treatment for several weeks, the population of circulating red blood cells has an increased percentage of relatively young cells with average life spans closer to the 120 day maximum, adjusting the mean for the entire population to some value significantly higher than 60 days. Because of this skewing of the average life span toward the high end by Epo treatment, even with the post-Epo treatment reduction in erythropoiesis, a decrease in hematocrit is not easily seen within the first week or two. Therefore, as Catlin *et al.* point out, "an athlete could be enhanced and yet have a negative urinary isoelectric focusing test" [8].

The problem of direct testing of athletes for recombinant Epo abuse its likely to get even more complex as additional Epo products and Epo analogs become available. Of special interest is epoetin delta (co-developed by Transkaryotic Therapies and Aventis as Dynepo^{TM}), an Epo produced by human cells, the glycosylation of which may more closely resemble that of endogenous human Epo. However, as of late 2003, there were no published scientific reports of the isoelectric focusing pattern of this protein either alone or in comparison to other Epos, including endogenous Epo.

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11
The Future of Erythropoietin

Given the great success that recombinant Epo has enjoyed in treating various forms of anemia and the increasing number of additional applications for the hormone, as well as its great commercial success, it is understandable that there are numerous efforts to improve on its pharmaceutical properties and/or to find replacement pharmaceuticals. For the most part, the object of these pursuits has been to reduce or to totally eliminate the need for intravenous or subcutaneous injections of the hormone. A reduction in frequency of the injections can be accomplished by prolonging the *in vivo* half-life of the molecule and/or by developing slow- or continuous-release formulations. The total elimination of injections requires alternative means of administration of the hormone or of molecules that can substitute for Epo in biological function. The reader is referred to the excellent recent review by Elliott as an additional source of information on this topic [1].

11.1
New Formulations

Sustained-release formulations of recombinant Epo have been developed both in academia and commercially. Morlock *et al.* prepared biodegradable microspheres containing Epo using triblock copolymers with encapsulation efficiencies ranging from 72–99% [2]. However, the authors noticed the formation of high molecular weight Epo aggregates, a phenomenon that would preclude administration to patients. Pistel and colleagues studied recombinant Epo loaded into microspheres prepared from linear and star-branched block copolymers [3]. Although the authors met with some success, Epo aggregation was again identified as a problem and was similar to that seen using linear polymers. Recently, Qiu *et al.* developed a hydrogel by *in situ* crosslinking of a thiol-containing polyethylene glycol-

based copolymer [4]. They noted that no organic solvent, elevated temperature or harsh pH was required for formulation or patient administration. Using recombinant Epo and other proteins as model compounds, they confirmed sustained-release for 2–4 weeks and prolonged biological activity *in vivo* of the released proteins.

Commercial attempts at sustained-release Epo formulations have also been reported. In the late 1990s, Alkermes, Inc. received United States Patent 5 674 534 entitled "Composition for Sustained-Release of Non-Aggregated Erythropoietin". Alkermes and Johnson and Johnson entered into an arrangement to develop this sustained-release formulation using Alkermes' ProLease™ technology for human use. However, after more than two years of collaboration, Johnson and Johnson stopped the project in June, 2000 with no public explanation offered [1)].

A question that must be faced by all developers of sustained-release formulations of recombinant Epo is the possibility that these formulations may function as adjuvants, thereby enhancing the immunogenic potential of Epo. This possibility is of special concern in view of the tendency of Epo and other proteins to be subject to subtle or major alterations in structure when formulated in this manner, thereby enhancing their antigenic potential [5]. This consideration may explain why a sustained-release formulation of recombinant Epo has not yet been approved.

Oral formulations of Epo have also been considered. The major problem with this approach is the instability of Epo and other proteins in the gut of adult humans. Nevertheless, in the early 1990s, Cortecs International in the United Kingdom (now Provalis) was developing an oral formulation of Epo and other therapeutic proteins. They worked with at least two pharmaceutical companies, one of which was Johnson and Johnson, but there was no public disclosure of the results of the project.

11.2 Erythropoietin with Enhanced Glycosylation

As described in Chapter 4 and Chapter 5, although the *in vitro* biological activity and receptor affinity of Epo is increased as oligosaccharides are removed or eliminated, the opposite is true with *in vivo* half-life and biological activity, which are increased with addition of oligosaccharides, especially terminal sialic acid residues. It should be recalled that erythroid cells respond best to Epo when it is present continuously, thus supporting

1) http://www.fool.com/news/2000/slks000612.htm

efforts to increase its half-life. Precisely why glycosylation increases Epo's *in vivo* half-life is unknown, but there is speculation that increasing the Stokes' radius or "hydrodynamic size" of the molecule in some way reduces its clearance. As discussed previously, the mechanism(s) of Epo's clearance from the circulation is/are poorly understood.

Based upon these considerations, scientists at Amgen, Inc. developed "darbepoetin alfa", a recombinant Epo mutant with an increased number of oligosaccharides attached [6–45]. After trying a series of compounds, they settled on introducing five mutations into the native sequence of recombinant Epo (Asn30-Thr32 and Val87-Asn88-Thr90) thereby adding two more N-linked glycosylation sites (Fig. 11.1). This increased the average carbohydrate content of the molecule from 40% to 51% and increased the maximum sialic acids from 14 to 22 [1]. As expected, *in vitro* studies indicated that the potency (activity/milligram) of the molecule was decreased somewhat from recombinant Epo *in vitro*, since increased glycosylation reduces the molecules affinity for the receptor just as removal of glycans increases its affinity. The scientists who developed darbepoetin alfa were aware of this consideration, and they introduced these two new N-linked oligosaccharide attachment sites in regions of the molecule that were less likely to interfere with receptor interaction or with protein structure. Preclinical studies showed that darbepoetin alfa, originally designated NESP (*n*ovel *e*rythropoiesis *s*timulating *p*rotein) had an *in vivo* half-life of 2–4-fold that of recombinant Epo. Also, it increased the hematocrits of normal animals and animals made anemic by a variety of methods. Further clini-

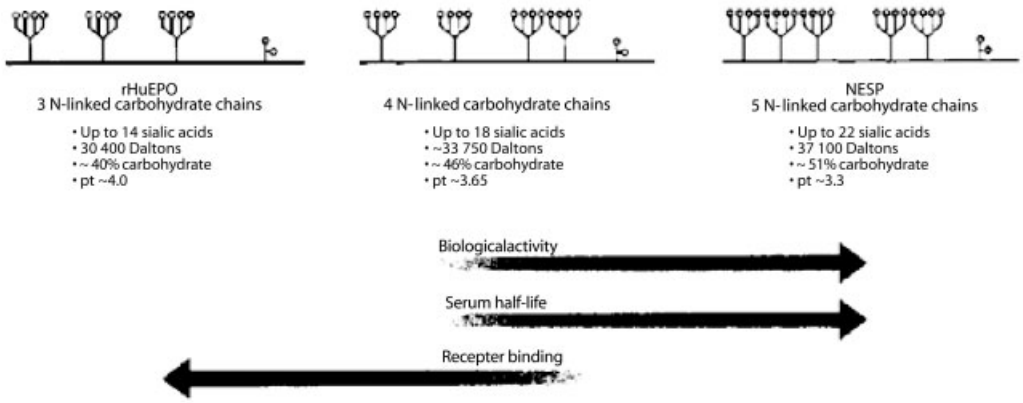

Fig. 11.1 Structural considerations during the design of NESP (darbepoetin alfa). Relationship of N-linked carbohydrate chains, molecular weight, carbohydrate content and pI, along with *in vivo* biologic activity, *in vivo* serum half-life and receptor binding affinity as recombinant Epo was mutated to contain 4 or 5 (NESP) N-linked carbohydrate chains. From reference [123].

cal studies confirmed the preclinical work, demonstrating increased *in vivo* half-life in humans with pharmacodynamic properties similar to that of recombinant Epo but requiring less frequent administration. Darbepoetin alfa (Aranesp, Nespo) has since been approved for treatment of a variety of anemias in several countries.

11.3 Alternatives to Glycosylation of Erythropoietin

There are two principal reasons why developers of enhanced erythropoietin therapeutics would seek alternatives to glycosylation. The first is that these methods can potentially provide greater control over the structure of the final product and, moreover, allow increasing of the *in vivo* half-life even beyond that of darbepoetin. The second is especially relevant to certain patent issues in the United States relating to recombinant Epo as a "glycoprotein". By producing an Epo molecule without attached sugars, but rather enhancing its half-life by attaching synthetic chemical moieties, companies with new technologies can "engineer around" existing patent claims that refer to recombinant Epo as a glycoprotein and, potentially, yield a product with enhanced pharmaceutical properties. Three approaches to these glycosylation alternatives described below serve to illustrate the potential of this type of technology.

Attachment of polymers of polyethylene glycol (PEG, "pegylation") has long been known to result in prolongation of *in vivo* half-life of proteins [46–49]. By attaching relatively bulky chemical "side chains" to the polypeptide backbone, the molecule exhibits an increased Stokes' radius (or hydrodynamic size) resulting in reduced clearance from the circulation, similar to that afforded by increasing the numbers of oligosaccharides. There is probably more than one mechanism involved in this phenomenon but the fundamental reason why pegylation or other methods that increase molecular size tend to reduce clearance require further investigation. Conventional pegylation chemistry targets amino groups on proteins, although other approaches exist. Since Epo has eight epsilon amino groups on its lysyl residues and one amino terminal group, the potential for nine sites of modification with varying combinations and degrees of efficiency exists. How this might affect *in vitro* activity in overall structural stability cannot be predicted *a priori*. Companies that have PEG-Epo in development include SunBio, Korea, Prolong Pharmaceuticals, Inc. and others. As pointed out by Elliott [1], the possibility exists to make pegylated Epo synthetically, that is, without depending upon recom-

binant DNA technology, allowing for the introduction of pegylated amino acids at precise locations along the molecule, thereby providing better control of overall pegylation and structure of the final product.

Scientists at Hoffmann-La Roche have used a similar approach in designing their new drug CERA (**c**ontinuous **e**rythropoiesis **r**eceptor **a**ctivator). Using as a base molecule what appears to be nonglycosylated Epo (as of this writing, the company has not revealed exactly what the polypeptide is), they tested a number of side chain polymers, varying chain length, chemistry and number of side chains [50, 51]. They screened candidate molecules for their effect on reticulocyte count in a normocythemic mouse assay. The most active product (CERA) contained a single 30 kDa side chain. Evaluation of pharmacokinetic properties revealed that the median terminal elimination half-life of CERA in dogs following intravenous injection was 49 hours, a seven-fold increase over that of recombinant epoetin beta. Affinity for the Epo receptor was lower than that of recombinant Epo, consistent with the increased Stokes' radius of the CERA molecule. However, *in vivo* activity was increased, as would be expected based upon its prolonged *in vivo* half-life. More complete preclinical pharmacokinetics and pharmacodynamics of CERA indicate that it is superior to recombinant Epo, based upon reduced frequency of dosing and, although not compared directly, may be superior to darbepoetin alfa in this regard. CERA has successfully passed Phase I clinical trials and preliminary results from Phase II trials are very encouraging [2)].

An approach using complete chemical synthesis of an Epo molecule has been described [52]. Kochendoerfer and colleagues reported the total chemical synthesis of "synthetic erythropoiesis protein", SEP, which consists of a 166 amino acid polypeptide chain and two covalently attached negatively charged, branched polar moieties. Because of total control over the chemistry, the molecule was synthesized precisely and has a defined single structure, in contrast to the multiple glycoforms that characterize glycosylated Epo or darbepoetin. The authors demonstrated SEP's *in vitro* and *in vivo* biological activity, which were significantly greater on a microgram basis than was that of recombinant Epo. The terminal elimination half-life of SEP in rats was 9.5 ± 0.7 hours compared with 5.1 ± 1.3 hours for recombinant Epo. Hoffmann-La Roche appears to be interested in developing this compound along with development of its own CERA [53].

2) http://biz.yahoo.com/rc/031107/health_roche_cera_1.html

11.4
Erythropoietin Oligomers and Fusion Proteins

Erythropoietin oligomers and fusion proteins have the potential for increased *in vivo* half-life or enhanced biological properties or both. Sytkowski *et al.* hypothesized that a recombinant Epo with a larger molecular size would exhibit increased plasma half-life and potentially enhanced biological activity [54]. The authors speculated that by dimerizing Epo it would both increase its *in vivo* half-life and facilitate its binding to the receptor, thereby enhancing its activity at the cell surface. They produced active Epo dimers and trimers by chemically crosslinking the monomeric form using a combination of heterobifunctional crosslinking agents. The Epo dimers exhibited a modest increase in specific activity *in vitro,* in contrast to darbepoetin and to Epo species with chemical substituents instead of oligosaccharides. Importantly, the *in vivo* half-life of Epo dimers in rabbits was greater than twenty-four hours, compared to approximately four hours for the monomers, a marked increase considering the relative simplicity of the chemical crosslinking procedure (Fig. 11.2). Evaluation of the biological activity of the Epo dimer in mice showed that it was greatly enhanced over that of conventional recombinant Epo monomer. The authors calculated that the dimer exhibited approximately 26-fold higher activity *in vivo* than did the monomer, and it was effective after only one dose in mice.

Based upon the success of the crosslinked dimer, Sytkowski *et al.* designed a fusion protein cDNA, encoding a novel fusion protein designated Epo-Epo, comprising two human Epo coding sequences linked in

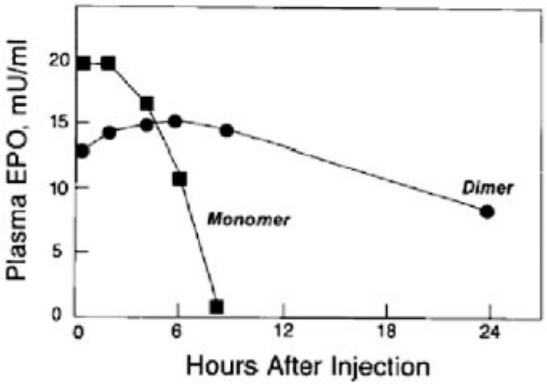

Fig. 11.2 Plasma clearance of Epo monomer or chemically cross-linked Epo dimer injected intravenously into rabbits. Each point represents the mean biologic activity determined in triplicate for groups of 3–4 animals. Standard deviation was equal to or less than ±20% for all values. Note markedly prolonged *in vivo* clearance of Epo dimer. From reference [54].

tandem by a sequence encoding a flexible polypeptide linker (Fig. 11.3) [55]. The authors constructed and expressed the Epo-Epo cDNA in COS cells and showed that the fusion protein migrated with an average apparent molecular mass of 76 kDa, slightly greater than twice the average for recombinant Epo. Enzymatic N-deglycosylation confirmed that the N-linked oligosaccharides of the fusion protein appeared identical to that of the conventional recombinant protein. No evidence of steric hindrance to full glycosylation was detected. The specific activity of the Epo-Epo fusion protein *in vitro* was nearly three-fold that of the Epo monomer, consistent with an enhanced activity at the cell surface, presumably due to facilitated binding of the second Epo domain after prior binding of the first. A single subcutaneous dose of Epo-Epo fusion protein to mice resulted in a significant increase in hematocrit within seven days, whereas administration of an equivalent dose of conventional recombinant Epo was without effect. A preliminary pharmacokinetic study indicated markedly prolonged *in vivo* half-life of the Epo-Epo fusion protein. The superiority of Epo-Epo fusion protein over recombinant Epo has led Sytkowski and colleagues to design and express additional fusion proteins comprising three or more Epo binding domains (unpublished).

Fusion proteins of Epo with other hematopoietic growth factors have been described [56–59]. They include GM-CSF/Epo and IL-3/Epo. Both of these molecules exhibited enhanced erythropoietic activity *in vitro* over that of recombinant Epo alone.

Another interesting possibility is a fusion protein of Epo with the Fc portion of immunoglobulin (Ig). The Fc portion of Ig imparts the prolonged *in vivo* half-life characteristic of Ig, due, at least in part, to the oligosaccharide attached to this portion of the molecule. Numerous fusion proteins using Fc have been described and their potential for therapeutic applications has been documented [60–66]. Attachment of protein molecules to Fc as a fusion protein results in markedly enhanced *in vivo* half-life. In an interesting application of the Fc technology, Spiekermann *et al.*

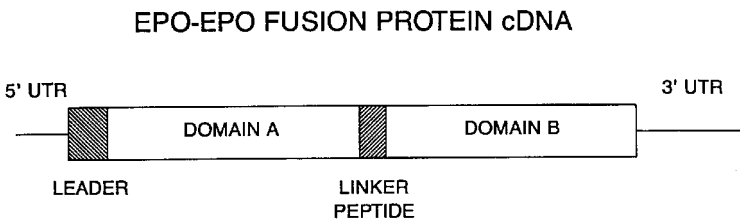

Fig. 11.3 Diagram of Epo-Epo fusion protein cDNA. Domain A and domain B each encode the complete, mature 166 amino acid Epo polypeptide. From reference [55].

reported on the production of an Epo-Fc fusion protein that was then administered by inhalation to allow systemic uptake of the fusion protein mediated by Fc receptors in the lung [67]. The authors found that bronchial epithelial cells from humans, nonhuman primates and mice expressed Fc receptor and demonstrated the *in vivo* absorption of biologically active Epo-Fc across the respiratory epithelium of mice. The potential for this means of administration of Epo and other biologically active protein therapeutics remains to be fully investigated.

11.5 Erythropoietin Mimetic Agents

Perhaps the most important reason for the pursuit of agents mimicking the action of Epo, especially small molecules, is the possibility of alternative means of administration including oral and inhalation. On the other hand, most small molecules used in medicine today have *in vivo* half-lives much shorter than that of recombinant Epo. This presents a problem given the physiology of erythropoiesis, which benefits from the presence of the erythropoietic active agent in the circulation for prolonged periods (days).

One of the first descriptions of a non-Epo molecule with erythropoietic activity was published by Elliott and colleagues, who developed antibodies that dimerized the Epo receptor and, hence, exhibited the biological activity of Epo [68]. In 1996, Wrighton *et al.* applied affinity-selective methods on random phage-displayed peptide libraries to isolate small peptides that bound to and activated the Epo receptor [69]. The authors identified several candidate agonists by *in vitro* and *in vivo* biological assay. The principal compound was a 14 amino acid disulfide bonded cyclic peptide with a minimum consensus peptide sequence of YXCXXGPXTWXCXP, X representing several possible amino acids. The sequence is not found in Epo. In a second study, Wrighton *et al.* synthesized a dimeric form of an erythropoietin mimetic peptide (EMP1) with markedly increased affinity for the Epo receptor and elevated potency *in vitro* and *in vivo* [70]. The authors showed that each member of this EMP1 dimer bound to a single EpoR resulting in EpoR dimerization. Because of the very short half-life of EMP1, Kuai *et al.* designed a fusion protein of EMP1 with plasminogen activator inhibitor-1 and showed increased *in vivo* activity [71]. However, the resulting fusion protein precluded oral administration. McConnell *et al.* also isolated cyclic peptides capable of activating the Epo receptor from phage display libraries [72].

Using a different strategy involving screening a library of in-house chemicals for inhibition of Epo binding to the EpoR, Qureshi *et al*. identified a compound (Compound 1) with inhibitory activity [73]. They synthesized a new compound, designated Compound 5, comprising 8 copies of Compound 1 held together by a central core. This new compound induced dimerization of the soluble EpoR. Compound 5 exhibited biological activities similar to those of Epo including activation of a STAT-dependent luciferase reporter gene, supporting proliferation of cells expressing the EpoR, and inducing *in vitro* differentiation of human erythroid progenitor cells [74]. Discovery of this nonpeptide molecule confirmed the possibility of the development of an orally active erythropoietic agent. In another effort to find a nonpeptide Epo mimetic small molecule, Goldberg *et al*. screened combinatorial libraries of dimeric iminodiacetic acid diamides and identified several small molecules that bound to EpoR [75]. After synthesizing dimer, trimer and tetramer libraries from these candidates, Goldberg *et al*. found several to be partial agonists *in vitro*. Further work toward developing nonpeptide Epo agonists has been carried out by Connolly *et al*. who showed the EpoR binding affinities of some N,N-disubstituted amino acids [76]. The search for nonpeptide Epo agonists continues.

11.6 Erythropoietin Gene Therapy

Another approach to the replacement of injectable recombinant Epo is gene therapy. Numerous studies have employed a variety of methods to show the potential for this approach [77–122]. They include direct injection of Epo expression plasmids into muscle or liver, introduction of the gene using various viral vectors, and implantable capsules containing cells expressing the Epo gene either constitutively or in a regulated fashion. None of these approaches has yet gone beyond the preclinical stage.

Obviously, regulated expression, as opposed to constitutive expression of the Epo gene, is to be preferred, and hypoxic regulation would be most desirable. Indeed, there have been attempts to establish the kidney as the site of expression of the introduced gene. Other approaches have included regulation using tetracycline, mifepristone and rapamycin. Each of these approaches has obvious advantages and disadvantages. Given the relatively slow rate of progress of gene therapy over the last twenty years in general, it is likely that this approach to Epo therapy will require many years of development.

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