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Erythropoietins and Erythropoiesis

Molecular, Cellular, Preclinical, and Clinical Biology

Edited by G. Molineux, M.A. Foote, and S.G. Elliott

Birkhäuser Verlag
Basel · Boston · Berlin

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Library of Congress Cataloging-in-Publication Data
A CIP catalogue record for this book is available from the Library of Congress,
Washington D.C., USA

Bibliographic information published by Die Deutsche Bibliothek
Die Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliografie;
detailed bibliographic data is available in the Internet at <http://dnb.ddb.de>.

ISBN-10: 3-7643-7542-6 Birkhäuser Verlag, Basel - Boston – Berlin
ISBN-13: 978-3-7643-7542-3

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Unchanged second printing of the hardcover edition published in 2003.

©2006 Birkhäuser Verlag, P.O. Box 133,CH-4010 Basel, Switzerland
Part of Springer Science+Business Media
Printed on acid-free paper produced from chlorine-free pulp. TCF ∞
Cover illustration:A secondary structure depiction of rHuEPO (see page 28).
Printed in Germany
ISBN-10: 3-7643-7542-6
ISBN-13: 978-3-7643-7542-3

9 8 7 6 5 4 3 2 1 www.birkhauser.ch

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Preface

Research on, and interest in, red blood cell formation spans several centuries and was thought to have peaked in the 1980s with the cloning of the erythropoietin (EPO) gene. In the years subsequent to the cloning of EPO and its expression as a recombinant protein, much was written about EPO. Although much has been learned and published, new, exciting data are becoming available on almost a daily basis. *Erythropoietins and Erythropoiesis: Molecular, Cellular, Preclinical, and Clinical Biology* compiles both pertinent historical and very recent research on this molecule and its clinical utility.

The book is divided into two sections: Background and Basic Science and Clinical Uses of Recombinant Erythropoietins. To begin, Israels and Israels describe the biology of red cells, the hierarchy of erythropoietic progenitor cells, their development to mature cells, and the effects of endogenous EPO on their development. Foote summarizes the historical interest in, and search for, an erythropoietic factor. Once EPO was identified, cloned, and expressed, the path was set for the study of other aspects of EPO biology both within erythropoiesis and other cellular systems.

The structures of recombinant human erythropoietin (rHuEPO) and its receptor (EPOR) have been studied and modeled using X-ray crystallography and other techniques, and the chapter by Osslund provides three-dimensional structural information. Activation of EPOR by EPO is essential for the survival, proliferation, and differentiation of red blood cells. EPOR is also expressed in many organs, including the brain, heart, endothelium, and ovaries, and may have physiological roles in these organs. Although studies are underway to establish the role of EPOR signaling in various organs, it is becoming increasingly apparent that red cells are not the only targets of EPO. Dame provides further data on the effects of both endogenous EPO and rHuEPO on hematologic and non-hematologic tissues. EPO has long been known to have a direct effect on the formation of red blood cells, and more recent work suggests that it may have a myriad of diverse effects that may allow the use of rHuEPO in clinical settings of neurological, cardiac, neonatal care, and as well as in other settings. Heatherington presents detailed information on the pharmacokinetics of EPO and rHuEPO in various patient populations. The pharmacokinetic properties of rHuEPO are some of the major factors that determine dosing regimens and mode of administration, and this literature review offers extensive information. Molineux reviews the basic biology of EPO and rHuEPO. The effects of treatment with rHuEPO are wide-ranging, especially in patients with degenerating kidney performance, suggesting effects beyond mere replacement of the missing endogenous EPO.

This section ends with a description by Chuck et al. of the production techniques for rHuEPO, from establishment of a cell bank to purification of the final clinical product.

Several clinical settings are discussed in detail, including treatment of the anemias of nephrology (Macdougall), oncology (Glaspy), and chronic diseases (Means) and also use in surgery (Cushner). Glaspy discusses the design of clinical trials using rHuEPO and offers insight into some published clinical data with rHuEPO and darbepoetin alfa, an erythropoiesis-stimulating protein that persists in the circulation three times longer than rHuEPO. The anemias of chronic diseases are among the most common syndromes in clinical medicine, but they often are under-recognized and under-treated. Clinical studies have demonstrated the efficacy of rHuEPO in the management of anemias of chronic diseases and have established a role for iron therapy as an adjunct to rHuEPO in this syndrome. Blood loss is inherent to the surgical setting. Using the orthopedic surgery model, Cushner focuses on the use of rHuEPO not only to decrease allogeneic transfusions but also to maximize blood parameters, such as hematocrit and hemoglobin concentration, during the peri-operative period.

Because rHuEPO is so effective in stimulating production of red blood cells, it has the potential for abuse by athletes, particularly Olympic athletes in endurance sports, such as running, cycling, and cross-country skiing. Catlin et al. describe how laboratories test for the illegal use of rHuEPO and how athletes are monitored and charged.

In 2001 and 2002, numerous cases of pure red cell aplasia, a rare but potentially life-threatening condition, were suddenly reported to health authorities worldwide. These cases of pure red cell aplasia were first noted in patients with kidney disease who were receiving rHuEPO. Mayeux and Casadevall describe this adverse event and how they test patients for the presence of anti-EPO antibodies, and they provide a possible explanation for the occurrence of pure red cell aplasia.

Finally, Elliott discusses current approaches in construction of EPO analogs to stabilize or increase activity, chemical modification of rHuEPO, gene delivery, and development of slow-release formulations. One successful strategy discussed is glycoengineering of rHuEPO, which involves construction of glycosylation analogs with increased content of sialic acid-containing carbohydrate. One such glycoengineered molecule, darbepoetin alfa, has been approved for marketing in the United States, the European Union, Australia, and Canada. Clearly, more than 20 years after the initial identification, isolation, cloning, and expression of the gene for EPO, research continues.

This book contains much information on erythropoiesis and red blood cell production, its regulation, and areas of continued or possible research, and is a resource for new and veteran researchers. Since different perspectives allow readers to arrive at their own conclusions and serve to stimulate scientific thought, we have not removed areas of controversy or overlap among chapters. We hope that this book proves useful and we invite your comments.

We have tried to acquire the necessary permissions and authorizations before publication, and great care has been taken in the preparation of the chapters. Nevertheless, errors cannot always be avoided. The editors and publishers, therefore, cannot accept responsibility for any errors or omissions that have inadvertently occurred. The views and opinions expressed in the book are those of the participating individuals and do not necessarily reflect the views of the editors, the publisher, Amgen Inc., or any other manufacturer of pharmaceutical products named herein. The package insert should be consulted before administration of any pharmaceutical product.

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Thousand Oaks, California
February 2003

I. Background and basic science

Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Erythropoiesis: an overview

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Erythrocyte production

Red blood cell production is dynamic and highly regulated. The normal red cell life-span is approximately 120 days requiring, at equilibrium, a daily replacement of 0.8% to 1.0% of the circulating red cell pool. Balanced production and destruction maintain the red cell mass within relatively narrow limits. An immediate erythropoietic response is triggered when tissue oxygenation is compromised due to blood loss or a shortened red cell life-span, or when demand increases for oxygen-carrying capacity.

Erythrocytes arise primarily from the $CD34^+$ pluripotent hematopoietic stem cells of the bone marrow. These progenitor stem cells constitute approximately 0.1% of the nucleated cells in the bone marrow, only about 5% of which are in cycle at any one time. The stem cell pool maintains itself, with little if any depletion, by asymmetric division into a committed colony-forming unit (CFU) and another stem cell. The quiescent stem cell remains in the G0 or G1 phase of the cell cycle, protected from genotoxic events and with extended time for DNA repair [1]. Cytokine binding to cell-surface receptors triggers stem cell activation. The early CFU have a higher proliferative rate but a more limited self-renewal rate than stem cells. The progeny of pluripotent CFU are heterogeneous, the result of a stochastic process with the possibility of more than one cell type evolving from early CFU progenitors; proliferation and survival are regulated by cytokines [2, 3]. Although most hematopoietic stem cells reside in the bone marrow, a small number circulate in the peripheral blood, as do some of the early CFU cells.

The micro-environment of the bone marrow consists of a network of sinusoidal vascular channels with hematopoietic cells, stromal cells, and fat in the spaces between the venous sinuses. The hematopoietic cells are sequestered in specific islands and are “nursed” by stromal cells that secrete the cytokines required for their development. The stromal cells include fibroblasts, macrophages, dendritic cells, T cells, and osteoblasts. The extracellular matrix provides a support grid and an adhesive substrate, consisting of collagen, fibronectin, reticulin, thrombospondin, proteoglycans, and laminin [4, 5]. A

number of adhesion molecules, including integrins, intercellular adhesion molecules (ICAM), and selectins, are expressed on the surface of hematopoietic cells, their density changing as the cells mature. Adhesive interactions between the cells and the extracellular matrix govern retention of the precursors within the bone marrow and may have a role in stem cell homing after bone marrow transplant [6, 7].

Hematopoietic stem cells respond to stem cell factor (SCF), a cytokine also referred to as Kit ligand or Steel factor. The SCF receptor (SCFR or c-kit) is a member of the superfamily of tyrosine kinase receptors; upon ligand binding, autophosphorylation of the cytoplasmic region of the receptor initiates an intracellular signal through the SH-2 recognition domains of the adaptor protein complex Grb2/Sos, followed by activation of Ras. The message continues through the MAP kinases and the early response genes, resulting in the activation of genes encoding the cyclins, notably the D cyclins and the cyclin-dependent kinases (CDK) 4/6, to stimulate G1 progression and cell proliferation [8]. Amplification of the SCFR pathway is dependent on stimulation by a network of the early-acting cytokines, e.g., interleukins (IL-3, IL-6, and IL-9), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), secreted by the local macrophages, monocytes, and lymphocytes [7]. After development of CFU, the erythroid line proceeds through the shared CFU-GEMM (granulocyte, erythrocyte, macrophage, megakaryocyte) precursor. The selection of lineage from this nodal point is probably random, but may be influenced by physiologic requirements.

The erythroid-committed units of CFU-GEMM proceed through the developmental stages shown in Figure 1. The nature of the early red cell progenitors, erythroid burst-forming unit (BFU-E) and erythroid colony-forming unit (CFU-E), has been determined by *in vitro* studies of the cells grown in methylcellulose. In culture, BFU-E give rise to large multiclustered "burst" colonies of up to 30,000 cells. Morphologically, they possess the fine nuclear chromatin, nucleoli, and abundant cytoplasm of a blast cell. They proliferate under the stimulus of IL-3, IL-9, GM-CSF, plus insulin-like growth factor (IGF-1). The early BFU-E have few erythropoietin receptors (EPOR) and, therefore, respond minimally to erythropoetin (EPO) [9]. As these cells mature, larger numbers of EPOR are expressed and the late BFU-E become EPO responsive. *In vitro,* their CFU-E progeny generate single small colonies of 10 to 50 cells expressing many EPOR; these cells respond strongly to EPO. EPO both stimulates growth and prevents apoptosis [9]; *in vitro,* cells deprived of EPO fail to replicate and undergo apoptotic death within a few hours.

At the CFU-E phase of development, cytoskeletal proteins begin to assemble and definitive membrane components appear, including red cell antigens and the surface adhesion molecules. Interaction of the adhesion molecules (ICAM)-1 and $\alpha_4\beta_1$ integrins with the extracellular matrix serves to retain the precursor cells within the bone marrow during proliferation and maturation. With the loss of these adhesive proteins, the reticulocytes and red cells are free to exit the bone marrow [7].

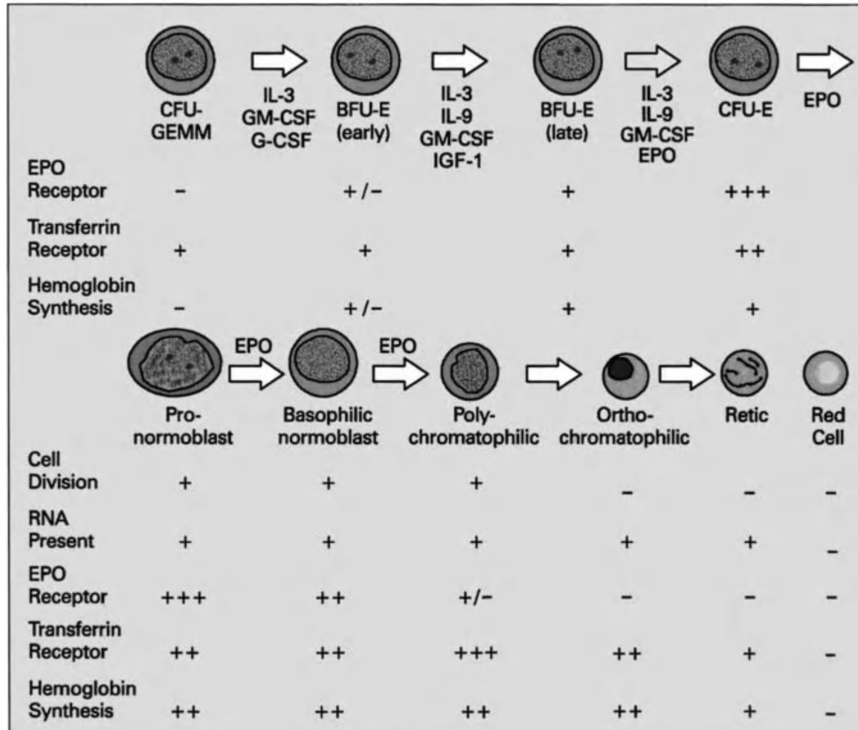

Figure 1. Erythropoiesis, from CFU-GEMM to erythrocyte; developmental stages, regulatory cytokines, cell-surface receptors, and hemoglobin synthesis.

Transferrin receptors (TfR) are common to most mammalian cells, providing access for transferrin-bound iron required for the synthesis of heme and other iron-containing proteins. Many TfR are present on hemoglobin-synthesizing cells, reaching a peak of some 800,000 per cell on polychromatophilic normoblasts, and decreasing to 100,000 on reticulocytes. TfR are absent from mature red cells: as hemoglobin synthesis has ceased, no further iron is required. TfR preferentially bind diferric transferrin, although monoferric also can be bound. With TfR binding of the iron-bearing transferrin, the complex is endocytosed and the iron is released into the cytoplasm. The receptor-transferrin complex then returns to the membrane where the transferrin is released into the plasma and the receptor again becomes available [10]. Hypoxia-induced upregulation of transferrin synthesis is controlled by the transcription factor hypoxia-inducible factor-1 (HIF-1) [11] (see below).

The earliest microscopically identifiable erythrocyte precursor in bone marrow aspirates is the normoblast, 15 to 20 μm in diameter with a large nucleus containing multiple nucleoli and basophilic cytoplasm. Further cell division results in the appearance of basophilic and then polychromatophilic nor-

moblasts; cell division ceases with development of the orthochromatic normoblast. In the next step, the nucleus is extruded and because of the presence of residual strands of RNA (reticulin), the anuclear cell is referred to as a reticulocyte. Over the next 24–48 hours, a further decrease in size and loss of some reticulin precedes the exit of the cell from the bone marrow. These reticulocytes may be retained in the spleen for 36–48 hours where adhesion molecules and residual reticulin are removed. The time from pronormoblast to mature erythrocyte is approximately seven days. When there is an acute demand for increased red cell production, this interval may be shortened by reducing the intermitotic interval or by skipping a mitotic division.

Failure of red cell production may occur as one component of a generalized bone marrow aplasia due to stem cell failure or appear as an isolated defect, a "pure red-cell aplasia" [12]. Congenital red cell aplasia (Diamond-Blackfan Syndrome) probably encompasses a number of gene mutations reflected as molecular defects in cells at the level of CFU-GEMM, BFU-E, and CFU-E [13]. Acquired red cell aplasia most commonly results from immunologic blockade of erythropoiesis at the level of BFU-E and CFU-E by T cells or antibody. IgG antibodies capable of inhibiting BFU-E or CFU-E colony formation can be demonstrated in about half of these patients [12]. Immunologically induced aplasia may occur in association with thymomas, lymphomas, and some non-hematologic tumors. A pure red cell aplasia associated with the development of anti-EPO antibodies inhibitory to erythroid colony formation has been documented in patients with renal disease receiving recombinant human erythropoietin (rHuEPO) [14]. (See Chapter 14 for further information.)

Parvovirus B19, a single-stranded DNA virus, is the etiological agent of the childhood exanthem fifth disease. The blood group P antigen on the red cell membrane is the receptor for the virus. B19 replicates only in P antigen-positive erythroid progenitor cells and is directly cytotoxic in these cells [15]. Suppression of erythropoiesis by B19 infection usually persists for only one to two weeks, until the viremia is cleared by the immune response. In the presence of a normal red cell life-span, this transient red cell aplasia is of little clinical importance. In patients with a compensated hemolytic process and a short red cell life-span, however, the decrease in hemoglobin concentration can be precipitous, resulting in an "aplastic crisis".

EPO synthesis

EPO, a 35 Kd glycoprotein, is the primary humoral regulator of erythropoiesis, promoting both proliferation and survival of erythroid precursors. EPO production is regulated by tissue oxygenation. Approximately 90% of EPO is synthesized in renal peritubular interstitial cells that respond to an oxygen-sensing mechanism, an hypoxia-response pathway that is upregulated by oxygen deprivation. In response to hypoxia, additional EPO-producing peritubular cells

are recruited to supplement the cells that constitutively synthesize EPO; when the hypoxic stimulus is removed, the recruited cells return to their non-secretory state [16, 17]. The mediator of this pathway is HIF-1, a transcription activator of at least 20 genes involved in erythropoiesis and angiogenesis, including EPO, transferrin, the vascular endothelial growth factor (VEGF), and a series of genes central to glucose metabolism. HIF-1 binds to the hypoxia-response element in the target hypoxia-response genes to activate their transcription [18].

HIF-1 is a heterodimeric transcription factor consisting of two sub-units: the oxygen-insensitive HIF-1β sub-unit is expressed constitutively at a relatively constant concentration independent of tissue oxygenation; the oxygen-sensitive HIF-1α sub-unit, also expressed constitutively, is degraded rapidly (with a half-life less than five minutes) in cells supplied with adequate oxygen, but accumulates when cells are exposed to low-oxygen stress. The transcriptional activity of the heterodimer HIF-1 depends upon the availability of the HIF-1α sub-unit [19, 20].

HIF-1α is regulated by two hydroxylases that require molecular oxygen as a co-substrate: targeted proline hydroxylation mediates HIF-1α degradation [21], and specific asparagine hydroxylation events regulate HIF-1α activity [22] (Fig. 2). Under normoxic conditions, proline residues within the oxygen-dependent degradation domain of HIF-1α are hydroxylated. The hydroxylation is catalyzed by an Fe^2-dependent prolylhydroxylase, where oxygen is the essential co-substrate and appears to be the rate-limiting factor in this reaction [21]. This event targets HIF-1α for proteasomal degradation as the hydroxylated proline sites are recognized by pVHL (von Hippel-Lindau protein): In the cytoplasm, pVHL is present in a complex with an E3 ubiquitin protein ligase [23]. Under hypoxic conditions, the lack of oxygen prevents proline hydroxylation and HIF-1α is not tagged by pVHL for degradation; HIF-1α rapidly moves into the nucleus and binds with HIF-1β to form the active heterodimeric HIF-1 transcription factor [24].

A second hypoxia-sensitive site is present in the carboxyl-terminal transactivation domain (CAD) of HIF-1α [25]. In the normoxic state, an asparagine residue in CAD is hydroxylated by an Fe^2-dependent arginine hydroxylase, with oxygen as co-substrate. Arginine hydroxylation prevents the association of CAD with the transcription co-activators, CBP and P300. In the hypoxic state and the absence of arginine hydroxylation, CBP and P300 association with CAD enhances the binding of HIF-1α to the hypoxia-responsive element sites on target genes. This increased transcriptional activity may provide the "fine tuning" for the enhanced synthesis of EPO, transferrin, VEGF, and other factors that maintain oxygen homeostasis.

In tumors associated with an absent or mutated pVHL (e.g., in clear cell renal carcinoma), degradation of hydroxylated HIF-1α is decreased, the HIF-1 dimer accumulates and continues to stimulate gene transcription even under normoxic conditions; the enhanced angiogenesis due to upregulation of VEGF and the increased glycolytic activity are of major significance in the develop-

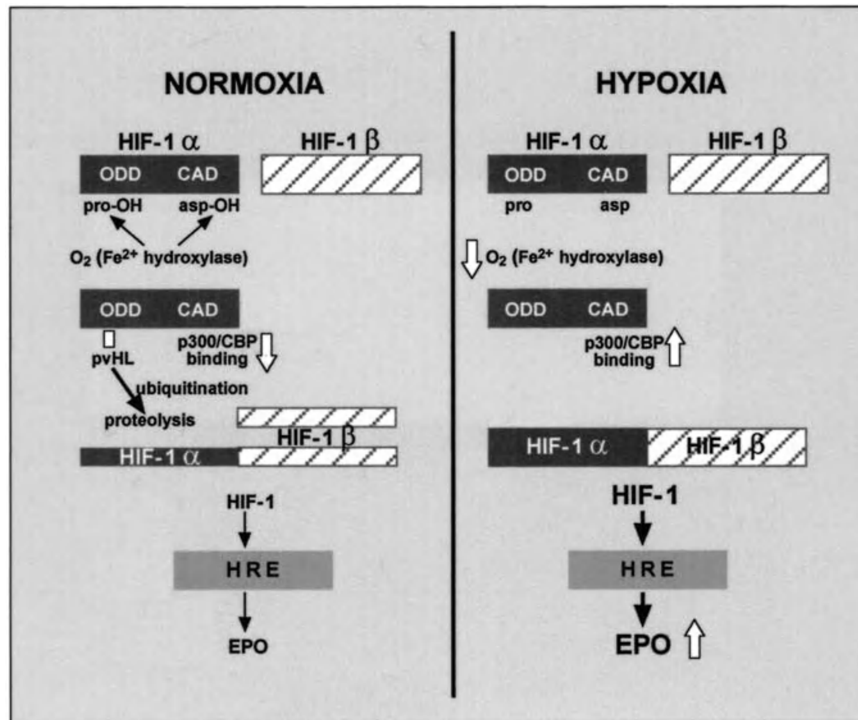

Figure 2. Regulation of transcription factor HIF-1: role of oxygen-dependent hydroxylases, co-activators of transcription and pvHL-targeted proteolysis. CAD = carboxy-terminal transactivation domain; ODD = oxygen-dependent degradation domain; pro = proline sites; asp = asparagine sites; P300/CBP = co-activators of transcription; pVHL = von Hippel-Lindau protein; HRE = hypoxia response element. (Israels LG and Israels ED, 2000. Used with permission of Core Health Services Inc.)

ment and growth of these tumors [26]. In the von Hippel-Lindau syndrome, the activity of the tumor suppressor protein pVHL is lost, resulting in the development of multiple vascular tumors from unregulated VEGF production and, in some patients, erythrocytosis due to increased EPO synthesis [27, 28].

The tumor suppressor protein p53 also is a regulator of HIF-1α: p53 recruits the E3 ligase MDM2 to promote ubiquitin-mediated proteasomal degradation of HIF-1α. Mutations in the p53 gene are present in more than 50% of human tumors. Loss of p53 may result in HIF-1α overexpression, increased VEGF synthesis, and the angiogenesis associated with some highly vascular renal tumors [29].

EPO stimulates red cell production, differentiation, and maturation, and prevents apoptosis. The focus for EPO regulation of erythropoiesis is probably at the level of the CFU-E. When there is an increase in circulating EPO, the existing CFU-E respond by proliferation and differentiation; when EPO concentrations decrease, CFU-E undergo apoptosis. A stable constitutive EPO

concentration maintains CFU-E production at a rate appropriate to physiologic demand. Anemia is a common finding in patients with chronic renal failure. EPO production is reduced as the renal cells responsible for EPO synthesis disappear. Inflammatory cytokines inhibitory to CFU-E colony formation contribute to the development of anemia [30].

Erythrocytosis with high plasma EPO concentrations most commonly is associated with lifestyle or disease that results in the development of hypoxia, including chronic lung disease, residence at high altitude, cyanotic congenital heart disease, or chronic carbon monoxide intoxication in cigarette smokers. Congenital familial polycythemia is associated with decreased oxygen release by high-affinity hemoglobins, congenital methemoglobinemias, or congenital 2,3-BPG deficiency with a left shift in the oxygen dissociation curve [31]. A familial autosomal-recessive erythrocytosis, with high EPO concentrations, has been described in the Chuvash region of the Russian Federation [32]. The specific etiology has not been determined but may be related to an abnormal renal oxygen sensor or HIF-1 deregulation.

Erythropoietin receptor (EPOR)

EPOR (Fig. 3) is a member of the cytokine-receptor superfamily, characterized by an extracellular-binding region, a transmembrane region, and an intracellular domain [33]. The receptor first appears in small numbers in early BFU-E, increases in CFU-E and pronormoblasts, and declines in the late erythropoietic cells. The binding of EPO results in homodimerization of the receptor, or spatial rearrangement of a preformed receptor dimer, to initiate transmission of a signal to the intracellular domain with subsequent activation of signal transduction pathways [33–35]. Although the receptor lacks intrinsic growth-promoting kinase activity, the activated receptor induces secondary tyrosine kinase activity in a number of associated cytoplasmic proteins, and recruitment of the cytoplasmic tyrosine kinase JAK2 results in JAK2 phosphorylation of specific tyrosine sites on EPOR. The phosphorylated tyrosine residues on the receptor serve as docking sites for the cytoplasmic transcription factor STAT/5 (signal transducer and activator of transcription); phosphorylation of STAT/5 facilitates dimerization and translocation to the nucleus where it activates gene transcription. Phosphorylation of other cytoplasmic effector proteins, including P13-kinase and Ras, induce additional signaling pathways that contribute to gene response and cell activation [36].

The EPO-initiated signal transduction pathway is highly regulated, with molecules in the signaling pathway activated within minutes of EPO binding, and the signal terminated within a few hours. The rate at which the signal is turned off is determined by the net effect of several factors, including deprivation of EPO, SHP-1 phosphatase dephosphorylation of a specific site in the cytoplasmic domain of EPOR, and activation of negative-regulators of JAK kinase and STAT/5 transcription [37].

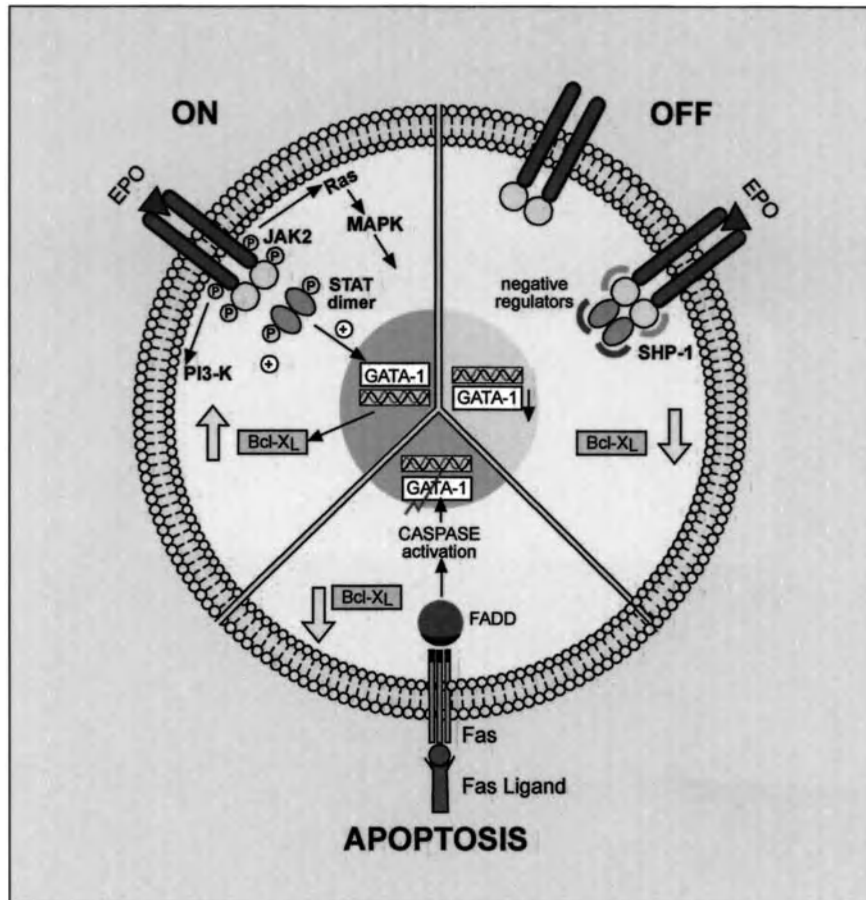

Figure 3. Regulation of the erythropoietic response. ON = EPO engagement of its receptor with transmission of signal through JAK2 and STAT/5 and amplification by PI3-K and Ras. OFF = absence of EPO; negative regulators of the EPO receptor (EPOR) including SHP-1 phosphatase. Apoptosis = Fas/FasL activation of death pathway. Presence or absence of GATA-1 and Bcl-X_L activity is indicated in each segment. (Israels LG and Israels ED, 2000. Used with permission of Core Health Services Inc.)

Primary familial erythrocytosis is an autosomal-dominant disease due to a mutated EPOR gene, the result of missense or frame shift mutations that affect the binding site for SHP-1. The abnormal cytoplasmic tail of the receptor is either truncated or is insensitive to the SHP-1 phosphatase that normally turns off the EPO signal [38, 39]. The mutated receptor is hyper-responsive to the EPO ligand and, as a result, the proliferative response is maintained in the presence of a minimal EPO stimulus. Patients are reported to have early cardiovascular disease (although one propositus of a Finnish family won three Olympic gold medals in cross-country skiing, possibly the advantage of an

increased hematocrit in this cardiovascular-demanding sport) [40, 41]. (See Chapter 13 for further information.)

Polycythemia vera, a myeloproliferative disease, is due to a clonal somatic mutation in hematopoietic precursor cells that results in red cell overproduction independent of EPO regulation. The amount of circulating EPO may be either normal or low. The increased red cell production, frequently associated with concomitant increases in platelets and granulocytes, represents a clonal proliferation at the level of the hematopoietic stem cell or CFU-GEMM. A deregulated increase in the expression of the apoptosis inhibitor bcl-X_L, possibly due to a functional deletion of a negative-regulatory mechanism in signal transduction, may allow these cells to escape normal apoptotic regulation [42]. Early in the disease, a mixed population of red cell precursors exists, but over time, the hyper-responsive polycythemia vera clone increases and dominates, exhibiting a survival advantage over that of the normal red cell precursors.

"Olympic polycythemia" describes the erythrocytosis associated with the use of rHuEPO to boost hemoglobin concentrations in sports requiring major cardiopulmonary output. Because rHuEPO differs from physiologic EPO at a significant number of glycosylation sites, the exogenous rHuEPO can be detected in the blood of these athletes [43]. (See Chapter 13 for further discussion.)

Other erythropoietic signaling pathways

The GATA-1 transcription factor expressed in erythroid precursors is essential for their development and survival as it regulates the amounts of heme synthetic enzymes, globin, membrane proteins, and other red cell components. GATA-1 recognizes conserved DNA-GATA (guanine, adenine, thymine, adenine) motifs in the promoter and/or enhancer regions of many erythroid-expressed genes. Both EPO and GATA-1 strongly upregulate expression of the anti-apoptotic gene bclX_L, thus enhancing erythroblast survival [38, 39].

Angiotensin II (ATII) is well recognized as a regulator of renal hemodynamics and blood pressure. *In vitro* studies have demonstrated that ATII can stimulate the proliferation of BFU-E colonies in the presence of EPO [44]. The ATII type-1 receptor (AT1R) has been identified on BFU-E–derived cells [45]. Whether ATII binding induces intracellular signaling by an independent or a shared pathway with EPO is not clear. (In rat aortic smooth muscle cells, binding of ATII to its receptor initiates signaling through the JAK/STAT pathway [46].) 10–20% of patients receiving a renal transplant develop erythrocytosis although EPO concentrations may remain within the normal range. The enhanced erythropoiesis can be controlled by the administration of angiotensin-converting enzyme (ACE) inhibitors. ATII concentrations decrease without reducing the amount of EPO. ACE inhibitors also have been found to reduce the hematocrit in high altitude-associated polycythemia [47].

The role of IGF-1 is unclear. In *in vitro* cell culture systems, IGF-1 can substitute for EPO, using a signaling pathway that appears to be independent of EPO. It may serve as a substitute for EPO as the erythropoietic stimulus in anephric patients who maintain a low but significant amount of erythropoiesis. Erythroid cultures from patients with polycythemia vera are hyper-responsive to IGF-1, suggesting it may have a role in the EPO-independent erythropoiesis in this myeloproliferative disease [48, 49].

Apoptosis

A network of cytokines, including EPO, are required for normal growth and differentiation of erythroid cells. The cytokines essential for the viability of these cells inhibit apoptosis by augmenting the transcription of apoptosis-suppressing genes such as bcl-2 and bcl-X_L [50]. In contrast, inflammatory cytokines such as tumor necrosis factor (TNFα) and interferon (IFNγ) can activate the death signal in erythroid progenitor cells through upregulation of the FasL/Fas apoptotic pathway [51]. Binding of the FasL to Fas activates the proteolytic caspases that cleave a number of intracellular proteins, including GATA-1, with the subsequent loss of bcl-X_L [52].

A second distinct Fas/FasL pathway is intrinsic to the erythroblast cell line. Fas is present on both early and late erythroid precursors, but its activating ligand (FasL) appears only on late erythroblasts. The expression of FasL may serve to regulate erythropoiesis within the erythropoietic islands of the bone marrow as the mature erythroblasts expressing FasL induce apoptosis of their Fas positive immature precursors [53].

Acknowledgements
The authors thank Marie Field for her secretarial expertise in the preparation of the manuscript.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Studies of erythropoiesis and the discovery and cloning of recombinant human erythropoietin

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Introduction

Patients who are anemic because of chronic kidney disease, cancer, arthritis, or chemotherapy or radiation therapy often report fatigue [1–6]. Before the introduction of recombinant human erythropoietin (rHuEPO), anemia and its sequelae fatigue were treated with red blood cell transfusion, androgen stimulation of red blood cell production, and/or iron supplementation, among other treatments [7]. While effective in increasing red blood cell counts, both transfusions and androgen therapy have inherent risks [8]. Transfusions of red blood cells can be complicated by blood-borne pathogens, iron overload, immunologic consequences, and lack of or delayed hemoglobin response. Transfusions often improve but do not correct anemia and usually must be given frequently, and androgen therapy can cause viralization or abnormal liver function. rHuEPO is an ideal therapy because it mimics the action of the endogenous hormone by stimulating the production of red blood cells. Patients with chronic kidney disease are unable to produce adequate amounts of endogenous erythropoietin (EPO) to stimulate red blood cell production. Patients with cancer often have damaged bone marrow, with or without the insult of chemotherapy, that does not completely respond to the endogenous hormone.

The cloning of the human *EPO* gene by Fu Kuen Lin and colleagues was a difficult and frustrating endeavor. This milestone and the subsequent creation and production of rHuEPO as a therapeutic option was a breakthrough that has enabled physicians to ameliorate anemia and its sequelae. Patients treated with rHuEPO report a return to more normal lives. rHuEPO is the standard of care for treatment of anemia in patients with chronic kidney failure or receiving chemotherapy, and in other disease settings.

This chapter is a literature review of the history of the early work in erythropoiesis and the discovery and cloning of EPO. Other chapters discuss its commercial production and the clinical uses of rHuEPO.

Early studies in erythropoiesis

Bright [9] is credited with being the first scientist to recognize that anemia was a complication of kidney disease, but Jourdanet [10] has been credited as the first scientist to observe the relationship between altitude and blood viscosity. Jourdanet noted the similarity of symptoms reported by patients with altitude sickness and the symptoms reported by patients who had experienced severe blood loss. Several years later, Viault [11, 12] expanded knowledge of red blood cells and the effect of altitude on them, and quantified the change in red cell counts as altitude increased. On a train trip from the city of Lima, Peru to the high-altitude tin mines of that country, he repeatedly sampled his blood, blood of willing fellow travelers, and blood of a dog, rooster, and llama. Viault noted an increase in his red blood cell count from $5 \times 10^6/mm^3$ to $8 \times 10^6/mm^3$ during the ascent to higher altitude.

Other early scientists continued studies in an attempt to understand the mechanism of erythropoiesis in rabbits [13] and immigrants to the high Alps [14, 15]. One theory proposed at the time to explain the polycythemia seen at high altitudes was that low oxygen pressure directly stimulated bone marrow to increase red blood cell production. This theory held for nearly 50 years.

In 1906, Carnot developed the concept of humoral regulation of erythropoiesis [16–18]. Serum from anemic rabbits was injected into normal rabbits, and caused an increase in the red blood cell counts of the normal rabbits. Carnot suggested that "hemopoietine" present in the serum of anemic rabbits was responsible for the increase in cell numbers. Many other investigators repeated these experiments in anemic rabbits or rabbits raised at high altitudes [19–23].

For almost 30 years, researchers continued to repeat Carnot's work. Because some investigators were successful and others were not, controversy continued about the mechanism of erythropoiesis. Finally, Erslev [24] modified Carnot's original study: He injected large amounts of plasma from anemic rabbits into normal rabbits and found that the number of nucleated red blood cells in the bone marrow, the number of peripheral reticulocytes, and the hematocrit of the normal rabbits increased. This study suggested that red blood cell production is mediated by a humoral factor in rabbits. Four years later, Jacobson et al. [25] demonstrated that this factor, EPO, was produced by the kidney. Progress was being made in understanding the relationship between oxygen supply and demand of the body and EPO and erythropoiesis; however, despite more than 100 years of research, nothing was known about the structure of EPO or the *EPO* gene. Some debate continued whether EPO was produced as an inactive precursor in the kidney that was activated in some other tissue or organ.

The role of kidney and bone marrow in erythropoiesis

In the fetus, the liver is the primary site of endogenous EPO production [26]. In the adult, EPO is produced primarily (i.e., >90%) in the adult kidney [25, 27]. The liver [28, 29] and the brain [30] both synthesize some EPO, but the amount produced by these tissues alone is insufficient to maintain adequate erythropoiesis. Thus, kidney disease causes anemia due to loss of the main source of EPO production.

An oxygen sensor within renal cells detects the oxygen content of the blood and the kidney regulates the amount of EPO released into the blood (Fig. 1). The hormone acts on red blood cell precursor cells in the bone marrow to stimulate their proliferation and maturation and to increase the number of red blood cells in the peripheral circulation. The feedback loop is completed when the kidney cells recognize the change in oxygen delivery secondary to the

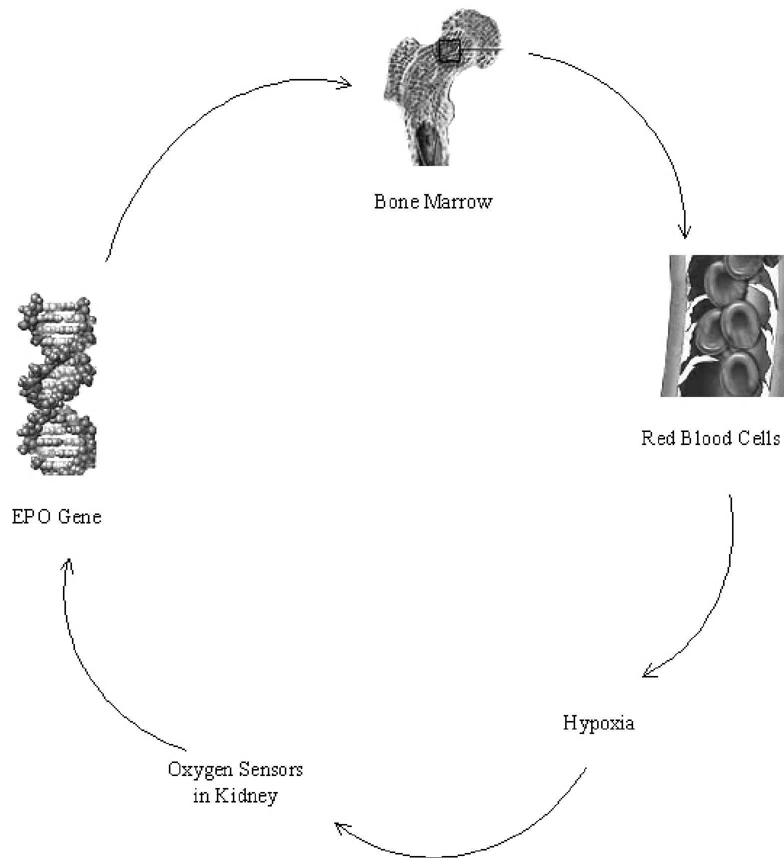

Figure 1. Relationship of kidney and bone marrow in production of red blood cells. (Figure courtesy of Amgen.)

change in the number of circulating red blood cells. Production of new red blood cell slows until the sensor cells recognize a need for increased erythropoiesis. Some EPO, albeit in trace amounts, is always detectable in the circulating blood, even in patients with total kidney failure, suggesting that a subset of cells provides a continuous output of EPO even when oxygen delivery is normal.

Isolation of EPO from urine

Even though the amounts of endogenous EPO increase under conditions of hypoxia or anemia, EPO represents a minor fraction of the total protein in the blood. A significant obstacle to the development of EPO as a therapeutic agent was the difficulty in isolating and purifying adequate amounts of the hormone to allow for its characterization. Several groups attempted to purify human [31, 32] and sheep [33, 34] EPO. The results were inconclusive, with the purity of the product questionable or produced in insufficient amounts to allow chemical characterization.

In 1977, Miyake, Kung, and Goldwasser succeeded in isolating and purifying milligram amounts of EPO from 1500 L of urine from patients with aplastic anemia [35]. Patients with aplastic anemia characteristically overproduce EPO, however the amounts present are still small. Large volumes of urine were essential for the recovery of a sufficient amount to purify EPO.

Miyake et al. [35], using a seven-step process that included ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography, produced a preparation with a potency of 70,400 units/mg protein, 21% yield, and a purification factor of 930. This method allowed the production of enough material to partially characterize the hormone. With a source of EPO now available, a strategy for the cloning and expression of the human gene was devised.

Cloning of the *EPO* gene

In the 1980s, the nucleic acid sequences for cynomalogous monkey and human EPO were finally isolated and characterized, each by a different method. Several hurdles needed to be overcome, including the limited amount of data about the primary structure of human or monkey EPO, lack of a known source of mRNA, no information about the genomic structure of the gene, lack of simple tests to confirm that the cloned sequence encoded the *EPO* gene, and controversy about the induction mechanism for EPO production due to hypoxia. The difficulty in cloning the gene was further complicated by an inability to determine which step in the process was responsible for the failure.

In 1981, Goldwasser presented the first 26 amino acids of the protein at a meeting. Sue and Sytkowski [36] published the sequence in a paper that

described the development of polyclonal antibodies to EPO. The Sue and Sytkowski paper was subsequently shown to have two errors, unknown at the time, in the amino acid sequence. Another group [37] published a putative amino acid sequence for the first 31 amino acids of EPO, but it was proved to be erroneous, as it had the same two errors reported by Sue and Sytkowski as well as three additional errors in the amino acid sequence, again unknown at the time of publication. Goldwasser had provided Lin and colleagues at Amgen Inc. with the sequence he and his colleagues had obtained for the first 26 amino acids of human EPO, but of course, none were aware at the time of the errors. They were aware of the possibility that the available peptide sequence may overlap the intron-exon boundary of the *EPO* gene thereby preventing successful cloning using oligonucleotide sequences based on the peptide sequence. Earlier, Goeddel et al. [38] had successfully sequenced, cloned, and produced recombinant human insulin. This protein has only 51 amino acids, compared with EPO's 165 amino acids, and required nearly 10 years of work to sequence. With newer biotechnology techniques, it was naively thought that the sequencing of EPO would be easy. Lin needed not only to isolate the gene with no knowledge of its structure or of a simple way of confirming that the gene was in hand, but also to express the gene in a suitable host cell to provide a product with the proper structure, including the carbohydrate and polypeptide components of the molecule.

Lin used many approaches, including the standard gene-cloning routes known at the time, all of which failed. He persisted and eventually succeeded only because he used a technique far more complex than any technique tried earlier. This novel approach involved the use of multiple sets of fully degenerate oligonucleotide probes to screen a human genomic library. Two small pools of oligonucleotides corresponding to short fragmented samples of EPO amino acid sequences were used. Both pools, one of 20 nucleotides and the other of 17 nucleotides, had low codon degeneracy. Because of the degeneracy of the genetic code, the same amino acid can be encoded by more than one codon; Lin and colleagues accounted for every possible codon that encoded these putative amino acid sequences necessitating 128 different probes in each pool. The probes were labeled with radioactive phosphorus to identify any matches of a single probe with the human genome. The gene library on which the probes were tested consisted of the total human genome, fragmented into pieces 10,000 to 20,000 nucleotides long. Lin and colleagues found that probes in both mixtures hybridized with four of the 1.5 million clones in a human fetal liver genomic library [39]. Analysis of these clones showed that at least one contained the entire coding region of the human gene for EPO and it was the basis for developing the expression system using transfected Chinese hamster ovary (CHO) cells. In parallel with this effort, cDNA from the kidneys of anemic monkeys was prepared using mixed probes based on the human EPO peptide sequences [40] and the monkey gene also was cloned.

After Lin and his colleagues successfully cloned the gene, Jacobs and colleagues [41] also cloned the human *EPO* gene using degenerate oligonu-

cleotides and a peptide sequence derived from urinary EPO supplied by Miyake. Cloning of additional *EPO* genes from other species used the sequence information from the human and monkey genes and proceeded rapidly. The mouse *EPO* gene was cloned using monkey and human EPO DNA segments as hybridization probes [42, 43]. Subsequently, *EPO* genes from other species were cloned by hybridization or by polymerase chain reaction using probes or primers based on the known EPO sequences, including the genes from the rat [44], pig [45, 46], sheep [45, 47], and cow [48], among other animals.

Expression of the *EPO* gene

Clones of the gene for EPO were inserted into CHO cells, which synthesized the 193 amino acid precursor protein, removed the signal peptide and carboxy-terminal arginine, added *N*- and *O*-linked carbohydrate to glycosylation sites, and released the mature protein into the culture medium. Immunologic, biologic, and biochemical assays showed that the recombinant hormone had the *in vivo* biologic activity and was immunologically equivalent to human EPO, as revealed within the limits of the available assays [39]. EPO expressed by CHO cells has a molecular weight of 30.4 Kd and contains 40% carbohydrate [49].

Discussion

The isolation and expression of the gene for human EPO was a major achievement that capped almost 155 years of exploration into the nature of anemia and the production of red blood cells. EPO is present in minute quantities in the blood, and difficulty in isolating and purifying the hormone in amounts that would allow investigation of its properties posed a significant obstacle to the development of EPO as a therapeutic agent. After Miyake, Kung, and Goldwasser developed a technique to purify urinary EPO, a strategy for the cloning and expression of the human gene could be devised. The innovative approach of Lin et al. allowed the successful isolation, cloning, sequencing, and development of the recombinant protein. Large-scale production began and clinical trials started. The recombinant protein, epoetin alfa, produced dose-dependent increases in erythropoiesis that paralleled the expected response to endogenous EPO. The commercial production of epoetin alfa has been translated into benefits for millions of patients (Tab. 1). Epoetin alfa ameliorates the debilitating symptoms of anemia and allows these patients to have more normal lives. Other chapters in this volume will elaborate on the clinical use, production, formulation, and other important and evolving aspects in the study of rHuEPO.

Table 1. Some benefits of epoetin alfa therapy

- Increased exercise tolerance
- Improved central nervous system function
- Reduced heart enlargement
- Reduced extreme fatigue
- Increased ability to perform daily functions of life
- Reduced risk of alloimmunization in transplant recipients
- Improved coagulation

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Structural basis for the signal transduction of erythropoietin

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Introduction

The structures of recombinant human erythropoietin (rHuEPO), erythropoietin receptor (EPOR), and several novel peptides have been extensively studied using a variety of methods including X-ray crystallography and nuclear magnetic resonance (NMR) [1, 2]. EPOR has been classified as a member of the class 1 cytokine receptor super-family [3, 4], and is activated when EPO binds to the extracellular portion of the receptor causing oligomerization [5]. The dimerization of the extracellular portion of the receptor induces tyrosine kinase proteins associated within the cytoplasmic side of the receptor to initiate the signal transduction events, which eventually cause the committed erythroid colony-forming units (CFU-E) progenitor cells to mature into red blood cells [6] (Fig. 1).

Studies on novel molecules, which cause two EPOR to form a dimer, have shown that EPOR is promiscuous, as a variety of structurally unrelated molecules can bind and activate the receptor. This oligomerization of the EPOR can be caused by an engineered extracellular disulfide bond in EPOR that can stimulate constitutive signal transduction [7]. Antibodies with epitopes that react with the extracellular domain of EPOR also stimulate EPOR [8], as well as several different peptides that show both agonist as well as antagonist activity [9–12]. In addition to rHuEPO, a novel hyperglycosylated form of EPO (darbepoetin alfa) has shown activation of EPOR with improved pharmacologic properties. The common modality for all the molecules that cause signal transduction is that they all result in the formation of an EPOR dimer. It was initially speculated that EPO functioned mechanistically as a cross-linker that brings the intracellular portion of EPOR into close proximity, producing the signal transduction events that eventually allow the cell to mature into a red blood cell. Recent data, however, suggests that EPOR may initially form a dimer on the cell surface [13], and that the function of EPO is to provide the proper orientation outside the cell, thereby allowing the intracellular kinases to be in the precise geometry for biologic activity.

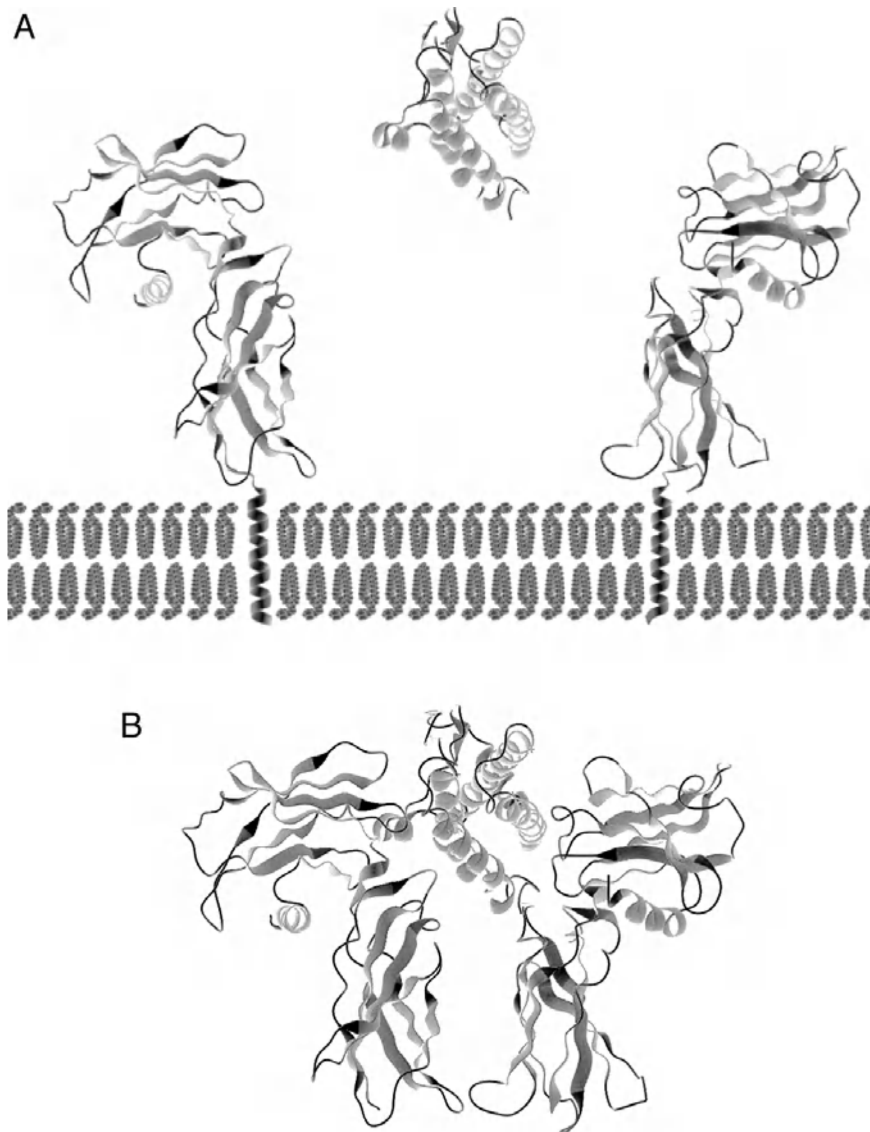

Figure 1. Panel A: Model depicting the structures the two EPOR embedded into the membrane of committed CFU-E progenitor cells. EPO, which is produced in the kidney and released into the bloodstream, functions by binding two receptors, causing dimerization. The complex of EPO and two EPOR allows the precise geometry required by intracellular portion of EPOR (not shown in this diagram) for biologic activity. The relative proximity of the two intracellular regions caused by the mechanical association of EPO with EPOR allows the association and phosphorylation of kinases, which is the intiating event that allows the cell to mature into a red blood cell. Panel B: The X-ray crystallographic structure of EPO bound to two EPOR. The structure resolved by Syed et al. [1] provides the empirical data about the details of the interaction between EPO and its natural receptors. Figures courtesy of Timothy Osslund.

rHuEPO structure

The fundamental issue in resolving the X-ray crystallographic structure of rHuEPO ligand and receptor was obtaining a construct of rHuEPO that was suitable for crystallization [14]. Proteins that are highly glycosylated, such as rHuEPO, are often not good candidates for crystallization; when proteins are expressed in bacteria, the unglycosylated protein often forms aggregates. Aggregate formation presumably occurs because the residues that were covered by the carbohydrate structures are now exposed. Multiple constructs were put through the screening process until a suitable crystallization candidate could be determined. The first significant structural data came when the structure of the receptor and an EPO peptide mimetic was first revealed [10]. Within a few years of discovering this breakthrough structure, the structures of the rHuEPO/EPOR complex and the NMR structure of the unbound ligand [2] were published. These data provided further insight into the structure of the ligand and its structural relationship to the receptor.

rHuEPO has a structural topology that is similar to other members of the cytokine hormone family. The characteristic topology of rHuEPO, and cytokines in general, is a core structure composed of a four helical bundle (Fig. 2). The structure comprises one pair of anti-parallel long helices, A (residues 8 to 26) and D (residues 137 to 161), and another pair of shorter helices, B (residues 56 to 83) and C (residues 90 to 112). A long loop connects the first and second helices (AB), and a short loop connects the second and third helices (BC) and another long loop connects the third and fourth helices (CD). This configuration has been termed the "up-up-down-down four-helical bundle topology" [15]. The core bundle has a left-handed twist and the crossing angles of the helices (–148° to 167°) [2] also provide a further classification of the cytokine family. Before the elucidation of the structure of rHuEPO, most cytokines could be classified as either having long helices and short crossing angles (e.g., granulocyte colony-stimulating factor [G-CSF] and interleukin-2 [IL-2]) or short helices with long crossing angles (e.g., granulocyte-macrophage colony-stimulating factor [GM-CSF] and stem cell factor [SCF]) [16]. Often the short helical cytokines have two anti-parallel beta strands that structurally link the long loops AB and CD. The rHuEPO structure illustrates a hybrid of the two classifications having longer helices and shorter crossing angles than shorter helical cytokines. Inconsistent with the longer helical classification, EPO has an anti-parallel beta sheet: β1 (residues 39 to 41) and β2 (residues 133 to 135).

The first of two disulfide bonds structurally link together the amino terminus and the carboxy terminus at residues Cys7 to Cys161. The proximity of this disulfide bond relative the amino terminus and carboxy terminus is presumable to hold the four helical bundles together at one end while a beta strand and second disulfide bond stabilize the quaternary structure of the protein at the opposing end. The second disulfide, Cys29 to Cys33, links the end of helix A with the AB loop. As predicted by Bazan [3], the hydrophobic core residues on helix D

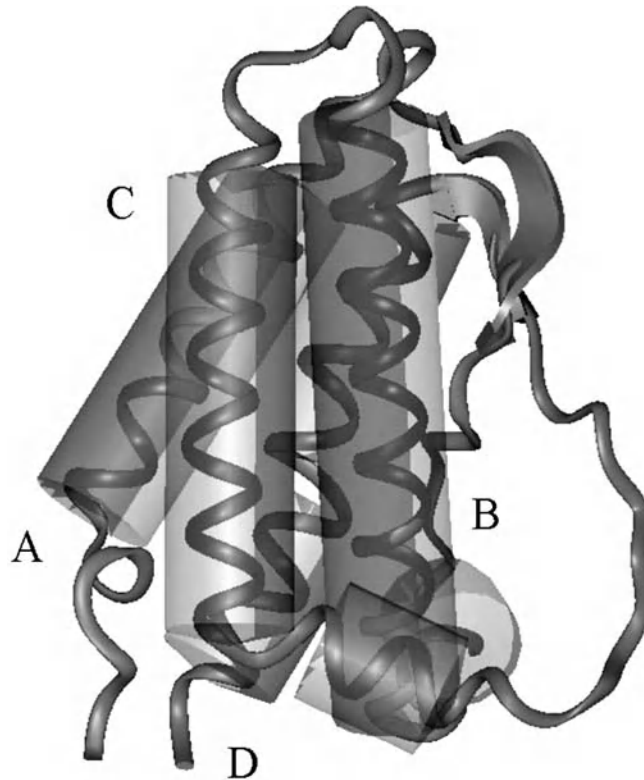

Figure 2. A secondary structure depiction of rHuEPO. rHuEPO has a structural topology that is similar to other members of the cytokine hormone family. The characteristic topology of EPO, and of cytokines in general, is a core structure composed of four helices. One pair of anti-parallel long helices, A (residues 8 to 26), and D (residues 137 to 161), and another pair of short helices, B (residues 56 to 83), and C (residues 90 to 112) complete the helical bundle.

pack against the hydrophobic residues of helices A, B, and C, which reflect the pattern of the approximate 3.5 residues per turn in a standard α-helix.

In the X-ray structure of rHuEPO bound to EPOR, two additional short helices are found: the B' helix (residues 47 to 52) orthogonal to B and the mini-helix C' (residues 114 to 121) following C with a 90° tilt beginning at Gly113. The second mini-helix is not found in the NMR structure and may be an artifact of the mutations made in this area to develop a protein suitable for crystallization.

Structure of EPOR

The extracellular domain of the rHuEPO receptor has two β sandwich domains, designated D1 (*N*-terminal) and D2 (*C*-terminal), each of which

shares an overall topology with the fibronectin type III fold. The D1/D2 domains are almost perpendicular to one another and form an inverted “L” with the ligand-binding site located between the monomers. Consistent with the cytokine family of receptors, the EPOR has a WSXWS box that is a sequence of amino acids containing tryptophan and serine that are essential for the biologic activity of the receptor [17].

In addition to the predominantly β-sheet structure, an α-helix is found at the amino terminus, which may have important structural implications (Fig. 3). Adopting the position inside the “elbow” of the D1 and D2 domains, the α-helix makes extensive contacts with both D1 and D2 domains of the receptor, with buried surface area of approximately 200 $Å^2$. The α-helix is amphipathic, having both hydrophobic and hydrophilic regions, and presents several residues towards a hydrophobic patch on the D1 domain of the receptor. Phe11 and Leu18 of the α-helix form the basis of hydrophobic interactions with Phe39, Leu27, and Phe29 in the D1 domain. *N*-terminal to the WSXWS box

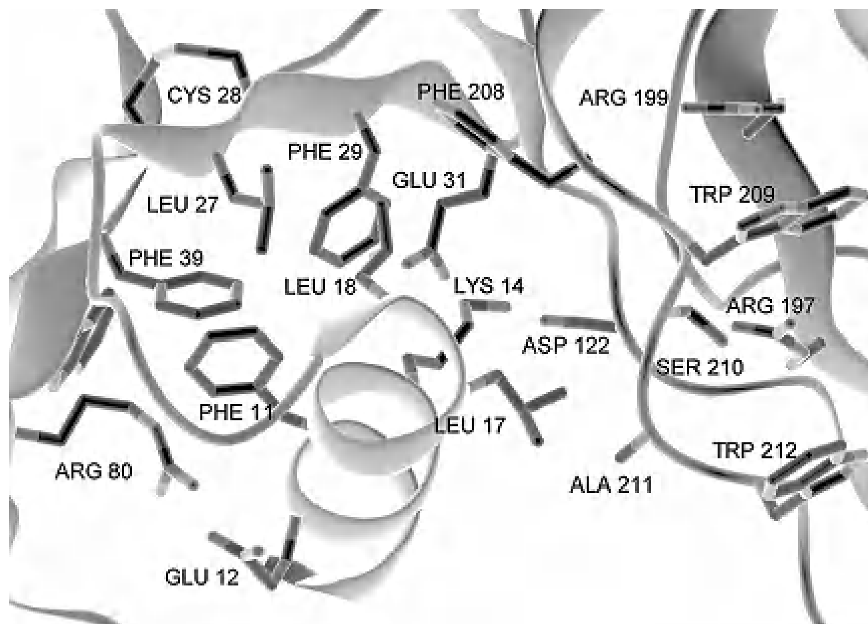

Figure 3. Amino acid contacts of amino terminal α-helix of EPOR. Ribbon representation of the soluble EPOR. Selected residues at the helical interface are depicted using a stick model. Residues 209 to 212 represent the putative cytokine family WSXWS box. The EPOR *N*-terminal α-helix is amphipathic and presents several hydrophobic residues towards a hydrophobic patch on the D1 domain of the receptor. Phe11 and Leu18 of the α-helix form the basis of the hydrophobic interactions with Phe39, Leu27, and Phe29 in the D1 domain. *N*-terminal to the WSXWS box on domain D2, Phe208 also makes contacts with this hydrophobic pocket. Two electrostatic interactions occur between the α-helix and the rest of the protein. One salt bridge is formed between Glu12 and Arg80, and the other salt bridge links all three domains by the electrostatic interactions of Lys14 with Glu31 (on domain D1) and Asp122 (on domain D2).

domain D2, Phe208 also makes contacts with the hydrophobic pocket. Two electrostatic interactions occur between the α-helix and other regions of EPOR. Several salt bridges function to link the α-helix with D1 and D2, including the electrostatic interactions of Lys14 with Glu31 (on domain D1) and Asp122 (on domain D2).

Alteration of the WSXWS sequence disrupts ligand binding and receptor signaling. The WSXWS motif has been shown to be critical for the folding and transport of the receptor to the cell surface, and an A211E mutation further improved the efficiency of the processes. In the structure of an rHuEPO-EPOR complex, Glu211 occupies the X position. The side-chain of Glu211 in the WSXWS motif is closest (<4.5 Å) to Leu17 of the *N*-terminal receptor helix. Additionally, the side chains of the tryptophans in the WSXWS motif, residues Trp209 and Trp212, sandwich the hydrophobic side chain of Arg197 in the receptor fold, while Ser210 and Ser213 are within hydrogen-bonding distance of Ala198 and Val196, respectively. The observation that the WSXWS motif interacts with with the *N*-terminal helix and the β-sheet residues (Val196, Arg197, and Ala198) in the rHuEPO-EPOR complex, suggests that the *N*-terminal helix may be important in stabilizing the folded EPOR through an interaction with WSXWS. The relationship of WSXWS, the α-helix, therefore, may be essential in holding D1 and D2 in the precise geometry allowing dimerization and signal transduction of the ligand.

The rHuEPO-EPOR complex is a 2:1 stoichiometry, and the receptor molecules are held together through two distinct surface areas on rHuEPO that are located on opposing faces of the molecule. These rHuEPO/EPOR interfaces have been identified as high-affinity (Kd approximately 1 nM) and low-affinity (Kd approximately 1 μM) binding sites and are referred to as site 1 and site 2, respectively. Site 1 comprises rHuEPO residues from helices A, B', D, and part of the AB loop; site 2 comprises the A and C helices, exclusively. Presumably site 1 binds more tightly than site 2 because of the larger number of residues participating in the interaction of the first site compared with the second site. Site 1 is characterized by a hydrophobic-binding region in the middle of the binding site with flanking amino acids that allow electrostatic interactions with EPOR. EPOR loops L1, L5, and L6 interact with the hydrophobic rHuEPO residues with Phe93 of EPOR as the dominant hydrophobic contact. Phe93 is a pivotal residue firmly held in place by hydrogen bonding to rHuEPO residues Thr44 and Asn147. Mutagenesis has shown that Phe93 on the receptor is a critical rHuEPO-binding determinant; and mutation of this residue eliminates any detectable binding [18]. The site-1 binding site of rHuEPO also has contributions from the A-B linker polypeptide residues (Thr44-Phe48) of rHuEPO. The hydrophobic binding surface of site 1 itself is surrounded by hydrophilic interactions with Asn147 of rHuEPO functioning as a major contributor to the electrostatic interactions. Asn147 binds to EPOR using three hydrogen bonds, one of which is between its Nδ atom and the carbonyl oxygen of Phe93. On either side of the Asn147 in EPO

are arginine residues, Arg143 and Arg150, which form salt bridges with acids Glu60 and Glu117 of rHuEPO, respectively.

rHuEPO site-2 interactions with EPOR are less extensive than site 1. Most of the site-2 interactions are between residues of the C helix of rHuEPO and the L3 loop of EPOR. The hydrophobic surface is created on EPOR by residues Phe93, Phe205, and Met150, although because of the positioning of the D1 domain relative to the site-2 interactions with rHuEPO, a relatively flat EPOR surface exists that allows interaction with rHuEPO residues. The closest non-polar contact in site 2 includes rHuEPO Leu108 and rHuEPOR Phe93 Cγ (3.9 Å). The rest of the C helix residues are positioned at a greater distance from the EPOR hydrophobic surface than at the site-1 interface. Phe93 is again the major contributor to the central hydrophobic-binding pocket (Leu5, Val11, Tyr15, Ser104, Thr107, and Leu108 of EPO are within 4.5 Å of EPOR Phe93). Met150 of EPOR, however, is buried more deeply here than in site 1, making van der Waals contacts with Arg^{10}, Val^{11}, and Arg^{14} of rHuEPO.

In general, the side-chain interactions at both interfaces are predominantly between positively charged lysines and arginines of rHuEPO and negatively charged aspartate and glutamate side-chains of EPOR. Site 1 contains almost twice as many side-chain – side-chain interactions as site 2. The number of hydrophilic contacts involving main-chain atoms from either rHuEPO or EPOR, however, is equal for both sites. In addition, site 1 contains two hydrogen bonds that involve main-chain atoms from both rHuEPO and EPOR. Hydrophilic interactions from three EPOR residues are common to both interfaces, two of which involve hydrogen bonding from a main-chain atom. Although most residues that interact across both interfaces are polar, the expected contribution to binding affinity is greatest from the hydrophobic contacts.

Structure of a rHuEPO peptide mimetic bound to EPOR

In an elegant series of experiments, Livnah and co-workers resolved the structure of a soluble EPOR complexed with an agonistic peptide, which had no sequence identity to the naturally occurring ligand (Fig. 4). Included in this body of work was the bacterial expression, purification, and refolding of a soluble form of EPOR (EBP) [19] and the isolation of the self-dimerizing 20 amino acid peptide EMP1 (GGTYSCHFGPLTWVCKPQGG) [9]. The peptide has β-sheet structure and a disulfide bond constraining the peptide. The stoichiometry of the EBP/EMP1 complex is one peptide homodimer bound to two EPOR monomers. EMP1 was originally identified using peptide phage display technology. The 20-amino acid peptide mimicked the biologic activity of rHuEPO and functioned as an agonist. The affinity of the peptide, compared with rHuEPO, binds approximately 1,000-fold less at 200 nM, compared with 200 pM.

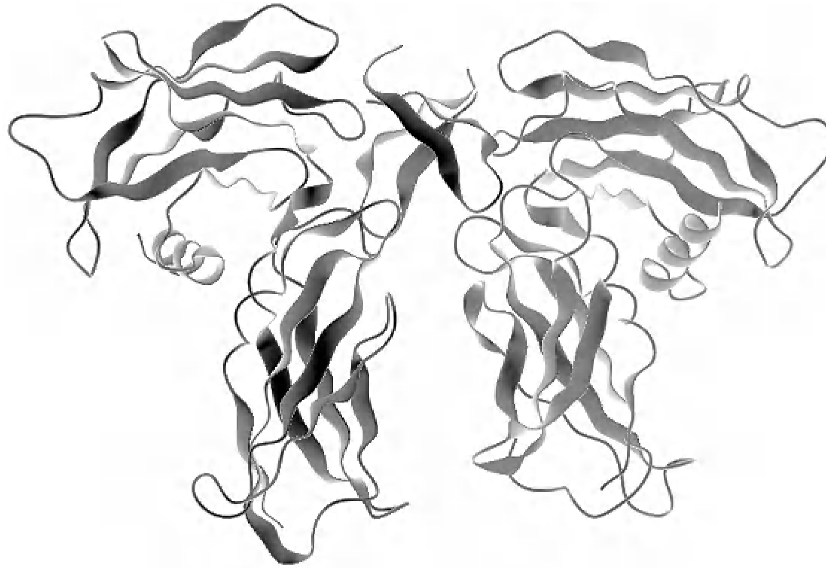

Figure 4. Ribbon depiction of the complex between the extracellular domain of rHuEPO and an agonist peptide. The X-ray crystallographic structure of rHuEPOR complexed with an agonist peptide was resolved by Livnah et al. [10]. The two-fold symmetry of the peptide allows the dimerization of two EPOR. The functional mimicry of EPO by a 20-residue peptide with a totally unrelated sequence was of considerable value in understanding the structure of the receptor and its signal transduction mechanisms.

The interface between the peptide and EPOR is mainly hydrophobic in nature with very few invaginations and charged residues that would be characteristic of a naturally occurring binding pocket. The three residues contributing the most binding energy on the receptor to EMP1 are Phe93, Met150, and Phe205. These amino acids contact Phe10 and Tyr4 of the peptide. The dimeric and symmetrical nature of the peptide is reflected in the nature of the binding interaction with EPOR. Both interaction sites on the EMP1 dimer are identical; therefore, the two receptors adopt orientations that are different than orientations found in EPOR dimerized by rHuEPO. If the two complexes (rHuEPO/EPOR and EMP1/EPOR) are viewed along a plane that is perpendicular to the membrane, almost a 60° difference is seen between the angles of the D1 domain of EPOR with respect to the rHuEPO and peptide complexes. In addition, a significant difference of about 45° in the angle of the hinge region is seen between D1 and D2 with respect to the binding of rHuEPO and EMP1.

Conclusion

Perhaps what is most surprising in this body of research is the number of different molecules that can effect the dimerization and signal transduction of

EPOR. Recent data suggest that the receptor may actually be associated on the cell surface without the natural ligand [13]. This finding suggests that it may not merely be the dimerization event but an even more subtle conformational change that occurs only after the natural ligand, rHuEPO, binds.

The quality and scope of the structural biology research in understanding the interactions of EPOR with several different molecules has provided a detailed analysis of the role of individual amino acids within the receptor. These structures provide a rational for the design of the next generation of rHuEPO biologics and may, in the future, provide the basis for a true small molecule that could effectively allow the dimerization and conformational change required for signal transduction comparable to the natural ligand.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Molecular biology of the erythropoietin receptor in hematopoietic and non-hematopoietic tissues

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Introduction

Reports on the identification, characterization, and cloning of human erythropoietin (EPO) were published in 1985 [1, 2]. Four years later the murine EPO receptor (EPOR) was identified and cloned, and this was followed soon by reports of cloning human EPOR [3–6]. The biology of the EPO/EPOR system remains of great interest, not only because of the importance in erythropoietic regulation, but because a broad range of non-hematopoietic effects of this system have become evident [7, 8]. Non-hematopoietic effects of EPO may have a significant potential in treating various disorders, including spinal cord injury, asphyxia, stroke, and neurodegenerative or retinal diseases [9–12]. This chapter summarizes current data on the molecular and cellular biology of EPOR as a key element in transmitting the effects of EPO. Specific focus is given to developmental-stage dependent, tissue-specific, and environmental aspects of EPOR in non-hematopoietic organs.

The implication of EPO and EPOR in hematopoiesis

The crucial role of EPO and EPOR for normal development is indicated by the fact that in mice, homozygous deletion of the *EPO* gene ($EPO^{-/-}$) or *EPOR* gene ($EPOR^{-/-}$) results in fetal death between day 10.5 and 13.5 of gestation [13–16]. In the absence of EPO or EPOR, commitment of hematopoietic progenitors (CFU-GEMM) to the erythroid lineage is intact, erythropoiesis progresses *in vitro* through the stage of erythroid burst-forming units (BFU-E), but terminates at the stage of erythroid colony-forming units (CFU-E). In mice with targeted homozygous deletions of the *EPO* or *EPOR* gene, "primitive" erythropoiesis is established, but "definitive" erythropoiesis is not [13, 15, 17]. The growth of early $EPOR^{-/-}$ erythroid progenitor cells can to some degree be compensated by thrombopoietin (TPO) alone or in combination with other growth factors such as stem cell factors (SCF) or interleukin (IL)-3 plus IL-11

[14, 17]. Other members of the cytokine receptor super-family have the potential to overcome the dominance of the EPOR in erythroid proliferation and differentiation. Granulocyte colony-stimulating factor (G-CSF) induces *in vitro* the growth of erythroid colonies generated from G-CSF receptor overexpressing hematopoietic cells derived from the fetal liver of $EPOR^{-/-}$ or $STAT5a^{-/-}$ mice (STAT = signal transducer and activator of transcription) [18].

These and other experiments give evidence that intact EPOR primarily mediate the expansion of late BFU-E and CFU-E into mature red cells. Approximately 300 EPOR are constitutively expressed on late BFU-E; receptor numbers rise in CFU-E to approximately 1,100 per cell. EPOR density remains high in erythroblasts, but EPORs are almost absent on reticulocytes [19–21]. Furthermore, EPO stimulates the early release of maturing normoblasts from their production site. The expression level of EPOR regulates erythropoiesis by controlling the sensitivity of erythroid progenitor cells to EPO [22]. EPO also increases the amount of hemoglobin synthesized per erythrocyte, and it suppresses programed erythroid cell death [23–25].

Molecular biology of the EPOR

Characteristics of the EPOR gene and structure of the EPOR

EPOR belongs to the cytokine receptor super-family, which is characterized by a common domain structure, consisting of an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular/cytoplasmic domain (Fig. 1) [26, 27]. The extracellular domain of members of the cytokine receptor super-family is further characterized by four conserved cysteine residues and the WSXWS (tryptophan-serine-X (X = any amino acid)-tryptophan-serine) motif. Also highly conserved are two motifs (box 1 and box 2) in the cytoplasmic domain [26]. The cytokine receptor super-family includes receptors for various hematopoietic growth factors, e.g., G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), TPO, several interleukins (including IL-2, -3, -4, -5, -6, -11, -15), growth hormone, prolactin, and leptin among others [26, 28–31].

EPOR is highly conserved, and transgenic mice with homozygous EPOR deletion can be rescued by the human *EPOR* gene [32]. In humans, the single copy *EPOR* gene is localized on chromosome 19p, spans approximately 6 kb, and contains eight exons. Exons 1–5 code for the extracellular domain, exon 6 for the transmembrane domain, and exons 7 and 8 for the intracellular domain (Fig. 2) [4, 33].

Human EPOR is a cell surface protein which consists of 508 amino acids. After a 24-amino acid leader sequence, the first 226 amino acids form the extracellular domain; the transmembrane domain contains 23 amino acids, and the intracellular domain has 235 amino acids. EPOR has a calculated molecular weight (MW) of approximately 55–56 Kd [5, 6, 33, 34]. Meanwhile mul-

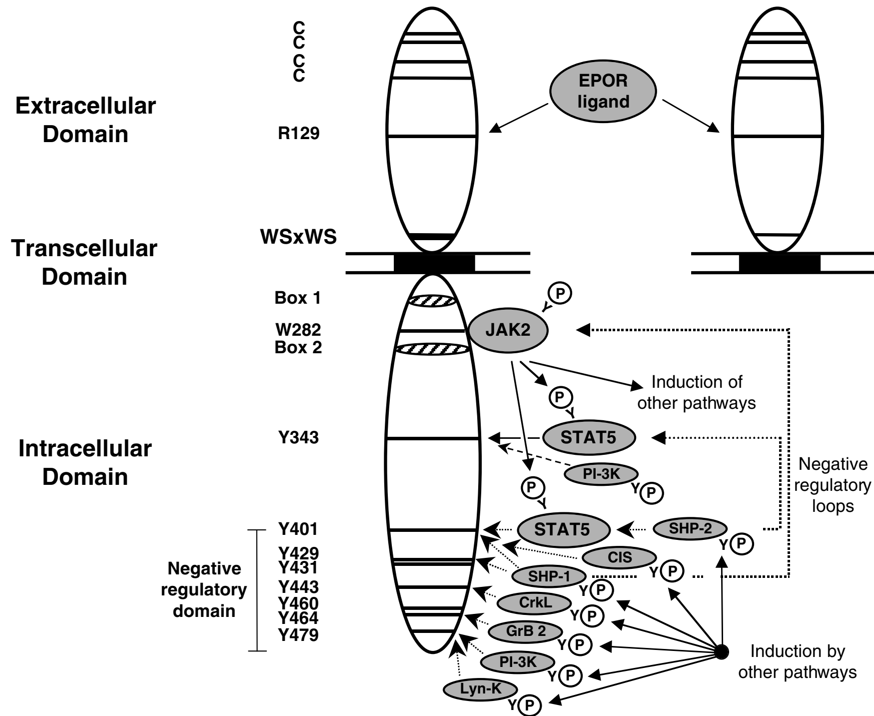

Figure 1. Model of the EPO receptor. Important residues or motifs are marked on the left side of the figure with their abbreviation (Cysteine: C; Tyrosine: Y; Arginine: R; Serine: S; Tryptophan: W) and position. Important signaling pathways and negative regulatory loops are indicated on the right side of the model. P indicates the phosphorylation necessary for the activation of the pathway. (Modified from [25a] and [86].)

tiple forms of EPOR have been identified in EPOR-bearing cells (64 to 78 Kd), which differ by the degree of glycosylation. Compelling evidence is given that preferentially EPOR forms with the highest MW bind EPO protein and induce signal transduction [35]. The extracellular domain of EPOR consists of two fibronectin type III-like domains (designated *N*- and *C*-terminal domain), each of them comprising seven beta strands that are connected by six loops. Most residues of the extracellular domain involved in the binding of EPO protein are located on the loops. Phe95 and Phe205 are most important for the binding of EPO, although other residues are critical for alternative agents activating EPOR (e.g., Met150 for EPO mimetic peptites) [36, 37]. Arg129, Glu132, and Glu133 are involved in the dimerization of EPOR. The WSXWS motif is important for ligand binding and internalization as well as signal transduction [38, 39].

The intracellular domain is divided in two functional regions. The membrane proximal region is encoded by exon 7 and contains the box 1 and box 2

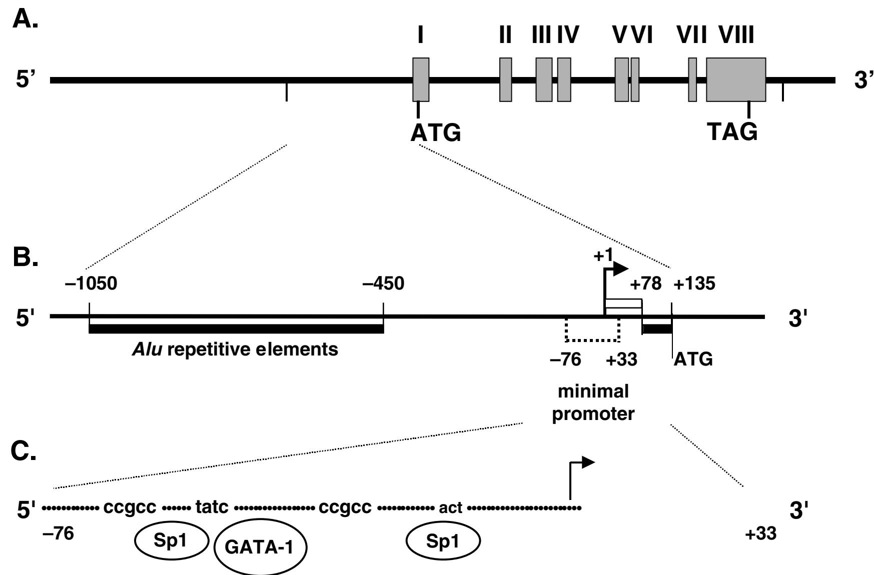

Figure 2. Genomic organization of human *EPOR* gene. Panel A shows the organization of the entire gene locus. In Panel B, the minimal promoter of *EPOR* gene is indicated by scattered lines. A positive regulatory domain (open box) from nt+1 to +78 contains potential binding sites for proteins of the helix-loop-helix family. Negative regulatory domains are indicated by filled boxes. Panel C refers to important binding sites in the minimal promoter region and the transcription start site (arrow).

motifs. Box 1 (residues 257–264) and box 2 (residues 303–309) subdomains together with a tyrosine site 343 (Fig. 1) are required for initiation of signaling cascades and further for the development of committed progenitor cells within both the erythroid and the megakaryocytic lineage [40, 41]. A region between box 1 and box 2 (residues 267–276) also plays a role in EPOR internalization [42, 43]. The distal region of the intracellular domain, encoded by exon 8, acts as a negative regulatory domain, where proteins bind that determine the signaling process [44]. Accordingly, the activation of the seven tyrosine residues (Y401 to Y479, Fig. 2) is not required for the growth and terminal differentiation of erythroid progenitors [45]. However, the distal cytoplasmic domain of EPOR can also induce a specific myeloid differentiation signal distinct from mitogenic signaling as shown in murine 32D cells, transfected with truncated EPOR [46].

Activation of EPOR, induction, and termination of signaling

Different mechanisms are identified for the binding of the ligand to the extracellular domain of EPOR. Usually, one EPO molecule binds to two EPOR and

forms a homodimer [47, 48]. Biophysical studies suggest that EPO binds to one of the two receptors with high affinity (approximately 1 nM) and to the other with low affinity (approximately 1 μM) [49]. Dimerization of EPOR can also be achieved from other molecules (different form and size) as long as two recognition motifs are provided in each molecule. Such a molecule can be a dimeric EPO, containing only one binding motif on each monomer (heterodimerization), a mutant EPOR which is cross-linked by a disulfide bound, a bivalent antibody against the extracellular domain of EPOR, or an EPO mimetic peptide [48, 50–55].

EPO mimetic peptides are a family of EPOR agonists which contain 9–22 amino acids. Although they do not share sequence homology with EPO, they mimic its erythropoietic effects *in vivo* and *in vitro* [54, 56]. X-ray crystallography gives evidence of their specific binding to EPOR and its dimerization. The extracellular domain folds into a L-shape with the long axis and aligned at approximately 90° to the other axis [55]. The three-dimensional structure of the complex of two extracellular domains of EPOR bound to EPO shows that the receptor orientation by the ligand is critical for the efficiency of signaling [37, 55]. It has also been shown that a major fraction of the full-length (murine) EPOR exists as a preformed dimer which is not constitutively active and requires EPO-binding to induce signaling.

Once EPOR is dimerized, the intracellular signaling pathway becomes activated by a rapid tyrosine phosphorylation of numerous proteins that associate with EPOR. EPOR itself possesses no endogenous tyrosine kinase activity [57, 58]. In this process, Janus kinase 2 (JAK-2) is the primary kinase responsible for the phosphorylation of EPOR (Fig. 2). JAK-2 is associated with EPOR before ligand binding, and rapidly activates it after the binding of the ligand and the dimerization of EPOR [58]. The EPO signaling pathways, which are induced following EPOR activation, include the JAK/STAT pathway, Ras/MAP (Ras protein/mitogen-activated protein) kinase, phosphatidylinositol-3 kinase (PI-3 kinase), and protein kinase C (PKC) pathways. After signaling is initiated, the receptor-ligand complex is subsequently internalized and degraded. The signaling is terminated by dephosphorylation of EPOR and JAK-2. In this process, negative regulatory loop mechanisms are included (Fig. 1) [59]. The EPOR signaling pathways are described in detail by Ghaffari et al. in Chapter 5.

Other factors binding to EPOR

Other members of the cytokine receptor super-family interact with EPOR, which may be relevant for its tissue-specific activation and function: In erythroid progenitor cells, EPOR is phosphorylated by the tyrosine kinase activity of the activated receptor for stem cell factor (*c-kit*). *C-kit* physically interacts with the extended box 2 region in the cytoplasmic domain of EPOR [13, 17, 60]. Moreover, the β-chain of the IL-3 receptor functionally interacts with

EPOR [61]. EPO has been shown to activate the common β-chain of the IL-3 and GM-CSF receptors through EPO-specific signaling pathways [62, 63]. These cross-talk phenomena need further investigation, particularly with regard to the biology of EPOR in non-hematopoietic tissues.

Regulation of EPOR expression

EPOR gene expression can be regulated at the transcriptional and post-transcriptional levels and by post-translational modification [64–66]. At the level of transcription, EPOR mRNA expression is controlled by *cis* acting elements located upstream of the *EPOR* gene that mediate positive and negative regulatory effects. The transcription start site of the EPOR gene is located 141 bp upstream of the initiation codon (Fig. 2). The minimal EPOR promoter region (nt–76 to +33 of the transcription start site) does not contain a TATA or CAAT box, but contains binding sites for the ubiquitous transcription factor Sp1 at nt–20 and GATA does include transcription factors at nt–45. A positive regulatory domain from nt+1 to +78 contains potential binding sites (CANNTG) for proteins of the helix-loop-helix family [67]. A negative regulatory domain has been identified upstream of the minimal EPOR promoter region from nt–1050 to –450 which carries Alu repetitive elements [67, 68]. A second negative regulatory domain exists downstream of the transcription start site (nt+79 to +135), where a potential binding site for Spl-like proteins exists (nt+85). Both negative regulatory domains have an independent and non-cumulative effect on the expression of the human *EPOR* gene [67]; however, erythroid-specific expression of *EPOR* gene can be achieved if sequences are available which are further downstream localized of the transcription start site (nt+1 to +135).

Since minimal promoters of several erythroid genes contain at least one GATA binding site in close association with a CACCC or Sp1 motif, an essential role in regulating EPOR on hematopoietic cells is suggested [69]. The minimal EPO promoter can be transactivated in non-erythroid cells by the co-transfection with GATA-1 [70, 71]. Maouche et al. concluded from reporter gene assays in different cell lines that the fragment of *EPOR* gene containing the GATA and Sp1 sites is not erythroid specific [67]. Mice with targeted $EPOR^{-/-}$ deletion which were transgenic for two different GATA-1 minigene cassettes with hematopoietic regulatory domains (GATA-1-HRD) were rescued from the lethal defect of EPOR deficiency [22]; however, further studies are necessary to determine the specific regulatory processes of EPOR expression in non-hematopoietic tissues [67].

EPOR expression also underlies other regulatory processes. In the human EPO-dependent leukemia cell line UT-7, treatment with EPO or GM-CSF results in a transient decrease of EPOR mRNA expression. The down-regulation of EPOR is preceded by a transient down-regulation of GATA-1 mRNA. The expression of both EPOR and GATA-1 concomitantly decreases at the G0/G1 phase and increases at the S and G2/M phases of the cell cycle.

Moreover, it is suggested that the increase of EPOR mRNA expression at the resting phase induced by growth factor starvation is dependent on other transcription factors than GATA-1 (Fig. 3) [72]. Furthermore, in a human EPO-independent acute megakaryoblastic leukemia cell line (HML/SE), SCF induces *EPOR* gene expression by activating its promoter [73]. Human *EPOR* gene expression can also be significantly upregulated by IL-1α and the translation inhibitor cycloheximide [74]. Moreover, it has been shown that IFN-γ down-regulates EPOR at the surface of erythroid progenitor cells, and the resulting inactivation of EPOR might reduce survival of these cells and induce apoptosis [75]. At the post-transcriptional level, EPOR expression can be regulated by chemical agents such as phorbol myristate acetate [64]. Post-translational modification of the EPOR is reported in IL-3 dependent (murine) cell lines [66].

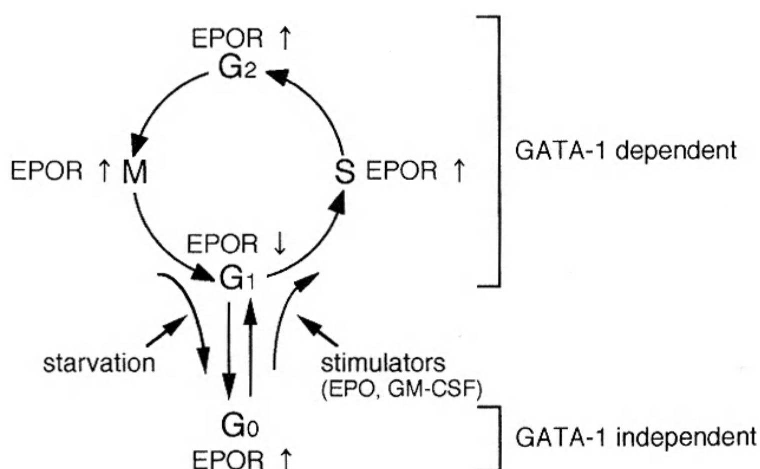

Figure 3. Model for relation between EPOR and GATA-1 expression during the cell cycle in human EPO-dependent hematopoietic progenitor cells. EPOR expression in the proliferating cell is mediated at the level of transcription through cell cycle-dependent GATA-1 expression. However, in the quiescent phase other factors appear to regulate EPOR expression. (From [72] with permission.)

Effects of EPOR activation on the expression pattern of other genes

Signals from EPOR activate genes that are important in cell proliferation, differentiation, or survival. They include GATA-2, *c-myc*, *c-myb*, and *c-Fos/c-Jun* (only in murine EPO-dependent cells) as regulators of proliferation [25, 76–78]. Bcl-x_L and Bcl-2 are activated as antiapoptotic genes [79]. However, in a human EPO-dependent erythroid cell line (AS-E2), EPOR signals also negatively regulate GATA-1, *c-myb*, α-globin, β-globin, and γ-globin [25].

EPOR biology in hematological diseases

Polycythemia vera, myeloproliferative and myelodysplastic diseases, erythroleukemia, Diamond-Blackfan anemia

Several studies focus on EPOR biology in hematological diseases in humans. Although in mice, a point mutation in the extracellular or transmembrane domain of EPOR results in a constitutively activation of the receptor, and deletions in the intracellular domain increase EPO-mediated proliferation of erythroid progenitors [47, 80, 81] and result in erythroleukemia, several studies in humans have failed to detect structural abnormalities of EPOR in polycythemia vera and related myeloproliferative diseases as potential cause for the increased sensitivity of cultured erythroid progenitors to recombinant EPO (rEPO) [82–84]. There is no evidence that EPOR mutations are involved in myelodysplasia, erythroleukemia, Diamond-Blackfan anemia, or pure red cell aplasia [85, 86].

Idiopathic erythrocytosis

In idiopathic erythrocytosis, the red blood cell mass is increased without other criteria of polycythemia vera. This disorder generally involves an autosomal dominant mode of inheritance. It is defined by erythrocytosis without evolution into leukemia or myelodyplastic syndrome, absence of splenomegaly, normal white blood cell and platelet counts, low or moderate EPO plasma concentrations, normal P_{50}, and hypersensitivity of erythroid progenitors to rEPO *in vitro*. Eight mutations in the cytoplasmic domain of EPOR have been identified in these patients [87–95]. They are clustered in a 122 bp region (between nt5881 and nt6002, numbering according to [33]) in one copy of the gene and result in the formation of a stop codon. Consequently, between 59 and 110 amino acids of the negative regulatory domain are lost. All deletions include tyrosine 429 that acts as SHP-1 binding site (Fig. 1). The lack of JAK2 dephoshorylation by the negative regulatory loop of SHP-1 may result in enhanced STAT5 activity and the pathomechanism for erythrocytosis. Other pathomechanisms of idiopathic erythrocytosis may exist, particularly if associated with high EPO plasma concentrations.

Biology of EPOR in non-hematopoietic tissues

Although the crucial role of EPO in hematopoiesis is known for decades, the biology of EPO in non-hematopoietic tissues became evident first during the last decade (Tab. 1). Experimental and first clinical data indicate the pharmacological potential of rEPO in various acute and chronic disorders. EPOR expression in non-hematopoietic cells seems to be regulated in a developmen-

tal-stage and tissue-specific manner which is only poorly understood. It has been postulated that non-hematopoietic EPOR expression results from the common origin of various cell types; for example endothelial and hematopoietic cells originate from the hemangioblast [96]. Based on this concept, the finding of common anti-apoptotic, proliferative, differentiative, and angiogenic effects of EPO in various tissues may be not surprising.

Vasculature

EPOR is expressed in human endothelial cells [97, 98]. By activation of EPOR, EPO stimulates cell migration, proliferation, and differentiation of endothelial cells into pro-angiogenic structures. It also stimulates endothelin and matrix metalloproteinase (MMP-2) release, and an increase in cytosolic-free calcium concentrations [97–102]. Studies on the *in vitro* and *in vivo* effects of EPO on endothelial cells resulted in new concepts for using rEPO in tissue regeneration or repair [8]. The affinity of EPOR to EPO is significantly lower in endothelial cells (K_d approximately 1 nM) than in erythroid cells (K_d approximately 50 pM) and may play a role in regulating the effects of EPO [103, 104]. However, further investigations on the specific regulation of EPOR expression on endothelial cells are necessary to understand how angiogenic effects of EPO are regulated in health under chronic high EPO concentrations (at high altitudes) or in disease (diabetes, artherosclerosis, or others).

EPO, expressed in bone marrow stroma cells, may also play a significant paracrine role in bone marrow angiogenesis, particularly during development [105–107]. EPOR is also expressed in (rat) smooth muscle cells [108, 109]. EPOR activation results in a direct vasopressive effect, suggesting that the increase in blood pressure observed in some rEPO-treated patients may not be only secondary to an increased blood volume. Recombinant EPO also normalizes the contractile response of rat aortic sections in a state of shock. This may be partly transmitted by ionic changes (Ca^{2+}) in smooth muscle cells after rEPO administration [110]. Moreover, rEPO inhibits the activity of the inducible nitric oxide synthase and reverts vascular dysfunction in a shock model of splanchnic artery occlusion [111].

Musculature

EPOR expression is reported in primary murine satellite cells which are localized at the basal lamina of adult skeletal muscle [112]. EPO increases the cytoplasmic calcium levels and enhances the proliferation of these cells, but reduces differentiation and fusion into myotubes. Since satellite cells become activated when a muscle is injured or diseased, EPO may participate in muscle development or repair. Maturation to myotubes results in a down-regulation of EPOR. EPOR are undetectable in mature myotubes or adult skeletal

Table 1. EPOR expressing human (H) non-hematopoietic organs

EPOR expressing organ	EPOR expressing cell	Developmental stage	Detection method	Reference
Vasculature	Endothelial cells	Umbilical cord	IHC, RT-PCR	[97, 98]
Musculature (M)	Satellite cells	Adults	IHC	[112]
Heart (H)	Myocardium	Early fetal development	RT-PCR, IHC	[161]
	Endothelial cells derived from the cardiac auricle	Adults		[116]
(M)	Endocardium (endothelial cells) Epicardium (mesothelial cells) Pericardium	E10.5 – E14.5 of gestation	IHC	[16]
Lung	Scattered, round interstitial cells	6 weeks *post conceptionem*	RT-PCR, IHC	[115]
	Basilar surfaces of respiratory bronchi	8 weeks *post conceptionem*		
	Epithelial cells lining the bronchi	Mid gestation		
	Unidentified cells	Late gestation and neonatal period	RT-PCR	[117]
Kidney	Mesonephros	Early gestation (<17 weeks *post conceptionem*)	RT-PCR, IHC	[115]
	Proximal tubulus cells	Mid gestation	IHC	
	Renal cortex, medulla, papilla	Adults	RT-PCR	[119, 121]
	Proximal tubular cells	Adults	RT-PCR	
Adrenal gland	Adrenal cortex and medulla, all zones	Early fetal gestation	RT-PCR, IHC	[115]
Gastrointestinal tract	Intestinal villi cells of the proximal and distal small bowel	Fetus and neonate	RT-PCR, IHC	[122]
Uterus	Glandular epithelial cells	Fertile, premenopausal women	IHC	[140]
Mammary gland	Tubular epithelial cells	All stages	IHC	[127]

(continued on next page)

Table 1. (continued)

EPOR expressing organ	EPOR expressing cell	Developmental stage	Detection method	Reference
Placenta (H)	Villous and extravillous cytotrophoblast cells	All developmental stages	IHC; Western blot; PCR	[145]
	Syncytiotrophoblast cells	All developmental stages	IHC; Western blot; PCR	
	Endothelial cells of feto-placental blood vessels	All developmental stages	IHC	
Fetal membranes (S)	Amniotic epithelium, chorionic cytotrophoblasts	Last trimester	IHC	[147]
Male reproductive organs				
Testis (R)	Ledyig cells	Adults	IHC	[152]
Epididymis (M)		Adults	IHC	[153]
Central nervous system	Undifferentiated neuroepithelial cells in the periventricular germinal zone	Embryo (5–6 weeks *post conceptionem*)	IHC	[162]
	Subventricular zone, neuropil, cortical plate	Fetus (10 weeks *post conceptionem*)		
	Neocortex	Midgestation		
	Astrocytes around and within brain capillaries	Increased after brain injury		[165]
	Neurons	All developmental stages		
	Choroid plexus	All developmental stages		
Eye (H)	Entire retinal cell layer	Early fetal gestation	RT-PCR, IHC	[161]
(R)	Retinal ganglion layer Inner and outer plexiform layers	Neonates	IHC	[187]
(M)	Rod inner segment and presynaptic complexes of photoreceptors, inner nuclear layer	Adults	IHC	[188]

If the respective organ has been investigated in non-human species only, the species is indicated as follows: (S), sheep fetus; (M), mouse; (R), rat. RT-PCR, reverse transcription-polymerase chain reaction; IHC, immunohistochemistry; ISH, *in situ* hybridization.

muscle. The presence of rEPO increases EPOR expression levels six-fold in murine myblasts (C2C12 cells) [112]. The associated two-fold up-regulation of GATA-3 expression levels by rEPO may mediate changes in the cellular proliferation and differentiation. GATA-3 may also limit EPO gene expression by binding on the EPO promoter as a negative regulatory element [113].

Heart

In fetal mice, EPOR is expressed in the heart in a temporal and cell type-specific manner. EPOR is initially detectable at day 10.5 of gestation, and its expression persists until day 14.5. During this period, EPOR expression is found in the endocardium (endothelial cells), epicardium (mesothelial cells), and pericardium, but not in the myocardium [16]. Evidence for the biologic relevance of EPO in the heart is given by the finding of ventricular hypoplasia coupled to defects in the intraventricular septum both in $EPO^{-/-}$ and $EPOR^{-/-}$ mice. These animals die at day 13.5 of gestation and also suffer from detachment of the epicardium and abnormalities in the vascular network. Immunohistochemistry indicates EPOR expression in the mycocardium at embryonic day 11.5 (E11.5) [114]. Although EPOR expression could not be detected at E13.5 by *in situ* hybridization, a reduced cell number is the most consistent and significant finding at this stage of development [16]. Lack of EPOR does not affect the initial commitment of cells to cardiomyocyte development, but the number of proliferating cells is markedly reduced in $EPO^{-/-}$ and $EPOR^{-/-}$ mutant fetal mice. Accordingly, rEPO significantly stimulates the proliferation of fetal murine cardiomyocytes *in vitro*. Furthermore, chimeric analysis provides evidence that EPO triggers murine cardiomyocyte proliferation in a non-cell-autonomous manner [16]. Therefore, it has been postulated that activation of EPOR in the endo- or epicardium is required for the initiation of a cascade of events leading to the secretion of a mitogenic factor in the myocardium. Such interactions are essential for other factors involved in normal cardiac development. Cardiac defects in $EPO^{-/-}$ and $EPOR^{-/-}$ mutant fetal mice result in suppressed cardiac function with fetal hydrops and appear to be independent of the general state of hypoxia due to the lack of onset in "definitive" hematopoiesis [16]. EPOR deficiency increases apoptosis in the endocardium and myocardium, which may be primarily and/or secondarily caused [114].

In humans, Juul et al. reported only weak EPOR immunoreactivity in the fetal myocardium at five weeks *post conceptionem*, but increasing EPOR staining by eight and 18 weeks pc [115]. In endothelial cells derived from human adult myocardial tissue (cardiac auricle), rEPO has a significant angiogenic potential which is equal to that of vascular endothelial growth factor (VEGF) [116]. These data suggest that EPO not only has a role in normal cardiac development, but also acts as an angiogenic and anti-apoptotic factor on endothelial cells in the heart, which makes it an agent with potential in myocardial repair in ischemic heart disease or in inflammatory processes of the epi- or endocardium.

Lung

EPO and *EPOR* gene expression has been found in the developing human lung between six and 32 weeks of gestation [115, 117]. In early gestation, EPOR immunoreactivity is weak or absent in the lung parenchyma, but weak to moderate in the capillary network as well as in the apical surface of the developing bronchi [115]. EPOR immunoreactivity shows a developmental change. With the exception of scattered round interstitial cells, only weak EPOR immunoreactivity is detectable at six weeks *post conceptionem*, while later in development, basilar surfaces of the respiratory bronchi become immunoreactive. At mid-gestation, epithelial cells lining the bronchi express EPOR, but mesenchymal cells of the interstitium remain negative in immunohistochemistry [115]. Further investigations on the biological effect of EPO in the development of the respiratory tract, particularly of the capillary bed, are necessary.

Kidney

As the primary site of EPO production in children and adults, the kidneys act as an endocrine organ for erythropoiesis [106, 118]. However, EPOR transcripts were found in human fetal (gestational age seven to 16 weeks *post conceptionem*) and adult kidney specimens [115, 119]. Immunohistochemistry reveals EPOR expression in the mesonephric stage of renal development, and becomes more localized to cells of the proximal tubules at 22 weeks *post conceptionem* [115]. In the adult kidney, EPOR mRNA has been detected specifically in the renal cortex, medulla, and papilla. *In vitro* studies showed that renal EPOR in murine proximal tubular cells are functionally intact [119]. It has been shown that EPO increases the Ca^{2+} influx in glomerular mesangial cells [120]. Data indicate that EPO stimulates mitogenesis *in vitro* as well as cell survival and motogenesis of injured tubular cells. *In vivo*, EPO acts cell-protective in a rat model of “ischemic” acute renal failure and accelerates functional recovery [121].

Adrenal gland

EPO and EPOR are expressed in the human fetal adrenal gland between six and 16 weeks *post conceptionem* [115]. Juul et al. described moderate immunoreactivity for EPO in the developing zona fasciculata of the adrenal cortex, and weak or absent EPO reactivity in the adrenal medulla. In contrast, EPOR immunoreactivity is strong in all zones of the adrenal cortex and medulla [115]. As in the lung, the function of EPO and EPOR in the adrenal gland is unknown.

Gastrointestinal tract

EPOR mRNA and EPOR protein have been detected in the intestinal villi of the proximal and distal small bowel of the human fetal and neonatal gastrointestinal tract, but no data are reported on adults [115, 122]. EPO protein is present in biologically relevant concentrations in amniotic fluid and mammalian milk, suggesting that gastrointestinal EPOR bind ingested EPO protein [123–127]. Simulated *in vitro* digestion studies give evidence that a significant portion of milk-borne EPO is protected against proteolytic degradation. Thus, a local effect of ingested EPO in the gastrointestinal tract can be proposed. Functional data on the local effects of systemic or enterally administered rEPO in the human gastrointestinal tract are not yet available. In rats, EPOR has been detected in gastric mucosal cells, intestinal epithelial cells, and intestinal and mesenteric microvascular endothelial cells [122, 128–130]. EPOR in the gastrointestinal tract are functionally intact, since ^{125}I-labeled rEPO fed to neonatal rats was found in the wall of the stomach [131]. *In vitro,* rEPO has mitogenic effects on rat gastric mucosal cells [128]. Moreover, rEPO increases the healing and stimulates the migration of rat intestinal epithelial cells without significant proliferative effects [122]. *In vivo*, systemic or enteral administration of rEPO to neonatal rats increases the small intestinal length, villous surface area and villous height, as well as the ileal crypt depth. These trophic effects are most pronounced in the ileum and greater if rEPO is enterally applied rather than parenterally. Recombinant EPO does not seem to change the specific activity of disaccharidases (lactase, maltase, or sucrase) [130]. Recent data show that rEPO stimulates the proliferation and microvascular tubular formation of rat intestinal mesentery endothelial cells [129].

The effects of enterally administered rEPO on erythropoiesis have been studied in human preterm babies and in animal (rat) models. The results are in part contradictory and do not allow a final conclusion on the erythropoietic effects of milk-borne EPO or enterally administered rEPO to neonatal rats. However, it is evident that enterally administered rEPO has no erythropoietic effect in adult rats, except when given rectally in the presence of salicylates that improve rectal mucosal permeability [132].

In humans, a non-controlled trial feeding high dose rEPO (1,000 U/kg body weight per day) showed increased plasma EPO concentrations in six preterm babies, but the reticulocyte number and hematocrit levels did not change [133]. In this study rEPO was given in a non-protein-containing buffered solution which may have significantly decreased the stability of the protein. In a controlled study, addition of rEPO (600 U/kg body weight per week in three doses) to formula in six preterm babies resulted in higher plasma EPO concentrations and a higher peak in reticulocytes compared with six controls, but no increase in hemoglobin concentration was observed. A decrease in ferritin concentrations suggested an increased iron utilization in the group fed rEPO [134]. In neonatal rats, 5% of enterally administered ^{125}I-labeled rEPO was

found intact in the plasma, and another 8–10% reached the bone marrow [131].

Current research focuses on the clinical effects of rEPO on the developing gastrointestinal tract. A retrospective study in preterm neonates (body weight < 1,250 g; n = 260), who received rEPO to prevent anemia of prematurity, showed a lower incidence of stage II and III necrotizing enterocolitis (4.6% *versus* 10.8%) compared with controls (n = 223) [135]. Feeding intolerance is a common problem in neonatal intensive care among ill preterm babies and may lead to intestinal villous atrophy of a result of being *nihil per os* during parenteral feeding. Recent studies investigate the effects of the application of a simulated amniotic fluid which contains rEPO beside recombinant G-GSF, on the maturation and repair of the epithelial of the gastrointestinal tract [136, 137].

Female reproductive organs

Uterus and oviduct

In the murine uterus, EPO and EPOR mRNA are expressed, and EPO protein is produced *in vitro* and *in vivo* in an estrogen-dependent manner. EPO mRNA levels increase one hour after estrogen administration, reach a peak after four hours, but decrease after eight hours [138]. Interestingly, this decrease is not the loss of responsiveness of the uterine EPO-producing cells to estrogen [139]. *EPO* gene expression in the uterus in response to hypoxia also needs the presence of estrogen which indicates a unique regulation of the *EPO* gene. *In vivo* experiments showed that endogenous or recombinant EPO induces uterine hypertrophy and endometrial growth [138]. Immunohistochemistry reveals EPOR expression in uterine microvascular endothelial cells, but changes in its expression level are not reported [138]. The down-regulation of the responsiveness to estrogen in uterine EPO-producing cells may be very important to prevent uterine angiogenesis in an estrogen cycle stage where it should not occur [139].

In human uterine tissue specimens of fertile, premenopausal women, EPO and EPOR are expressed in isolated epithelial, but not stroma cells [140]. During menstrual cycle, EPO expression in these cells is cycledependent and higher during the secretory phase than in the proliferative phase. As shown by immunohistochemistry, EPO and EPOR protein expression in glandular epithelial cells is increased during the mid-proliferative phase and maintained during the late proliferative phase and the secretory phases [140]. These data suggests that also in humans ovarian steroids may stimulate EPO production in human endometrial glandular epithelial cells.

Ovary and oviduct

EPO is also expressed in the mammalian ovary and oviduct [141]. In contrast to the uterus, *EPO* gene expression in the murine oviduct can be induced by

hypoxia in the absence of estrogen, although in general EPO mRNA in the oviduct is also inducible by estrogen [141]. EPOR biology and implication of EPO expression in the oviduct are unknown.

Placenta

Expression of *EPO* and *EPOR* genes has been described in the placenta of humans, mice, rats, and sheep [142–146]. The expression of *EPOR* gene in human placental tissue and trophoblast–derived choricarcinoma Jar cells has been detected by RT-PCR [144, 145]. Using immunohistochemistry, EPOR expression has been identified in the villous and extravillous cytotrophoblast cells as well as in the syncytiotrophoblast of the human placenta at all gestational stages [145]. EPOR is expressed by cells within the villous core, including endothelial cells of feto-placental blood vessels [145]. The presence of EPOR protein and its phosphorylation in response to rEPO application in trophoblasts has been confirmed by Western blot analysis [145].

The function of EPO in the placenta is not totally understood. The macroscopic examination of the placenta in mice with heterozygous maternal and homozygous fetal deletion of *EPO* or *EPOR* gene has been unrevealing. The finding of EPO mRNA, protein, and EPOR localized to the fetal-maternal interface within the cotyledon in a region populated with binucleate cells, suggests that EPO may potentially mediate cellular function in both the maternal and the fetal portions of the placenta [146]. Based on these data, an autocrine role for EPO in the survival, proliferation, or differentiation of placental trophoblasts or in angiogenesis is proposed.

EPO and EPOR are expressed in ovine membranes, where EPO may mediate the development and/or function of the amnion and chorion [147]. EPO mRNA expression was detected in both the amnion (amniotic epithelium) and the chorion (chorionic cytotrophoblasts), but was absent from the connective tissue layer (extracellular matrix) between the amnion and chorion [147]. The EPO protein is localized exactly at the same sites. EPOR expression shows a developmental expression pattern. While EPOR is not detectable in the membranes early in gestation, it is found late in gestation both in the amniotic epithelium and chorionic cytotrophoblasts [147]. Further studies are necessary to determine the function of EPO in fetal membranes.

Mammary gland

Conflicting data are reported regarding whether EPO is expressed in the mature, non-lactating breast of non-pregnant women [127, 148]. However, EPO immunoreactivity in mammary tubular epithelial cells increases during pregnancy, and robust EPO production is obvious during lactation [127]. EPO concentrations in human milk increase during the lactating period, with highest levels after the day 50 *post partum*. EPO concentrations do not differ in fore, mid, or hind milk [127]. EPO concentrations in milk are higher in mothers who were smokers or who had significant peripartum anemia [125]. Furthermore, EPO concentrations in milk and plasma increase during subcu-

taneous rEPO treatment in mothers with postpartum anemia [126]. These data suggest that maternal circulating EPO concentrations influence milk EPO concentrations or that EPO production – after the onset by a tissue-specific regulator – can also be stimulated by hypoxia or anemia. The implication of milk-borne EPO was discussed earlier. EPO may also have an autocrine or paracrine function in the breast, since weak EPOR immunoreactivity is present in mammary duct epithelial cells regardless of the lactating state [127]. Further studies may elucidate the para- or autocrine function and regulation of EPO in the mammary gland.

Male reproductive organs

While clinical effects of rEPO on the hypothalamic-pituitary-testicular axis in men are reported, no data on the expression of EPO and EPOR in the human male reproductive organs are available [149]. Animal data suggest a role of EPO in male reproductive organs.

In rats, EPO mRNA is expressed in testis and localized in Sertoli and peritubular myoid cells, but no EPO mRNA is detected in Leydig cells [150, 151]. In both Sertoli and peritubular myoid cells, *EPO* gene expression can be induced by $CoCl_2$. *EPO* gene expression responds to hormones *in vitro* in a cell-specific manner: While follicle-stimulating hormone induces EPO mRNA levels in Sertoli cells, testosterone suppresses *EPO* gene expression in peritubular myoid cells (LC-540 cells) [151]. Rat Leydig cells express EPOR, and binding of rEPO stimulates testosterone production *in vitro* [152].

Another insight into the biology of EPO in the male reproductive system comes from studies in mice. EPO is expressed in the murine testis and epididymis. EPO mRNA levels in epididymis are approximately seven-fold higher than in testis, both independent of the presence of testosterone or estrogen. EPO expression levels increase more significantly in the epididymis than in the testis in response to hypoxia, indicating a tissues-specific regulatory mechanism for *EPO* gene expression. Under continuous hypoxic stimulation, EPO mRNA expression in testis and epididymis peaks after four hours, but decreases at eight hours. In the murine epididymis, EPO mRNA expression increases three-fold during three and nine weeks of postnatal development, but remains on a plateau between six and nine weeks when male mice are capable of fertilization. After induction of EPO gene expression, EPO producing cells were identified by *in situ* hybridization in the interstitial space of ductus epididymis, but not in the duct epithelium. Data on the expression of EPOR are very limited. So far, EPOR mRNA expression is detected in murine epididymis, without changes in the expression levels during sexual maturation [153]. The exact identification of EPOR-expressing cells in the epididymis will be crucial to understand the physiological function of EPO in epididymis. It has been speculated that EPO may not be directly involved in sperm maturation, but may rather support the duct function through paracrine action [153].

Central nervous system

Among non-hematopoietic tissues, the biology of EPO in the central nervous system (CNS) has been most intensively investigated, and recent reviews report on these findings [7, 154]. EPO expression in the CNS underlies a tissue-specific regulation under normoxia and hypoxia as well [139, 155, 156]. The EPO protein produced by neurons and astrocytes is biological active [157]. By immunochemical analysis in PC12 cells (an EPO-expressing rat pheochromocytoma cell line which can differentiate into a neuronal phenotype), solubilized EPOR showed to be smaller (62 Kd) than that on rat erythroid cells (68 Kd). Moreover, the affinity of rEPO to EPOR on neuronal cells was found to be lower as on erythroid cells [158].

The effects of EPO are mediated by the expression of EPOR, which is abundant in the embryonic, fetal, and adult CNS as it has been shown in rats, mice, monkeys, and humans [155, 156, 159–161]. A temporal and cellular distribution of EPOR in the human embryonic, fetal, and adult CNS has been identified. In the embryonic cerebral hemisphere, EPOR expression has been localized to undifferentiated neuroepithelial cells in the periventricular germinal zone at five to six weeks pc [161, 162], from where neuronal regeneration takes place [163]. At 10 weeks of gestation, EPOR is localized primarily to the subventricular zone, the neuropil, and the cortical plate, while only low expression was found in the ventricular and matrix zone. Immunoreactivity of the hippocampus and nucleus caudatus is weak or not found at this stage of development. As development proceeds, the diffuse staining of broad zones of the developing neocortex is replaced by more specific cellular staining of increasingly differentiated neurons. EPOR expression can be detected in sub-populations of astrocytes within and around brain capillaries [161, 164]. EPOR immunoreactivity has been found within the astrocytic foot processes surrounding the capillaries and also within endothelial cells [164]. Intense immunoreactivity for EPOR has been found in neurons (in a pattern restricted to the somata and proximal dendrites) and the choroid plexus [162, 164]. The pattern of cellular staining seems to be shifted from astrocytes, which are predominantly stained for EPOR early in development, to neurons in the mature brain [161, 162, 165]. In adult humans, *EPOR* gene expression has been described in the hippocampus, amygdala, and temporal cortex [156, 162]. However, the level of EPOR expression in human adult bone marrow is two-fold higher than the level detected in adult brain as determined by quantitative RT-PCR analysis [166].

The developmental regulation of EPOR expression in the CNS has been confirmed in animal models. While EPOR expression can be detected at day E 10.5 in the embryonic mouse brain at similar levels as in hematopoietic tissue, but EPOR mRNA levels in the CNS decrease significantly with further development [167]. Because EPOR transcripts have a relatively short half-life of about 90 minutes in erythroid cells, the gene must be continuously transcribed to maintain high expression levels [168]. A region flanking the human EPOR

proximal promotor (exon 1 to exon 2) is necessary to actively drive EPOR expression [160]. The proximal promotor of the EPOR gene is functional intact in the CNS, but underlies an alternate processing or reduction in splicing efficiency compared with EPOR on erythroid progenitor cells. In the human brain, EPOR transcripts can be initiated by a region far upstream of the EPOR proximal promoter (exon A and exon B). The expression of these transcripts in comparison to transcripts from the EPOR encoding regions (exon 1 to exon 2) is developmentally regulated. In the adult brain, transcripts of the proximal promoter region are expressed 10-fold lower than in the developing brain [166]. These data indicate that the biological effectiveness of EPO in the CNS may be regulated – at least in part – by EPOR expression levels. *In vitro* studies in NT2 cells, a human neuronal precursor cell line that expresses EPO, showed that EPOR expression in neuronal cells can be induced on the transcriptional level by GATA-3 binding to the minimal EPOR region. Conversely, lack of EPOR down-regulates GATA-3 expression in cortical cells, suggesting specific and complex mechanisms of EPOR gene regulation in neuronal cells [114].

Experimental studies indicate a crucial role of the EPO/EPOR system in the normal CNS. Binding of endogenous EPO by soluble EPOR renders EPO less available to the developing CNS cells, and treated adult rats show neuronal degeneration and impaired learning [169]. Mice with targeted homozygous deletion of the *EPOR* gene suffer from extensive apoptosis in the developing brain. As early as day E10.5 the number of neuronal progenitor cells has been reported as reduced, while apoptosis increased. *In vivo*, neuronal generation from cortical cells is decreased, while the sensitivity to low oxygen tension is increased, resulting in cell death [114]. In contrast, the proliferation of brain derived cells from $EPOR^{-/-}$ mice, taken at day E11.5–13.5 of gestation, is normal [16]. Differences between these both studies may be due to the fact that EPOR expression peaks in the murine CNS during mid-gestation or that different cortical cell lineages were selected. Another study gave evidence that EPO regulates the *in vivo* and *in vitro* production of neuronal progenitors by mammalian forebrain neural stem cells [170]. A recent study shows surprisingly normal brain development in $EPOR^{-/-}$ mice rescued from the lethal hematopoietic defect by using a blood-specific regulatory element of the GATA-1 promoter to drive EPOR expression [22]. Further analysis of these animals is necessary to elucidate the cause for this discrepancy and answer the question whether EPOR in the CNS is primary relevant under stressful conditions as hypoxia or ischemia in the non-rescued $EPOR^{-/-}$ fetuses [171].

Various *in vivo* and *in vitro* studies indicate that EPO exerts its trophic and cell-protective effects in the injured CNS by a variety of mechanisms, including antiapoptotic and anti-inflammatory mechanisms, and immune or electrophysiological modulation [7, 11, 154, 158, 159, 164, 169, 172–181]. Changes in EPOR expression play a key regulatory element in activating these mechanisms. *In situ* hybridization analysis showed the up-regulation of EPOR and its continuous expression (at 24 hours after brain injury) in the periphery (ischemic penumbra) of the cerebrocortical infarct area in the adult murine

CNS [182]. Compared with neuronal specific EPOR immunoreactivity in the human adult brain, EPORs have been detected in microvessels and neuronal fibers after acute injury, and in astrocytes of old infarct areas [165]. In response to hypoxia or anemic stress, neuronal EPOR expression is modified by a relatively higher increase in transcripts containing the appropriately spliced 5' coding region (exons 1 to 2) than that of the upstream transcripts (exon A). This indicates a shift under injury towards increased sensitivity to EPO [166]. *In vitro*, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α also up-regulate EPOR expression in human neuronal cells [183].

The activation of EPOR in neuronal cells is associated via JAK-2 with the phosphorylation of I-κB and results in the cytoplasm-to-nucleus translocation of NF-κB. Hereby, the transcription of genes which are associated with neuronal cell protection becomes activated [184]. Such a cross-talk phenomenon between JAK-2 and NF-κB signaling has not been described in non-neuronal cells, suggesting that neuro-specific proteins link both pathways together [11].

In transmitting the cell-protective effects of EPO in the injured CNS, EPOR expression on vascular smooth muscle cells is important, because EPO normalizes the auto-regulation of the cerebral blood flow after acute brain injury [185]. EPOR expression on capillary endothelial cells in the CNS may contribute to the neuroprotection as well, because these cells may be stimulated to produce neutrophins such as brain-derived neurotrophic factor (BNDF) [186]. The specific role of a second, soluble form of EPOR expressed in (murine) brain capillary cells needs additional investigations [103].

Eye

Expression of both EPO and EPOR in the human eye has been detected by RT-PCR analysis in tissue specimens from fetuses between six and 16 weeks of gestation [161]. While further data on the biology of EPO in the human eye are not available yet, animal studies indicate an important role of EPO as an endogenous retinal survival factor [12, 102].

In neonatal rats, immunohistochemistry revealed EPOR expression in different layers of the retina, most intensive in the retinal ganglion cell layer, and less intensive in the inner and outer plexiform layers and the inner portion of the inner segments [187]. *In vitro*, rEPO stimulates the neurite outgrowth in neurons of the rat retina [187].

In adult mammalian (mouse) retina, EPOR is localized in the rod inner segment of photoreceptor cells and in presynaptic complexes of photoreceptors. EPOR has also been detected, but with lower immunohistochemical staining intensity in the inner nuclear layer [188]. EPO expression in the retina increases after exposure to hypoxia, and hypoxic pretreatment protects murine photoreceptor cells from apoptotic cell death after exposure to damaging light levels *in vivo*. The systemic application of rEPO one hour before or after light

insult protects photoreceptors – demonstrating that rEPO, given in high dose of 5,000 U/kg, crosses the blood-retina barrier [188].

Cell-protective effects of rEPO on neurons of the retina have been found in a rat model of transient global retinal ischemia induced by raising intraocular pressure. Acute retinal ischemia followed by reperfusion results in a time-dependent (maximum at 72 hours), up to four-fold induction of EPOR in the retina and was identified in murine ganglion cells, amacrine cells, and astrocytes [189]. Recombinant EPO reduces the histopathological damage in transient global ischemia and promotes the functional recovery of the retina if systemically administered in a high dose of 5,000 U/kg, before or immediately after induction of tissue damage. Data generated with TUNEL staining suggest that rEPO has cell protective effects by inhibiting apoptosis [189].

These data allow new concepts for rEPO as a potential therapeutic agent for several retinal diseases including acute glaucoma, acute retinal occlusion, diabetic retinopathy, and hypertensive retinopathy. New experimental strategies are being developed to achieve a local effect of EPO by using an intraocularly administered, inducible adeno-associated virus (AAV) vector construct [190]. However, angiogenic effects of EPO must be considered, for example in ventilated pre-term babies who may be treated with relatively high doses of rEPO to prevent transfusions during the anemia of prematurity, but who are at high risk for retinopathy of prematurity.

Conclusions

The expression and regulation of EPOR in mammalian non-hematopoietic organs involves developmental-stage and tissue-specific mechanisms. Further studies are necessary to elucidate these regulatory mechanisms. A more complete understanding of this system might broaden the therapeutic use of rEPO toward repair of chronically or acutely damaged tissue.

Acknowledgement
I thank Dr. Robert D. Christensen, University of South Florida, St. Petersburg/FL, USA, and Dr. Joachim Fandrey, University of Essen, Germany, for the critical review and stimulating discussion of this manuscript. Author's work was partially supported by a grant from the Deutsche Forschungsgemeinschaft (DA 484/1-1).

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Erythropoietin receptor signaling processes

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Introduction

Activation of erythropoietin receptor (EPOR) by erythropoietin (EPO) is essential for the survival, proliferation, and differentiation of red blood cells, as has been demonstrated definitively by the generation of EPO and EPOR-deficient mice [1–3]. Signaling by other cytokine receptors cannot replace EPO since EPO and EPOR-deficient mice die of severe anemia at days 13 to 15 (E13–E15) of embryogenesis due to lack of differentiation of their mature erythroid progenitors (erythroid colony-forming units [CFU-E]) [1–3]. Fetal livers of $EPO^{-/-}$ and $EPOR^{-/-}$ animals contain normal numbers of primitive (erythroid burst-forming units [BFU-E]) and mature CFU-E progenitors indicating that EPO signaling is not required for lineage determination and commitment, but is essential for terminal maturation of red blood cells. EPO binding induces conformational changes of an already dimerized EPOR on the cell surface and triggers the transphosphorylation and activation of receptor-associated JAK-2, a member of Janus protein tyrosine kinase family (Fig. 1). JAK-2 phosphorylates several tyrosine residues on EPOR, providing docking sites for binding of intracellular signaling proteins and initiating EPOR signaling pathways. (See Chapter 3 by Osslund for further information.) These pathways converge presumably on a common set of targets resulting in cell proliferation, differentiation, and survival. It is not known, however, whether any signal emanating from EPOR specifically regulates transcription of genes required for red cell differentiation and maturation.

For historical and physiological reasons, EPOR signaling has been well studied in red cell progenitors and erythroblasts. It has become clear more recently that EPOR is expressed in many organs including brain, heart, endothelium, and ovaries and may have physiological roles in these organs [4–9]. In particular, it has been suggested that EPOR signaling may be required for survival of neuronal cells [5, 10]. Although studies have begun [11] to establish the role of EPOR signaling in these organs, it is becoming increasingly apparent that red cells are not the only targets of EPO.

EPOR activation

EPOR dimerization

Dimerization has been shown to be crucial for EPOR activation. For example, bivalent, but not monovalent, monoclonal antibodies directed to the extracellular domain of the EPOR, as well as small noncovalently dimerized peptides, can activate EPOR [12, 13]. An R129C point mutation in the extracellular domain renders the receptor constitutively active as a result of the formation of an intermolecular disulfide bond that connects two receptor polypeptides [14, 15]. Based on the crystal structure of the growth hormone receptor (GHR) extracellular region and comparison between EPOR and GHR amino acid sequence, Arg129 maps to a region near the dimer interface. Two other mutations in this region, E132C and E133C, also constitutively activate the receptor and form disulfide-linked dimers [16]. Fusion proteins of the extracellular domain of c-kit or epidermal growth factor (EGF) receptor to EPOR cytoplasmic domain can be activated by stem cell factor (SCF) or EGF, respectively, which are known to dimerize their corresponding receptors [17]. Cytoplasmic truncation mutants of EPOR, which are inactive in cell proliferation assays when expressed on their own, dominantly inhibit EPO-dependent proliferation and differentiation when co-expressed with wild-type receptor [16, 18, 19]. In 1998, the crystal structure of a complex between EPO and the extracellular domain of EPOR was obtained [20]. This structure shows a 1:2 EPO/EPOR stoichiometry, and leads to a model that after EPO binding, two monomeric receptors are brought together into a dimer and activate the receptor. (See Chapter 3 for further information.)

Recent lines of evidence however, reveal that EPOR exists as a preformed dimer on the cell surface before ligand binding. The crystal structure of the soluble extracellular domain of human EPOR in its unliganded state adopts a dimeric configuration, although with a geometry different from that of the EPO-bound receptor [21]. Results of antibody-mediated immunofluorescence copatching (oligomerizing) of epitope-tagged receptors at the surface further demonstrated that unliganded EPOR forms dimers or higher-order oligomers on living cells [22]. Consistently, when two complementary dihydrofolate reductase (DHFR) fragments were fused to the cytoplasmic domain as a fusion protein through a long linker (30 amino acids, 120 Å), complementation between the DHFR sub-units ocurred between unliganded receptors [23].

Specific homophilic interactions between the transmembrane domains have been shown to play important roles in the oligomerization of EPOR. Kubatzky et al. showed that the expression of the transmembrane domains of EPOR in a chimeric bacterial protein triggers transmembrane-mediated dimerization and the activation of a reporter gene [23, 24]. Abrogation of dimerization by mutagenesis in EPOR transmembrane domain also impaired EPOR signaling in mammalian cells. The researcher concluded that interactions between transmembrane domains may drive EPOR signaling. Constantinescu et al. showed that oligomerization of EPOR is dependent on its transmembrane domain.

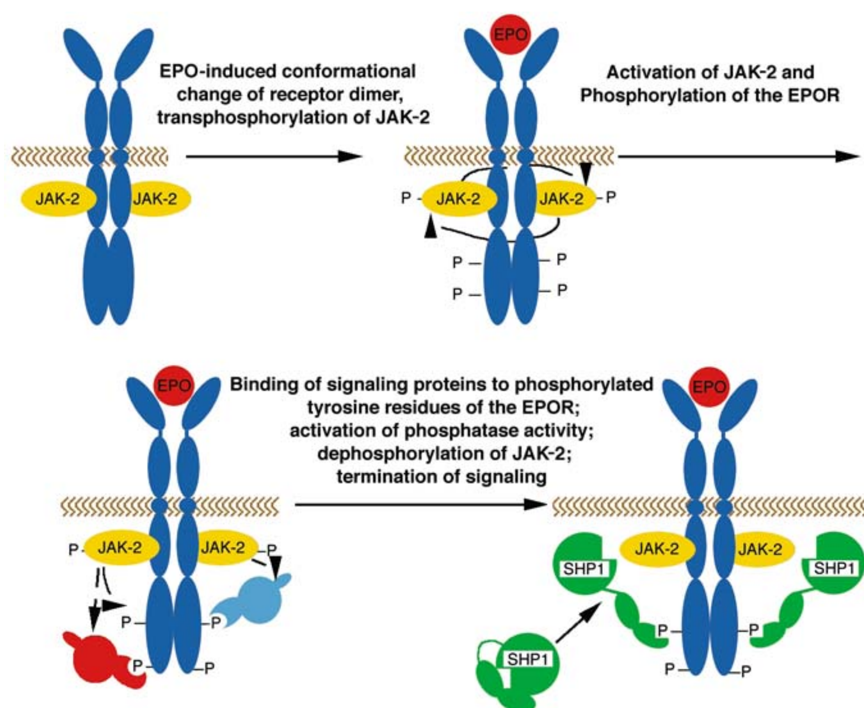

Figure 1. Binding of EPO induces a conformational change in the receptor dimer that is transmitted to the cytosolic domain, leading to auto- or transphosphorylation of JAK-2. JAK-2 kinase then phosphorylates several tyrosine residues in EPOR, creating docking sites for the SH2 domains of several signal transduction proteins. These signaling proteins become activated and downstream signal transduction is initiated. One of the mechanisms of terminating EPOR signal transduction is through the protein tyrosine phosphatase SHP1. SHP1 binding to a segment of EPOR containing phosphotyrosine 429 induces activation of the phosphatase. SHP1 then removes the activating phosphate from JAK-2, and terminates signal transduction.

Preformed unliganded EPOR oligomers on the surface of living cells are not active, and a fusion protein of EPOR with its transmembrane domain replaced with the transmembrane domain of glycophorin A, shown to mediate dimer formation, also requires EPO for activation [25]. The latter paper concludes that interactions between the transmembrane domains of unliganded EPOR maintains them in an inactive state. These and other results [20] suggest that inactive EPOR are converted to an active state upon EPO binding through a rotational movement instead of a lateral movement of the two transmembrane domains to achieve functional proximity of the cytoplasmic domains (Fig. 2). This model contradicts one based on crystallographic structures of liganded and unliganded EPOR that proposes a decrease in the distance between the two transmembrane domains upon ligand binding to preformed EPOR dimers [21, 23]. Thus, the mechanism by which EPO binding leads to receptor activation remains unknown.

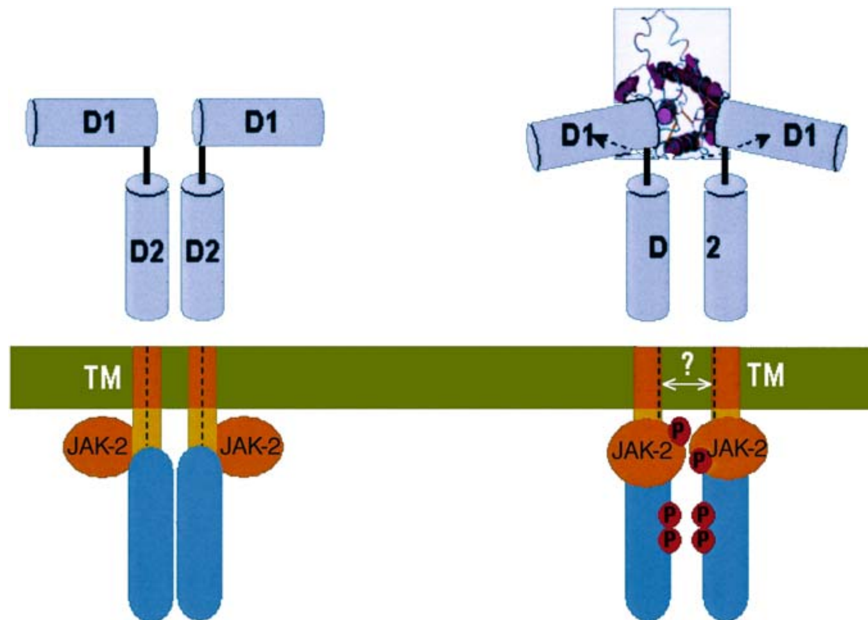

Figure 2. The unliganded EPOR exists as a symmetric dimer; dimerization is mediated mainly by the transmembrane (TM) domains. The associated JAK-2 kinases are apart when EPOR is in its inactive form. The dotted lines represent the middle of the TM domains in the inactive state (A). Binding of an asymmetric EPO molecule causes a rotation of the extracellular domains, and the two D1 domains adopt a 120° angular orientation. Coupled by the TM domain, this rotation causes similar movements in the juxtamembrane (JM) part of the intracellular domains, thus brings the associated JAK-2 kinases to a close functional organization resulting in JAK-2 activation and EPOR phosphorylation (B). The structure in the boxed area is determined by crystallography [20]. It is not clear (as indicated by the question mark) whether the transmembrane domains move further away from each other in the activated state.

Orientation of EPOR dimer

The fact that EPOR in the unliganded state exists as a dimer strongly indicates that receptor activation is achieved by a distinct conformational change in response to EPO. An *in vivo* fragment complementation assay shows dramatic ligand-induced enhancement of proximity of the cytoplasmic domains of EPOR dimers [23]. Furthermore, results from EPO mimetic peptides showed that the relative orientation of EPOR extracellular domains in a receptor dimer is directly related to the efficiency of signaling through the cytoplasmic domain [26]. For example, the EPO-mimetic peptide EMP dimerizes EPOR with two-fold symmetry and triggers activation of EPOR signaling cascades. A derivative of EMP, EMP33, acts as an antagonist of EPO. The inactive complex of two EPOR extracellular domains with EMP33 differs from that of the active complex formed with EMP by a 15º deviation from the axis of symmetry. The difference

in orientation was interpreted as related to whether the intracellular domains of EPOR would be brought into productive or non-productive orientations.

Results from our laboratory highlighted the productive or non-productive orientations of EPOR dimer. A conserved hydrophobic motif (L253, I257, W258) in EPOR juxtamembrane cytoplasmic region just *N*-terminal to the conserved Box 1 motif is necessary for receptor signaling [22]. Using a combination of computer modeling and mutagenesis, we showed that this motif is conformationally rigid and is contained within an α-helix continuous with the transmembrane α-helix. An insertional mutation of three alanines, which maintains the register of the α-helix encompassing the hydrophobic motif, signals like wild-type EPOR. Alteration of the register of the α-helix by inserting one alanine results, however, in a change in JAK-2 positioning on EPOR. In this case, EPO induces JAK-2 transphosphorylation but these kinases are positioned in a way that they cannot phosphorylate EPOR and are inactive in EPOR signaling.

R129C

A constitutively active form of EPOR, R129C, was isolated by its ability to support EPO-independent proliferation of Ba/F3 cells [14]. Unlike the wild-type EPOR, this mutant forms disulfide-linked homodimers in the absence of EPO [15].

Mice infected with a virus carrying the R129C mutant EPOR develop polycythemia and splenomegaly, demonstrating that R129C stimulates expansion of the erythroid compartment *in vivo*, with a concomitant increase in the number of circulating erythrocytes [27]. When factor-independent cells were isolated from the spleen of the infected mice and injected into syngenetic mice, a fatal leukemia developed with a massive expansion of erythroblast-like cells that populated the bone marrow and spleen of the recipient animals [27]. R129C also stimulates EPO-independent development of CFU-E cells into mature erythrocytes *in vitro* [28]. Artificial overexpression of R129C EPOR mutant in different lineages induces leukemia, suggesting that once expressed, EPOR generates signals that induce proliferation of many types of progenitors additional to erythroid progenitors [27]. This report described the first oncogenic mutation in a member of the cytokine-receptor family.

Friend virus-induced erythroleukemia

Friend virus consists of a replication-competent murine leukemia virus (FMuLV) and a replication-defective spleen focus-forming virus (SFFV). Genetic studies have shown that the transmembrane protein gp55 of the defective SFFV is responsible for Friend virus-induced erythroleukemia [29]. The two known strains of SFFV, the anemic or “A” strain and the polycythemic or

“P” stain, encode two types of gp55 (gp55-A and gp55-P, respectively). Both bind to and abnormally activate murine, but not human, EPOR [30, 31]. gp55-P is more potent than gp55-A in activating EPOR and leads to EPO-independent proliferation of host cells [32]. The interaction of gp55 and EPOR is through their transmembrane domains [33, 34]. Mutagenesis studies of both murine and human EPOR transmembrane domains identified that Ser238 in murine EPOR is critical for EPOR-specific interaction with gp55 [35]. In addition, computational modeling and mutagenesis studies showed that gp55-P Met390, which interacts with Ser238 of EPOR, is the key amino acid in the transmembrane domain essential for the full function of gp55-P [35]. gp55-P and gp55-A are thought to activate EPOR signaling by inducing receptor oligomerization, which constitutively activates downstream Raf-1/MAPK and PI-3/Akt pathways [36, 37].

Kinases interacting with EPOR

JAK-2 signaling

Activation of the cytoplasmic tyrosine kinase JAK-2 appears to be the initiating event of EPOR signal transduction [38]. Activated JAK-2 phosphorylates various substrates, including multiple tyrosine residues on EPOR. These phosphorylated tyrosines serve as docking sites for SH2 domain-containing proteins. Binding of SH2-containing proteins to EPOR phosphotyrosines activates signaling by the signal transducer and activators of transcription STAT5, phospholipid modifying enzymes (PI-3-Kinase, PLC-γ, and SHIP), regulators of Ras and MAP kinase signaling, tyrosine phosphatases (SHP1 and SHP2), suppressors of cytokine signaling (CIS and SOCS3), and Src-family kinases [38].

Constitutive activation of human JAK-2, caused by either overexpression or chromosomal translocation, results in leukemias, e.g., acute lymphoblastic leukemia or chronic myelogenous leukemia [39–41]. Consistently, a mutation in the JAK homolog in *Drosophila*, hopscotch, causes abnormal proliferation and differentiation of the larval hematopoietic system and leads to lethality [42].

In addition to the hematopoietic system, EPOR signaling through JAK-2 is important in neural progenitor cell survival and neuronal protection in models of ischemic and degenerative damage [4, 5].

JAK-2 knockouts

The physiological importance of JAK-2 in EPOR signaling is best demonstrated through JAK-2-deficient mice. JAK-2 knockout mice die about E12.5 of embryogenesis due to severe anemia, a phenotype similar to, but more severe than, EPOR knockout mice [43, 44]. Analysis of JAK-$2^{-/-}$ fetal liver cells in culture demonstrated that myeloid progenitors are decreased in number in these mice and they fail to respond to a variety of cytokines and growth factors including EPO.

Processing of EPOR by JAK-2

Only low numbers of EPOR (200 to 3,000 receptors/cell) are expressed on the surface of primary erythroid cells or on cells engineered to overexpress the receptor [14, 35–47]. Most newly synthesized molecules are retained in the endoplasmic reticulum and appear to be degraded without further processing [14, 15]. *In vitro* experiments have suggested that unliganded EPOR normally is tethered to JAK-2 [23], and it was thought that their interaction occurs only at the plasma membrane. Our results showed that JAK-2 assembles with newly synthesized EPOR in the endoplasmic reticulum, and that this assembly results in efficient expression of the receptors at the cell surface [48]. This interaction is specific to the *N*-terminal domain of JAK-2, as JAK1 has no effect on EPOR surface expression. Therefore, in addition to its signaling ability, JAK-2 is essential for optimal expression of the signaling EPOR/JAK-2 complex at the cell surface.

JAK-2 activation in the EPOR/JAK-2 complex

JAK-2 contains an *N*-terminal domain followed by a pseudokinase domain, and a kinase domain at its *C*-terminus. The *N*-terminal domain is responsible for functional EPOR interaction and EPOR surface expression [48]. JAK-2 physically associates with the membrane-proximal Box1/Box2 region of EPOR cytoplasmic domain [49]. EPO induces auto- or transphosphorylation of a tyrosine in the kinase activation loop and triggers JAK-2 activation [50]. Figure 2 illustrates our current model for EPOR induction of JAK-2 activation.

In light of a preformed dimeric EPOR/JAK-2 complex in the absence of EPO on the cell surface, JAK-2 activation in this complex may involve the transmission of the presumed conformational change in the extracellular domain of EPOR upon EPO binding to the intracellular activation of pre-associated JAK-2 and the subsequent phosphorylation of EPOR cytoplasmic domain. Three hydrophobic residues, Leu253, Ile257, and Trp258, in the juxtamembrane region of EPOR constitute a hydrophobic motif critical for JAK-2 activation and EPOR signaling [48]. Mutations of the individual residues in this motif generated EPOR that form cell surface EPOR/JAK-2 complexes, however, these mutant EPOR are unable to activate JAK-2 upon EPO stimulation. We propose that this motif acts as a molecular switch to turn on JAK-2 tyrosine kinase activity upon EPO binding to the EPOR/JAK-2 complex. Since this motif is highly conserved among cytokine receptors, this mechanism may be used by other cytokine receptors and their cognate JAK. Further studies are required to test this hypothesis.

Results from studies using knock-in and transgenic mice showed that a truncated EPOR without any tyrosine residues can support erythropoiesis *in vivo*, although much poorer than the wild-type receptor [51, 52]. These receptors retain the Box 1/Box 2 region that binds and activates JAK-2, highlighting the essential role of JAK-2 in EPOR signal transduction in erythropoiesis. Furthermore, these experiments and others [53] underscore the contribution of

JAK-2-dependent but receptor phosphotyrosine-independent pathways in EPOR signaling. For instance, JAK-2 may directly phosphorylate STAT5 independently from STAT5-binding to EPOR, since EPOR (F8) where all EPOR cytosolic tyrosines are mutated to phenylalanine, can still support 10% of the STAT5 activity that is normally generated by EPO stimulation of wild-type EPOR [54].

Lyn tyrosine kinase

Three protein tyrosine kinases (Lyn, Syk, and Tec) have been reported to associate with activated EPOR complexes [55–57]. Among them, Lyn tyrosine kinase has been studied most extensively. In a mutant erythroleukemic J2E cell line that fails to differentiate in response to EPO, mRNA and protein expressions of Lyn are reduced and wild-type Lyn rescues the mutant defect [58]. In 32D cells, Lyn associates with EPOR at (P)Y464 and/or (P)Y479 and phosphorylates both EPOR and STAT5 [55]. Additional signaling proteins have been identified that bind Lyn in response to EPO, including SHP-1, SHP-2, ras-GAP, CrkL, Raf-1, MAPK, and HS1 [59–62]. A dominant-negative Lyn mutant that inhibits the association of Lyn to most of these signaling proteins also blocks erythroid differentiation of cultured cells [60]. In normal erythroblasts, EPO enhances phosphorylation of Lyn; moreover, overexpression of Lyn increases the number of CFU-E–derived colonies but has no effect on BFU-E colonies [60]. Although Lyn has been shown to phosphorylate EPOR in the presence of EPO, it is considered as a secondary kinase in EPOR signaling [60]. Consistent with this notion, Lyn$^{-/-}$ mice, in marked contrast to EPO$^{-/-}$, EPOR$^{-/-}$, and JAK-2$^{-/-}$ mice, do not die with embryonic anemia and do not exhibit major erythroid defects [1, 43, 44, 63]. Detailed analyses of Lyn-deficient mice are required to determine the precise role of Lyn in EPOR signaling *in vivo*.

Phosphorylation of EPOR and activation of downstream signaling pathways

Binding of EPO to its receptor activates many signaling pathways. All these pathways are activated by other cytokines and to date, the specificity of these pathways in terms of cellular outcome is not clear. In particular, we do not know if EPOR activates different pathways to different extents in CFU-E, erythroblasts, and neuronal cells.

EPOR and STAT5

STAT proteins

STAT proteins are identified as major players in cytokine signaling [64]. They were first identified as interferon-responsive transcription factors. STATs are

present in the cytoplasm of unstimulated cells, and directly link activated cytokine receptors to gene transcription. The STAT are recuited to the receptor complexes through SH2 domain recognition of specific phosphorylated tyrosine residues on the receptors (or JAK-2 or other proteins), are phosphorylated by JAK, undergo homodimerization or heterodimerization and translocate to the nucleus where they drive the transcription of a wide range of genes.

Stimulation of EPOR specifically activates STAT5 [54, 65, 66]. EPOR mutants that contain only one tyrosine, either Y343 (F7Y343) or Y401 (F7Y401), activate STAT5 to the same extent as the wild-type receptor [54, 67]. These mutant receptors can support differentiation of only 50% and 30% of the normal numbers of fetal liver CFU-E [68], respectively, suggesting that STAT5 activation is not sufficient to support normal erythroid differentiation. STAT5 is activated at least to some extent (approximately 10%) after stimulation of a mutant EPOR lacking all tyrosine residues (EPOR F8), indicating that there are multiple pathways leading to EPOR activation of STAT5, and that JAK-2 tyrosine kinase may directly activate STAT5 through STAT5 directly binding to phosphotyrosines on JAK-2. Interestingly, the constitutively active EPOR (R129C) mutant does not induce constitutive STAT5 activation in BaF_3 cells, indicating that signaling by EPOR and its constitutively active mutant may be distinct [69].

STAT activity as a result of ligand stimulation is transient and is rapidly downregulated by several mechanisms including ubiquitin-mediated degradation of STAT5 [70], competition by induced cytokine-inducible SH2-containing molecule for the STAT binding site on the cytokine receptor [71], or direct binding of STAT5 to the negative regulator protein inhibitor of activated STAT [72, 73]. A role for protein inhibitor of activated STAT in down-regulation of STAT5 activity in particular has not been reported.

Known STAT5 target genes include tissue-specific genes and genes that regulate general cell growth. Overexpression studies using dominant negative and constitutively active forms of STAT5 in erythroid cell lines and cultured fetal liver cells have demonstrated an anti-apoptotic role for STAT5. In a number of erythroid and hematopoietic cells, STAT5 induces the immediate early gene expression of the anti-apoptotic Bcl2 family member gene Bcl_{XL}, by directly binding to STAT5 consensus binding site in the Bcl_{XL} promoter [74–77]. A role for STAT5 in both proliferation and differentiation of erythroid cells has been suggested; however, strict correlation of STAT5 activation with EPOR proliferative or differentiative potential appears to depend on the cell context [54, 66, 68].

In addition to STAT5, STAT1 and STAT3 are activated in response to receptor stimulation of EPO-dependent cells [78, 79]. Activation of STAT1 and STAT3 may negatively regulate EPO-induced differentiation of the human EPO-dependent UT7/EPO cells [79].

Knock-outs

STAT5A and B are 96% identical in amino acid composition, and form homodimers or heterodimers upon phosphorylation. STAT5A- and STAT5B-deficient mice show distinct phenotypes and reveal important functions for these proteins in prolactin and GH signaling, respectively [80, 81]. Alterations in hematopoiesis of $STAT5A^{-/-}$ or $STAT5B^{-/-}$ mice have not been reported. Deletion of both STAT5A and STAT5B in mice [82] results in anemia during embryogenesis and marked increase in apoptosis of erythroid cells [74]. In addition, some adult $STAT5A^{-/-}/STAT5B^{-/-}$ mice have near-normal hematocrit but are deficient in induction of erythropoiesis during stress-induced anemia [83]. In these mice, erythropoiesis is blocked at early erythroblasts that undergo apoptosis and fail to mature to late erythroblasts.

PI3-kinase/AKT

Activation of PI3-kinase and downstream AKT pathways

PI3-kinase signaling is activated in response to EPO stimulation in several EPO-dependent cultured cells. EPOR Y479 is essential for binding to the P85 sub-unit of PI3-kinase that activates PI3-kinase in response to EPO [84–86]. In particular, EPO stimulation of the F7Y479 EPOR mutant, in which all cytosolic tyrosines but Y479 are mutated to phenylalanine, activates PI3-Kinase and ERK but not STAT5. (P) Y479 recruits PI3-kinase to EPOR, resulting in RAS-independent activation of a MAPK cascade (ERK1/2) [86]. A role for protein kinase C as an intermediate has also been suggested [86–88]. It is not clear whether (P) Y479 also supports the activation of other MAPK pathways (JNK, p38). Each of two tyrosines, Y464 and Y479, alone can mediate a proliferative signal in Baf3 cells. Y479 but not Y464 is also capable of inducing the differentiation of over 85% of CFU-E. (P) Y479 is the only tyrosine residue on EPOR that can support both proliferation and differentiation of erythroid cells to the same extent as the wild-type receptor [68], suggesting that pathways additional to ERK may also be activated by PI3-kinase in response to EPO. Both PI3-kinase/AKT and MAPK pathways appear to be required for EPO-independent leukemic transformation of proerythroblasts [88, 89]. Inhibition of PI3-kinase activation prevents differentiation of primary human erythroid progenitor cells in culture, suggesting an important role for PI3-kinase activation in generation of erythroid cells [90, 91]. Less is known about the contribution of PI3-kinase activation to erythroid cell formation *in vivo.*

It has been proposed more recently that additional pathways activated by EPO and distinct from (P) Y479, may result in PI3-kinase activation. For instance, in the primitive hematopoietic UT7 cells, EPOR is constitutively associated with insulin receptor substrate (IRS)-2. After EPO stimulation, IRS-2 is rapidly tyrosine phosphorylated and associates with the P85 sub-unit of PI3-kinase and with phosphatidylinositol 3,4,5 triphosphate 5-phosphatase

(SHIP) [92]. EPOR-associated IRS-2 may provide an alternative mechanism for activation of PI3-kinase in response to EPO in UT7 cells. This mechanism of PI3-kinase activation may be used in a subset of EPO-dependent cells expressing IRS-2 [92]. Alternatively in K562 cells, PI3-kinase is activated by binding of its P85 sub-unit to EPOR phosphorylated by Src tyrosine kinase. In these cells, Src also associates directly with P85 sub-unit of PI3-kinase and may enhance its activation [93].

Activation of AKT pathway

More recent work has clearly demonstrated activation of the AKT pathway in a PI3-kinase-dependent manner in response to EPO [90, 94]. Upon EPO stimulation, AKT serine threonine kinase is phosphorylated and activated in both primary human [91] and cultured erythroid cells [90, 94]. The activation of the PI3-kinase/AKT pathway has been shown to over-ride the DNA damage-induced cell cycle arrest of hematopoietic cells [95].

EPO also induces phosphorylation of the pro-apoptotic FKHRL-1 transcription factor, a known downstream target of AKT, and induces its cytoplasmic relocalization [90, and Ghaffari, Kitidis, and Lodish, unpublished data]. Whether phosphorylation of FKHRL-1 in response to EPO is through AKT or other PI3-kinase-dependent kinases is not clear. In addition, the role of AKT in erythroid development is unknown.

PI3-kinase/AKT knock-outs

Although PI3-kinase/AKT activation seems to be important for erythroid development, mice lacking AKT1 or AKT2 do not exhibit any obvious erythroid phenotype [96–98], possibly due to functional redundancy of AKT isoforms. Mice lacking either AKT3 alone or all AKT isoforms have not been generated and hematopoietic development in AKT1 and AKT2 knock-outs have not been closely examined. The status of red cell development in PI3-kinase$^{-/-}$ mice is also unknown.

Ras/MAPK pathways

Signaling

In mammalian cells, Ras activates three groups of MAPK, including ERK, JNK, or SAPK, and p38 [99]. In various EPO-dependent cell lines, different groups of MAPK are activated in response to EPO [100–104]. Activation of the Ras/ERK pathway may be through the canonical Grb2 binding site at (P)Y464 of EPOR [105], as cell proliferation promoted by this pathway in response to EPO is inhibited by Raf-1 antisense oligonucleotides [106, 107]. Synergestic activation of ERK1/2 by EPO and SCF may be important for proliferation of human erythroid progenitors [108]. Alternatively, the ERK pathway may be activated independent of Ras through the PI3-K pathway [86]. JNK and p38 are implicated in induction of erythroid differentiation of EPO-

dependent SKT6 cells [103, 104]. The role of JNK and p38 in inducing apoptosis of erythroid cells remains controversial [104, 109, 110].

Knock-outs

To date, all the three *ras* genes (*H-, N-,* and *K-ras*) have been knocked out in mice [111–114]. *K-ras*$^{-/-}$, *N-ras*$^{-/-}$, and *K-ras*$^{+/-}$ mice die at early embryonic stages with anemia [112]. The cellular mechanisms underlying this phenotype remain elusive. On the other hand, mutations in *N-* and *K-ras* genes that create constitutively active/oncogenic Ras proteins are frequently identified in patients with myeloid disorders [115]. In these disorders, the erythroid lineage is often affected, suggesting a role for altered Ras signaling in dysregulated erythropoiesis seen in these patients. Terminal erythroid differentiation is blocked in human umbilical cord blood cells expressing oncogenic N-ras [116]. It is not clear whether N-ras is blocking downstream signaling of EPOR in these cells.

Negative regulation and hyperactive EPOR

SOCS

The amplitude and duration of EPOR signaling is modulated by negative regulators. The cytokine-inducible SH2-containing protein, currently known as CIS1, was the first member of a large family of cytokine-receptor inhibitors identified [71]. CIS1 was cloned originally as an immediate early gene induced in response to interleukin (IL)-2, IL-3, and EPO [71]. Upon EPO stimulation, CIS1 binds EPOR, specifically on Tyr401 [117], and may interfere with STAT5 binding to the receptor. Overexpression of CIS1 partially inhibits STAT5 activation and EPO-stimulated proliferation [118]. CIS1 inhibition of STAT5 may be part of a feedback loop where expression of CIS1 is induced by activated STAT5. The CIS promoter contains two STAT5-binding sites that can be induced by EPO in cells transfected with EPOR [118]. In addition, lack of CIS1 expression in several organs of STAT5a$^{-/-}$/b$^{-/-}$ mice supports the hypothesis that STAT5 activates CIS expression [82, 119]. Available data suggest that CIS1 may also attenuate EPOR signaling by accelerating the ubiquitination and degradation of activated EPOR [117, 120].

Two other members of the CIS family bind JAK-2 tyrosine kinase. The JAK-binding protein interacts with the kinase domain of JAK-2 [121] by specifically binding to the phosphorylated tyrosine pY1007 in the activation loop [122]. JAK-binding protein was cloned as suppressor of cytokine-signaling-1 (SOCS-1), an inhibitor of IL-6-induced differentiation of the murine myeloid M1 cells, as well as STAT-induced STAT inhibitor (SSI-1) [123]. SOCS-1 is a negative regulator of interferon (IFN)-γ signaling. The phenotype of SOCS-1$^{-/-}$ mice is reminiscent of that seen in IFN-γ transgenic mice [124, 125]. SOCS-3 binds the kinase domain of JAK-2, and inhibits JAK-2 tyrosine

kinase activity *in vitro* [126]. SOCS-3 is highly expressed in the erythroid lineage during embryonic development, and deletion of SOCS-3 results in embryonic lethality at E12 to E16 associated with erythrocytosis [127]. These studies identify SOCS-3 as a major negative regulator of fetal liver erythropoiesis possibly through negative regulation of EPOR/JAK-2 signaling. The exact mechanism of negative regulation of EPOR signaling by SOCS-3 requires additional investigation.

Phosphatases

Three phosphatases interact with EPOR, including SH2-containing protein tyrosine phosphatase 1 (SH-PTP1 or SHP1), SHP2, and SH2-containing inositol-5-phosphatase (SHIP) [128]. Among them, only SHP1 is a negative regulator of the phosphorylated EPOR. SHP1 binds to the (P)Y429 of EPOR after EPO addition [129]. Cells expressing EPOR Y429F mutant are hypersensitive to EPO and show a prolonged EPO-induced autophosphorylation of JAK-2, suggesting that SHP1 dephosphorylates and inactivates JAK-2 [129, 130]. Furthermore, mice with the deletion of EPOR distal region (including Y429) display erythrocytosis when challenged with continuous EPO injections [52]. In *motheaten* mice, which carry a spontaneously occurring SHP1 mutation, many hematopoietic cell lineages show hyperproliferation, including splenic CFU-E [131–133]. This phenotype is reminiscent of familial polycythemia observed in human patients carrying mutated EPOR. To date, among the ten published EPOR mutations associated with erythrocytosis, six have *C*-terminal truncations missing Y429, supporting the negative regulatory role of the EPOR Y429 and its associated SHP1 [134–144]. In contrast, SHP2 and SHIP act as positive effectors in erythropoiesis. In embryonic stem cells carrying a SHP2 mutant with a disrupted *N*-terminal SH2 domain, the production of erythroid cells in embryoid bodies is suppressed [145, 146]. Similarly, in $SHIP^{-/-}$ mice, the number of bone marrow-derived CFU-E is decreased [147].

CD45 is another phosphatase with a potential role in EPOR signaling. CD45 is a transmembrane phosphatase that is highly expressed on hematopoietic cells and has been shown as a key regulator of antigen receptor signaling in T and B cells [148, 149]. Targeted disruption of CD45 leads to enhanced activation of JAK-2 signaling [150]. *In vitro* studies showed that CD45 could directly dephosphorylate JAK-2 and negatively regulate EPOR signaling [150]. CD45 is present only on a small, relatively immature subset of EPOR-expressing erythroid cells in the bone marrow [151, 152]. In particular, CD45 is not expressed on CFU-E or erythroblasts, suggesting that the potential physiological role of CD45 in the regulation of EPOR signaling occurs in pre-CFU-E erythroid progenitor cell.

Summary

Many signaling pathways are activated in response to EPO stimulation of erythroid cells. Activation of STAT5 is required for erythroid survival. Specific roles of other EPOR-activated signaling pathways in erythroid development remain to be addressed. In addition, the nature and the role of signaling pathways activated in response to EPO in nonerythroid cells should be investigated.

Acknowledgements
We apologize to those whose work could not be cited due to space considerations. We would like to thank Dr. Xiaohui Lu for providing Figure 2.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Clinical pharmacokinetic properties of rHuEPO: a review

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Introduction

As with all therapeutics, the clinical endpoints for recombinant human erythropoietin (rHuEPO) are the most important considerations for usage. Understanding the pharmacokinetics of an agent allows consideration of clinical questions such as:.

- If the dose is doubled, will the blood concentration double?
- If the drug is administered long-term, will it accumulate?
- Should the dose be changed if route of administration is changed?
- Should the dose be altered if the patient has liver disease?

Hence, having a mechanistic understanding of the factors that influence the serum concentrations of a drug allows for more informed decisions when treating patients. An assumption in this argument is that a relationship between drug serum concentration and efficacy has been established. In the case of rHuEPO, a pharmacokinetic-pharmacodynamic relationship has indeed been established [1] wherein hemoglobin increase is delayed in response to increased serum rHuEPO concentrations. An additional benefit of understanding the pharmacokinetic properties, and their relationship to efficacy, is the potential ability to improve patient/physician convenience and/or efficacy by manipulation of the dose administration schedule, route, or even the molecule.

This review focuses on clinical studies that have determined the pharmacokinetic properties of rHuEPO in healthy volunteers, nephrology patients (adult and pediatric), cancer patients, and others. For each population, the available data were considered with the following questions in mind:

- What are the average values and associated variability of the pharmacokinetic parameters in that population for both intravenous and subcutaneous dosing?
- Do the pharmacokinetic properties remain the same over a wide dose range, i.e., are they dose independent?

- Do the pharmacokinetic properties remain the same upon repeated administration over time, i.e., are they time linear?
- How do the pharmacokinetic parameters vary from one population to the next?

In addition, the preclinical and clinical literature relating to clearance mechanisms is reviewed. Discussion of the relationship of rHuEPO serum concentrations to efficacy outcomes is beyond the scope of this review. For clarity purposes it should be noted that rHuEPO is currently available as two molecules – epoetin alfa and epoetin beta. Epoetin alfa is marketed as Epogen, Procrit, Eprex, and Erypo (depending on region) in most of the developed world, including the United States, Europe, Canada, Australia, and Asia. Epoetin beta is marketed as NeoRecormon in Europe and Japan. Each of these marketed products is available in a range of concentrations and, in some cases, formulations. This review does not address concentration-specific or formulation-specific effects. Although minor differences in both pharmacokinetic and pharmacodynamic properties have been noted between the two molecular forms (to be discussed), the molecules are generally used interchangeably. All values in this review are given as mean (SD) unless explicitly noted otherwise.

Challenges in determining and comparing properties

The accurate determination of the pharmacokinetic properties (and associated variability) of a drug in a given population has a number of prerequisites. The most important of these are summarized. Blood samples should be collected for a duration sufficient to fully characterize the serum-concentration time profile. The exact dose, as well as the exact sampling times relative to dosing times, should be recorded and used in the analysis. The assay should be validated with a sufficiently low quantification limit and limited cross-reactivity with interfering substances. The pharmacokinetic analysis should take advantage of all available information, and characterization of the terminal phase should use relevant data points. The most suitable pharmacokinetic analysis technique should be used. In addition, the use of treatment-naive subjects, crossover studies, and larger numbers of subjects allows for a better estimate of certain pharmacokinetic characteristics.

In the case of rHuEPO, the most blatant, unavoidable rule-breaker is the use of assays that cross-react with endogenous erythropoietin (EPO). Due to the structural similarity between endogenous EPO and rHuEPO, most (if not all) of the commonly used assays measure both the endogenous and exogenous hormone. The most common method of correcting for the inability to differentiate between the two products is by baseline-correction and all the articles cited in this review have used baseline-corrected values. At least one, and up to three samples, are collected pre-dose and these are used as a baseline estimate to correct (by direct subtraction) subsequent concentrations for the

endogenous component [1, 2]. This method of correction assumes that the endogenous EPO concentration remains constant over the course of the study. This assumption ignores the natural diurnal fluctuation of endogenous EPO, which has been shown to be lowest at 8:00 AM and increase by an average of 60% at its peak at 8:00 PM [3]. This method also ignores the potential impact of exogenous administration on endogenous EPO through feedback mechanisms. Two assay systems are generally used – radioimmunoassay (RIA) [1, 4–11] and enzyme immunoassay (EIA) [12, 13]. The limit of detection is generally in the range 2–15 mU/mL, which covers the endogenous range.

It should be noted that cross-study comparisons of the pharmacokinetics of any drug have the inherent issues of differences in study design, including duration and frequency of sample collection, and differences in bioanalysis, including methodology, accuracy, and specificity.

Pharmacokinetics in healthy volunteers

Pharmacokinetic studies conducted in healthy volunteers tend to use treatment-naive subjects for the sole purpose of determining single-dose parameters, either over a dose range or for different routes of administration. In the studies reviewed, the sampling duration after intravenous dosing was generally sufficient (48 hours) with 10 or 11 samples (up to 16 or 18) collected over that time. The sampling duration after subcutaneous dosing should be longer for full characterization of the terminal phase; it varied greatly from 48 hours to 672 hours [4] with generally nine to 13 samples, and as many as 23 or 26, collected during that time. In general, the higher the dose investigated, the longer the test article is quantifiable in the serum, resulting in a more accurate determination of the full pharmacokinetic profile. A sampling duration of at least 72 hours would allow reasonable determination of the parameters of a subcutaneously administered dose.

Intravenous dosing

General

Generally, one [9, 14–17] or two [18] phases were evident in the serum concentration-time profiles. In cases where only one phase was observed, it is likely that the early rapid phase was not captured (due to sampling schedule and/or inter-individual variation) and that the slower, later phase dominated. The volume of distribution was generally similar to plasma volume (40–60 mL/kg), indicating limited extravascular distribution [14, 15].

Single dose, dose range

Key pharmacokinetic parameters determined after single-dose, intravenous administration to healthy volunteers are presented in Table 1. In healthy vol-

Table 1. Single-dose pharmacokinetic parameters of intravenously (IV) administered rHuEPO in healthy volunteers

Ref	IV dose (U/kg)	n	CL (mL/hr/kg)	$t_{1/2,z}$ (hr)
[11]	300 U [5 U/kg][a] [epoetin alfa]	12	13.8[a,b]	12.0 (SEM 5.3)
[14]	10 [epoetin beta]	6	14.68 (3.94)	4.42 (1.18)
	50 [epoetin beta]	6	5.38 (0.44)	5.34 (0.52)
	150 [epoetin beta]	6	4.98 (0.81)	6.10 (0.88)
	500 [epoetin beta]	6	3.70 (0.87)	8.49 (0.83)
	1000 [epoetin beta]	6	4.08 (0.94)	11.02 (0.03)
[9]	50 [epoetin alfa]	6	11.9 (3.1)	5 (0)
[15]	150 [epoetin alfa]	6	6.0 (1.0)	5.3
	300 [epoetin alfa]	6	5.5 (0.6)	6.1
[7]	100 [epoetin alfa]	9	8.12 (1.00)	6.77 (2.71)
	100 [epoetin beta]	9	7.94 (1.24)	8.79 (2.19)
[18]	100 [epoetin beta]	12	6.8[c]	4.92[d] [3.58–9.20]
[17]	100 [epoetin beta]	10	6.7[c]	5.37[d] [3.58–9.20]

Values given as mean (SD) [range] unless otherwise indicated. CL, clearance; IV, intravenous; SEM, standard error of the mean. $t_{1/2,z}$, terminal half-life.
[a] assume mean body weight of 60 kg. [b] recalculated from mean value in L/hr by *(1000/60).
[c] recalculated from median value in mL/min per 1.73 m^2 by *(60/70). [d] median.

unteers, assessments of dose-linearity are easier to determine as the drug is being used, not as a therapeutic, but as an investigational agent. Accordingly, more extensive sampling is done as well as testing of a greater range of doses. Flaharty et al. [14], at doses of 10, 50, 150, 500, and 1,000 U/kg, found a trend of decreasing clearance with increasing dose, with the most dramatic difference being between 10 and 50 U/kg (approximate three-fold decrease). The reduction in clearance resulted in longer half-lives as dose increased, for example from 4.42 hours at 10 U/kg to 5.30 hours at 50 U/kg and 8.50 hours at 500 U/kg. For studies conducted with single-dose groups, the results tended to be quite variable and not always in strict agreement (Tab. 1). Over the dose range of 100 to 150 U/kg, however, clearance was approximately 7 mL/hr/kg and terminal half-life was approximately five hours.

Although the pharmacokinetic properties of rHuEPO are generally considered to be nonlinear with dose, there has been little attempt in the literature to further characterize and describe this nonlinearity. In one study, Veng-Pedersen et al. [19] simultaneously analyzed data from three dose levels of intravenously administered epoetin alfa obtained in a three-way cross-over study in ten subjects (10, 100, and 500 U/kg). These authors observed that clearance decreased as dose increased, which they ascribed primarily to a saturable nonlinear mechanism described by the Michaelis-Menten expression. They determined V_{max} (theoretical maximum rate of elimination associated with the nonlinear pathway) to be 902 (175) mU/mL/hr and K_m (plasma concentration at which half-maximal effect is observed) to be 4,814 (1,184) mU/mL.

Multiple dose

In healthy volunteers, limited information is available on the impact of multiple dosing on the pharmacokinetic properties of rHuEPO. McMahon et al. [15] examined the single-dose and multiple-dose pharmacokinetics of epoetin alfa at intravenously administered doses of 150 and 300 U/kg in a parallel design (n = 6/group). At both dose levels, the mean clearance increased by 25% after five doses, resulting in a comparable reduction in terminal half-life. The authors indicated that the biggest change in clearance occurred between the first and second dose.

In summary, after intravenous dosing to healthy volunteers, the pharmacokinetic properties of rHuEPO are non-linear with dose wherein clearance is faster at lower doses. Additionally, clearance appears to increase over the initial period of multiple dosing.

Subcutaneous dosing

General

After subcutaneous dosing, the rate and extent of absorption are the determining characteristics of the serum concentration time profile for rHuEPO. The rate of absorption, which is slower than the rate of elimination, becomes limiting upon subcutaneous dosing, hence, it is represented by the terminal phase. The extent of absorption, as represented by bioavailability, represents that percentage of the dose that is measured in the circulation after extravascular administration.

Single dose; dose range

Key pharmacokinetic parameters determined after single-dose subcutaneous administration in healthy volunteers are presented in Table 2. The most comprehensive dose-ranging study has been reported by Cheung et al. [4] who studied single dose administration of epoetin alfa at eight dose levels ranging from 300 to 2,400 U/kg over an extended sampling duration (672 hours post-

Table 2. Single-dose pharmacokinetic parameters of subcutaneously (SC) administered rHuEPO in healthy volunteers

Ref	SC Dose (U/kg)	n	T_{max} (hr)	$t_{1/2,z}$ (hr)	F (%)
[20]	50 [epoetin alfa]	6	13 (6)	25 (12)	36 (23)
[15]	150 [epoetin alfa]	6	[8–24]	ID[a]	ID
	300 [epoetin alfa]	6	[12–24]	ID	ID
[7]	100 [epoetin alfa]	9	15 (8)	19.39 (10.71)	31.9 (9.1)
	100] [epoetin beta]	9	15 (7)	24.18 (11.16)	32.7 (8.2)
[18]	100] [epoetin beta]	12	11.0[b] [8.0–24.0]	22.58[b] [10.30–26.76]	38.5[b] [9.0–60.3]
[21]	50 [epoetin alfa]	2[c]	9,12	10.8, 12.3	NA
[4]	300 [epoetin alfa]	5	22.2 (8.1)	68.2 (52.2)	NA
	450 [epoetin alfa]	5	15.6 (5.8)	24.2 (3.2)	NA
	600 [epoetin alfa]	5	27.6 (9.1)	29.3 (9.4)	NA
	900 [epoetin alfa]	5	22 (13.0)	36 (13.5)	NA
	1200 [epoetin alfa]	5	26.4 (7.8)	78.5 (95.4)	NA
	1350 [epoetin alfa]	5	23 (9.0)	33.4 (2.4)	NA
	1800 [epoetin alfa]	5	28.8 (7.8)	32.4 (8.4)	NA
	2400 [epoetin alfa]	5	25.2 (6.2)	43.6 (25.9)	NA
[22]	30 [epoetin beta]	3	13 [12–15]	25.2 [18.0–33.2]	NA
	60 [epoetin beta]	3	11 [5–12]	20.5 [14.4–24.4]	NA
	100 [epoetin beta]	3	13 [12–15]	18.5 [12.5–23.6]	NA
[12][d]	150 [epoetin alfa]	24	15.4 (7.5)	19.7 (12.8)	NA
	750 [epoetin alfa]	24	16.6 (4.8)	25.7 (14.9)	NA

Values given as mean (SD) [range] unless otherwise indicated. Bioavailability (F) given for cross-over studies unless otherwise indicated. NA, not available. T_{max}, time at which peak concentration observed.

[a] insufficient duration of sample collection to fully characterize profile. [b] median. [c] individual values presented. [d] values reported are for the human serum albumin-containing formulation.

dose). The mean terminal half-life varied up to three-fold, with up to 100% interindividual variability, however, no consistent pattern with dose was seen, which is not unexpected given the rate-limited absorption of rHuEPO. For groups with lower variability (%CV < 50%), the mean half-life was between 24–36 hours. Mean peak concentration generally occurred between 16–29 hours post-dose and the authors established a linear relationship between C_{max} (peak concentration) and dose. These findings combined indicate that the absorption rate and extent of epoetin alfa are not dose dependent.

In another dose-ranging study, Sans et al. [22] determined the single-dose pharmacokinetics of epoetin beta at 30, 60, and 100 U/kg (n = 3/group). They noted a dose-proportional increase in area-under-the curve (AUC) as well as a constant T_{max}. They also noted no dose-dependency for terminal half-life nor mean residence time. In a third study, Hayashi et al. [23] used population pharmacokinetic analysis (non-linear mixed effects modelling) to describe the data from a bioequivalence study with epoetin beta in healthy volunteers. Using a one-compartment model with first-order absorption, the authors did not observe a dose-dependency in the parameter estimates obtained from 48 subjects (1,500 and 3,000 U). These three studies consistently demonstrate that the rate and extent (proportion) of absorption of rHuEPO are not dose-dependent, however, higher exposure is obtained with higher doses.

Bioavailability

Despite the apparent non-linearity in the pharmacokinetics of rHuEPO, no attempt appears to have been made to consider this when determining the subcutaneous bioavailability (F). To gain an accurate determination of F for a non-linear drug, it is necessary to initially determine the underlying intravenous kinetics then quantify the subcutaneous bioavailability by modeling. All estimates in the literature have been obtained by administration of the same dose intravenously and subcutaneously with comparison of AUC; this method would tend to under-estimate F, especially at lower doses where the non-linearity in clearance is most evident. Estimates of F for subcutaneously administered rHuEPO, as determined in the literature, tend to lie between 20% and 40%, when determined in a cross-over study [7, 16, 20].

Multiple dose

The findings regarding the impact of multiple subcutaneous dosing on the pharmacokinetics of rHuEPO are disparate. McMahon et al. [15] reported that C_{max} after five doses of 150 U/kg epoetin alfa was much increased relative to single dose; however, after five doses of 300 U/kg, C_{max} was similar to its single-dose value. In a more recent study, Sans et al. [22] found a significant decrease in AUC (between 57% and 90% of single-dose values) and C_{max} (between 47% and 76% of single-dose values) after three doses of epoetin beta at 30, 60, or 100 U/kg (n = 3/group). For each group, there was a concomitant increase in $t_{1/2,z}$ (by one- to two-fold) and mean residence time (MRT) and a 5–9 hour prolongation in mean T_{max}. After four weekly doses of 600 U/kg

(n = 5), however, Cheung et al. [4] essentially found the pharmacokinetic properties of epoetin alfa unchanged.

Other considerations

Although the International Olympic Committee has banned the use of rHuEPO since 1989, it has been misused by athletes. (See Chapter 13 by Catlin et al. for further information.) It would be expected that the pharmacokinetic properties of rHuEPO in athletes would be similar to those determined in healthy subjects. In one study, the concentrations in the control group of endogenous EPO remained at approximately 10 mU/mL indicating that regular-to-moderate training did not influence endogenous EPO concentrations [21]. The same authors also determined that serum and urinary measures of rHuEPO were markers for abuse only if measured within seven and four days after the final injection, respectively.

In summary, after subcutaneous dosing of rHuEPO to healthy volunteers, the absorption rate is slow, resulting in an extended terminal half-life with bioavailability ranging from 20% to 40%. Dose- or time-dependencies are not apparent.

Pharmacokinetics in nephrology patients

In the nephrology population, most pharmacokinetic studies have been done in patients with end-stage renal disease who are undergoing either peritoneal dialysis (which may be subclassified as continuous ambulatory peritoneal dialysis or continuous cycling peritoneal dialysis) or hemodialysis. As most patients studied had not previously received rHuEPO (i.e., they were rHuEPO naive), they were generally anemic (hematocrit <30%). Few studies have been published for patients with chronic renal insufficiency wherein some degree of kidney function is maintained. For intravenous dosing, blood samples were generally collected out to 48 hours (range: 21–72 hours); for subcutaneous dosing, sampling was sometimes extended to 96 hours [2]. As the drug in patients is not simply an investigative tool, but also a therapy, the studies are limited in their ability to assess dose ranges and even the impact of multiple dosing due to the confounding impact of dose adjustments over time, as well as other factors previously outlined.

Intravenous dosing

General

As for healthy volunteers, two phases were generally evident in the serum concentration-time profiles of nephrology patients [2, 13, 24, 25] and the volume of distribution was generally similar to plasma volume (40–60 mL/kg), indi-

cating limited extravascular distribution [1, 8, 24–26]. Three phases (tri-exponential) have also been reported [27].

Single dose; dose range

Single-dose intravenous pharmacokinetic parameters in rHuEPO-naive nephrology patients are summarized in Table 3. Due to the nature of treatment, few studies investigate the pharmacokinetics of rHuEPO over a wide dose range. Even in studies that did investigate a range of doses, it is sometimes difficult to draw conclusions. For example, Egrie et al. [28] and Lim et al. [31] each investigated a range of doses, however, only summary pharmacokinetic parameters across all dose groups were provided. In another study by Nielsen et al. [8], few subjects were enrolled and it was possible that the factors of prior treatment and different sampling durations were confounded, as those subjects who received the low dose (50 U/kg, n = 4) had previously been receiving rHuEPO (i.e., were non-naive) and those receiving the highest dose (150 U/kg, n = 2) were rHuEPO-naive. Yamazaki et al. [34], using doses of 3,000 U (60 U/kg) and 600 U (120 U/kg) stated that they observed dose-dependent increases in AUC and C_{max}, however, mean clearance at the low dose was slightly higher than at the high dose (3,000 U: 7.26 mL/hr/kg; 6,000 U: 5.84 mL/hr/kg).

Table 3. Single-dose pharmacokinetic parameters of intravenously administered rHuEPO in rHuEPO-naive nephrology patients

Ref	IV Dose (U/kg)	n	CL (mL/hr/kg)	$t_{1/2,z}$ (hr)
[28]	15, 50, 500 [epoetin alfa]	6	NA	9.3 (3.2)
[29]	12 [epoetin alfa]	11	NA	4.90 (1.7) [2.3–7.3]
[25]	80 [epoetin beta]	19	6.0[a]	8.75[b] [5.66–17.23]
[26]	120 [epoetin beta]	8	2.82[c] [1.92–5.1]	8.2 [6.2–10.2]
[10]	300 [epoetin alfa]	6	3.1 (SEM 0.5)	11.2 (SEM 0.4)
[30][d]	100 [epoetin beta]	8	9.4[e]	8.4[b] [4.8–19]
	100 [epoetin beta]	7	6.8[e]	8.3[b] [6.6–13]
[31]	50, 100, 150 [epoetin alfa]	7	5.69[f]	7.69 (SEM 1.11)
[32]	100 [epoetin beta]	12	6.9[f]	5.6 (SEM 0.3)

(continued on next page)

Table 3. (continued)

Ref	IV Dose (U/kg)	n	CL (mL/hr/kg)	$t_{1/2,z}$ (hr)
[14]	25–225	15	8.1[f]	6.9 (SEM 0.8) [4–44]
[8]	50 (non-naive), [epoetin beta]	4	9.7[g]	5.4 (0.9)
	150 (naive) [epoetin beta]	2	5.1[h]	7.6
[9]	24, 48 [epoetin alfa]	6	9.8[f]	5.3 (1.3)
[33]	100 [epoetin beta]	6 HD	NA	6.0
	100 [epoetin beta]	6 CAPD	NA	6.1
[1]	50 [epoetin alfa]	12	10.09 (3.54)	5.40 (1.7)
[2]	40 [epoetin beta]	8	13.5[i]	6.67 (1.18)
[34]	3000 U [epoetin beta]	3	7.26[j]	9.99 (SEM 1.59)
	6000 U] [epoetin beta]	4	5.84[j]	10.41 (SEM 0.79)
	9000 U [epoetin beta]	3	NA	NA
[13]	100 [epoetin alfa]	8[k]	4.8[c]	7.91 (4.2)
[18]	100 [epoetin beta]	12	4.3[l]	8.23[b] [6.05–13.7]

Values given as mean (SD) [range] unless otherwise indicated. CL, clearance; IV, intravenous; NA, not available; n, number of subjects; SEM, standard error of the mean.
[a] recalculated from median clearance in mL/min using median body weight (57 kg); *60/57. [b] median. [c] calculated from clearance in mL/min/kg by *60. [d] IV data from two groups reported separately. [e] calculated from median AUC using CL = Dose (mU/kg)/AUC (mU*hr/mL). [f] recalculated from mL/min assuming 70 kg body weight; *60/70. [g] recalculated from mL/min using mean body weight (60.1 kg); *60/60.1. [h] recalculated from mL/min using mean body weight (86.3 kg); *60/86.3. [i] recalculated from mL/min using mean body weight (58.2 kg);*60/58.2. [j] recalculated from mL/hr using mean IV body weight (50.0 kg). [k] subjects were mixture of EPO-naive and non-EPO naive. [l] recalculated from median value in L/hr per 1.73 m^2 by *(1000/70).

Multiple dose

Studies comparing single-dose and multiple-dose pharmacokinetics, as reflects clinical practice, have generally found that the pharmacokinetic properties change to a limited degree upon multiple dosing; these studies are summarized in Table 4. Most studies have found that clearance increases and/or terminal half-life decreases after multiple dosing [1, 25, 28, 31]. Additionally,

Table 4. Multiple-dose pharmacokinetic parameters for intravenously (IV) administered rHuEPO in rHuEPO-naive nephrology patients

Ref	IV Dose (U/kg)	n	No. Doses	CL	$t_{1/2,z}$
[28]	15, 50, 500 [epoetin alfa]	6	7 3 months	NA NA	32% decrease No further decrease
[29]	Initial: 12 (then dose-adjusted) [epoetin alfa]	11	Steady state	NA	14% decrease
[25]	80 [epoetin beta]	10	TIW for 3 months	14% increase	22% decrease
[31]	50, 100, 150 [epoetin alfa]	7	TIW for 8 wks	45% increase	40% decrease
[1]	Initial: 50	12	3 months	10% increase (NS)[a]	14% decrease (Sig)[b]
[9]	48–192	9	TIW "several weeks"	31.6% decrease[c] (NS)	NA
[18]	100 [epoetin beta]	12	4.1–17 months	3.2% increase (NS)	3.3% decrease (NS)
[34]	3000 U [epoetin beta]	3	8 weekly doses	25% decrease	8.6% decrease
	6000 U [epoetin beta]	4	8 weekly doses	15% increase	8.6% increase

Values calculated as % change in mean/median from single-dose estimate. CL, clearance; NA, not available; TIW, three times a week.
[a] not significantly different from single dose estimates. [b] significantly different from single dose estimates. [c] single and multidose patients were different cohorts.

some investigators have reported that after an initial change (i.e., after two to three weeks), no further changes were noted upon more long-term administration [28]. Conversely, other investigators reported no substantiative change in pharmacokinetic properties from single-dose values after reaching a new steady-state hemoglobin value [29] or after 56 days [34]. Jensen et al. [18], who did not find a change in the pharmacokinetic properties (except for a reduction in volume of distribution) of epoetin beta after long-term treatment, hypothesized that after hemoglobin has been stabilized for a prolonged duration, the elimination rate may return towards the pretreatment value. In a number of studies, the single-dose pharmacokinetics were not assessed; however, the authors have reported no difference upon short-term treatment and long-term treatment. For example, Hughes et al. [27], in patients receiving intravenous epoetin alfa, found no difference in terminal half-life after the seventh or ninth dose at 100 U/kg (5.1 ± 0.6 h, $n = 10$) and after 11 to 20 weeks of treatment (5.6 ± 0.6 h, $n = 3$). Gladziwa et al. [6], again did not conduct studies in naive-patients, but concluded through literature comparison that the

pharmacokinetic properties of epoetin beta at 150 U/kg administered intravenously at steady state were similar to those upon single dose.

In summary, after intravenous dosing to nephrology patients, the pharmacokinetic properties of rHuEPO are non-linear with dose wherein clearance is faster at lower doses. Additionally, clearance appears to increase over the initial period of multiple dosing.

Subcutaneous dosing

General

All studies that investigated both intravenous and subcutaneous dosing have shown that absorption is rate limiting, i.e., the terminal half-life after subcutaneous dosing is longer than that after intravenous dosing [13, 16]. This slow absorption of rHuEPO relative to elimination has been advantageous in the nephrology setting by allowing a lower dose to be delivered by the subcutaneous route [5].

Single dose; dose range

Pharmacokinetic parameters after single-dose, subcutaneous administration to r-HuEPO-naive nephrology patients are presented in Table 5. In the very few studies that have investigated a range of doses, no apparent dose-dependency in pharmacokinetic properties was determined.

Bioavailability

Similar to the literature available for healthy volunteers, no studies were found that took into consideration the nonlinearity in the disposition of rHuEPO. Average bioavailability estimates for subcutaneously administered rHuEPO tend to lie between 30% and 50%, when determined in a cross-over study [1, 30]. Several investigators have reported lower estimates, ranging from 14.1% to 23.0%.

Multiple dose

A small number of studies have investigated the pharmacokinetics of subcutaneously administered rHuEPO after multiple dosing in nephrology patients. Kampf et al. [30], after six weeks of 40 U/kg three times weekly, concluded that there was no evidence for a change in the time course or extent of subcutaneous rHuEPO absorption after long-term treatment. Yamazaki et al. [34], after eight weekly doses of 3,000 or 6,000 U epoetin beta, also reported no changes, although they had inconsistent results with a small number of patients. In a well-controlled, cross-over study, Jensen et al. [16] observed no statistical change in any of the subcutaneous pharmacokinetic parameters (including absolute bioavailability) after a median treatment of 7.8 months. Schouten et al. [5], after one year of treatment, found no statistical difference in the pharmacokinetic parameters compared with those obtained after the first dose.

Table 5. Single-dose pharmacokinetic parameters for subcutaneously (SC) administered rHuEPO in rHuEPO-naive nephrology patients

Ref	SC Dose (U/kg)	n	T_{max} (hr)	$t_{1/2,z}$ (hr)	F (%)
[28]	15, 150 [epoetin alfa]	5	8–12	NA	NA
[26]	120 [epoetin beta]	8	18	27.7[a]	21.5 (11.3–36.0)
[25]	40 [epoetin beta]	9	18.0[b] [6.0–24.1]	11.16[b] [5.31–13.98]	25%[c]
[10]	300 [epoetin alfa]	6	24–36	NA	18.2[d] (SEM 2.1)
[32]	100 [epoetin beta]	12	28.0 (SEM 5.0)	30.2 (SEM 5.3)	14.9 (SEM 4.8)
[14]	25–225 [epoetin alfa]	15	21.7 (SEM 2.2)	NA	48.8[e] (14.5–96.3)
[8]	50 (non-naive) [epoetin beta]	4	27.3 (8.6)	NA	14.1
	150 (naive) [epoetin beta]	2	19.5	46.2	31.7
[33]	100 [epoetin beta]	6	NA	NA	23
	100 [epoetin beta]	6	NA	NA	24
[1]	50 [epoetin alfa]	12	NA	NA	43.7 (6.7) [28–100]
[34]	3000 U [epoetin beta]	5	23.4 (SEM 4.3)	23.28 (SEM 5.09)	36.3
	6000 U [epoetin beta]	5	24.0 (SEM 5.4)	26.12 (SEM 5.28)	31.0
[13]	100 [epoetin alfa]	8	17.1 (5.0)	24.4 (27)	22.8 (14)
[5]	60 [epoetin beta]	8	17.2 (6.5) [8.1–30.8]	21.9 (9.7) [10.3–35.8]	NA
[18] median/ range	100 [epoetin beta]	12	19.3 [8–24]	25.1 [14.0–40.1]	20.8 [11.6–35.6]

Values given as mean (SD) [range] unless otherwise indicated. F given for cross-over studies unless otherwise indicated. F, bioavailability; NA, not available; SEM, standard error of the mean.
[a] estimated as $\ln 2/k_{el}$ where k_{el} given as 0.025 h^{-1}. [b] median. [c] estimated from parallel arms with different doses. [d] likely an underestimate as $AUC_{(0-72)}$ for SC dose. [e] unknown if cross-over study.

In summary, after subcutaneous dosing of rHuEPO to nephrology patients, the absorption rate is slow, resulting in an extended terminal half-life with bioavailability ranging from 20% to 40%. Dose- or time-dependencies are not apparent.

Other issues related to nephrology patients

Extent of renal function

Most pharmacokinetic studies in the nephrology population have been conducted in patients with end-stage renal disease receiving dialysis. In a study aimed at investigating the impact of varying degrees of renal function (as determined by creatinine clearance, range: <3 to >80 mL/min per 1.73 m^2) on the pharmacokinetics of epoetin beta, Kindler et al. [24] investigated intravenously administered doses of 130 to 152 U/kg in rHuEPO-naive subjects (n = 10). They determined that neither the terminal half-life nor total clearance were related to renal function. Using a 48-hour urine collection, they also determined (by RIA) that renal clearance contributed only 1.86% (n = 8) to total clearance.

Mode of dialysis

A number of studies have demonstrated that loss of rHuEPO in the dialysate is minimal. After intravenous dosing, Macdougall et al. [26] and Boelaert et al. [10] reported that 2.3% (range: 1.7%–3.0%) (n = 8, 120 U/kg) and 2.63% (n = 6, 300 U/kg) of the dose, respectively, was collected in the peritoneal dialysis fluid over the first 24 hours. Petersen et al. [35] compared the loss of epoetin alfa in dialysate when administered intravenously at the end and the beginning of the dialysis session. These authors concluded that the overall clearance of rHuEPO is not affected by the presence of a dialysis system, with approximately 7% administered dose in the dialysis system at the end of the study (4 hours). Gladziwa et al. [6] determined, by comparison of rHuEPO concentrations from the arterial and venous lines of the hemofilters and dialysers, that rHuEPO was not eliminated by either of these means. Additionally, although few direct comparisons are available, several authors have concluded that the dialysis mode, i.e., hemodialysis *versus* peritoneal dialysis, has no impact on the pharmacokinetics of intravenous or subcutaneous rHuEPO [10, 26, 33]. Stockenhuber et al. [33], in a direct comparison of epoetin beta by both intravenous and subcutaneous routes, found no statistically significant differences in pharmacokinetic properties between patients on hemodialysis (n = 6) *versus* those on peritoneal dialysis (n = 6). Jensen et al. [16] found no difference between hemodialysis (n = 7) and peritoneal dialysis (n = 5) for either intravenous or subcutaneous dosing after single or multiple dosing. In summary, the mode of dialysis does not appear to impact the pharmacokinetic properties of rHuEPO.

Intraperitoneal dosing for peritoneal dialysis patients

Given the use of peritoneal dialysis, intraperitoneal administration of rHuEPO has been considered. Reported bioavailability estimates are generally very low (3%, possibly an underestimate as the samples were only collected to 24 hours) [26] and 6.8% [2] with a large proportion of the dose (80%) being lost in the effluent [10]. The feasibility of the intraperitoneal route increases if

rHuEPO is injected into a dry peritoneum as Krömer et al. [32] obtained a bioavailablility estimate of 41.4% (SEM 7.2, n = 12) and Ateshkadi et al. [13] obtained an estimate of 11.4 (6.6)% (n = 8). In a comparison study, Bargman et al. [36] obtained a nine-fold increase in AUC after administration into a dry peritoneum compared with that injected with dialysate. Additionally, increasing the dwell time within the peritoneum is thought to increase the extent of absorption [13, 37].

Comparison of healthy volunteers and nephrology patients

When the pharmacokinetic characteristics of rHuEPO in healthy volunteers and nephrology patients are compared across studies, it appears that the parameters are similar. However, in the only within-study comparison study found, Jensen et al. [18] found considerable differences in the pharmacokinetics of 100 U/kg epoetin beta after both intravenous and subcutaneous dosing in healthy (n = 12) *versus* uremic/dialysis (n = 21) subjects. After intravenous dosing, clearance was statistically significantly reduced (from approximately 6.8 to 4.3 mL/hr/kg) and terminal half-life was increased (from 4.92 to 8.31 hours) in the uremic patients compared with the healthy volunteers. Distribution parameters did not change. After subcutaneous dosing, peak concentrations were significantly reduced (113 *versus* 153 mU/mL) and bioavailability was significantly lower (23.7% *versus* 38.5%) in uremic patients. The rate of absorption, however, did not appear to differ between the two groups. The differences were not attributed to loss in dialysate. This single comparison would need confirmation in independent studies.

Nephrology studies in pediatric patients

The pharmacokinetic properties of rHuEPO have been determined in a number of studies in pediatric nephrology patients; Table 6 summarizes the available literature for intravenous, subcutaneous, and intraperitoneal dosing. After intravenous administration of rHuEPO to pediatric patients, the mean terminal half-life ranged from 5.6 to 10.9 hours with mean clearance values ranging from 6.0 to 10.1 mL/hr/kg. After subcutaneous dosing, absorption was rate limiting such that the mean terminal half-life ranged from 13.3 to 25.2 hours with bioavailability estimates ranging from 34% to 40%. Braun et al. [41] concluded, using 20 patients, that the pharmacokinetic data of young and older children aged 7 to 20 years were not significantly different. For intraperitoneal dosing, Kausz et al. [45], by comparison to literature values, concluded that (similar to adults) injection into a dry peritoneum increases bioavailability. Evans et al. [39] concluded that, after intravenous administration in pediatric patients (9–16 years), clearance was increased by two-fold and terminal half-life decreased by 30% to 86% compared with adults, and after subcutaneous

Table 6. Pharmacokinetic parameters for rHuEPO in naive pediatric patients with chronic renal failure

Ref	Route	Age (yr)	n	Dose (U/kg)	Parameter estimates			
					T_{max} (hr)	$t_{1/2,z}$ (hr)	CL (mL/hr/kg)	F (%)
[38]	SC	[1.8–10.4]	5	100 [epoetin beta]	12–24	NA	NA	NA
[38]	IP	[0.6–12.5]	8	100 in 20mL/kg dialysis fluid [epoetin beta]	12	NA	NA	42[a]
[38]	IP	[3.3–14.1]	8	100 in 50mL dialysis fluid [epoetin beta]	12	NA	NA	88[a]
[39]	IV	[9–16]	9	40 [epoetin beta]	NA	5.63 [4.39–6.86]	10.1 [7.07–14.9]	NA
[39]	SC	[9–16]	9	40 [epoetin beta]	10	14.9[b] [6.24–51.7]	NA	40 [20–64]
[39]	IP	NA	3	40 [epoetin beta]	14	10.7 [8.75–14.1]	NA	17 [9.6–28]
[40]	SC	[0.25–18]	15	25 [epoetin alfa]	9	25.2 [6.2–58.7]	17.7[c] [3.4–43.5]	NA
[41]	SC	[6.8–19.9]	20	4000 U/m^2 (133 U/kg) [epoetin alfa]	14.3 (9.4)	14.3 (7.2)	NA	34[d]
[41]	IV[e]	15	2	4000 U/m^2 (133 U/kg) [epoetin alfa]	NA	10.85 [10.8–10.9]	6[f] [4.76–7.14]	NA
[42]	IV		3		NA	10.4 [8–14]	0.63 [g]	NA
[42]	SC	[9–17]	3	100	[1–24]	NA	NA	NA
[43]	SC	11.62 (1.42)	8	50 [epoetin alfa]	18.5 (SEM 2.6)	13.3 (SEM 1.9)	NA	NA
[43]		12.75 (0.6)	8 CAPD	50 [epoetin alfa]	26.8 (SEM 7.7)	13.5 (SEM 3)	NA	34[h]
[44]	IV	[7–18]	8	50 (1st dose) [epoetin alfa]	NA	7.5 (0.9) [6.2–8.7]	NA	NA
[44]	IV		3	50 (7th dose) [epoetin alfa]	NA	4.5[i] (0.1)	NA	NA
[45]	IP	9.1 (5.1) (non-naive)	10	100 in 50mL dialysate [epoetin alfa]	12	21.1 [j]	NA	NA
[46]	SC	13.5 [9–18]	10	75 [epoetin alfa]	No difference in presence or absence of EMLA cream			

dosing, that bioavailability was increased by two-fold. Geva et al. [42], however, concluded that the pharmacokinetic parameters in children (9–17 years) were similar to those of adults. Additionally, Jabs et al. [44] reported a decrease (45%) in half-life upon multiple dosing in children, which is similar to that reported in adults. However, due to the wide range in values, comparison to literature estimates for adults is difficult and no dedicated studies comparing the parameters for adult and pediatric patients were found.

In summary, the pharmacokinetic properties of rHuEPO in pediatric nephrology patients appear to be broadly similar to those of adults, although no controlled studies were reported in the literature.

Pharmacokinetics in oncology patients

Despite the widespread use and licensure of rHuEPO in oncology patients undergoing chemotherapy, no studies could be found in the literature detailing the pharmacokinetics of rHuEPO in this population. It would appear that this is an area worthy of investigation given the evidence that endogenous EPO levels are increased, both in an acute and chronic sense, by chemotherapy administration [47, 48]. Additionally, given the apparent dose-dependent pharmacokinetics in other populations, the pharmacokinetics of rHuEPO in patients with cancer may be different given the higher doses that are routinely used (e.g., 150 U/kg three times weekly or 40,000 U weekly). A challenge in this area would be differentiating between the changing endogenous and exogenous EPO given the cross-reactivity of endogenous EPO in the assays.

Pharmacokinetics in other populations

The pharmacokinetic properties of rHuEPO have been studied in very few other populations. In a comparison between patients with liver cirrhosis and healthy volunteers, Jensen et al. [17] did not find any difference in the pharmacokinetic properties of epoetin beta (intravenous, 100 U/kg). Patients with chronic obstructive pulmonary disease had similar mean concentrations and no significant difference in terminal half-life (5.98 ± 0.67 hours, n = 7) compared with healthy volunteers (5.87 ± 0.35 hours, n = 6) [49]. The determination of the pharmacokinetic properties of rHuEPO in patients with myelodysplastic

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← Values given as mean (SD) [range] unless otherwise indicated. Bioavailability (F) given for crossover studies unless otherwise indicated. CAPD, continuous peritoneal dialysis; CL, clearance; F, bioavailability; IP, intraperitoneal; IV, intravenous; NA, not available; SC, subcutaneous.
[a] F_{IP} calculated relative to SC. [b] calculated based on Ka ($ln2/K_a$). [c] relative clearance (CL/F). [d] F calculated for n = 2 only. [e] mean values calculated from individual values presented. [f] calculated from C (L/hr) by *(1000/BW) where BW 38.8 and 58.6 kg. [g] recalculated from CL (mL/min/kg) by *60. [h] F_{CAPD} calculated relative to predialysis value from mean [$CL_{predial}/CL_{CAPD}$]*100. [i] represents 45% decrease from mean 8.2 hr for n = 3. [j] estimated from 4 timepoints over 24 hours only.

syndromes, which are characterized by ineffective hematopoiesis, was complicated by the extremely high baseline EPO concentrations (210 to 5,984 mU/mL, n = 10) for the hemoglobin range (5.5 to 10.8 g/dL) [50]. After an intravenous bolus of 150 U/kg, peak observed total EPO concentrations were approximately 2- to 10-fold higher than baseline. The reported half-lives ranged from 3.9 to 20.0 hour with the average value (11.3 hour) being longer than that generally reported for healthy volunteers or patients with chronic renal failure. The authors reported a significant correlation between clearance rate and basal serum EPO and speculated that reduced clearance by the bone marrow remains a possibility.

Potential confounding factors

Site of injection

It has been reported that site of injection for subcutaneous administration of rHuEPO can have an impact on both the rate and extent of absorption. Macdougall et al. [51] demonstrated more extensive absorption when radiolabelled rHuEPO was injected in the thigh *versus* either the abdomen or arm. Compared with $AUC_{(0-\infty)}$ obtained after injection into the thigh, injection into the arm represented 89% (not significant) and injection into the abdomen represented 77% (significantly different, $p < 0.005$).

Jensen et al. [52], however, by comparing the pharmacokinetic properties after subcutaneous dosing with epoetin beta (100 U/kg) in the anterior thigh and the abdomen concluded that the overall differences in pharmacokinetics appeared to be too small to recommend a general preference of the injection site. The only notable difference they observed was a longer mean residence time after injection into the thigh (median: 32.7 hours) than abdominal injection (median: 26.2 hours), which they attributed to slightly slower absorption. Hence, the available evidence suggests that the thigh may be a preferable administration site.

Epoetin alfa vs epoetin beta

Both epoetin alfa and epoetin beta are both classified as rHuEPO, however there are slight differences in the isoform composition. Isoelectric focusing revealed that epoetin alfa had five distinct components whereas epoetin beta had six or seven components [53] with the additional components being more basic, hence containing a lower proportion of sialic acids. (See Chapter 13 by Catlin et al. for further discussion of isoelectric focusing.) In a four-way crossover study (Tab. 7), Halstenson et al. [7] found essentially similar pharmacokinetic parameters for epoetin alfa and epoetin beta upon either intravenous or subcutaneous administration.

Table 7. rHuEPO pharmacokinetic parameters from a four-way cross-over study at 100 U/kg in healthy volunteers (n = 9/group)

Route	Parameter (Units)	Epoetin alfa	Epoetin beta
IV	CL (mL/hr/kg)	8.12 (1.00)	7.94 (1.24)
	$t_{1/2,z}$ (hr)	6.77 (2.71)	8.79 (2.19)
	V_{ss} (mL/kg)	63.8 (6.08)	70.0 (10.4)
SC	T_{max} (hr)	15 (8)	15 (7)
	C_{max} (mU/mL)	141 (72.2)	131 (55.2)
	$t_{1/2,z}$ (hr)	19.4 (10.7)	24.2 (11.2)
	F (%)	31.9 (9.1)	32.7 (8.2)

Values presented as mean (SD). IV, intravenous; SC, subcutaneous [7].

Mechanisms of clearance

Despite the 20 years of clinical usage of rHuEPO, the exact mechanism(s) by which it is cleared is largely unknown. Given the similarity in structure between endogenous EPO and rHuEPO, it is highly likely that the clearance mechanisms are similar, if not identical. Based on the clinical data wherein higher clearance values were evident at lower doses, it is likely that there are at least two clearance mechanisms, one of which is saturable. Veng-Pedersen et al. [19] proposed that rHuEPO is cleared solely by nonlinear, saturable mechanism(s). Kato et al. [54] demonstrated both a saturable and nonsaturable component in rats. It is thought that the sialic acid residues serve to extend the residence time of the protein in the circulation [55], helping to explain the three-fold longer half-life of darbepoetin alfa relative to rHuEPO [56].

Role of kidney

Both preclinical and clinical data suggest that the kidney is not a major clearance organ, at least for the intact protein. Steinberg et al. [57] demonstrated that the clearance of rHuEPO in nephrectomized rats was unchanged relative to controls. Fu et al. [58] obtained a 30% decrease in clearance in bilaterally nephrectomized dogs compared with control animals; this finding may be confounded by the use of total counts for ^{125}I-labeled material that would not account for any degradation products. Dinkelaar et al. [59] observed a prolongation in plasma disappearance time in bilaterally nephrectomized rats compared with control animals (266 min *versus* 105 min). Kato et al. [54] suggested that the kidney contributes, at least in part, to the nonsaturable clearance of rHuEPO. Clinical studies, wherein urine samples were collected 24 to 48 hours post-dose, have indicated that <5% dose is recovered in its intact form [14, 19, 34]. Flaharty et al. [14], however, found that renal clearance was

approximately six-fold higher at 10 U/kg than at 1,000 U/kg. It should be noted that none of these clinical studies addressed the potential for degradation products to exist in the urine.

Role of liver

The liver has been suggested as another possible clearance organ, perhaps of a metabolite. One theory is that the sialic acids are removed by sialidases in the blood and/or tissue, thus exposing the penultimate galactose residues that makes the desialylated form a substrate for the asialo-glycoprotein receptor in the liver [55]. *Ex vivo* desialylation of rHuEPO resulted in much more rapid clearance in rats after intravenous administration than the intact form [55, 60]. Little evidence suggests that intact rHuEPO is cleared directly from the liver [54]. In a rat disease-state model (induced by d-galactosamine-HCl), although Dinkelaar et al. [59] found a small but significant elongation in the plasma disappearance time compared with control animals (164 min compared with 105 min), they dismissed the role of the liver in EPO catabolism as minor at best, as their own *in vitro* perfusion studies did not support the findings. Additionally, evidence for the *in vivo* action of sialadases is lacking. Although sialic acid removal may not be the *in vivo* mechanism of clearance, it is clear that the presence of sialic acids on these molecules enables them to remain in circulation [60].

Role of bone marrow

The bone marrow has been suggested, not only as the effector organ but also as a clearance organ. It has been demonstrated that rHuEPO is internalized and degraded by its target cells [61]. These authors demonstrated with mice spleen cells, which have cell-surface EPO receptors (EPOR), that between 40% and 60% of total radiolabeled EPO specifically bound to the cell was internalized and that degradation occurred, most likely through the lysosomes; the fate of EPOR was not determined, i.e., recycled or degraded. Kato el al. [54] demonstrated saturable uptake of rHuEPO by the bone marrow (and spleen), which they postulated was mediated by EPOR. Bowen et al. [50] found reduced clearance of rHuEPO for patients with myelodysplastic syndromes. A potential implication of EPOR-mediated clearance is that any organ with EPOR (e.g., liver, brain, spleen) has the potential to be a clearing organ to some extent. The involvement of progenitor cells, or more broadly EPOR-bearing cells, may help explain observations of increased clearance upon multiple dosing wherein the pool of cells has expanded.

Discussion and conclusions

The pharmacokinetic properties of rHuEPO have been extensively studied in healthy volunteers and nephrology patients after both intravenous and subcutaneous dosing. Combining the clearance values for these two populations enables a broader picture regarding dose-linearity to evolve. Despite the inter-study differences, it is clear from Figure 1 that clearance of rHuEPO is dose dependent. At doses >200 U/kg, clearance of rHuEPO is approximately constant at 5 mL/hr/kg. At doses <200 U/kg, clearance increases as dose decreases with a three-fold increase in clearance observed at 10 U/kg. This relationship appears independent of population (healthy or with chronic renal failure) or type of rHuEPO (epoetin alfa *versus* epoetin beta). Considering subcutaneous dosing, no clear patterns regarding dependence of absorption properties (rate or extent) on dose have emerged from this literature review, and bioavailability (although perhaps erroneously estimated) consistently lies between 20% and 40%. Upon multiple dosing, most of the data suggest that (at least with intravenous dosing), clearance of rHuEPO increases (approximately 10%

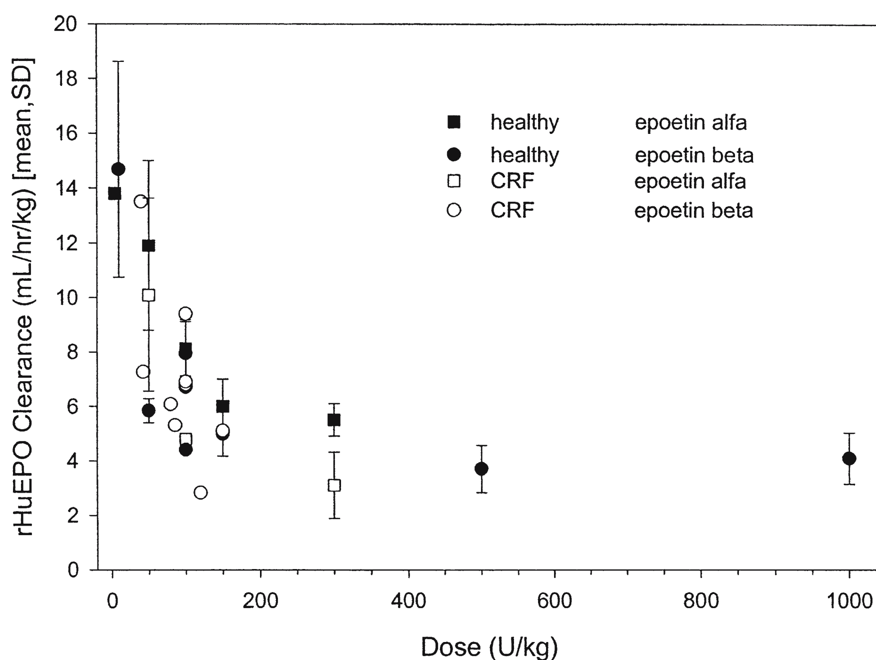

Figure 1. Clearance of rHuEPO over a range of doses in adult healthy and nephrology subjects, based on literature estimates. Values are presented as mean ± standard deviation (where available). Criteria for inclusion were: single-dose IV bolus studies, sample collection over sufficient period to characterize profile (at least 48 hour), clearance values given or calculated for individual dose level, rHuEPO-naive subjects. All data are included in Tables 1 and 3.

to 30%) with an accompanying decline in terminal half-life (approximately 10% to 40%) within several weeks. Generally no further changes were seen with more chronic dosing. For the populations for which data are available, no significant interpopulation differences were noted in pharmacokinetic properties of rHuEPO.

Application of pharmacokinetic principles and understanding the pharmacokinetic-pharmacodynamic relationship can explain one apparently anomalous situation for rHuEPO. That is, the reduced dose requirement (approximately 30% lower) in patients with CRF when rHuEPO is administered subcutaneously *versus* intravenously [62] despite the low subcutaneous bioavailability. This finding can be explained by the slow absorption after subcutaneous administration from the injection site that acts to prolong the blood concentrations for a longer time. For erythropoetic agents, it appears that maximal benefit is obtained by maintaining a therapeutic concentration of the drug for most of the dosing interval. Molecules that have an extended residence time (such as darbepoetin alfa) have the advantage of prolonged maintenance of therapeutic concentrations at a reduced frequency.

Despite the extensive body of work available, there are two main areas, namely absorption and clearance, where understanding the underlying mechanisms would greatly enhance our ability to rationalize and interpret data. For subcutaneous dosing of rHuEPO, new evidence from sheep models indicates that lymphatic uptake is the dominant uptake route, as opposed to direct entry into the vasculature, as a high proportion of the dose (>80%) is absorbed through the peripheral lymphatics. Additionally, a high proportion of this (75%) is recovered in the central lymph, indicating minimal lymphatic clearance [63]. Clarifying and quantifying the roles and relative contribution of various clearance pathways to the overall clearance of rHuEPO is crucial for further development. The implications that the target organ (i.e., EPOR-bearing cells) are at least partially responsible may help explain the limited increase in clearance upon multiple dose administration.

In addition to these basic unresolved mechanistic issues, other more applied questions exist:

- Do the pharmacokinetics of rHuEPO vary with hemoglobin/hematocrit values?
- What is the true bioavailability of rHuEPO when administered subcutaneously?
- What is the impact of rHuEPO treatment on endogenous EPO?
- What are the pharmacokinetic properties of rHuEPO in patients with cancer, either with or without chemotherapy?
- Should we expect the pharmacokinetic properties of rHuEPO to vary in currently unstudied populations, and if so, can we explain these differences mechanistically?
- What are the differences in the critical pharmacokinetic characteristics between rHuEPO and second-generation erythropoeitic agents, such as dar-

bepoetin alfa, which allow for reduced dosing frequency of the newer agents?

The value of answering questions such as these would be in improving the understanding between pharmacokinetics and pharmacodynamics with the ultimate goal of optimizing and individualizing patient care.

Acknowledgements
Bhavna Thapar and Robert Rovetti assisted in the preparation of this manuscript.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Biology of erythropoietin

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Roles of EPO

Recombinant erythropoietin (EPO) is a 165-amino acid glycoprotein hormone with a molecular weight of 30.4 Kd that contains 39% carbohydrate and is the biologic equivalent of the endogenous product of human chromosome 7q22 [1, 2]. Both the endogenous material (EPO), and the recombinant human glycoprotein (rHuEPO) bind a single receptor (EPOR) [3–5]. This receptor is widely distributed within the body. The significance of EPOR distributed outside the bone marrow will be discussed, but the bone marrow is the major therapeutic target of the recombinant hormone. The erythropoietic potential of the bone marrow and the non-erythropoietic potential of EPO will be discussed, including the biologic phenomena about EPO, its receptor, and its use in various settings.

Roles suggested by overexpression and knockout studies

Deliberately overexpressing or deleting the gene in "transgenic" or "knockout" mice provides insight into the potential roles of various proteins. EPO transgenic mice have high hematocrits (80%), increased blood volume, enlarged hearts, reduced exercise capacity, and premature death [6]. Although emerging data may suggest a direct role, it is likely that the latter four changes are compensatory in nature and not the result of a direct action of EPO on the cardiovascular machinery. In contrast, EPO or EPOR knockout mice lack embryonic erythropoiesis, have heart problems, and die at approximately gestational day 13.5 (E13.5) [7–9]. The latter can be rescued by insertion of human EPOR, offering formal confirmation of the initial observations [10]. Thus, the studies are relatively uninformative beyond confirming the role of EPO and EPOR in erythropoiesis; however, the similarity between the EPO and EPOR knockout phenotype is strong evidence that only a single ligand exists for EPOR and only a single receptor exists for EPO.

Control of EPO production and elimination

In the 19th and 20th centuries, researchers used the effects of hypoxia to formulate theories about the existence of EPO. (See Chapter 2 by Foote for further information.) EPO is widely recognized as a product of interstitial cells localized around the proximal tubule of the kidney in the adult [11, 12]; however, several other quantitatively less-significant sites of production have been documented including fetal and adult liver [11, 13–16], brain, testis, lung, spleen, placenta, bone marrow, and ovary [11, 13–23]. Although the precise nature of the oxygen-sensing mechanism has yet to be defined, the generation of reactive oxygen intermediates has been suggested as a preliminary step in upregulating EPO production. In hypoxia, a DNA sequence 3’ to the *EPO* gene is activated by a transcription factor, hypoxia-inducible factor (HIF) that appears to be responsible for switching several genes regulated by hypoxia, including EPO [24–26]. In addition to HIF, other signals such as HNF4, p300/CREB, and the COUP family of transcription factors are known to play a role in EPO regulation [27, 28]. Post-transcriptional events have also been reported to be involved [29].

Elimination of both recombinant and endogenous EPO remains somewhat of a mystery. (See Chapter 6 by Heatherington for more information.) A small amount of EPO is detected in the urine and a role for erythroid cells in clearance has been suggested [30–32]. Other mechanisms, perhaps involving the asialoglycoprotein receptor on liver cells have been considered [33]. It is possible that a combination of clearance pathways, some specific, some non-specific, some yet to be defined, account for the removal of EPO from the body.

EPO and erythropoiesis

The major function of red blood cells (erythrocytes) is to transport oxygen to the tissues of the body. The erythrocyte component represents about 40% to 45% of the blood by volume. A normal adult may have 5 L of blood and an erythrocyte count of 5×10^9/L. The red cell life-span in such a normal adult is approximately 100 to 120 days. To maintain a stable number of erythrocytes, replacing those lost by normal aging alone, requires replacement of 1% erythrocytes per day, i.e., production of 2.5×10^{11} cells per day or more than 10^{10} cells per hour. This prodigious rate of production is maintained in most circumstances for the lifetime of the individual. Thus, erythropoiesis is a highly dynamic process. EPO has a pivotal role in this process.

As discussed elsewhere in this volume (see Chapter 1 by Israels and Israels), the cellular target of EPO in erythropoiesis was traditionally referred to as the erythropoietin responsive cell. This population is now known to represent progenitor cells in late erythroid burst-forming unit (BFU-E) stage through the erythroid colony-forming unit (CFU-E) stage and the morphologically recognizable erythroblasts (Fig. 1 and Chapter 1). EPOR is, however,

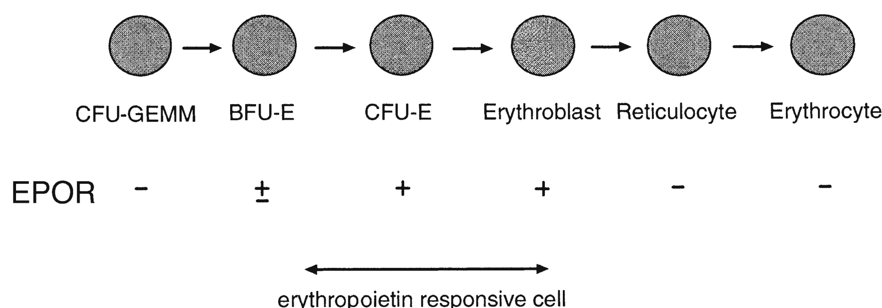

Figure 1. Cellular erythropoiesis. Erythroid cells are produced from a cellular lineage that progresses from cells which are multipotential (CFU-GEMM = colony-forming unit–granulocyte, erythrocyte, megakaryocyte, macrophage: a cell detectable by its ability to form a colony of cells in culture which contains all of these cell lineages), to lineage committed cells of high proliferative capacity (BFU-E = erythroid burst-forming unit), to those lineage committed cells of lower proliferative potential (CFU-E = erythroid colony-forming unit), to those cells that are recognizably hemoglobinized (erythroblast, reticulocyte and erythrocytes). Figure courtesy of Amgen Inc., Thousand Oaks, California.

absent on reticulocytes and mature erythrocytes [34]. $EPOR^+$ progenitor cells have somewhat different cytokine dependencies in culture, with CFU-E requiring the addition of only EPO, and BFU-E requiring EPO and more primitive-acting cytokines previously known collectively as burst-promoting activities. The burst-forming activities are now molecularly identified as materials such as stem cell factor (SCF), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6 among others, or occasionally mixtures of all or some of these.

Once EPO has engaged EPOR, a cascade of events is set in motion including activation of the dimerized receptor [35], and signal transduction through protein kinase C [36, 37], JAK-2, and Stat5 (reviewed in Frank [38] and Cheung and Miller [39]). (See Chapters 3 and 5 for further information.) The actions of EPO include promotion of the survival of sensitive progenitors through prevention of apoptotic processes [40, 41], stimulation of proliferation [42, 43], and differentiation into large numbers of hemoglobinized cells. To maintain homeostasis and supply the necessary number of erythrocytes, the degree of proliferation required is quite remarkable, and occurs in parallel with the acquisition of features of the specialized functions of erythrocytes – accumulation of hemoglobin and disposal of the cell nucleus.

As discussed, EPO does not act alone to stimulate erythropoiesis in the bone marrow. A host of other agents have been shown to affect EPO-driven erythropoiesis. As outlined in Chapter 1 and Figure 1, the hierarchy of cell populations leading to erythrocyte production includes successively more specialized cell types. Many of the co-factors in erythropoiesis act on these more primitive cells, although at least one (erythroid cell-stimulating factor) appears to act in concert with EPO on late-stage erythroid cells [44]. Among the prim-

itive-acting factors, the product of the mouse Steel locus, i.e., SCF, the functional ligand for the c-kit cell-surface receptor, is one of the better understood factors. In mice anemic because of defects in either SCF or c-kit, administration of exogenous EPO still increases hemoglobin concentration [45, 46], although to a modest degree. This result would suggest that the few CFU-E that survive functional SCF deficiency *in vivo* can still respond normally to exogenous EPO, though their numbers are reduced by limitations imposed on the cellular pathway at more primitive levels. The interaction of SCF and EPO, which is of obvious significance in these earlier populations, has been elegantly examined at the molecular level and shown to result from a subtle interplay of survival, proliferation, and differentiation signals [47].

These effects at the cellular level lead consistently to increases in hemoglobin in response to increases in circulating EPO, whether exogenous rHuEPO or endogenous EPO produced in response to hypoxia. The relationship between serum concentrations of EPO is relatively straightforward (Fig. 2). A significant temporal mismatch exists between the residence time of rHuEPO ($t_{1/2}$ = approximately eight hours), the life-span of reticulocytes (approximately five days), and the life-span of erythrocytes (approximately 120 days). A more thorough discussion of the pharmacokinetics of EPO is to be found in Chapter 6, but in summary, an increased EPO serum concentration of relatively short duration (measured in hours or days) can produce a very large number of reticulocytes. The flux through the reticulocyte compartment is huge under conditions of accelerated erythropoiesis and yields a large number of erythrocytes to the circulation. The erythrocytes formed, however, live a comparatively long time (on the order of three months). This process, in effect, leads to a disproportion between the duration of accelerated erythropoiesis (sustained by increased amounts of EPO and measured in hours) and the longevity of the hemoglobin response (measured in months). This information is of little value beyond curiosity until new therapeutic derivatives of EPO are considered (see Chapter 15), where an increase in circulating half-life of an erythroid molecule will have a large effect on hemoglobin – on a scale out-of-proportion to the increased residence time of the EPO analog.

Overall, much of the machinery involved in translating an increase of either endogenous or recombinant EPO into an increase in circulating hemoglobin has been defined. At the opposite end of the response is the effect of EPO withdrawal. In addition to the predictable cessation of erythropoiesis when EPO is in short supply, the precipitous decrease in circulating EPO concentrations associated with a return from altitude, the return from space flight, and, perhaps, the end of a course of EPO treatment in the clinic, is associated with an accelerated rate of hemoglobin loss from the blood. The seminal work in this area of Rice and colleagues has led to the proposal of a mechanism of neocytolysis, or the premature destruction of newly formed erythrocytes after interaction with endothelial cells or macrophages [48–50]. The precise role a mechanism such as neocytolysis may play in therapeutic intervention with rHuEPO has yet to be defined.

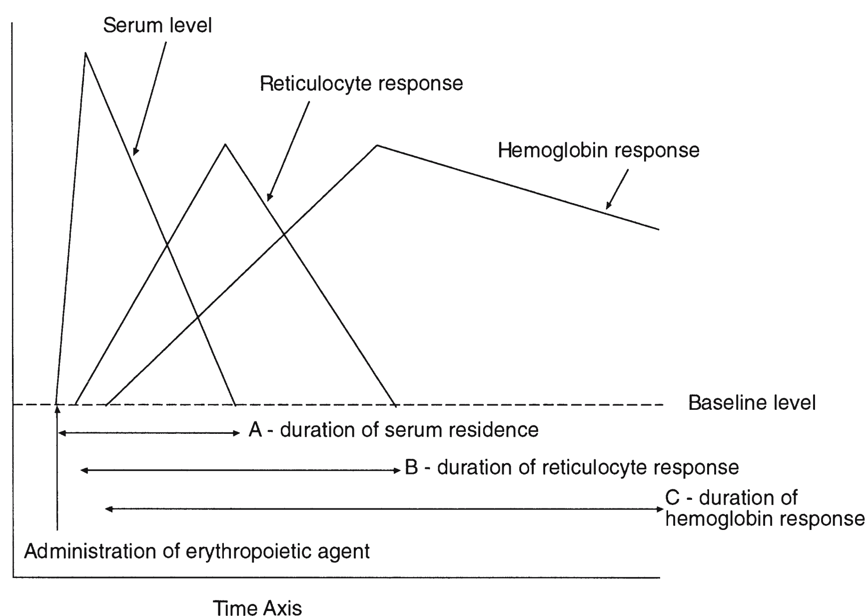

Figure 2. The relationship between residence time of EPO, or any other erythropoietic agent, reticulocyte response and hemoglobin response. The disproportionate effect on hemoglobin from elevated amounts of an erythropoietic agent. The duration A is controlled largely by three factors: the dose, the route of injection which controls the gradient of the upward slope, and the rate of clearance which controls the gradient of the downward slope. The half-life of rHuEPO is approximately eight hours (see Chapter 6 by Heatherington). The duration B of the reticulocyte response is determined by the duration of A – the time when serum levels of the erythropoietic agent are above baseline. Once serum levels of the erythropoietic agent return to baseline, reticulocyte numbers will begin to decline at a rate proportional to their half-life in the circulation. Reticulocyte life-span is of the order of five days. The duration C is controlled again by the duration B. During the time supranormal levels of reticulocytes are being produced the rate of production of hemoglobin exceeds the rate of loss, so hemoglobin will be increasing. When reticulocyte numbers return to baseline, the hemoglobin curve will inflect and hemoglobin will be lost at a rate proportional to the life-span of erythrocytes. Normal erythrocyte life-span in humans is around 120 days. Overall, an erythropoietic agent with a half-life measured in hours can influence hemoglobin levels on a timescale measured in months. Thus changes in the duration A, influenced by dose, route of administration and rate of clearance, will have a disproportionate effect on the duration C. See Chapter 15 by Elliott, for exploitation of this paradigm in the evolution of improved erythropoietic agents.

Biologic basis for the use of EPO in various diseases

Anemia

Anemia of renal failure

As the kidney represents the major site of EPO production in the adult, the progressive destruction of this organ might be intuitively linked to anemia of an EPO-limited nature. Indeed, since shortly after the gene was cloned in 1983

(see Chapter 2), the anemia of renal failure has been successfully treated with rHuEPO [51, 52]. In fact, rHuEPO represents a routine treatment for patients receiving regular hemodialysis or peritoneal dialysis and for many patients not receiving dialysis (i.e., predialysis patients) [53, 54]. Biologically, this use represents a form of hormone replacement therapy, although the benefits to the patient extend beyond the reduced need for transfusions and the associated risks of iron overload, incompatibility reactions, and viral infections and include the benefits of increased exercise tolerance, cognitive and psychomotor changes, and relief from hyperdynamic cardiac states. In total, these changes produce a significant impact on the quality of life of patients [55]. Beyond the surprisingly wide ramifications of correction of anemia in these patients, the biologic basis for rHuEPO therapy in patients with renal disease remains replacement of a hormone deficiency secondary to failure of the organ that produces the hormone. Other uses for rHuEPO, for instance in anemia of cancer, anemia of chronic disorders, or in chronic heart failure, have lead to extensive use outside the dialysis clinic.

Anemia in oncology

Anemia in patients with cancer results from a complex interaction of various factors that make treatment somewhat less predictable than the hormone replacement-like use in renal failure states. Included in the list of contributing factors are hemodilution, bleeding, hypersplenism (and hemophagocytosis), hemolysis, nutritional deficiencies, marrow damage, chemotherapy and radiotherapy, and the anemia of cancer itself (included in the broader classification of anemia of chronic disorders [ACD]) [56–59].

Addressing any one of these factors alone represents a significant challenge, yet the perceived benefits of managing anemia in patients with cancer has led to a number of successful therapeutic strategies. Perhaps the most obvious rationale for rHuEPO use in anemic cancer patients is in patients with documented low endogenous serum EPO concentrations. Concentrations of endogenous EPO are inherently variable and use of the data is further confounded by a phenomenon widely documented in ACD where the absolute EPO concentration is within the normal range and is low only when considered relative to the degree of anemia – the so-called "blunted" EPO production [60]. For the most part, studies of endogenous EPO concentrations in animals have not illustrated principles to be applied to clinical practice. For instance, inappropriately low EPO concentrations in tumor-bearing mice did not confirm the earlier observations made in tumor-bearing rats [61, 62]. Later work, however, showed that endogenous EPO concentrations were predictable and consistent with the degree of anemia [63]. In addition, the effects of chemotherapy on endogenous EPO concentrations have been variously reported to increase, decrease, or remain unchanged [64–66].

Patients with cancer frequently have circulating endogenous EPO amounts that are lower than would be expected for the degree of anemia. In early studies in patients with cancer, inappropriately low endogenous EPO concentra-

tions were found, yet only in association with other problems such as infection or inflammation [67] while other studies showed normal concentrations [68, 69]. Blunted EPO production, however, has been seen in more recent work in patients with cancer without complications, in contrast to patients with iron-deficiency anemia [70–74]. Overall, a low endogenous serum EPO concentration, or at least a concentration lower than might be expected, would suggest that rHuEPO use is warranted in cancer patients with anemia even before they receive chemotherapy.

Chemotherapy-induced or radiotherapy-induced anemia is the most frequently encountered anemia in patients with cancer [75]. Chemotherapy or radiotherapy cause systemic changes outside the bone marrow that result in anemia, e.g., hemolysis, blood loss, nutritional deficiency, damage to the erythroid populations in the bone marrow (Fig. 1), or other conditions may reduce hemoglobin concentration [76]. The endogenous EPO concentration in such patients can be variable depending on a host of factors including, but not limited to, tumor type and chemotherapy agent. Most patients (>50%) respond to administered rHuEPO [77]. Whether this responsiveness results from the correction of low endogenous EPO values or a heightened response from supernormal circulating EPO values (combining both endogenous and exogenous material) is unclear. The observation remains that the proportion of patients responding to rHuEPO after chemotherapy increases with increasing dose. In the case of limited marrow damage, the problem can be overcome by rHuEPO administration, though when marrow damage has become extensive, it is unlikely that rHuEPO will be effective.

Other anemias

Although the most appealing indication for a recombinant protein therapeutic may be where amounts of the endogenous prototype are limited, the discussion earlier suggests that, in cancer at least, an endogenous EPO-deficit condition is not the only time when using rHuEPO may be useful. In general in those cases where endogenous serum EPO concentrations are reduced, administration of rHuEPO is most effective. Thus, the rationale for rHuEPO therapy in iron-deficiency anemia due to limited dietary iron intake, for example, is weak. Table 1 summarizes some anemic conditions and the rationale for rHuEPO use in those conditions. These conditions range from an apparent EPO-deficit condition documented in neonates through premature erythrocyte destruction noted in elderly patients. Despite the different cause of these two types of anemia, rHuEPO appears to be effective in both. In contrast to these anemias are the hemoglobinopathies, where an rHuEPO-induced increase in hematocrit, without the accompanying conversion of hemoglobin type from diseased to fetal (e.g., with hydroxyurea therapy), would be of limited benefit. Anemia in the various intensive care patient populations responds, in general, quite well to rHuEPO and appears to have many features in common with ACD. Similarly, the anemia noted in patients with AIDS, despite the multifactorial causation, responds predictably to rHuEPO.

Table 1. The rationale for use of rHuEPO in various anemic conditions

Disease	Rationale			Ref
	Endogenous EPO	Target tissue	Notes	
Iron-deficiency anemia	High	Bone marrow	Iron is vital for hemoglobin synthesis. Correcting iron deficiency may reveal problems elsewhere. Absolute deficiency (where total body load of iron is low) contrasts with functional deficiency (where the body load of iron may be adequate but erythropoiesis lacks sufficient iron due to it being locked in tissues elsewhere).	[114]
Renal failure Hemodialysis Peritoneal dialysis Predialysis	Low	Bone marrow		[51, 115]
Oncology				
Anemia of cancer	Normal to low (low relative to hemo-globin), possibly tumor specific	Bone marrow	Tumor-derived inhibitors, inflammatory cytokines, marrow infiltration, stem cell defects. Complex, multifactorial causation; hemolysis, blood loss, erythrophagocytosis, autoimmune RBC destruction, nutritional deficiency etc.	[56, 58, 116]
Chemotherapy-induced anemia	Conflicting data – could be drug-specific	Bone marrow	Extensive marrow damage results in no EPO target cells. Nutritional limitations. EPO clearance could be prolonged; chemotherapy also affects other organ systems, limiting responses.	[75, 117]
Chronic heart failure	Unknown	Bone marrow	Multiple comedications. Correction of cardiac performance may be secondary to correction of anemia.	[118, 119]
Intensive care medicine	Low	Bone marrow	Seriously ill patients, multiple causes of anemia, and various co-medications and co-morbidities. ACD-like symptoms are common (functional iron limitation due to maldistribution). Time to EPO response slower than transfusion.	[120, 121]

(continued on next page)

Table 1. (continued)

Disease	Rationale			Ref
	Endogenous EPO	Target tissue	Notes	
Hemoglobinopathies				
Sickle-cell anemia	Low	Bone marrow	Increased hemoglobin is not beneficial unless accompanied by a switch to fetal hemoglobin. EPO has a modest influence as a single agent fetal hemoglobin and increased hematocrit may be associated with increased crises	[122, 123]
Thalassemia	Inconsistent reports	Bone marrow		[124, 125]
Sports anemia	Variable		Catch-all phrase for different anemias, mainly due to mechanical RBC destruction and/or volemic changes. Use of rHuEPO would appear unwarranted, or unethical	[126]
Parasitemia	Conflicting data, high or low relative to anemia (?)		Mostly disorders of RBC destruction. Parasite and host species dependent. Economic influences. EPO can influence sex of malaria parasites. Largely untested	[127]
AIDS anemia	Low	Bone marrow	Multiple causes of anemia–viremia, comedications, opportunistic infections, autoantibodies to EPO	[99, 128, 129]
Anemia of the elderly	Unknown		Probably associated with increased RBC destruction	[130]
Anemia of prematurity	Low	Bone marrow	Iron deficiency is common.	[131]

ACD, anemia of chronic disorders; RBC, red blood cell.

A variety of anemias exist, many of which will respond well to rHuEPO administration, but other cases exist where rHuEPO may not be a good therapeutic choice. In settings of anemia where endogenous EPO concentrations are low or even normal, rHuEPO has a good chance of being effective. In other conditions where EPO concentrations are supernormal, the administration of rHuEPO can help. In these cases it might be reasoned that EPO is not the only limiting factor in the development of anemia, but that supplementing EPO can make up for the limitation elsewhere in erythropoiesis [45].

Nonanemic conditions

Given the relatively wide distribution of EPOR expression in the body, it would be surprising if effects were not seen outside the erythroid machinery. The central nervous system is known to be the site of both EPO and EPOR expression in the fetus and adult [20, 78, 79] and has been widely discussed as a therapeutic target for rHuEPO therapy [80]. It is not clear whether EPO passage across the blood/brain barrier is a process facilitated by a specific mechanism, but neural cells are stimulated to grow *in vitro* in response to EPO [81–84]. EPOR is expressed in rodent capillaries at the blood/brain interface [82]. These data have lead to much speculation that rHuEPO may offer a therapeutic advantage in various neurological injury–speculation borne out by preclinical experience [82, 85–88].

EPO and EPOR are expressed in peripheral nerves before injury and show differential regulation after crush injury, which suggests EPO may have peripheral effects in the nervous system [89]. Effects of EPO on vascular function, angiogenesis, fatigue, preadaptation to altitude, hemoglobin oxygen dissociation, and cardiac performance have been documented (Tab. 2). Among these areas, the effects of EPO on angiogenesis both in culture and in intact endometrium have suggested a role in neovascularization [90, 91]. Whether these studies have implications for vascularization of neoplastic tissues is unknown. Direct effects upon the vascular tissues have been shown *in vitro* as have changes in blood pressure after longer term rHuEPO administration [91–94]. A direct effect of EPO on blood pressure has not been reported, however. Effects of EPO in fatigue separate from the changes mediated by hemoglobin increase have been suggested but have proven difficult to substantiate. Several of these areas may prove productive in future clinical research, but may be confounded by the major effect of EPO, which is, almost inexorably, to increase the hematocrit. It may prove somewhat difficult to separate the role of endogenous EPO from that of rHuEPO in the non-anemic condition where normal hematocrit is presumably maintained by an adequate endogenous production of EPO.

Table 2. The rationale for rHuEPO use in various nonanemic conditions

Neural effects	Stroke, subarachnoid hemorrhage	Controversy over whether EPO crosses the blood/brain barrier. Mechanism of action may be anti-apoptotic or mitogenic.	[132]
Cognitive effects	Secondary to hemo-dynamic changes? Oxygenation?		[133, 134]
Vascular effects	Changes in blood pressure often parallel hemoglobin, however, direct effect of EPO alone is unclear.		[135]
Angiogenesis	Demonstrated effects in endometrium, other setting less well documented.		[91]
Blood donation	Autologous donation before surgery.		[136, 137]
Oxygen dissociation	Modulation of erythrocyte 2,6 DPG (and oxygen dissociation) directly by EPO.		[138]
Effects on tumors	Receptor shown in some breast and renal tumor cells. Proliferation shown in renal carcinoma.		[139, 140]
Effects on fatigue		Difficult to deconvolute from anemia, cognitive function etc.	[141]

DPG, diphosphoglyceric acid.

Control of rHuEPO response rates

The administration of rHuEPO in a situation where every other constituent of the biologic response is in excess and EPO is the only limiting factor in erythropoiesis will yield the most marked response. Such a situation is extremely rare. Even in the case of renal disease where compromised endogenous EPO production is an obvious feature, other factors such as uremia, general inflammation, or iron deficiency can limit the effectiveness of injected rHuEPO by placing other constraints on the response [95]. These constraints may be the demonstrated inhibition of erythropoiesis by tumor necrosis factor (TNF), IL-1, and interferon (IFN), the pro-inflammatory cytokines [96], or by limited supply of iron for hemoglobin synthesis. Recognizing that such a clear-cut situation is not likely to present itself, the effectiveness of administered rHuEPO will depend on a milieu of negative influences including those above in addition to anti-EPO antibodies and soluble EPOR. The latter two factors have direct consequences for rHuEPO therapy. Antibodies to EPO have been noted in diseases such as systemic lupus erythematosus and HIV and are capable of neutralizing the action of administered rHuEPO [97–99]. Antibodies may be

Table 3. Factors affecting rHuEPO response rates

Condition	EPO-response limiting factor [Ref]
Renal failure [142]	Iron Deficiency [143] Overload [144] Soluble EPOR [101] Inflammatory cytokines; inhibition of CFU-E [145] Endogenous inhibitors [146] Oxidative damage to red blood cells [147] Aluminum toxicity [148]
Oncology	Marrow infiltration by tumor, hemolysis, nutritional deficiency [59] Inflammatory cytokines [96] Iron [149] Anemia-inducing factors [150] Marrow injury [151]
Systemic lupus erythematosus	Multiple causes: anemia of chronic disease, iron deficiency, hemolysis [97] Antibodies to EPO [152]
Concurrent therapies	Inhibition of erythropoiesis by Cyclosporin [153] IFN [154] AZT [155]
Anemia of chronic disorders	Reduced EPO production and effectiveness, iron metabolism [156]
Pure red cell aplasia	Antibodies (Mayeux and Casadevall, this volume) Multiple causes [157]

AZT, zidovudine; IFN, interferon; CFU-E, erythroid colony-forming unit; EPO, erythropoietin.

raised in response to administered rHuEPO with serious consequences (see Chapter 14) and may include life-long refractoriness to rHuEPO and endogenous EPO and a long-term dependence on blood transfusions for survival. Soluble EPOR is a truncated version of the full-length membrane-bound version that has been suggested to act as a competitive inhibitor of the cell-borne form of the receptor and can limit the response to injected rHuEPO [100, 101]. Several such confounding factors likely to be found in various indications are listed in Table 3.

Interaction with iron

Iron is a vital component of hemoglobin and limitations on its supply can compromise the ability of erythropoietic cells to synthesize hemoglobin. Paradoxically, iron is a poisonous metal for which most mammals, including humans, have highly evolved strategies to ameliorate its toxicities. Iron deficiency is commonly discussed, although the body's treatment of iron as a toxin can lead to its deposition as relatively inert forms (such as ferritin or hemosiderin) in various tissues [102]. This iron maldistribution situation, where the

body load of iron may be excessive yet the presentation is one of functional iron deficiency, is typical of ACD, the anemia of cancer, and perhaps other anemias with an inflammatory component. When deficient, and depending upon the reason for the deficiency, supplementation with oral or intravenous iron may be relatively straightforward. The move from oral administration of simple salts like ferrous sulfate towards more complex intravenous forms of iron resulted from a series of clinical studies, although agreement is not complete [103, 104]. EPO has not been shown to have a direct role in mobilizing iron from storage organs, but can act indirectly by stimulating hemoglobin synthesis and drawing iron from storage pools. Although iron may often be a limiting step in erythropoiesis, it may be corrected by supplementation. The converse may prove an interesting new use for rHuEPO – in managing inappropriate iron distribution often seen in conditions such as chronic inflammation and associated with oxidative damage, cognitive impairment, overt toxicity.

The use of EPO in different species

EPO from mammalian species as diverse as human, hamster, rat, mouse, cat, lion, dog, horse, sheep, dolphin, and pig has been cloned and sequenced and found to be remarkably conserved with a minimum identity of 79.8% between any two of these species [105, 106]. This finding has lead to the experimental use of rHuEPO in cross-species settings, for instance in anemic feline immunodeficiency virus (FIV)-infected cats [107], for idiopathic anemia in dolphins [108], in blood doping of racehorses [109], in transient brain ischemia in gerbils [110], for subarachnoid hemorrhage in rabbits [111], and to study erythrokinetics in sheep [112]. Even with the high degree of conservation across species, the administration of rHuEPO can raise antibodies that may cross-neutralize endogenous EPO (e.g., in horses [113]). Despite the precise species match between recombinant and endogenous forms of EPO, instances of cross-neutralizing antibodies have been reported in humans receiving some forms of rHuEPO, but not others with identical amino acid sequence. (See Chapter 14 for further information). It may be the case that even though these antibodies bind to similar epitopes on the protein backbone of rHuEPO and endogenous EPO, their induction is dependent on other factors such as autoimmune disease or manufacturing or formulation methods resulting in protein aggregation or degradation. (See Chapter 15 by Elliott for further information.)

Conclusions

rHuEPO is, for the most part, an erythroid stimulus that has found widespread use in patients with EPO-limited anemia. It has proven useful in treating anemias where endogenous EPO concentrations may not be abnormally low.

rHuEPO may find utility in non-anemic conditions to treat neural and cardiovascular symptoms. Many variables affect the response to rHuEPO, suggesting that treatment paradigms must be designed individually and can be expected to be different in different disease settings.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Commercial production of recombinant erythropoietins

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Introduction

For the production of recombinant products from genetically engineered cells, a number of cell hosts may be used, but the ones generally used belong to one of five categories: plant, bacterial, yeast, insect, or mammalian. Depending on the type of product that is desired, the features of that product, the intended use of the product, and the preferred method of manufacture, an appropriate host cell can be selected. With the appropriate genetic engineering, the gene of interest such as erythropoietin (EPO) can be produced by a host cell. In the case of EPO production, the sequence of amino acids as well as the amount of glycosylation must be correct to achieve the desired efficacy *in vivo*. This chapter describes one method of EPO production using cells genetically engineered to secrete recombinant human erythropoietin (rHuEPO). In this method of production, mammalian cells, which are capable of producing glycosylated forms with the desired efficacy in humans, are typically selected as hosts. The host mammalian cells secrete the rHuEPO product into the medium environment in which they are cultured, making the remainder of production a matter of separating the rHuEPO product from the cells and other components in the cell culture broth. This chapter summarizes the generation of recombinant rHuEPO-producing cell lines, the production of rHuEPO, the separation of rHuEPO from components of the cell culture broth, and the packaging of the final rHuEPO drug product.

Cell line development

The development of a cell line generates a consistent source of cells that is capable of satisfying commercial demand and that adheres to regulatory guide-

lines for genetically engineered cells. Development begins with the creation of genetically engineered cells that produce the desired product, and ends with a cell bank, a homogeneous population of genetically engineered cells frozen at the same population doubling level.

Cell bank

Once a cell line has been identified as the manufacturing line, a cell bank must be generated. The cell bank provides the starting material for all lots of product manufactured. For this reason, it is carefully scrutinized from both a business and regulatory perspective.

Once a homogenous population of cells ("cell bank") is created, a master cell bank is made. The master cell bank is the base starting material for the lifecycle of the product. Cell banks are created in a two-tier system (Fig. 1). Each vial of master cell bank material can be used to create a working cell bank. Likewise, a single vial provides the starting material for a commercial cell culture lot.

Single-cell cloning

The industry standard for generating a homogenous population is single-cell cloning. Single-cell cloning is accomplished by placing one cell in a microculture environment, and then expanding that cell through increasingly larger cultures to generate a greater number of cells (e.g., 1×10^9 cells) (Fig. 2). Doubling of a single cell should produce two identical cells. Doubling again results in four cells that are assumed to be identical. Within a limited number of generations, expansion of the cells in this manner results in a homogenous culture of cells with identical genomes. When sufficient cells are amassed, a premaster cell bank of approximately 25 vials is frozen.

Two-tier system: creation of master and working cell banks

A two-tiered approach is commonly used in industry to generate a supply of cell bank vials for the lifetime of a product. The master cell bank is generated first and typically consists of 200 to 400 vials. The master cell bank is not used by the manufacturing facility directly. Instead, the second-tier cell bank, the working cell bank, is used by the manufacturing facility. The working cell bank is generated by expanding one vial of material from the master cell bank. The working cell bank typically contains 500 to 1,000 vials.

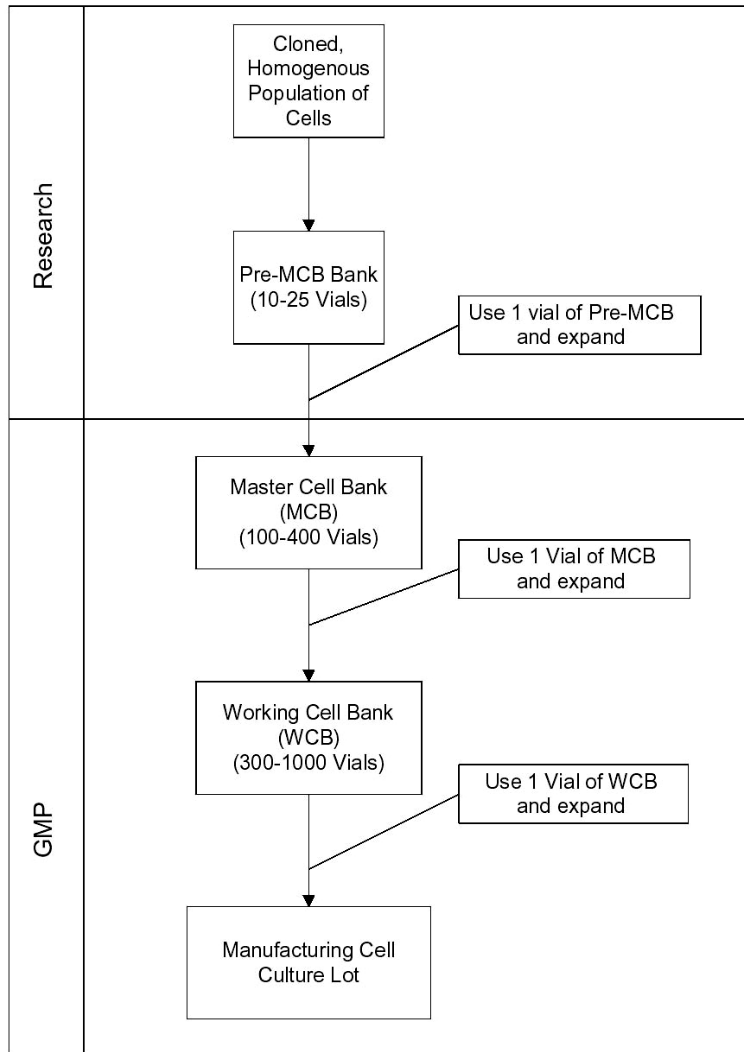

Figure 1. Cell banking is a two-tiered system. GMP, good manufacturing processes; MCB, master cell bank; WCB, working cell bank.

Safety assessment

Once cell banks are generated, they must be characterized before they can be used to manufacture a drug for clinical use, as per FDA industry guidelines [1]. Characterization is critical to ensure that the correct protein is being produced by the cells and that the cell line is not contaminated by adventitious agents.

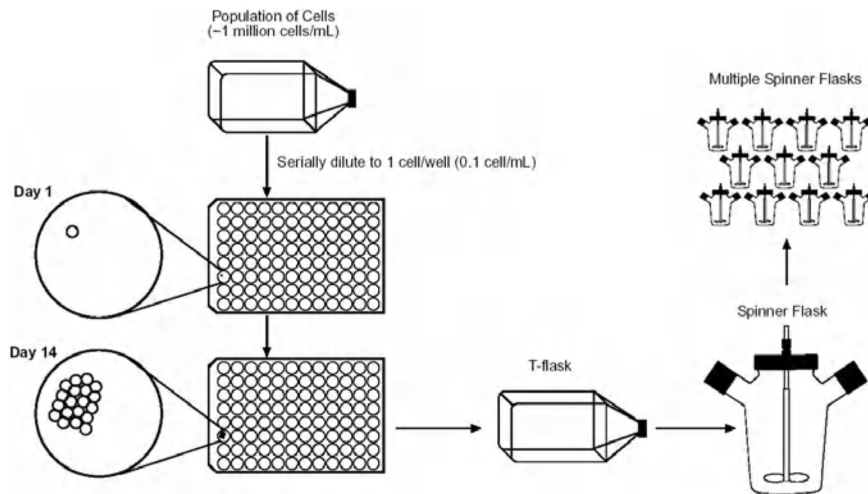

Figure 2. An example of single-cell cloning technique for mammalian cells.

The cell bank is the primary source for the recombinant protein produced, so verification that the correct molecule is being produced must be done at the inception of the bank. Confirmation of the DNA and/or RNA coding sequence ensures that the cells encode the required genetic sequence for the protein. Verification of the genetic sequence within the cell must be followed with verification of the amino acid sequence of the purified protein. Additionally, the cell bank must be examined to confirm that it is not producing any altered forms of the protein. For example, during gene integration in mammalian cells (after transfection or amplification), the cell's genome is rearranged, and rearrangement possibly may involve the coding sequence of the protein of interest. One concern is that the rearrangement could occur at a site that results in a molecule containing a portion of the correct protein and a portion of another protein. In this event, the rearranged molecule could retain some of the correct protein's characteristics and be carried through a purification process; however, the net result may be either the incorrect protein or a mixture of correct and incorrect proteins. Therefore, the cell bank is examined in great detail to ensure the absence of rearrangements that could lead to unwanted proteins being carried through manufacturing.

Ensuring that adventitious agents are not present is crucial. When a cell bank comprises mammalian cells, which theoretically can act as a host to viruses and is the starting material for every lot of protein, the cell bank is examined for mycoplasma, bacteria, fungi, and viruses. Numerous *in vitro* and *in vivo* assays are used to assess viral contamination. In the end, all of the aforementioned tests for adventitious agent must be negative. If they are not, the cell bank cannot used to make material intended for clinical use.

Cell bank stability assessment

The master cell bank is designed to last the lifetime of the product, so monitoring it and the working cell bank for storage stability is crucial. Cell banks are generally stored at –130 °C or below to ensure cell stability. The cell banks are monitored by testing cells for viability upon thawing. If the cell bank's viability is stable, then no other testing is necessary. If viability is not stable, the bank is re-examined, and another master cell bank may need to be generated.

Cell culture process

One method of EPO manufacture uses cell lines genetically engineered to produce rHuEPO. In this chapter, an example of rHuEPO production in a fermentation process is presented, where cells from a mammalian cell bank are grown and secrete product into the cell culture medium. The ability of the cell culture to produce product is affected by the nutrient environment and other physical parameters, such as temperature, pH, osmolality, and concentrations of dissolved gas. The cell culture fermentation process is designed to support the production of rHuEPO of a consistent quality, tailored to enable the product specifications and efficacy targeted by the manufacturer.

Raw materials

The raw materials for the fermentation process consist of the cell bank and the nutrients used to sustain and expand these cells. Nutrients in the cell culture medium generally are amino acids, additional carbon sources (e.g., glucose), vitamins, trace elements, growth factors, hormones, and salts. A review of media composition is given in [2]. Historically, cell culture medium has also contained serum from bovine sources, which may vary in composition depending on its source or the processing. Guidance for the industry for use of raw materials for the manufacture of biologicals is provided by CBER/CDER.

Process options

Many process options are available to produce a recombinant protein. One of the simplest fermentation options is batch mode, where the cells and the nutrient-containing media are added to a production vessel, and no further additions are made. Physical parameters (e.g., temperature, pH, and dissolved oxygen concentration) may be controlled within set ranges. The cells grow to the extent that can be supported by the initial nutrient concentrations; cells will stop growing and may begin dying when nutrients become insufficient and/or

if the amounts of waste products (the by-products of nutrient consumption by the cells) increase.

Fed-batch fermentations differ from batch fermentations by providing for the addition of nutrients into the culture as required. This method allows a much greater number of cells to be supported than batch fermentation.

The significance of cell number to product made results from the constitutive production feature engineered into most commercial cell lines. With constitutive production, one can reasonably assume that as long as a cell is present and viable, it will continue to make product. Thus, cell number correlates closely with amount of recombinant protein produced.

In addition to supplementing a culture with nutrients as required, spent medium in the culture may be removed to reduce the amount of waste products. The process of feeding fresh nutrients and concurrently removing spent media is called perfusion. The spent medium, called the harvest, contains the protein product; the device used to separate the spent medium from the cells is called a cell retention device.

Operationally, batch and fed-batch processes are relatively easy to execute and perform reliably; perfusion processes are relatively difficult to execute and in general use longer (i.e., >two-fold longer) production times. The number of pieces of equipment that must be operated in a sterile manner within the sterile envelope are depicted in Figure 3, Figure 4, and Figure 5. The number of pieces and complexity of equipment in the sterile envelope correlates with the difficulty in executing the three types of processes. A schema of the equipment (bioreactor, media feed tank, and harvest tank) used for each of these process

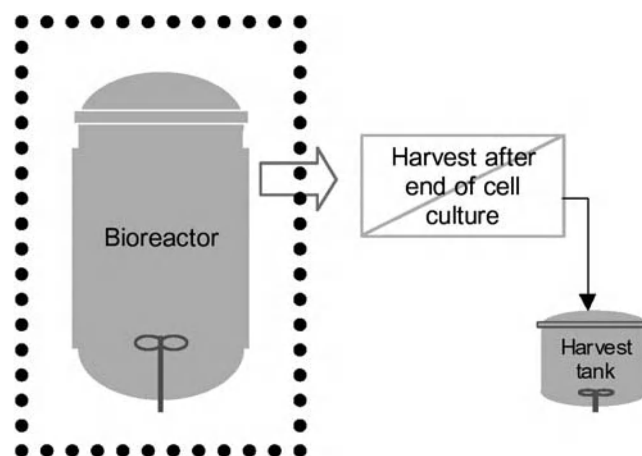

Figure 3. Schematic of a bioreactor operating in batch mode. The sterile envelope, depicted by the dotted line, encompasses only the bioreactor. The harvest operation, which includes cell separation from the conditioned medium and concentration of the conditioned medium, is not performed under sterile conditions.

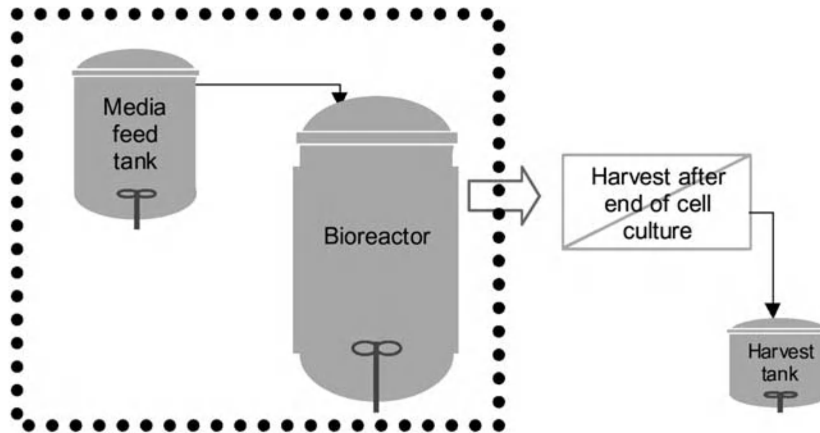

Figure 4. Schematic of a bioreactor operating in fed-batch mode. The sterile envelope, depicted by the dotted line, includes the media tank and the bioreactor. The harvest operation, which includes cell separation from the conditioned medium and concentration of the conditioned medium, is not performed under sterile conditions.

options and the relative scale of tanks and production vessels is depicted. For batch and fed-batch processes, the production vessel is typically the largest piece of equipment used in the cell culture process; for perfusion processes, the feed and harvest tanks are typically the largest equipment used.

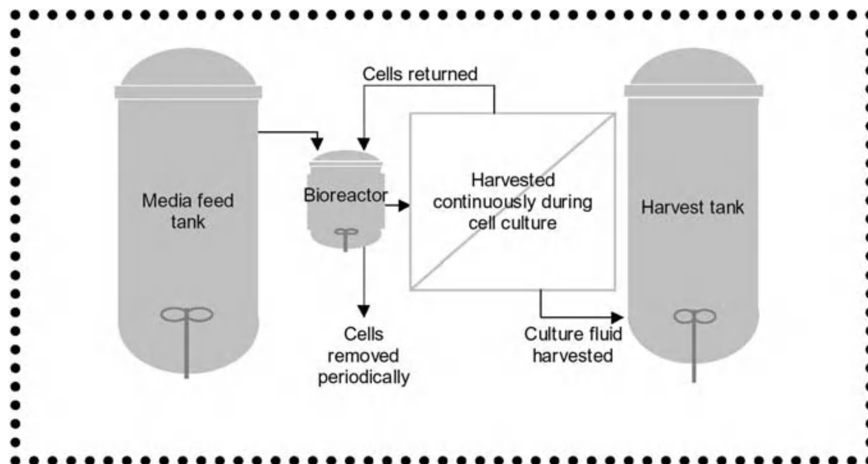

Figure 5. Schematic of a bioreactor operating in perfusion mode. The sterile envelope, depicted by the dotted line, includes the media tank, bioreactor, cell separation device (harvest unit), and harvest tank. The harvest operation here does not include any concentration of the conditioned medium.

The size of the equipment and the time over which each piece of equipment needs to be occupied for a given production run will influence the design of a manufacturing facility and the number of fermentations a manufacturing plant can execute. The manufacturing facility is designed to comply with regulatory guidelines for GMP manufacture (i.e., CFR parts 210 and 211 are used in the US; guidelines differ slightly in other countries). Additional information on process options can be found in [3] and [4].

Commercial manufacture

A commercial manufacture cell culture process consists of two parts: A seed train is used to generate a sufficient number of cells with which to initiate production in the production vessel; the required process conditions are imposed on a production vessel to generate optimal quantity and quality of product.

Seed train

A seed train describes the expansion of cells from a frozen cell bank vial to the inoculum of the production reactor. The volumes involved may range from 1 mL to 10,000 L. The vessels involved may include T-flasks, shaker flasks, roller bottles, Cellbags® (Wave Biotech, Bridgewater, NJ), and stirred tank reactors (Fig. 6). The reactors may be operated in batch, fed-batch, or perfusion modes. The purpose of each step of the seed train is to generate a sufficient number of cells of known quality to enable inoculation of the subsequent step. The culmination of the seed train occurs at inoculation of the production reactor.

Given a cell bank vial containing 1×10^7 viable cells, the initial seed train time to the production reactor may be estimated. First, the doubling time for the cell line must be established. For mammalian cells, doubling time typically ranges from 16–48 hours, with 48 hours used as a conservative basis for planning. Second, the requirements for cell number generated at the end of the seed train must be established. For example, if the process is envisioned to go towards commercial production with a 10,000-L production reactor and the production reactor requires an inoculum density of 1×10^6 viable cells/mL and an initial volume of 7,500 L, then 7.5×10^{12} viable cells are required. The number of cell doublings (n, or population doubling level) from vial thaw to production reactor inoculation can be given by the formula:

$$n = LN(XS/X0)/LN(2)$$
$$n = LN(7.5 \times 10^{12}/1 \times 10^7)/LN(2)$$
$$n = 19.5$$
$$n \sim 20$$

where LN = natural logarithm and XS final cell number = 7.5×10^{12} and X0 = initial cell number = 1×10^7.

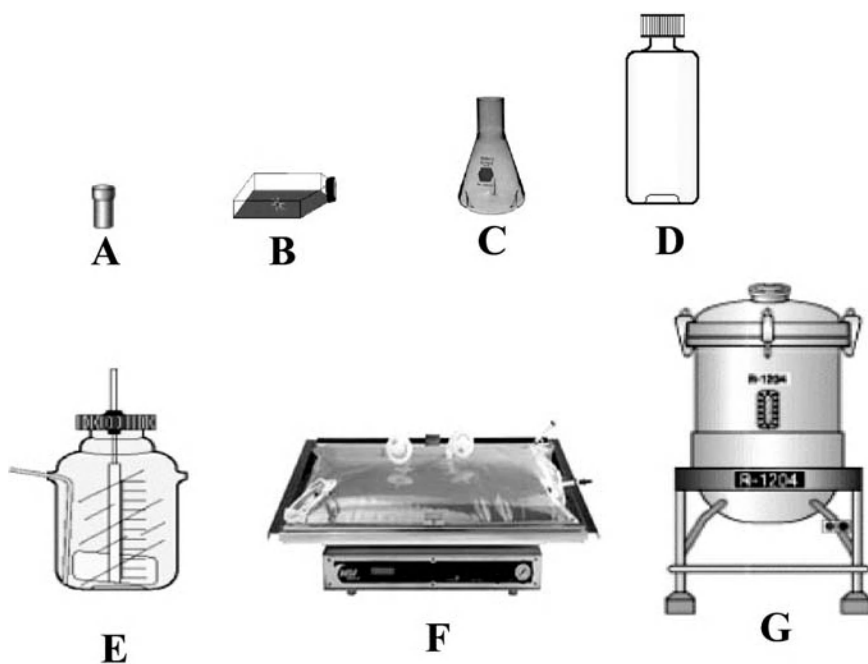

Figure 6. Vessels used in the preparation of a seed train: (A) freezer vial, (B) T-flask, (C) shaker flask, (D) roller bottle, (E) spinner flask, (F) Cellbag®, (G) stirred tank reactor.

Using the conservative estimate of two days for the doubling time (td), the overall seed train time is $n \times td = 20 \times$ two days = 40 days, or approximately six weeks.

A backup seed train using cells spun off from an existing seed train may be initiated if cell numbers allow and if the cell line's productivity remains stable. Thus, multiple production reactors stemming from a single cell bank vial may be considered to shorten the overall time to inoculate production reactors. This approach is an option to the more typical practice of scheduling each production reactor to be derived from a single vial thaw.

Production

Once the seed train has generated a sufficient number of cells from the cell bank, these cells can produce rHuEPO in either adherent mode or suspension mode. In adherent mode, cells adhere to a solid substrate and secrete rHuEPO into the liquid cell culture medium. Examples of solid substrates include tissue culture-treated plastic, such as roller bottles or microcarrier beads that may be made of ceramic or other porous materials. In suspension mode, cells are freely suspended in liquid medium where they secrete rHuEPO. In cell culture processes, the three production modes may be used for both adherent and sus-

pension cultures. Adherent cultures in roller bottles and suspension cultures in stirred tank vessels are shown in Figure 7 and Figure 8, respectively.

Commercial cell lines are designed and selected for constitutive production; therefore, the amount of rHuEPO produced correlates with the viable cell number (Fig. 9). For adherent culture, the number of cells is limited by surface area. To increase production of rHuEPO, the surface area is increased, typically by increasing the number of roller bottles or microcarrier beads. Since nutrients are supplied in the liquid phase and products are secreted (by the cells) from the solid phase into the liquid phase, the successful mass transfer

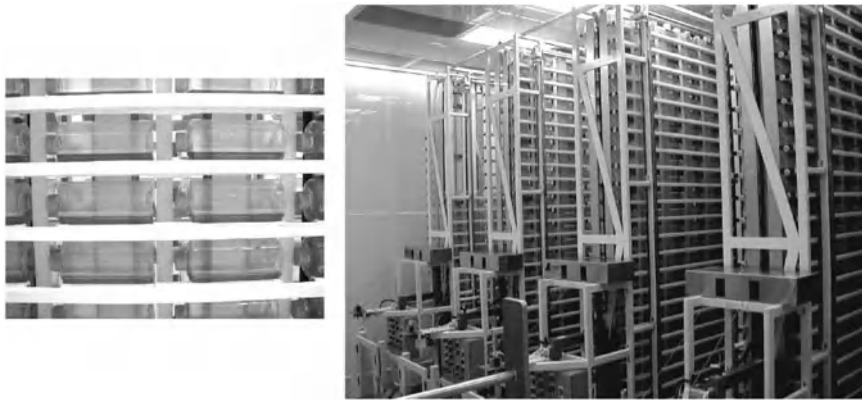

Figure 7. Production of rHuEPO in adherent culture. The left panel shows roller bottle cultures of rHuEPO. The right panel shows large racks containing many of the same type of roller bottle cultures.

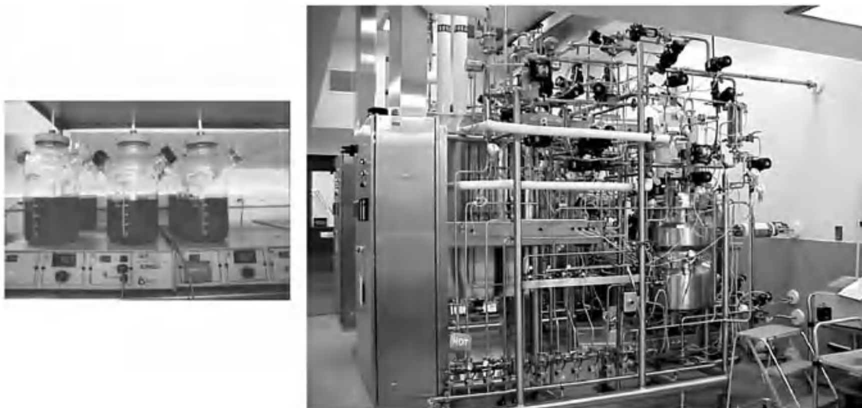

Figure 8. Production of rHuEPO in suspension culture. A suspension of rHuEPO-producing cells is shown in the spinners on the left. The stirred tank reactor shown on the right also contains a suspension of rHuEPO-producing cells.

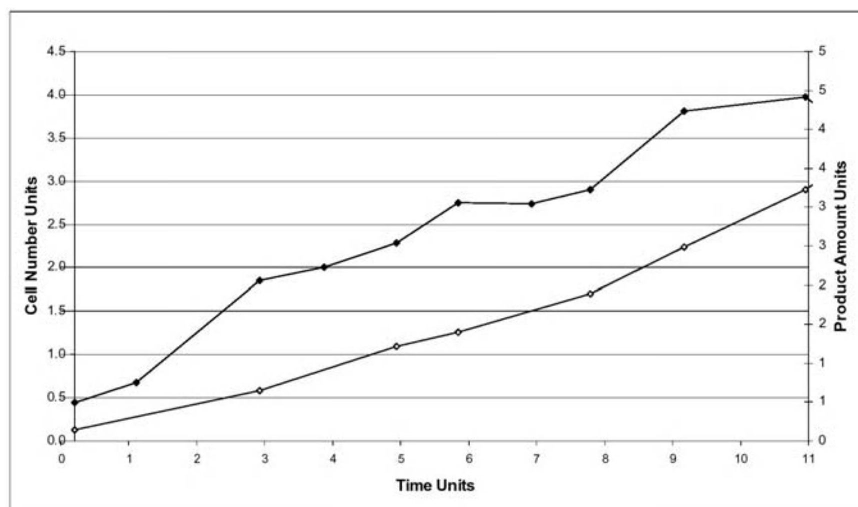

Figure 9. Correlation between number of cells and amount of product produced. Solid diamonds = cell number profile; hollow diamonds = product amount profile.

of nutrients to the cells, and rHuEPO and metabolic waste products from the cells must be carefully considered in the design of the process. A commercial production lot from adherent cultures typically entails using many small production vessels; the challenge of such a process for commercial scale production is to demonstrate equivalent control (i.e., monitoring) of each of the multiple production vessels. The degree of control required is imposed to achieve the growth and productivity performance from each production vessel. After product is produced, it is separated from the cells in the harvest process. Since cells are attached to the solid substrate, harvesting the rHuEPO product in the liquid conditioned medium is operationally straightforward and involves pouring off or decanting the liquid before volume reduction and purification.

For suspension cultures, the number of cells itself generally limits the productivity of the production. To increase production, the number of cells per unit volume (i.e., cell density) may be maximized, and/or the volume of culture may be maximized. To maximize the cell density, limiting nutrients within the culture environment are identified and supplements are added to the culture. The medium is sufficiently enriched to support the maximal number of cells. To maximize the culture volume, a larger scale of production vessel may be used. Increasing the scale of production for suspension cultures within a single vessel is fairly straightforward. Both cells and product are distributed homogeneously in well-stirred reactors so that the growth and production performance within each volume of liquid is consistent. The volume of liquid may be increased as long as homogeneity is achieved (i.e., mixing is adequate). Stirred tank reactors for suspension cultures of mammalian cells typically

range between 1,000 L and 20,000 L. To harvest the product, the cells must be separated from the liquid medium. Separation is achieved by microfiltration or centrifugation. The clarified liquid-containing product is then concentrated for volume reduction and purification.

Within any of the process modes described above, a cell line that has been engineered to produce rHuEPO may grow and secrete product encoded by the *EPO* gene. Not all of the secreted product may qualify as the rHuEPO sold by a manufacturer. The EPO molecule has three *N*-glycosylation sites and one *O*-glycosylation site, leading to the possibility of differently charged molecules with different isoelectric points (i.e., isoforms). Those molecules with the lowest isoelectric points, typically corresponding to those having the greatest amount of sialylation and glycosylation branching, have the greatest *in vivo* efficacy. Molecules with higher isoelectric points, or less sialylation and less glycosylation, have less therapeutic efficacy, and can be removed in purification. (See Chapter 13 for further information about isoforms.) Additionally, even if the cell produces product of the desired charge profile, this product may be degraded in culture by proteases. The product itself may form undesired complexes such as aggregates, which must be separated from the desired monomer. The cell may be influenced to produce increasing amounts of the desired charge profile by controlling process conditions, such as temperature, pH, osmolality, and nutrient and waste product concentrations. Similarly, undesired forms of the product may also be minimized.

Recovery and purification of erythropoietin

A number of purity criteria must be met for rHuEPO to be a viable human pharmaceutical. Host cell and other contaminants, such as DNA, host cell proteins, and endotoxin, must be removed to appropriate levels. The removal or inactivation of any theoretical endogenous or adventitious viruses must be shown. Amounts of product-related contaminants, such as aggregates, proteolytically degraded, under-glycosylated, or oxidized forms, are minimized. A targeted purity is specified by the manufacturer, so each product lot must meet the criteria described in the manufacturer's product specification. Product specifications may differ among manufacturers.

The clearance rate of rHuEPO in the human body is determined by the number of sialic acid residues on the carbohydrate portion of the molecule. Under-sialylated rHuEPO are cleared in the liver by the asialoglycoprotein receptor [5]. Selecting for molecules with the greatest degree of sialylation gives a product with the desired pharmacokinetic properties, which affect the biological potency of rHuEPO. Different manufacturers may have different selection criteria (and therefore different product quality characteristics) for the EPO molecules that are retained in the recovery and purification processes.

In addition to having a well understood and reproducible recovery process, a number of regulatory requirements exist for the manufacture of biopharma-

ceuticals. The product must be produced under Good Manufacturing Practices (GMP), which encompasses a range of topics from plant design, which affects air and water quality, solvent usage, and waste disposal, to day-to-day operations, which include operator training, batch record writing and review, quality assurance, and cleaning validation. Many of these guidelines can be found in "Q7A Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients" [6].

Concentration and diafiltration

After the clarified cell culture media is harvested, it must be concentrated, and the buffer salts and other high conductivity/low-molecular-weight components must be removed. Concentration is an important step, since it is difficult to store and handle large-pool volumes. The expense of storing large volumes of harvest media in a frozen state can be prohibitive. In addition, the time required to load a large volume of media on to a column decreases plant productivity. Prolonged column load times can also lead to proteolytic degradation of product in the harvest media as it awaits loading onto the column. Removal of buffer salts and other high-conductivity, low-molecular-weight components is important because these may interfere with binding to the purification columns. For example, ion-exchange chromatography depends on ionic interactions between charged groups on the protein and oppositely charged groups on the chromatography media. The high conductivity of the harvest media would prevent protein from binding to the ion-exchange chromatography media.

Concentration and diafiltration are done using ultra-filtration membranes. This operation allows for the passage of low-molecular-weight solutes and water through membrane pores and the retention of larger-molecular-weight solutes, like rHuEPO and other proteins. The driving force for the passage of these solutes and water through the pores is the pressure difference across the membrane. The membranes can be made of cellulose, polyether sulfone, or other polymers. Membranes are available that have different "nominal molecular-weight cut-offs," ranging in molecular weight from 5,000 to 500,000. Proteins and other high-molecular-weight materials that do not pass through the membranes are referred to as the retentate; the material that passes through the membranes is called the permeate. The rate at which the water and solutes go through the membrane is called the permeate flux rate and is usually measured in liters per minute.

During the concentration phase (Fig. 10a), both water and solutes are forced through the pores, leading to a volume reduction in the harvest medium. The range of this volume reduction may be 5- to 100-fold depending on what is required for a given protein. During the diafiltration phase, the concentrated harvest continues to be fed through the membrane; at the same time, however, a lower conductivity buffer (diafiltration buffer) is pumped into the harvest

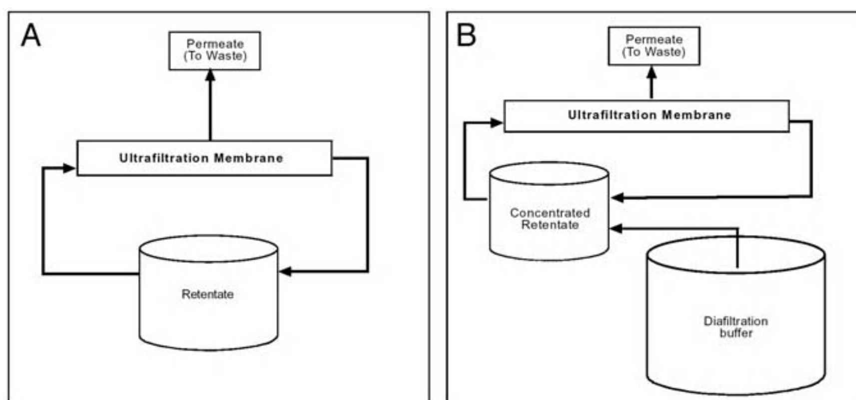

Figure 10. Panel A – Concentration mode. Clarified cell culture medium volume is reduced in retentate to make concentrated retentate. Panel B – Diafiltration (DFM) mode. Removal of high-conductivity components in concentrated retentate to make DFM.

media retentate at approximately the same rate as the permeate flux rate (Fig. 10b). This process causes a buffer exchange of the original high conductivity buffer for the lower one. The greater the number of retentate volumes pumped though the membrane during the diafiltration step, the more complete the buffer exchange. This product pool is referred to as diafiltered media, which either can be immediately processed through the subsequent purification steps or stored frozen and processed later.

Chromatography: isoform selection

An anion-exchange media can be used to separate molecules on the basis of molecular charge. This chromatography may be used to separate rHuEPO from host cell proteins, nucleic acids, and endotoxin.

Chromatography: removal of contaminant proteins

A reversed-phase chromatography step can be used to remove host cell proteins and additional nucleic acids. The interactions of the column matrix with proteins are mostly hydrophobic in nature. Once bound to the column, a protein can be eluted with another hydrophobic, low-dielectric organic solvent. The protein portion of rHuEPO is quite hydrophobic because of the large proportion of hydrophobic residues and its solubility characteristics after removal of the polysaccharide portion of the molecule. Gel filtration chromatography, also known as size-exclusion chromatography or molecular sieving, may also be used to separate a protein mixture based on molecular weight. The media

consists of agarose or acrylic beads of defined pore sizes. Proteins too large to penetrate the pores are excluded from the interior volume of the beads and have a shorter elution path through the column (hence they elute sooner). Smaller proteins either partially or completely penetrate the pores, resulting in a longer elution path and longer elution times.

Viral clearance

If rHuEPO is made in mammalian cells, the ability to remove theoretical endogenous or adventitious viruses from the product must be shown. Because virus titers are well below the level of detection, bench-scale studies are done in which different types of model viruses are deliberately added to process streams to demonstrate the ability of the process to remove viruses. Usually several types of viruses are used, including enveloped and non-enveloped viruses.

Dosage form (drug product) manufacturing

In general, dosage form manufacturing of rHuEPO, as with all recombinant protein products, is governed by national governmental regulatory agencies to ensure that biologic production facilities adhere to sound quality control and current GMP. Facilities that produce rHuEPO are routinely audited and inspected to ensure compliance and patient safety. In the United States, biologic production for parenteral applications is governed by the Food and Drug Administration (FDA CBER/CDER); regulations are covered under section 21 of the *Code of Federal Regulations* Parts 210 and 211. These regulations differ slightly from country to country. All manufacturing plants and processes are validated and filed with the FDA (or regional authority) before marketing approval is given. All operations are conducted in an aseptic, temperature-controlled, highly monitored environment, with final filling operations done under conditions that typically allow only 100 particle counts/cubic foot of air in the filing operation suite. For dosage form/drug product manufacturing, maintaining validated processes, quality control, and aseptic conditions are critical to patient safety and product integrity.

In addition to regulatory compliance, a primary concern for recombinant proteins, and in particular rHuEPO, is the maintenance of product integrity by minimizing physical or chemical degradation. Product integrity is maintained from the bulk stage through the final dosage form by adjusting process parameters and the composition of the final formulation. To mitigate process impacts, rHuEPO is formulated in solutions that often contain human serum albumin or polysorbate that act as a protector against surface adsorption, surface denaturization, shear forces, chemical degradants, and other deleterious factors. Because of the relatively low concentrations of rHuEPO used clinical-

ly, additives are important to protect the product during processing. Formulations are optimized for stability and parenteral delivery. Ingredients such as citrates, chlorides, and phosphates at relatively neutral pH are often used. Non-optimized formulations can result in degradants that arise before the end of shelf life. The formation of degradants can lead to loss of activity or by-products that may potentially be antigenic.

rHuEPO is marketed in a variety of formulations and concentrations, providing the practitioner with a wide selection of dosages for achieving optimal hematocrit values for patients. The final dosage form available in clinics is typically an aqueous formulation in a vial. Pre-filled syringes, lyophilization, frozen liquid, or other delivery forms can be used. This variety of formats can complicate production because of the need to formulate at different strengths, different fill volumes, and different delivery vehicles.

Process

Typical processing of rHuEPO consists of several unit operations: buffer preparation, formulation of the purified bulk, filtration of the formulated product, filling into a delivery vehicle, lyophylization (i.e., freeze drying if applicable), unit inspection, and packaging. Figure 11 depicts a generic process flow from bulk though packaging operations. A formulation-and-fill process may comprise several vessels, a fill line, and inspection packaging lines. One vessel may be used for buffer preparation, another for active addition to the buffer, and a third for holding the final formulated product before filling. The set up can vary depending on batch size and the formulation being produced.

General processing conditions must be closely examined to ensure product compatibility. Typically, the following items must be examined: materials in the processing vessels and transfer lines; duration of product exposure to various temperature and sterility conditions; and shear forces associated with mixing, filtration, and filling. During the formulation-and-filling operation, rHuEPO primarily comes in contact with stainless steel. Exposure to silicone tubing, various filtration membranes, ceramics, and glass can occur, however. Generally, process conditions are tested, and the product is placed on a stability testing protocol to ensure that no deleterious effects result from processing.

The initial step of the dosage form manufacturing is bulk dispensing and addition of materials associated with the final formulated product. Product amounts are adjusted according to batch size and concentration (potency). Additional excipient raw materials are added before production. Before use, each material is examined to ensure that safety, quality standards, and product-formula requirements are met. Buffer preparation is completed by the addition of the excipients to purified Water For Injection in a processing vessel. The buffer can be filtered to clarify and to remove particulates. After buffer preparation, the bulk rHuEPO is added. Additional components that protect or stabilize the rHuEPO can also be added. The formulations or stock-keeping units

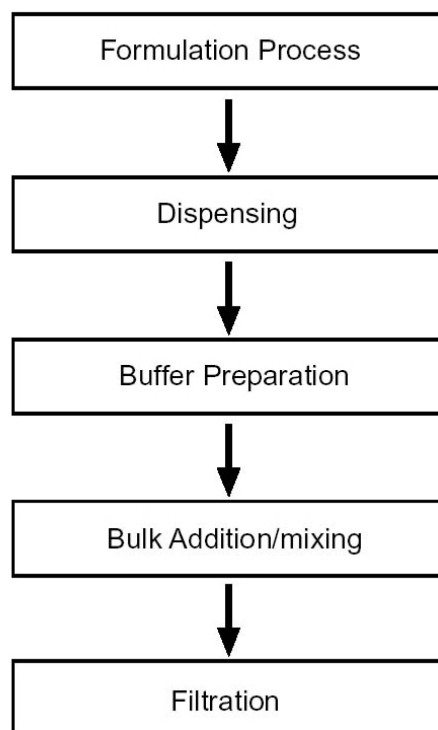

Figure 11. Generic process flow from bulk to packaging operations.

that are used in multidose settings will contain an antibacterial agent to minimize microbial growth after initial breech of the stopper septum. The bulk rHuEPO product together with the excipients added to make up the formulation is referred to as the formulated bulk.

The formulated bulk is sterile filtered into a holding vessel and stored there until filled. Dosage-form components such as vials or syringes are filled using automated equipment in a clean-room environment. The filled vials and syringes are immediately stopper and capped (in the case of vials). Product contact components such as stoppers, vials, and syringes used are washed and depyrogenated at high temperature or sterilized in the presence of steam. Some components can be purchased from vendors pre-sterilized and ready to use.

Each filled vial or syringe is inspected for particulate matter in solution or cosmetic defects, such as marks or scratches. Additionally, each unit is inspected to ensure that proper lot number and expiration date have been applied to the label.

Release

Before the release of any rHuEPO lot, the final material is thoroughly tested to ensure sterility, concentration, lack of endotoxin and bioburden, and product integrity according to quality control requirements. Additionally, batch records from the manufacturing run are reviewed to ensure that no deviation was made during the production run. Finally, as each lot is released, the product is shipped to wholesalers according to validated procedures.

Summary

An example of commercial production of rHuEPO is discussed in this chapter, where the manufacture starts with a cell line that is engineered to produce product in commercial quantities of required quality. The cell line is banked, and the cell bank is used as the starting material for the manufacturing process. In this example, recombinant HuEPO is secreted from genetically engineered mammalian cells in a fermentation process and then recovered and purified as rHuEPO bulk in a purification process, to achieve the desired product characteristics specified by the manufacturer. The bulk rHuEPO is formulated to achieve the required dosage forms and put into dosage forms that are used in the clinic. The manufacturing process is performed in a cGMP facility and monitored for consistent performance. Regulatory and other safety requirements are followed to produce a safe, efficacious product.

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II. Clinical use of recombinant erythropoietins

Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Use of recombinant erythropoietins in the setting of renal disease

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Introduction

Recombinant human erythropoietin (rHuEPO) therapy was introduced for the treatment of renal anemia in the mid-1980s, and since that time, several million patients have been treated worldwide. The market for this product has expanded considerably, and is currently valued at approximately US$ 7 billion. In the context of renal disease, the treatment has proved highly effective, largely because the major cause of renal anemia is a relative deficiency in circulating amounts of endogenous EPO. The history and development of rHuEPO is discussed elsewhere in this book (see Chapter 2), as are the pharmacokinetics (see Chapter 6) and biological effects of this therapy (see Chapter 10). This chapter will, therefore, discuss the clinical use of rHuEPO in patients with renal disease, focusing in turn on the hematologic response, the secondary benefits associated with this treatment, and possible adverse effects.

Currently available erythropoietic agents for treating renal anemia

Worldwide, quite a number of erythropoietins/erythropoietin analogues are manufactured using recombinant DNA technology for the treatment of anemia in patients with kidney disease. Several of these products breach patent regulations but are produced for local use in their country of origin, and/or for limited export to some of the less industrialized countries of the world. Excluding these, there are three major erythropoietic agents produced for the developed world, and these include two rHuEPOs (epoetin alfa and epoetin beta), and a second-generation erythropoietin analogue (darbepoetin alfa). Epoetin alfa and epoetin beta share structural homology with the endogenous hormone, while darbepoetin alfa is a modified EPO molecule that was designed to be longer acting. All erythropoietic agents act on the erythropoietin receptor (EPOR)(see Chapters 3 and 5 for further information), and all enhance the proliferation and survival of erythroid progenitor cells in the bone marrow, thus

increasing the circulating red cell count and hemoglobin concentration in patients receiving this therapy.

Hematologic response to erythropoietic therapy

Within a few days of starting erythropoietic therapy, circulating reticulocyte counts are measurably increased in the blood approximately two to three times over baseline value, depending on the dose administered. The erythropoietic response is dose dependent, as shown in the first clinical study done with rHuEPO in patients with renal disease [1]. The increase in circulating reticulocytes is followed by a more delayed increase in hemoglobin and red blood cell count, which is evident after approximately two to three weeks of therapy in most patients. The large multicenter clinical trials of rHuEPO therapy conducted in the United States [2] and Europe [3] suggested that 90% to 95% of patients with anemia due to renal disease will respond to treatment with a significant improvement in their anemia. In addition to stimulation of the bone marrow by erythropoietic therapy, erythropoiesis is also dependent on an adequate supply of vitamins and minerals, particularly iron, vitamin B_{12}, and folic acid. Thus, patients receiving erythropoietic therapy should have an adequate supply of these nutrients, which may also be supplemented if required.

Vitamin B_{12} and/or folate deficiency affects the response to rHuEPO much less commonly than iron deficiency, but replacement of these vitamins, if required, will certainly enhance the response to erythropoietic therapy [4]. The role of iron deficiency in inhibiting the response to erythropoietic therapy has been increasingly recognized over the last decade [5, 6], and this may be either absolute (where there is a deficiency of iron stores in the body), or functional (where there are adequate iron stores, but a failure of these stores to release iron rapidly enough to satisfy the demands of the proliferating bone marrow). In both types of iron deficiency, supplementation with iron is mandatory to ensure an optimal response to erythropoietic therapy, and this may be administered orally, intramuscularly, or intravenously. Oral iron is simple and relatively inexpensive, but unfortunately is ineffective in most patients with renal disease, partly due to poor absorption from the gastrointestinal tract and partly due to the amount of iron required in patients receiving erythropoietic therapy. Thus, in many patients, intravenous iron supplementation is required to optimize the response to rHuEPO [7, 8]. Now that non-dextran-containing intravenous iron preparations are available, this has become the mainstay of iron supplementation in many patients receiving rHuEPO therapy.

Other factors that may affect the response to rHuEPO include acute or chronic infections, other inflammatory disease, malignancy, and inadequate dialysis. Hyperparathyroidism may inhibit the response to rHuEPO [9], partly due to its role in causing osteitis fibrosa cystica: parathyroidectomy may improve the response to erythropoietic therapy in some patients. Aluminum toxicity is now less common than it was previously due to the decreased use

of aluminum-containing phosphate binders and improved dialysate water purification. Aluminum toxicity can cause a severe rHuEPO-resistant microcytic anemia. Despite improved water technology, contamination by such substances as chloramine may cause resistance to rHuEPO due to hemolysis [10], and should be suspected if an entire dialysis population shows hyporesponsiveness to rHuEPO.

The presence of a hemoglobinopathy may also impair the response to erythropoietic therapy, particularly in homozygous sickle cell disease, where there is an inherent genetic defect in hemoglobin synthesis. Likewise, bone marrow disorders unrelated to the underlying renal disease such as myelodysplastic syndrome or myeloma, may cause some resistance to erythropoietic therapy. Finally, pure red cell aplasia with anti-erythropoietin antibodies causes severe resistance to rHuEPO therapy; this rare but important condition will be discussed later in this chapter and in Chapter 14.

Secondary benefits of anemia correction by erythropoietins

Cardiovascular system

Longstanding severe anemia has profound effects on the cardiovascular system, many of which have been shown to be reversed or improved after correction of anemia by rHuEPO (Tab. 1). The increased cardiac output returns towards normal values with correction of the anemia [11], the compensatory hypoxic vasodilatation is reversed (producing an increase in peripheral vascular resistance), and the mean arterial blood pressure increases in 20% to 30% of patients. Oxygen delivery to the myocardium is improved, reducing symptoms of angina and exercise-induced myocardial ischemia [12, 13]. Left ventricular mass also decreases progressively after rHuEPO therapy, particularly when the left ventricle is grossly hypertrophied before treatment [12, 14, 15]. This latter finding may have long-term implications for cardiovascular mortality, since left ventricular hypertrophy has been shown to be an independent determinant of survival in patients receiving dialysis [16]. The internal dimen-

Table 1. Cardiovascular effects of recombinant human erythropoietin (rHuEPO therapy)

- Increased exercise tolerance
- Normalization of increased cardiac output
- Increased peripheral vascular resistance
- Increased blood pressure (20% to 30% of patients)
- Decreased symptoms of angina
- Reduction in myocardial ischemia
- Reduction in left ventricular hypertrophy
- Reduction in left ventricular internal dimensions
- Decreased cardiac size on chest radiograph

sions of the left ventricle in both systole and diastole decrease after rHuEPO therapy, and cardiac size therefore progressively diminishes [15]. Finally, exercise physiology improves after rHuEPO therapy: exercise capacity, maximum oxygen consumption, anaerobic threshold, and carbon monoxide transfer factor have all been shown to increase [12, 17].

Other systems

The list of secondary effects associated with rHuEPO therapy is impressive (Tab. 2). Studies on the coagulation and hemostatic pathways, prompted by the early observation of possible increased vascular-access thrombosis with rHuEPO, have documented a reduction in bleeding time along with improvements in platelet function, both aggregation and adhesion to endothelium [18]. The standard coagulation tests are unaffected by rHuEPO, as are measurements of the coagulation factors. A prothrombotic state may develop, however, possibly contributed to by increased blood viscosity [19], reductions in protein C and protein S concentrations [20], and increases in thrombin-antithrombin III levels [21], Factor VIII-related activities [22], and plasminogen activator inhibitor-1 production after rHuEPO therapy.

The hematocrit is the major determinant of whole blood viscosity, and thus an rHuEPO-induced increase in red cell mass inevitably causes an increase in blood viscosity. Furthermore, the relationship between hematocrit and blood viscosity is exponential, such that a linear increase in the former results in a disproportionate increase in the latter. Detailed rheological studies have indicated that the increase in blood viscosity occurs solely as a result of the larger quantity of circulating red cells, without any change in plasma viscosity or the rheology of the red cells themselves in terms of their deformability or aggregability [19].

Objective assessments of quality of life parameters [23] and of brain and cognitive function [24, 25] have shown improvements after rHuEPO therapy. Patients report subjective improvements in memory, concentration, and other cerebral functions. Electrophysiological studies have shown an increase in amplitude of the P3 component of the brain event-related potential [24], and

Table 2. Non-cardiovascular effects of recombinant human erythropoietin (rHuEPO) therapy

- Improved quality of life
- Improved brain/cognitive function
- Decreased uremic bleeding tendency
- Improved platelet function
- Improved sexual function
- Improved endocrine function
- Enhanced immune function
- Decreased uremic pruritus

higher scores in various neuropsychological tests have been recorded. These findings suggest that anemia may be an important factor in the etiology of uremic brain dysfunction.

Impaired sexual function is common in patients receiving dialysis. Women may report anovulation, amenorrhea, and infertility, while men may experience impotence, reduced libido, oligospermia, or gynecomastia. rHuEPO therapy has been shown to improve libido, potency, and sexual performance in men [26, 27]. Return of regular menstruation and even pregnancy [28] have been reported in women receiving dialysis and rHuEPO. These effects may be partly mediated by changes in prolactin or testosterone concentrations since reductions in the former and increases in the latter have been found after rHuEPO treatment. Other diverse endocrine effects that have been reported in association with rHuEPO include suppressive effects on the renin-angiotensin system, the pituitary-adrenal axis, growth hormone, glucagon, gastrin, follicle-stimulating hormone, and luteinizing hormone, while increases in plasma insulin, parathyroid hormone, and atrial natriuretic peptide have been reported [29].

Erythropoietin also appears to have effects on the immune system and it has even been suggested to be a physiologic regulator of immune function. Concentrations of circulating cytotoxic antibodies progressively decline in patients receiving rHuEPO therapy [30] and this effect is only partly due to the avoidance of blood transfusion. Immunoglobulin production and proliferation of B cells increase, and seroconversion response to hepatitis B vaccination is enhanced [31]. Phagocytic function in neutrophils is increased [32]. Uremic pruritus is lessened after commencement of rHuEPO therapy, possibly due to a reduction in plasma histamine concentrations [33]. The nutritional status of patients treated with rHuEPO has been shown to improve.

Adverse effects of rHuEPO

Most of the reported complications associated with rHuEPO therapy are thought to be due not to the recombinant product *per se* but to the resultant increase in hematocrit and blood viscosity (Tab. 3). Hypertension is the most common and potentially most worrying adverse effect associated with

Table 3. Adverse effects of recombinant human erythropoietin (rHuEPO) therapy

- Hypertension
- Seizures/encephalopathy
- Vascular access thrombosis
- Clotting of dialysis lines
- Hyperkalemia
- Myalgia/influenza-like symptoms
- Skin irritation (epoetin alfa only)
- Pure red cell aplasia (with anti-EPO antibodies)

rHuEPO therapy, occurring in approximately 20% to 30% of patients treated. The risk of developing a significant increase in blood pressure appears to be independent of a previous history of hypertension, the rate of increase of the hematocrit, or the target hemoglobin achieved. Most hypertensive problems occur during the acute correction of anemia, however, rather than during the maintenance phase. Interestingly, this side-effect of rHuEPO appears to be peculiar to patients with renal disease and is very rare in other groups of patients receiving this treatment, such as those with rheumatoid arthritis.

The mechanism of rHuEPO-induced hypertension remains poorly understood although factors that have been suggested to contribute include an inadequate reversal of the increased cardiac output of anemia, a relative increase in peripheral resistance as the compensatory hypoxic peripheral vasodilatation of anemia is reversed, an increase in blood viscosity, increased endothelin production, and possibly a direct pressor effect of EPO [34]. In most instances, blood pressure is easily controlled by fluid removal and the use of standard antihypertensive drugs; it is unusual to need to discontinue rHuEPO therapy for severe uncontrollable hypertension.

A number of the early studies had anecdotal reports of seizures or hypertensive encephalopathy occurring in patients receiving rHuEPO, usually within the first three months of treatment [35]. The pathogenesis of these adverse effects remains poorly understood, although loss of autoregulation of cerebral blood flow and/or reduced cerebral perfusion may play a part. This complication of erythropoietic therapy no longer seems to be a problem, perhaps because patients treated now have a less severe anemia, and are managed at an earlier stage in the course of their disease.

In the early studies, up to 10% of patients receiving hemodialysis who were treated with rHuEPO developed thrombosis of their vascular access [3]. This event may be more common with prosthetic grafts than with native fistulae [36], and possible pathogenetic factors include an increase in blood viscosity, shortening of the bleeding time, enhanced platelet aggregation and adhesion, a reduction in protein C and protein S concentrations, an increase in thrombin-antithrombin III levels, enhanced Factor VIII-related activities, and a marginal increase in platelet count in some patients (see above).

Occasionally, patients receiving rHuEPO therapy show an increase in serum potassium, phosphate, and creatinine [1, 3] that may be due to enhanced dietary intake and/or reduced dialyzer clearance of these molecules secondary to the increased hematocrit. Heparin requirements for hemodialysis may increase in some patients. Other adverse effects of rHuEPO therapy include transient myalgia or influenza-like symptoms after the first few injections only, and skin irritation around the injection site caused by citrate buffer in one of the early formulations of the drug [37].

Recently, a new and potentially serious adverse effect of rHuEPO therapy has been identified, that of pure red cell aplasia with detectable neutralizing antibodies against EPO [38]. The features of this condition are summarized in Table 4, and the exact cause of this problem, which was extremely rare before

Table 4. Features of recombinant human erythropoietin (rHuEPO)-induced pure red cell aplasia

- Severe transfusion-dependent anemia despite erythropoietic therapy
- Reticulocyte count 0 to 10×10^9/L
- Bone marrow shows absence of red cell precursors (<5% erythroblasts), but normal white cell and platelet maturation
- Normal circulating white cell and platelet counts
- Neutralizing antibodies to human erythropoietin (detected by RIP or ELISA)

RIP, radioimmunoprecipitation; ELISA, enzyme-linked immunosorbent assay.

1998, remains unclear. The anti-EPO antibodies, which are mainly of IgG class, are directed against the protein backbone of the molecule, and they cross-react with all three erythropoietic agents currently available in Europe, as well as the endogenous hormone. Management of this condition consists of immediate cessation of erythropoietic agents, along with possible immunosuppressive therapy.

The future of erythropoietic therapy

The future of erythropoietic therapy appears bright. It is well established as a highly effective treatment and, apart from the recently recognized problem of pure red cell aplasia, the treatment generally has an excellent safety record. Large multicenter studies (such as CHOIR, CREATE, and ACORD) are examining the impact of anemia correction on long-term morbidity and mortality in dialysis, as well as pre-dialysis, patients. There has recently been a treatment paradigm shift away from aiming for higher target hemoglobin concentrations to earlier correction of anemia, particularly in the pre-dialysis phase of treatment. The contribution of other cytokines and growth factors in erythropoiesis and their interaction with EPO is the subject of ongoing research, as is the putative role of EPO as a neuroprotective agent. Finally, at a molecular level, much has still to be learned about mechanisms controlling EPO production, hypoxic sensing, and gene expression. Novel strategies for stimulating erythropoiesis, such as EPO-mimetic peptides [39] and EPO gene therapy [40], have been assessed in animal experiments, but their translation into clinical practice, if ever, is at least a decade away.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Erythropoietic therapy in the practice of oncology

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Introduction

It is well recognized that patients with cancer are frequently anemic [1]. The basic underpinning is the anemia of chronic disease (ACD), with its associated reduction in endogenous erythropoietin (EPO) concentrations [2]; cytokine-induced suppression of bone marrow function; disturbances in ferrokinetics, including both decreased gastrointestinal absorption and increased reticuloendothelial retention of iron; and decreased red cell survival [3]. In addition, patients with cancer are often subject to other important contributing factors, including myelosuppressive chemotherapy, radiotherapy, bleeding, hemolysis, bone marrow tumor infiltration, and poor nutrition. It is not surprising that the reported frequency of severe anemia (hemoglobin concentration <8 g/dL and/or red cell transfusion dependence) across a broad spectrum of cancers and treatment regimens is 5% to 20%, and of mild anemia (hemoglobin concentration 8–11 g/dL) is 25% to 45% [1]. Before the introduction into the clinical practice of oncology of recombinant human erythropoietin (rHuEPO) in 1991, the only available treatment for anemia was red blood transfusion, with all of its inherent disadvantages. Based upon a desire to limit risk to patients and protect the blood supply, physicians were trained to overlook mild and moderate anemia and to transfuse only in response to relatively severe, potentially life-threatening symptoms. Implied in this approach was the assumption that less-profound levels of anemia were clinically insignificant and relatively asymptomatic. Over the last five years, significant changes have occurred in our understanding of the impact of anemia on patients with cancer and previously held beliefs have been challenged or disproven. To a significant extent, this discovery process has paralleled the earlier experience of nephrologists treating patients with chronic renal failure and it is likely that we have more to learn from these colleagues, especially regarding the use of iron in rHuEPO-treated patients and the role of physicians in advocating for standards of excellence in patient care. (See Chapter 9 for further information.)

Worldwide, several erythropoietic agents are currently available for the treatment of anemia in oncology practice. (See Chapter 15 for further information.) Two forms of rHuEPOs, epoetin alfa and epoetin beta, are in clinical

use. These two preparations have identical amino acid sequences and they differ slightly in glycosylation pattern and resulting isoform composition [4]; they have similar half-lives and per-unit efficacy in the oncology setting. Darbepoetin alfa is the product of a human *EPO* gene modified by site-directed mutagenesis to differ from rHuEPO in five amino acids; these modifications provide two additional glycosylation sites resulting in a greater carbohydrate content, an approximately three-fold longer half-life and greater per-molecule *in vivo* potency [5].

Erythropoeitic therapy for cancer patients receiving chemotherapy

As a chronic inflammatory illness, cancer is associated with an inappropriately low endogenous EPO response to anemia. It was logical to predict that therapy with rHuEPO might be effective in the treatment of anemia in the oncology setting. Although early studies of rHuEPO in patients with cancer suggested that patients receiving chemotherapy were somewhat less responsive to rHuEPO therapy than patients with cancer who were not receiving chemotherapy, the design of the pivotal randomized, placebo-controlled Phase III studies was such that the clinical benefit to patients was only shown in patients receiving chemotherapy [6]. Regretably, the focus of erythropoietic therapy in oncology since has been almost exclusively on patients receiving chemotherapy, and these agents are much less frequently used to treat the ACD associated with cancer, a setting in which they may be more effective than they are in the treatment of the chemotherapy patient [7]. (See Chapter 11 for further information on ACD). Moreover, because cisplatin-based chemotherapy is associated with renal impairment and further compromise of endogenous EPO response [8, 9], some studies have focused on this subset of patients. In many European countries, cisplatin-based therapy is the only oncology setting in which erythropoietic therapy is used.

In the initial clinical trials in patients receiving cancer chemotherapy, rHuEPO was administered in dose-escalation studies at 25, 50, 100, 200, or 300 U/kg five times weekly for four weeks (approximately 10,000 U to 120,000 U/week total dose) [10, 11]. The data suggested a relationship between dose and the proportion of patients in whom a 2 g/dL increase in hemoglobin concentration was observed, with the highest proportion of responders observed at the highest doses. Subsequently, small randomized studies comparing very low doses of rHuEPO (2,000 to 3,000 U) to higher doses (6,000 to 10,000 U) three times a week suggested that the higher doses were more effective in terms of hematologic response [12, 13]. In two studies, patients receiving chemotherapy were randomized to rHuEPO 150 or 300 U/kg three times a week or to a control group [14, 15]. In both studies, the treated group had higher hemoglobin values and a trend favoring decreased transfusion rates than the control group, with no clear advantage of the higher starting dose. In a study in patients with B-cell malignancies, weekly rHuEPO

doses of 70,000 U appeared to be superior to doses weekly doses of 14,000 U [16]. In most studies, patients receiving chemotherapy have been treated three times a week with starting rHuEPO doses of 150 U/kg, sometimes with a doubling of dose in non-responders, with 2 g/dL increases in hemoglobin concentration observed in approximately 60% of patients [17–24]. Possibly because of cost considerations, subsequent work did not pursue the suggestion in the initial dose finding studies that weekly rHuEPO doses >100,000 U may be associated with optimal hematologic responses in patients with cancer receiving chemotherapy. Data have not been published to show that patients benefit from the frequently used dose increases to doses <100,000 U/week for patients who have not responded to the initial starting dose. It is possible that patients in whom responses are observed after dose increases to a 60,000 U weekly dose increase would have responded had they remained on the initial lower starting dose.

Impact of erythropoietic agents on transfusion requirements

In Phase I and II studies, rHuEPO therapy was shown to be associated with increases in hemoglobin concentration in patients receiving cancer chemotherapy. For pivotal, placebo-controlled Phase III studies, it was necessary to choose a more clinically meaningful outcome variable. For a variety of reasons, including historical precedent in earlier nephrology studies, sufficient statistical power achievable with modest sample sizes, and the ambient bias in clinical oncology that the major deleterious effects of anemia was an increase in transfusion requirements, the outcome chosen was transfusion requirement. Randomized, placebo-controlled trials of rHuEPO at doses of 150 U/kg given subcutaneously three times a week to patients with cancer receiving chemotherapy, with doubling of dose after six to eight weeks in non-responding patients, reduced red blood cell transfusion requirements [6, 20, 25]. Similar results have been reported without the dose increase for non-responders [21, 26]. More recently, in an uncontrolled trial, weekly subcutaneous dosing with epoetin alfa at a weekly dose of 40,000 U, increasing to 60,000 U after four weeks in non-responding patients, has been associated with hematologic responses similar to those observed with three-times-a-week dosing [27]. The necessity of the 33% increase in weekly dose with once-weekly *versus* three-times-a-week treatment has not been shown in randomized trials, and weekly dosing has not been compared with placebo in randomized clinical trials. In the United States, rHuEPO is usually given weekly, starting at 40,000 U, because of the increased convenience for patients. More recently, studies using similar doses have been done and show the ability of rHuEPO to prevent rather than treat anemia in the setting of cancer chemotherapy [28].

In a randomized, placebo-controlled trial of darbepoetin alfa in patients with lung cancer receiving cisplatin-based chemotherapy, subcutaneous doses of 2.25 μg/kg administered weekly, with doubling of dose permitted in non-

responding patients, was associated with a significant decrease in transfusion requirements [29].

It is clear from the available data that erythropoietic therapy is associated with a reduction in transfusion requirements in patients with cancer receiving chemotherapy [30]. These data, however, have several limitations [31]. First, the doses and schedules of erythropoietic agents used in these placebo-controlled studies have not been chosen to be optimal in terms of patient benefit or cost-effectiveness, forcing the clinician to integrate data from open-label and/or active-control trials into his/her approach to patient care. Early studies with rHuEPO were focused on identifying doses that would be superior in terms of transfusion rates compared with placebo in randomized trials, not on ensuring that the chosen doses and schedules would be associated with the highest proportion of responding patients, the most rapid responses, or the greatest cost-benefit. To date, no studies have been published of the pharmacokinetics or pharmacodynamics of rHuEPO in patients with cancer undergoing chemotherapy and the dose-response curves have not been fully explored, especially at the higher doses that appeared promising in terms of optimal benefit in early dose-finding studies. (See Chapter 6 for further information.) Active-controlled, dose-finding studies of darbepoetin alfa done after the randomized trial was underway suggest that doses lower than those used in the placebo-controlled trial, including 1.5 µg/kg per week or 3 µg/kg every two weeks produce hematologic effects indistinguishable from those observed with epoetin alfa given 150 U/kg three times a week or 40,000 U weekly, or from weekly darbepoetin alfa doses of 2.25 µg/kg [32, 33]. These studies have shown that this agent can be administered every two weeks without increasing the total dose administered over time. In the United States, this finding has led to a situation similar to that for epoetin alfa, in which clinicians use a dose and schedule (3 µg/kg every two weeks) that is different from that which was used in pivotal Phase III studies. The recent interest in every-other-week chemotherapy for patients with lymphoma and for the adjuvant therapy of breast cancer increases the importance, in terms of patient convenience, of effective and efficient every-other-week erythropoietic support. Identifying doses and schedules that are superior to placebo is a simpler matter than determining doses and schedules that are superior to all other doses and schedules, or equal in terms of efficacy and of lower cost, and much of this work remains to be done.

Impact of erythropoietic agents on quality of life

The early development of rHuEPO for oncology was focused on the efficacy of this agent in preventing transfusions during cancer chemotherapy. Nevertheless, in the randomized clinical trials, patients treated with rHuEPO consistently had a higher mean hemoglobin concentration than patients receiving placebo from the second month of therapy onward, despite having fewer

transfusions. In part because anemia was assumed to be relatively asymptomatic in patients with cancer, the potential benefit to patients of these higher hemoglobin concentrations was not initially explored.

In the first large, open-label, uncontrolled, study of rHuEPO done in the United States, chemotherapy patients receiving epoetin alfa at a starting dose of 150 U/kg three times a week completed a simple linear analog scale assessment of their energy level, ability to do daily activities, and overall quality of life at the initiation and termination of epoetin therapy [22]. The observed mean score for each of these domains increased significantly during epoetin alfa therapy over a time frame in which the mean hemoglobin concentration for these patients was increasing from approximately nine to approximately 11 g/dL. Simultaneously, the results of a large survey of patients with cancer suggested that fatigue was as great a contributor to symptom burden in these patients as was pain, and that fatigue impaired several domains of quality of life [34, 35]. Both observations, that fatigue decreased and quality of life increased when mild and moderate anemias were successfully treated in this patient population and that fatigue was an unappreciatedly important cause of functional decline in cancer patients, were subsequently confirmed in similar studies [23, 27, 36]. These findings led to a paradigm shift in the approach to erythropoietic therapy. Severe anemia (hemoglobin concentration <8 g/dL) is often treated with transfusions; because erythropoeitic agents decrease transfusion risk, they can be thought of as alternatives to transfusions. If improvements in hemoglobin concentration in the range of mild and moderate anemias (<8 to <11 g/dL), which are not treated with transfusions, are associated with improvements in functional status and a symptom important to patients, erythropoietic agents are much more than alternatives to red cell transfusion and their use may be beneficial even to patients whose anemia will not become severe enough to warrant transfusion.

The effects of erythropoietic therapy on fatigue level and quality of life have been assessed in several appropriately powered randomized trials in patients with cancer receiving chemotherapy [20, 21, 29]. In these studies, erythropoietic therapy has been associated with a significant improvement in quality of life and decrease in fatigue compared with the cohorts receiving placebo. In general, the magnitude of the quality of life improvement has correlated with the magnitude of the improvement in hemoglobin value. A retrospective analysis of data from two large, community-based trials in the United States demonstrated that, although quality of life is measurably improved for each incremental increase in hemoglobin concentration, the greatest increases are observed with the treatment of mild as opposed to moderate or severe anemia. The greatest improvements in quality of life were predicted to occur as hemoglobin concentration increased from 11 to 12 g/dL [37]. Because quality of life improvements are observed particularly with the treatment of mild anemia, where transfusion is infrequently an issue, these observations are potentially much more important in terms of the number of patients benefited than the transfusion data.

The observation that treatment of anemia not sufficiently severe to require red cell transfusion is associated with measurable improvements in functional status and quality of life had been previously made in patients with chronic renal failure undergoing dialysis and had been correlated with measurable changes in mood, cognitive function, and exercise capacity [38–43]. (See Chapter 9 for further discussion.) In light of this previous experience, the later discoveries in the oncology setting were not surprising. Nevertheless, unlike dialysis physicians, oncologists have not uniformly accepted the importance of treating anemia to optimize quality of life. Currently <40% of the patients in the United States who could clearly benefit from an increase in hemoglobin concentration are treated. The reasons for this disparity are complex, but probably include both the higher doses (and hence per week cost) of erythropoietic agents required to treat patients receiving chemotherapy and profound shortcomings in our current approach to treating anemia in oncology (see below) that need to be addressed.

Safety

In the early experience with rHuEPO in patients with renal failure receiving dialysis, a rapid rise in hemoglobin concentration was associated with an increased incidence of diastolic hypertension and, in severe cases, with resultant seizures. This syndrome was due to increases in blood volume associated with increasing red cell mass in the absence of normal volume homeostasis. The syndrome was found to be preventable by adherence to treatment paradigms that were associated with gradual rather than rapid increases in hemoglobin concentration, an appropriate approach in the setting of renal failure. When erythropoietic agents were developed in patients with human immunodeficiency (HIV) infection or cancer, in whom very rapid rises in hemoglobin concentrations are routinely encountered during red cell transfusions uncomplicated by hypertension or convulsions, no rational basis was found to assume that the much more modest rates of rise associated with even optimal therapy would be a problem. Nevertheless, clinical trials of rHuEPO and darbepoetin alfa have usually excluded patients with a history of seizures or uncontrolled hypertension. In placebo-controlled trials of erythropoietic agents in patients with cancer, epoetin alfa, epoetin beta, and darbepoetin alfa have been safe and well tolerated, without a demonstrable increase in the incidence of hypertension, seizures, or thrombotic events. Similarly, pure red cell aplasia due to the development of cross-reacting antibodies to EPO that has been observed in some patients on dialysis treated with Eprex-brand of epoetin alfa has not been observed in patients with cancer [44]. (See Chapter 14 for further information on pure red cell aplasia.)

Erythropoeitic therapy for patients not receiving chemotherapy

ACD associated with cancer

As noted above, data from the initial Phase II studies of rHuEPO for anemic patients with cancer not receiving chemotherapy suggested that this population of patients is more responsive to erythropoietic therapy than patients with cancer receiving chemotherapy, and therefore more likely to benefit from therapy [45, 46]. These observations were buttressed by the results of trials of preoperative treatment of cancer patients to increase hemoglobin concentration or increase autologous blood harvested [47–49]. (See Chapter 12 for more information on autologous blood donation.) The experience in these clinical trials suggests that a relationship exists between the dose of rHuEPO administered and the observed proportion of responding patients. In the Phase III, placebo-controlled trial in patients with cancer not receiving chemotherapy, lower doses of rHuEPO (100 U/kg three times a week) were given and, more importantly, the study drug was given for only eight weeks [6]. As a result, the transfusion requirements of the two groups, while different in favor of the rHuEPO group, did not achieve statistical significance.

Dose-finding studies of darbepoetin alfa have been done in patients with cancer not receiving chemotherapy; these data exhibit a greater proportion of responding patients to a given dose than has been observed with patients receiving chemotherapy [50]. These studies clearly show a relationship between the administered dose of darbepoetin alfa and the proportion of patients with a hematopoietic response. At the higher doses, response rates as high as 100% are observed. Moreover, these studies have shown a relationship between the dose administered and the rapidity of response. In this setting, hemoglobin concentration increases have been associated with increases in measured quality of life and productivity. Dosing intervals as infrequent as every four weeks are feasible with this agent in this setting.

As of this writing, no erythropoietic agent is registered in any country for the treatment of the anemia of cancer for patients who are not receiving chemotherapy and most patients with cancer who are treated for anemia are receiving chemotherapy. Placebo-controlled trials documenting the ability of erythropoietic agents to provide meaningful benefits, including quality of life, to patients not receiving chemotherapy are among the most acute needs in the field of erythropoietic therapy.

Anemia associated with myelodysplastic syndromes

Anemia due to the myelodysplastic syndromes (MDS) is a common and vexing problem in oncology because the anemia is frequently severe enough to require transfusion support and of sufficient duration to allow the development of alloimmunization. Although myeloblasts have been reported to bear ery-

thropoietin receptors (EPOR), rHuEPO therapy appears to be safe in these patients, without any evident increase in the risk of progression to acute leukemia. The obstacle to widespread benefit to patients with MDS has been that higher doses are required than for chemotherapy patients and despite high doses, only approximately 33% of patients appear to benefit. Intravenous rHuEPO doses of 210 to 3,200 U/kg/week [51–57] and subcutaneious doses of 150 to 2,100 U/kg/week [58–63] have been studied with an aggregate response rate of approximately 30%. One strategy to increase response has been the addition of a myeloid growth factor to rHuEPO therapy in this setting. Promising results have been reported with recombinant human granulocyte-macrophage colony-stimulating factor (rHuGM-CSF) [64] and with recombinant human granulocyte colony-stimulating factor (rHuG-CSF) [65, 66] given simultaneously or sequentially with rHuEPO for anemic patients with MDS. It is important to note that not all investigators have reported enhance rHuEPO efficacy in conjunction with myeloid growth factors [67, 68] and that the safety of maintenance therapy with myeloid growth factors in this patient population has not been established. In a randomized, placebo-controlled clinical trial, 150 U/kg/day (approximately 70,000 U/week) rHuEPO produced responses in 37% of treated patients and, at least over an eight-week treatment period, was not associated with an increased progression to acute leukemia [69].

Taken in aggregate, the available literature suggest that it is a subset of patients with MDS who appear to benefit from rHuEPO therapy; these patients typically have refractory anemia without excess of blasts, normal platelet counts, normal lactate dehydrogenase levels, and a short duration of disease. This subset of patients probably responds to rHuEPO therapy in a fashion similar to that of patients with cancer receiving chemotherapy and it is very reasonable to attempt to relieve transfusion burden or fatigue in this subset of patients [70, 71].

Future directions

Improvements in clinical trials methodology

As the field moves toward optimizing erythropoietic therapy, more sensitive endpoints for assessing the relative erythropoietic efficacy of different doses or schedules of drugs will be required. Until recently, most studies of erythropoietic agents in oncology were designed with a fixed starting dose administered to all patients, sometimes with a single dose increase permitted for non-responders. Studies reported a hemoglobin response rate (usually defined as a 2 g/dL increase in hemoglobin concentration not due to a transfusion) for the drug. When quality-of-life data emerged suggesting that a hemoglobin concentration of 12 g/dL may be a particularly important target, later publications expanded the definition of a hematopoietic response rate to include either a 2 g/dL increase or an increase to 12 g/dL. There are two issues with this approach to

measuring efficacy. First, it implies that some patients are biologically non-responsive to erythropoietic therapy and that defining who will benefit (predicting response) would be an important advance in cost-effectiveness. The available data for both rHuEPO and darbepoetin alfa suggest that the higher the dose, until very high doses are achieved, the higher the observed response rate. Second, response rate is a relatively insensitive measure of the erythropoietic behavior of a cohort of patients. Increases of <2 g/dL are not captured, and even substantial decreases in hemoglobin concentration are not distinguished from modest increases. The need for a more sensitive measure of center for the red cell production response to a given dose, schedule or drug has led to the inclusion of mean change in hemoglobin concentration for the each group as an important approach to comparison. Although this endpoint can be complicated by transfusions and missing data point (see below), when properly analyzed, it is an important addition to clinical trial methodology in this area.

With the introduction of new erythropoietic agents and emerging initiatives to improve established doses and schedules of existing agents, it is becoming necessary to compare the hematopoietic effects observed in cohorts of patients. Two vexing problems complicate these efforts. First, patients receiving erythropoietic agents still may require red cell transfusions, especially during the first four weeks of erythropoietic therapy. This requirement presents the investigator with mean hemoglobin values that are contaminated by transfused red blood cells. Three approaches can be used to handle these values. First, the measured hemoglobin values can be accepted as valid, with the highly suspect assumption being that, with a sufficiently large sample size, transfusions will contaminate all cohorts equally. This approach will increase the apparent change in hemoglobin concentration attributed to the agent, especially early in therapy when transfusions are most frequent. Second, the values from transfused patients can be censored for a period of time, and the measure of center (mean or median) calculated for the remaining patients. Unfortunately, this means excluding the least responsive and most anemic patients at that time point, which will tend to artificially inflate the effectiveness of the drug. Finally, hemoglobin values for transfused patients can be excluded for a period of time (usually four weeks) after transfusion, with the pre-transfusion value imputed in its place. This approach makes the conservative assumption that all of any increase in hemoglobin concentration observed for four weeks after transfusion is due to that transfusion, and probably tends to underestimate the hematopoietic effectiveness of the agent at that dose.

The second methodological issue has to do with the handling of patients who drop out of study, deaths, and other causes of missing data points. Studies of treatment of anemia during cancer chemotherapy, even when diligently executed, typically will have 20% to 30% of the hemoglobin data unavailable when the study is complete. The problem can be handled one of two ways. First, the analysis can be limited to those patients who completed therapy, usually a relatively select subset of the patients initially registered on study. This approach usually inflates the apparent effectiveness of the agent, because non-

responders are more likely to discontinue and the most anemic patients are often the most ill and most at risk to die. A more conservative approach uses the intent-to-treat principal in which all patients who begin therapy remain in the analysis whether they remain on study or not. Again, the last available hemoglobin level is imputed for each patient for each time point for the remainder of the study. This approach probably under-estimates the efficacy of the agent, because some patients who would have benefited withdraw from the study or die before that response occurs.

Given these complexities and the importance of developing approaches to the treatment of anemia in oncology that are superior to the current standard, it is important that the literature be interpreted critically and that important comparisons be done on randomized data sets using consistent and clearly articulated approaches to transfused hemoglobin concentrations and missing data.

Toward optimal patient management

Despite mounting evidence that successful treatment of anemia in patients with cancer provides benefits in terms of prevented transfusions and enhanced functional status and quality of life similar to those enjoyed by dialysis patients, most patients with cancer who have symptomatic anemia are not currently treated. One reason for the disparity between nephrology and oncology may be rooted in the different approaches to drug development taken in the two clinical settings. When rHuEPO was introduced for the treatment of patients with renal failure undergoing dialysis, patients were treated with increasing doses until all patients had responded, although individual patients differed as much as three-fold in terms of dose requirements. The result is an approach to the treatment of anemia in the dialysis setting in which the physician can be assured that all patients will ultimately benefit. When rHuEPO was developed for patients with cancer, the result was that patients were treated with a fixed dose, sometimes with a single dose increase at least one month later for non-responding patients; the doses chosen were not those associated with the highest response rates. Because patients receiving erythropoietic support during chemotherapy are usually treated for five months or less, this approach to rHuEPO therapy has resulted in therapeutic algorithms in which 40% or more of patients benefit minimally or not at all. This research has led to extensive efforts to identify predictors of response [72–74] that might permit non-responders to forgo therapy or discontinue therapy earlier, thereby conserving resources. Moreover, once the effects of transfusions are eliminated from the analysis of hemoglobin change data, the response to currently favored doses of erythropoietic agents (30,000 to 60,000 U weekly of rHuEPO, 3–5 μg/kg every two weeks of darbepoetin alfa) is slow, with median times to response of approximately 10 weeks [32]. The failure of current treatment paradigms to provide rapid relief to all anemic, fatigued patients with cancer, coupled with the higher dose requirements and per week costs,

might explain the lack of uniform adoption of an aggressive anemia treatment standard analogous to the one used in nephrology.

It may be possible to design more successful approaches to initiating erythropoietic therapy in patients with cancer. The relationship between dose and both response rate and time to response for darbepoetin alfa during cancer chemotherapy has been studied in a dose-escalation trial in which all data were analyzed with imputation to eliminate the effects of transfusions and an intent-to-treat approach to missing data points [32]. Similarly designed and analyzed dose-escalation trials, including very high doses (>100,000 U/week) of rHuEPO have not been reported. The resulting dose-response curve, shown in Figure 1, suggests a biphasic relationship of dose to the proportion of patients who demonstrate a hematopoietic response. For some doses of darbepoetin alfa, including those used in current clinical practice, a proportion of patients will respond and a significant remainder will be termed non-responders. While producing responses indistinguishable in a randomized trial from current approaches to rHuEPO use, these doses were found to be suboptimal. At doses of 4.5 μg/kg/week or greater, substantially higher response rates were achieved. Given that these data were analyzed using an intent-to-treat approach, few, if any, patients remain on therapy and have not responded. Hence, the dose of an erythropoietic agent is the best and most important predictor of response in oncology and, as in the renal failure setting, most patients will respond if treated with sufficient doses. It may be more appropriate to seek predictors of dose than predictors of response. Importantly, when the optimal dose of darbepoetin alfa is administered, the time to response is shortened by approximately 30% compared with standard approaches to patient treatment (Fig. 2).

Unlike patients receiving dialysis, in whom rapid volume expansion can be associated with hypertension, a rapid rise in hemoglobin concentration in the oncology setting is safe and beneficial to the patient. The minimum dose of darbepoetin alfa associated with optimal response rate and time to response in cancer patients is 4.5 μg/kg/week; this dose has not yet been determined for rHuEPO. It is possible that administering the optimal dose as the initial dose, with dose decreases as patients respond, will be both better for patients with cancer and more cost effective as well (Fig. 3). To explore this possibility, a randomized pilot trial compared the effects of darbepoetin alfa, given with frontloaded dosing (4.5 μg/kg/week with dose decreases in some cohorts after-four weeks) to epoetin alfa given by the conventional backloaded approach, with the usual starting dose and escalation for non-response in the treatment of anemia during cancer chemotherapy [76]. In this pilot trial, patients treated with the frontloaded approach enjoyed both more rapid improvement in hemoglobin concentration and more substantial gains in quality of life, as well as less dependence on caregivers. A large, randomized trial is underway comparing the effects of frontloaded darbepoetin alfa to conventional therapy. It is hoped that by defining more appropriate, consistently effective, rapid, and cost-effective approaches to the treatment of anemic patients with cancer will increase enthusiasm for erythropoietic therapy in oncology.

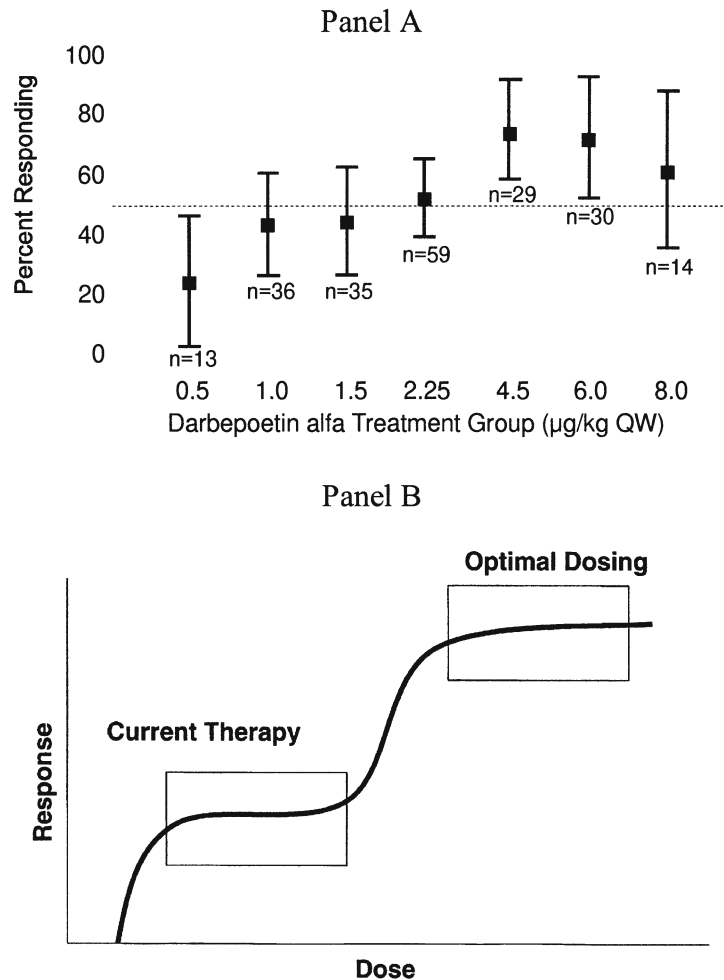

Figure 1. Panel A: The relationship between darbepoetin alfa dose and response in terms of the proportion of patients in whom a 2 g/dL or greater increase in hemoglobin concentration was observed that was not related to a red cell transfusion. All data were analyzed using an intent-to-treat approach to missing data points and all hemoglobin values within four weeks of a red cell transfusion were replaced with the imputed pretransfusion hemoglobin value. Adapted from [75]. Panel B: A schematic representation of the relationship between dose and response for erythropoietic agents in patients with cancer receiving chemotherapy. Current dosing, including both current starting doses and the doses that are used for dose increases in non-responding patients, appear to result in suboptimal hematologic responses, at least over the relatively short time frames (20 weeks) for which these patients are currently treated.

Another important and under-appreciated issue in erythropoietic therapy in oncology concerns the role of iron supplementation [77]. During chronic hemodialysis, iron loss during dialysis is significant and compromises the

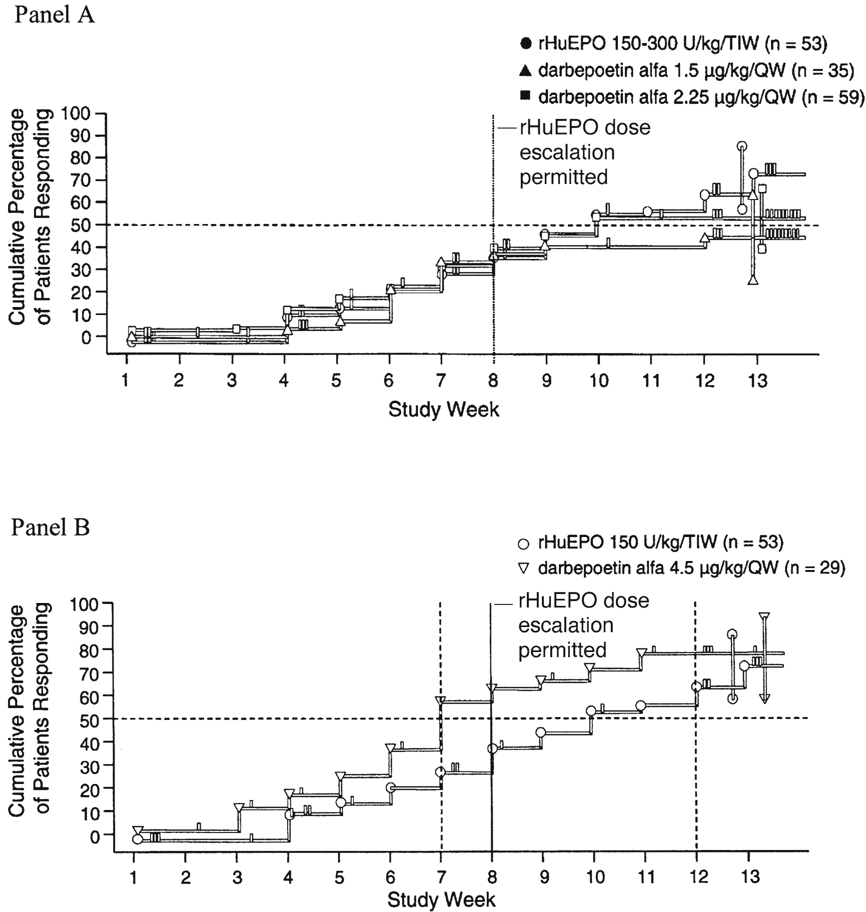

Figure 2. Panel A: The time to response data for darbepoetin alfa given weekly at doses of 1.5 or 2.25 µg/kg and for epoetin alfa given three times weekly at a dose of 150 U/kg, with a dose increase to 300 µg/kg after eight weeks in non-responding patients. When data are analyzed using an intent-to-treat approach to missing data points and all hemoglobin values within four weeks of a red cell transfusion are replaced with the imputed pretransfusion hemoglobin (eliminating the effects of transfused red blood cells on outcomes) it is apparent that with current dosing, the median time to response to erythropoietic therapy is approximately 10 weeks. Adapted from [75]. Panel B: The time to response data for darbepoetin alfa given weekly at a dose of 4.5 µg/kg and for epoetin alfa given three times weekly at a dose of 150 U/kg, with a dose increase to 300 µg/kg after eight weeks in non-responding patients. When data are analyzed using an intent-to-treat approach to missing data points and all hemoglobin values within four weeks of a red cell transfusion are replaced with the imputed pretransfusion hemoglobin values, the response to higher doses of an erythropoietic agent is significantly faster. Adapted from [75].

response to erythropoietic agents. In that setting, iron replacement is important, and the introduction of safer parenteral preparations has been associated with an increase in the routine use of parenteral iron in conjunction with ery-

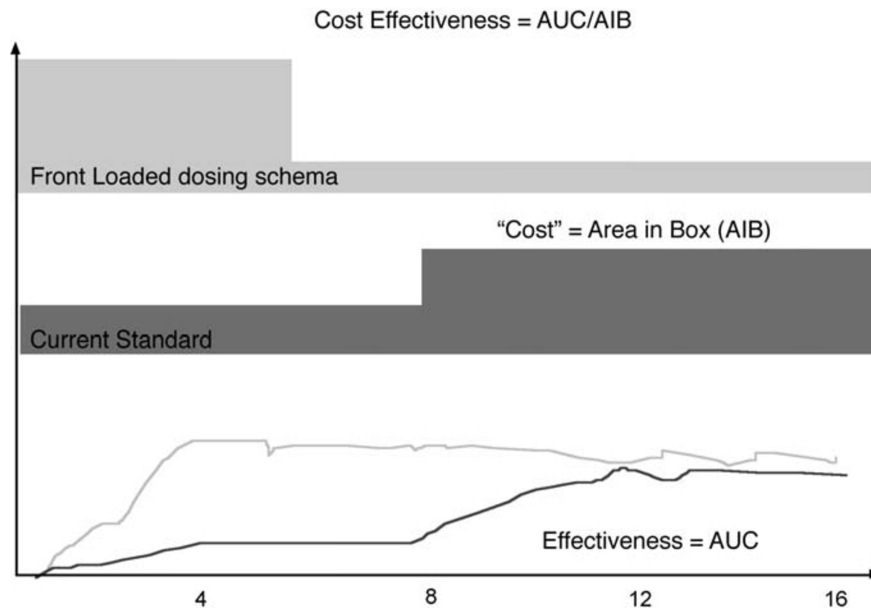

Figure 3. A theoretical schema for improving both patient benefit and cost effectiveness of erythropoietic therapy in oncology. For oncology patients, for whom rapid increases in hemoglobin are desirable, it may be more appropriate to begin therapy with optimal dosing and titrate down rather than to adopt an approach of starting suboptimal and titrating up, an approach better suited to dialysis patients.

thropoietic therapy, and a decrease in the doses of erythropoietic agents required to maintain target hemoglobin concentrations [78–81]. Because cancer is a chronic inflammatory illness, with both decreased gastrointestinal absorption of iron and an inflammatory blockade, compromising access by the erythron to body iron stores, the potential of parenteral iron to decrease the required doses of erythropoietic agents and to enhance hematologic response must be seriously considered. In one randomized trial in anemic patients with cancer who were not severely iron deficient, parenteral iron was associated with enhanced response to epoetin alfa, compared with either oral iron or no iron therapy; oral iron had no apparent effect [81]. Whatever the optimal schedule and dose of erythropoietic agents that emerges from future clinical trials, it appears likely that the addition of parenteral iron will enhance patient benefit and/or lower the requisite dose of the erythropoietic agent.

New outcomes

An important issue in erythropoeitic therapy in oncology is the cost of these agents, which is substantial and relatively simple to calculate. To ration-

ally approach the overall cost of erythropoietic therapy, however, one must know the cost of anemia in oncology, which is certainly substantial but much more difficult to estimate. In addition to the direct healthcare costs of preventable transfusions and their complications, randomized, placebo-controlled trials suggest that patients who are fatigued because they are not treated for anemia consume more hospital days and other direct healthcare resources [82, 83]. Moreover, evidence exists that patients who are not treated for anemia, or who are treated suboptimally, are depressed and less productive in terms of employment and family responsibilities [84–87]. These indirect, health-related costs have value for society. One of the most important directions for erythropoietic research will be to provide reliable estimates of the cost offsets, directly to the healthcare system and indirectly to society, associated with erythropoietic therapy. When these are known, they can be subtracted from the acquisition costs of the drugs and the incremental cost, if any, of treating patients to improve fatigue and quality of life can be meaningfully estimated.

Because anemia is a negative prognostic factor for most tumor types [88], an area of intense interest is the potential for erythropoietic therapy to improve survival for patients with cancer [89]. Even mild and moderate degrees of anemia may diminish the effectiveness of treatment with radiotherapy, chemotherapy, or modern targeted therapies. It has long been understood that mild anemia (hemoglobin concentration <12 g/dL) may compromise the effectiveness of radiotherapy for cervical and head-and-neck carcinomas by inducing demonstrable areas of hypoxia and radio-resistance in these tumors [90–100]. More recently, it has been shown that sub-lethal hypoxia confers *in vitro* resistance to several chemotherapeutic agents, including alkylating agents, platinum-containing complexes, and anthracyclines, and that anemia decreases the *in vivo* efficacy of cyclophosphamide [101, 102]. Moreover, it is now recognized that the normal cellular response to hypoxia, mediated by hypoxia-inducible factor-1 (HIF-1), increases the expression of vascular endothelial growth factor (VEGF) and may mediate some of the effects of HER-2/neu and other pathways relevant to tumor progression and modern targeted therapies [103–109]. One plausible explanation for the repeated observation that anemia is associated with decreased survival is that even mild anemia may compromise the efficacy of all forms of cancer treatment. It is possible that anemia decreases survival through its effects on the host, for whom severe fatigue may be a barrier to completing aggressive therapy and to surviving. Even for cancers for which treatment does little to change survival, quality of life remains one of the best predictors of prognosis [110].

Two clinical observations have increased awareness of the potential of erythropoietic therapy to improve survival. In a randomized, placebo-controlled trial of epoetin alfa in patients with cancer receiving chemotherapy, Littlewood and colleagues observed a trend favoring increased survival in the patients receiving epoetin alfa [111]. This study was not designed or powered to address the effects of anemia on survival, but it represents a hypothesis-gener-

ating starting point for future well-designed survival studies. In a randomized, placebo-controlled trial of darbepoetin alfa for patients with lung cancer receiving cisplatin-based chemotherapy, Vansteenkiste et al. observed a longer survival in patients with small-cell lung cancer receiving darbepoetin alfa with chemotherapy compared with patients receiving chemotherapy alone [29]. This trial is similarly hypothesis-generating, especially when considered in light of the preclinical evidence, cited above, that hypoxia compromises the cytotoxic effects of cisplatin. Several randomized trials of erythropoietic agents for patients undergoing radiotherapy for cervical, head-and-neck, and stage III lung cancers are underway or planned, as are trials in patients undergoing chemotherapy for breast and lung cancer.

EPO and EPOR are expressed in non-hematopoietic cells in the central nervous system, where EPO is believed to play a role in maintenance, protection, and damage-repair. When administered systemically, both rHuEPO and darbepoetin alfa penetrate the cerebrospinal fluid in concentrations believed to be protective [112]. In murine and rat models, systemically administered epoetin alfa has been shown to decrease the extent of brain injury resulting from ischemia, blunt trauma, and the administration of neurotoxins [113, 114]. In the oncology setting, these considerations have resulted in speculation that erythropoietic agents, in addition to decreasing fatigue and transfusion risk, may decrease the incidence of chemobrain, a syndrome of decreased memory and executive function detectable on high-sensitivity cognitive screens for up to two years after chemotherapy, which has been observed primarily after adjuvant chemotherapy for breast cancer [115]. It is not clear whether the syndrome results entirely from a direct chemotherapy effect on the brain or the effects of sleep disturbances associated with chemotherapy-induced menopause. Nevertheless, a sound pre-clinical basis exists for postulating a role of erythropoietic therapy in decreasing the incidence of cognitive dysfunction after chemotherapy. Parenthetically, the kinetics of erythropoietic agents crossing the blood-brain barrier and the available pre-clinical models suggest that higher systemic doses, such as those proposed in front-loading approaches, may be more effective in neuro-protection. Optimal hematopoietic support may provide optimal central nervous system protection.

New agents

Several erythropoietic molecules, including modified EPO and peptides unrelated to EPO that interact with EPOR, exist. Continuous EPOR activator is a pegylated EPO molecule with a serum half-life in normal volunteers of at least 80 hours. It is likely that results from clinical trials in oncology with new molecules will be available soon and may shed additional light on the optimal approach to treatment.

Conclusions

Over the last five years, it has become clear that anemia is a major problem in oncology because it compromises quality of life and possibly survival of patients with cancer. It is highly treatable. As the perceived importance of addressing the anemia problem has increased, the need for approaches to management, in terms of drug, dose, schedule, and co-therapy with iron, that insure that all patients will benefit as rapidly as possible has become more acute. At the same time, the question of the cost impact of erythropoietic therapy has become one of the most pressing health services issues in cancer medicine. New approaches, tailored to the needs of patients and consistent with the growing understanding of the biology of this anemia and its treatment, including higher initial doses of erythropoietic agents and more rapid responses are likely to emerge and to have advantages over current approaches that are associated with unacceptably high non-response rates and times to response. Rigorous clinical trials and health services research will be required if these optimized approaches are to be delivered in a uniformly accepted, affordable, and cost-effective manner.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Use of recombinant erythropoietins for the treatment of anemia of chronic disease

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Introduction

The term "anemia of chronic disease" (ACD) is used to describe a hypoproliferative anemia syndrome typically observed in patients with chronic infectious, inflammatory, or neoplastic disorders, and which is characterized diagnostically by hypoferremia despite adequate reticuloendothelial iron stores. ACD is often considered an unsatisfactory name, given that it does not include the anemias associated with many chronic conditions (such as chronic thyroid deficiency or end-stage renal disease), but does include the anemia observed in the context of acute infection or inflammation [1]. Unfortunately, most of the proposed alternatives are similarly deficient, being either purely descriptive (anemia of infection, anemia of inflammation, anemia of cancer), or, if they attempt to define ACD pathophysiologically, do so in an incomplete manner (anemia of defective iron reutilization). The most pathophysiologically accurate alternative name, "cytokine-mediated anemia", has never achieved common use [2].

Regardless of what it is called, ACD is clearly one of the most common hematologic syndromes observed in clinical medicine. In one series from the mid-1980s, ACD was diagnosed in more than 50% of the anemic patients admitted to the wards of a busy urban hospital [3]. Approximately 25% of outpatients followed for rheumatoid arthritis [4], and more than 50% of newly diagnosed inpatients on rheumatology services have ACD [5]. It is also strongly associated with chronic infections such as tuberculosis, empyema and lung abscess, endocarditis, cellulitis, and osteomyelitis [6, 7]. Most of patients with tuberculosis or endocarditis in modern series have ACD [8–10]. A syndrome essentially indistinguishable from ACD is observed in 5% to 17% of children with acute infections [11] during the first month after surgery [12], and also in critically ill patients in intensive care units [13].

Diagnosis

The diagnosis of ACD is typically made by the demonstration of anemia with inappropriately low reticulocyte counts with a low serum or plasma iron concentration despite adequate reticuloendothelial iron stores. Although it is most commonly seen in patients with a concurrent infectious, inflammatory, or neoplastic syndrome, in one series 40% of ACD patients lacked a traditional chronic disease [3]. This finding is a consequence of the cytokine-mediated basis of this syndrome.

The decreased serum iron concentration may lead to confusion between ACD and iron-deficiency anemia. Although typically a normocytic anemia, 20% to 50% of patients with ACD will have decreased erythrocyte mean corpuscular volume (MCV) [3, 4]. Serum total iron-binding capacity and/or transferrin concentration is traditionally said to be increased in iron deficiency and normal or decreased in ACD; however, in a recent multicenter study involving patients with complicated medical histories, only one of 24 patients with absent marrow iron stores had an increased serum total iron-binding capacity [14]. Although the serum ferritin concentration is the single best biochemical indicator of reticuloendothelial iron status, it has limited sensitivity in patients with concurrent inflammatory disorders. Serum ferritin is frequently increased disproportionately to iron stores. In a single-institution study correlating marrow iron stores to biochemical iron parameters, approximately 50% of patients without stainable marrow iron had serum ferritin concentrations in the normal range; a third of these iron-deficient patients had serum ferritin concentrations >100 g/L [15].

The determination of serum soluble transferrin-receptor concentration (TfR) appears to provide a solution to the problem of distinguishing iron deficiency with inflammation from ACD. TfR are predominantly expressed on erythroblasts; iron-deficiency erythroblasts express an increased number of TfR/cell [16], while those from patients with ACD express fewer TfR [17]. In addition, iron-deficient individuals often exhibit marrow erythroid hyperplasia, resulting in a further increment in cellular TfR numbers. Since the serum TfR concentration reflects the total body quantity of erythroblast cellular TfR [18], it would be predicted that this test could effectively distinguish iron deficiency and ACD; and initial reports supported this prediction [19]. More recent studies (using a different TfR assay), however, suggest that this picture is somewhat more complicated. A significantly increased proportion of increased TfR concentrations was noted in individuals with serum ferritin concentrations well above the upper limits of the normal range in one study of 77 patients [15]. The same phenomenon was observed in a larger study prospectively correlating marrow aspirate iron stores to serum TfR concentrations in 145 patients with anemia [14]. The authors proposed an algorithm in which patients with serum ferritin concentrations below the normal range were predicted to lack stainable marrow iron, patients with serum ferritin concentrations outside the normal range were predicted to be iron replete; and patients

with serum ferritin concentrations in the normal range undergo serum TfR determination. In this limited setting, an increased TfR concentration identified patients who lacked stainable marrow iron [14]. Other investigators have confirmed the value of TfR in the diagnosis of ACD, either alone [20] or as a ratio with ferritin [21].

Pathophysiology

It has been known for many years that the severity of ACD is correlated with the activity of the associated disease [4, 22]. This observation, as well as the need to find a common pathophysiologic mechanism encompassing disorders of microbial, autoimmune, and malignant origins, led investigators to consider mediators of the immune and inflammatory response, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and the interferons (IFN) as factors potentially involved in the development of ACD. Concentrations of these cytokines increase in patients with disorders associated with ACD [23–28].

Three major processes are involved in the pathogenesis of ACD. A modest (<10%) shortening of red-cell survival creates a demand for a slight increase in red cell production by the bone marrow. The marrow cannot respond adequately, however, because of impaired erythropoiesis and impaired mobilization of reticuloendothelial system iron stores [29]. The impairment of erythropoiesis, in turn, results from two processes: blunting of the expected increment erythropoietin (EPO) production in response to anemia; and a decreased response of the erythroid progenitors to EPO. The inflammatory cytokines can be implicated in all of these processes [30]. A physiologic correlation is provided by the observation that TNF blockade by a monoclonal antibody in anemic patients with rheumatoid arthritis results in increased hemoglobin concentrations [31]. Indeed, the effects of neutralizing antibodies to TNF and IFN-γ on erythroid colony formation *in vitro* in patients with renal failure has been used to implicate ACD mechanisms in the anemia of uremia [32]. It has been suggested that a relative deficiency of the anti-inflammatory cytokine IL-10 may contribute to the pathogenesis of ACD [33].

Shortened red cell survival

Anemic patients with rheumatoid arthritis (a widely used clinical example of ACD) show an inverse correlation between IL-1 concentrations and red cell survival [34]; similar results occur in mice after exposure to TNF *in vivo* [35]. The mechanism of this inhibition is unclear. Possible explanations include neocytolysis (hemolysis of the youngest red cells) in response to a reduction in EPO concentrations (discussed later) [36], or the effects of nitric oxide, a known second messenger of cytokine effects, on erythrocyte rigidity [37].

Impaired marrow response

The inability of the marrow to compensate for the modest reduction in red cell life-span seen in ACD results from the combination of an impaired EPO response to anemia and from impairment of the ability of the erythroid progenitors to respond to EPO. An inverse relationship exists between serum or plasma EPO concentrations and hemoglobin. As the hemoglobin concentration decreases, the EPO concentration increases [38]. A similar inverse relationship between hemoglobin and EPO was observed in anemic patients with rheumatoid arthritis [39]; however, for any given anemic individual with rheumatoid arthritis, the EPO concentration was lower than that found in equally anemic individuals with iron deficiency. This result indicated that the EPO response to anemia was blunted in patients with rheumatoid arthritis. Similar results have been reported in anemic patients with systemic lupus erythematosus [40]. This impaired EPO response appears to be a cytokine-mediated effect. IL-1, TNF-β, and transforming growth factor (TGF)-β inhibit production of EPO *in vitro* by hepatoma cell lines exposed to hypoxia or by isolated perfused rat kidneys [41, 42].

Although the EPO concentrations of patients with ACD are not as high as those found in equally anemic iron-deficient individuals, these concentrations are still higher than those found in normal individuals who are not anemic. This finding indicates that EPO concentrations are not the sole determinant of impaired erythropoiesis in ACD. Clinical studies in various syndromes have supported this concept [31, 43]. TNF, IL-1, and the IFN have been reported to inhibit erythroid colony formation and erythropoiesis *in vivo* and *in vitro* [25, 44–53].

The mechanisms by each of these cytokines act appears to differ widely. Erythroid colony-forming cells (CFU-E) appear to be relatively resistant to direct inhibition by TNF, IL-1, and IFN-γ. These cytokines appear to act primarily by inducing other cytokines that are directly inhibitory to CFU-E, such as IFN-β or IFN-γ [54–57].

Considerable attention has been devoted to the elucidation of the cellular mechanisms by which IFN-β and IFN-γ exert their effects on CFU-E formation. rHuIFN-γ induces apoptosis in CFU-E. This process appears to require *Fas* [58]. Ceramide, a product of sphingomyelin hydrolysis, is a known mediator of apoptotic effects of TNF, IL-1, and IFN-γ, and is frequently implicated in *Fas*-mediated events. Endogenous ceramide produced by exposure to bacterial sphingomyelinase (0.2 to 2.0 U/mL) and exogenous cell-permeable ceramide (C2-ceramide; at concentrations <10 mM) significantly inhibited bone marrow CFU-E formation. This effect was reversed by the ceramide antagonist sphingosine-1-phosphate. Inhibition of CFU-E by rHuIFN-γ, but not rHuIFN-β, was also reversed by sphingosine-1-phosphate. In addition, recombinant human (rHu)EPO 10 U/mL reversed CFU-E inhibition by C2-ceramide 10 mM. Exposure of marrow cells to rHuIFN-γ led to a 57% increase in ceramide content. These findings strongly suggest that ceramide is involved

in the inhibition of human CFU-E formation by IFN-γ [59]. Another potential second messenger implicated in the inhibitory effects of TNF and/or IL-1, nitrous oxide, appears to directly inhibit erythroid colony-formation *in vitro* [60]. A different approach to understanding the cellular effects of IFN-γ on CFU-E has been to evaluate changes in growth-factor receptors during erythroid development. Exposure to very high (2,500 U/mL) concentrations of rHuIFN-γ *in vitro* results in a decrease in EPO and stem cell factor (SCF), but not insulin-like growth factor-1 (IGF-1), receptors. This decrease is observed at both the protein and mRNA levels [61]. Although this finding has only been demonstrated at rHuIFN-γ concentrations significantly greater than those that inhibit CFU-E formation (100 to 1,000 U/mL), it is the most mechanistically appealing explanation for the effect of IFN-γ.

As will be discussed subsequently, treatment of patients with ACD with rHuEPO can increase hemoglobin concentration. The effects of supraphysiologic rHuEPO concentrations on the inhibitory effects of IFN-β- and IFN-γ on CFU-E *in vitro* have also been studied. The *in vitro* inhibitory effect of rHu γIFN, but not βIFN, can be reversed at increased concentrations of rHuEPO [57, 62]. It is possible that these observations provide a mechanism to explain the heterogeneity of the clinical response to rHuEPO in patients with ACD.

Impaired mobilization of reticuloendothelial iron stores

A diagnostic feature of ACD is hypoferremia in the setting of adequate or increased iron stores [29]. The impaired iron mobilization implied by these findings may also result from the effects of cytokines. A correlation between the immune activation marker neopterin and increasing ferritin concentrations in patients with malignancies has been reported, suggesting a role for immune activation in the altered iron metabolism of ACD [63]. Other investigators have reported that rodents injected with recombinant TNF develop a hypoferremic anemia associated with impaired reticuloendothelial iron release and incorporation into erythrocytes [35, 64]. IL-1 increases translation of ferritin mRNA, and it has been suggested that this additional ferritin could act as a trap for iron that might otherwise be available for erythropoiesis [65]. The acute phase reacting protein α-1-antitrypsin appears to inhibit erythropoiesis by impairing transferrin binding to TfR and subsequent internalization of the TfR-transferrin complex [66]. Nitrous oxide may have a major role in cytokine-mediated regulation of cellular iron metabolism [67–72].

Treatment of ACD with rHuEPO

Traditionally, ACD has been considered a cause of moderate anemia, with fewer than 2% of patients having an hematocrit <25% [73]. A series based on current diagnostic methodology, however, indicates that 20% to 30% of

patients with ACD are sufficiently anemic to potentially require red cell transfusions [3, 4]. The usual approach to ACD has been to direct treatment to the underlying disorder, since the degree of anemia generally reflects the activity of the associated disease. Since the anemia is generally not sufficiently severe to merit specific therapy, this remains the best general recommendation. For individuals who would potentially benefit from a higher hematocrit, however, the use of rHuEPO has proved highly effective.

rHuEPO in ACD

The use of rHuEPO in ACD was first reported as an abstract in December 1987 and as a full paper in 1989 [74]. Two patients with anemia and rheumatoid arthritis were treated with rHuEPO 100 to 300 U/kg intravenously three times a week for four to five months with correction of the hematocrit into the normal range. Comparable results were obtained with similar doses of intravenous rHuEPO in two larger studies (six and thirteen patients, respectively) involving patients with rheumatoid arthritis [75, 76].

About the same time, data began to emerge from the nephrology literature indicating that rHuEPO administered subcutaneously was of comparable or greater efficacy than rHuEPO administered intravenously [77]. In addition to offering a greater degree of convenience, this route of administration offered the possibility of self-administration, an issue of great importance in ACD. Table 1 summarizes several studies using subcutaneous rHuEPO in ACD.

Although subcutaneous rHuEPO is clearly effective in ACD, the size of the response and the dose required to achieve it in these studies differs considerably. In the study of patients in intensive care units [83], the comparatively small response in the context of large doses of rHuEPO likely reflects the short

Table 1. Use of subcutaneous recombinant human (rHuEPO) in anemia of chronic disease (ACD)

Ref	Setting	n	rHuEPO/wk	Doses/wk	Mean Hgb (g/dL)	Duration
[78]	Rheumatoid arthritis	11	250 U/kg		2.7	6 weeks
[79]	Rheumatoid arthritis	34	720 U/kg	3	1.3	6 weeks
[80]	Rheumatoid arthritis	36	300 U/kg	2	1.2	12 weeks
[81]	Rheumatoid arthritis	30	300 U/kg	3	2.5	12 weeks
[82]	Crohn's disease	4	450 U/kg	3	2.9	12 weeks
[83]	ICU	80	1500 U/kg × 1; then 900 U/kg	5; then 3	4.9%[b]	2–6 weeks
[84]	Congestive heart failure	26	5227[a]	1	1.9[a]	4–15 months

hgb, hemoglobin; ICU, intensive care unit.
[a] mean dose. The dose was adjusted to achieve a target hemoglobin of 12 g/dL.
[b] expressed as hematocrit in this study.

duration of treatment. In contrast, the excellent response observed in patients with congestive heart failure reflects both the long duration of treatment and the presence in many patients of concurrent renal insufficiency induced by low cardiac output [84].

Of more immediate relevance is the difference in degree of response between the two studies of patients with rheumatoid arthritis treated with rHuEPO 300 U/kg weekly [80, 81]. This difference almost certainly reflects the difference in iron supplementation. Only six of 36 patients studied by Nordstrom and colleagues received iron supplementation [80], compared with 23 of 30 patients treated by Kaltwasser et al. [81]. The median serum ferritin in the Nordstrom population (46.5 g/L) was below the definition chosen for iron deficiency in the Kaltwasser study (50 g/L). Clearly iron supplementation is a predictor of the hemoglobin response to rHuEPO.

Another predictor of the rHuEPO response in patient with ACD is the degree of disease activity. Patients with rheumatoid arthritis with lower C-reactive protein concentrations and erythrocyte sedimentation rates have been reported to have better responses to rHuEPO [80]. Subcutaneous rHuEPO administration has been reported to decrease disease activity in patients with rheumatoid arthritis [79, 81, 85] and to improve functional classification of heart failure patients [84]; comparable results are not reported in patients with rheumatoid arthritis treated with intravenous rHuEPO [74–76]. The adverse effects reported with rHuEPO in renal failure (exacerbation of hypertension, seizures, pure red cell aplasia) are not described in patients with ACD treated with rHuEPO.

Most of the studies evaluating the treatment ACD with rHuEPO use 300 to 450 U/kg subcutaneously in divided doses, generally three times weekly. Failure to respond at eight to 10 weeks should prompt a 50% dose increase. Although it has been studied primarily in cancer, there is no reason why the commonly used single weekly dose of 40,000 U rHuEPO subcutaneously should not be of equal efficacy in ACD. Failure to respond at six weeks should lead to an increase in dose to 60,000 U/week. For reasons made clear in the preceding paragraph, ferrous sulfate 325 mg three times a day should also be administered to patients with ACD who do not show laboratory evidence of iron overload. A trial of intravenous iron and rHuEPO should be considered in patients with ACD with a poor response to rHuEPO and oral iron [86]. Treatment with iron by itself (not as an adjunct to rHuEPO), whether oral or intravenous, is only useful in those patients with concurrent iron deficiency [87–89].

The use of rHuEPO to allow autologous blood donations by patients with ACD undergoing elective surgery (particularly orthopedic surgery) has been reported [90, 91]. This setting represents a particular challenge, since the ability to donate autologous blood is dependent on a normal hemoglobin or hematocrit. The administration of rHuEPO to otherwise healthy individuals allows collection of a larger quantity of autologous blood [92]. The quantity of autologous blood collected from patients with rheumatoid arthritis after

rHuEPO treatment is comparable to that obtained from healthy donors after rHuEPO [91].

Darbepoetin alfa in ACD

Darbepoetin alfa is an EPO analog with modified glycosylation permitting a longer half-life in the circulation. Darbepoetin alfa is able to correct anemia in an animal model of cytokine-mediated anemia [93]. Although it has not been studied in patients with ACD and classic inflammatory disorders such as rheumatoid arthritis, darbepoetin alfa has been shown to be effective in anemic patients with cancer not receiving chemotherapy (another clinical model for ACD) [94]. The optimal dosing schedule for ACD is not yet established.

Acknowledgments
Supported in part by the US Department of Veterans Affairs Veterans Health Administration Research funds and grant HL HL69418 from the US National Heart, Lung, and Blood Institute.

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Use of erythropoietins in the surgical setting

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Introduction

Modern conservation techniques have reduced the amount of blood lost during surgery, but acute anemia resulting from blood loss during surgery remains a problem and can cause severe damage to organs such as the heart, kidney, and brain. Prolonged post-operative anemia may delay recovery from surgery, leading to increased direct and indirect cost to the patient, healthcare providers, and payers. While allogeneic blood transfusions have traditionally been used to ameliorate the anemia of surgery, reducing exposure to allogeneic blood can reduce the risk of transmission of infections, allergic reactions, transfusion reactions, or immunosuppression [1–5]. In addition, frequent red blood cell transfusions with allogeneic blood may adversely effect the immune system of patients with cancer, thereby increasing the tendency to develop infections, hastening the time to relapse, or shortening survival [4–6].

Risk of infection

Nearly 50% of all anemic patients scheduled for single-joint replacement surgery receive allogeneic red blood cell transfusions. The risk of allogeneic transfusions is well described in the literature [7–13]. While often these concerns have focused on the transfusion of viral agents such as human immunodeficiency virus (HIV) or hepatitis, newer concerns have been raised such as West Nile virus transmission [14].

Allogeneic transfusions have been implicated in an increased incidence of bacterial infection after surgery. Houbiers et al. [15] evaluated 697 patients undergoing colorectal surgery in a randomized clinical trial. Multivariate analysis identified the transfusion of red blood cells as a significant independent risk factor for post-operative bacterial infection ($p < 0.01$). Kendall et al. [16] described immunosuppression secondary to allogeneic transfusions in 34 patients undergoing total hip arthroplasty. One of the goals of the study was to determine whether allogeneic transfusions resulted in post-operative immunosuppression. The authors concluded that immunosuppression does occur and

that lymphocyte function is impaired and that the immunosuppression may increase the risk of deep prosthesis infection.

While the Kendall et al. study documents the immunosuppression that occurs with allogeneic transfusions, Murphy et al. [17] demonstrated increased risk of post-operative infection in patients receiving total hip arthroplasty. Higher infection rates were noted if the patients received allogeneic blood transfusions. Aside from the infection risks, other authors have demonstrated a benefit of higher blood parameters (i.e., hematocrit and hemoglobin concentrations) during the peri-operative period.

Quality of life and other parameters

Another benefit of higher blood values is post-operative vigor. Keating et al. [18] described the assessment of patient vigor and defined a concise patient-based and caregiver-based instrument. Among the objective measurements studied, a statistically significant correlation was noted between vigor and hematocrit value. The question remains whether this increased vigor translates into shorter hospital stays and/or facilitates the ability of the patient to participate in the rehabilitation process.

Disease transmission is only part of the fear with blood management in the surgical setting. With growing evidence of possible negative consequences of immunosuppression as well as the benefits of increased vigor and fewer medical complications, the goal of the surgeon should be to not only minimize allogeneic transfusions but also to maximize the blood parameters with an aggressive peri-operative management program.

Risk factors for transfusion

The need for and the rate of transfusion have been historically correlated with the level of anemia in patients before surgery and the amount of blood lost during surgery [19–21]. One of the best ways to predict which patients will require allogeneic transfusion is to assess the pre-operative blood values, e.g., hematocrit and hemoglobin concentration. In our original study evaluating transfusion risks in patients receiving orthopedic surgery [22], we found that the pre-operative hemoglobin and hematocrit values were the best predictors of transfusion requirements in patients. Patients who received a transfusion lost the same amount of blood as patients who did not receive a transfusion. The important factor was pre-operative blood values: Patients who began with higher pre-operative hemoglobin concentrations were better able to tolerate the 10% hematocrit decrease because of surgery.

The relationship between pre-operative hemoglobin values and transfusion requirements was not only noted in total knee arthroplasty but was also seen in total hip arthroplasty. Nuttall et al. [23] evaluated 299 patients who had

undergone total hip arthroplasty to help determine the predictors for risk for red blood cell transfusion. They found that in many cases most of the cross-matched blood was not transfused, leading to wastage and increased costs. It was thought that if patients at risk of needing transfusions could be identified before surgery, unnecessary cross-matching and preparation could be eliminated. Pre-operative hemoglobin concentration was judged to be the most significant indication for preventing allogeneic transfusions, although age, sex, aspirin use, and estimated blood loss were other factors. Sculo and Gallino [24] evaluated 1,405 patients who had total joint replacement. Hemoglobin concentrations measured before surgery were inversely related to the frequency of allogeneic transfusions. In other words, the lower the pre-operative blood parameter values, the higher the allogeneic transfusion rate.

In one of the largest studies, Bierbaum et al. [25] evaluated 9,482 patients undergoing major orthopedic surgery. The authors not only demonstrated increased risk of transfusion for patients with a hemoglobin <13 g/dL but a more complicated post-operative course was also noted in patients receiving a transfusion. Transfusions were associated with an increased infection rate, fluid overload, and a longer hospital stay. These studies suggested that the presurgical goal should be to maximize the patients' blood levels before surgery.

Use of predonated autologous blood (PAD)

In the United States, the current standard of care for patients undergoing orthopedic surgery is predonated autologous blood (PAD), but the use of PAD is limited and variable in Europe. PAD cannot be used in patients who are anemic (i.e., hemoglobin concentration <11 g/dL in the United States and <10 g/dL in Europe) or for patients undergoing cardiac surgery. PAD is convenient for the physician and relatively convenient for the patient. Some studies report that PAD may cause further anemia and may be wasteful, at least in orthopedic setting [26–28].

Hatzidakis et al. [29] performed a retrospective analysis of 489 consecutive patients undergoing total joint arthroplasty. The study was limited since pre-donation blood values were only available for 149 of the 489 patients. These authors reported a decrease in hemoglobin concentration from the time of donation to the time of surgery (average of 1.22 g/dL). They questioned the benefit of PAD in patients with pre-donation hemoglobin concentrations >15 g/dL (>13 g/dL in patients <65 years of age).

Lotke et al. [30] evaluated medical complications after total knee arthroplasty. The study was initially designed to evaluate the controversy over the timing of administration of predonated autologous blood, i.e., should allogeneic transfusion and autologous transfusion have the same transfusion criteria. The results of the study suggested that patients who received immediate transfusion of PAD had fewer non-surgical complications compared with

patients who did not receive their PAD until their hemoglobin concentration was <9 g/dL ($p < 0.002$). The immediate transfusion of the PAD was suggested to be particularly critical for elderly patients undergoing total knee arthroplasty because they have an increased risk of cardiac and non-surgical complications and increased hemoglobin level is thought to improve outcome.

One concern noted by other authors is an observed decrease in the pre-operative blood values secondary to a PAD program [22, 24]. In addition, a one- to two-unit PAD program did not cause a significant erythropoietic response; therefore anemia was noted before surgery. Post-surgical transfusions may not always result in the desired hemoglobin concentrations because stored autologous units may contain suboptimal numbers of red blood cells.

We recently evaluated our PAD program. Between 1993 and 1995, two units of PAD were obtained on average compared with years between 1995 and 1997 when one unit was obtained. Our study showed a 3% decrease in hematocrit values for every unit donated before surgery. While the hematocrit values were similar with one- and two-unit protocols, increased wastage was noted in the two-unit PAD program [22].

We reviewed our results of the one-unit PAD program with automatic infusion of the donated unit and found it to be the best procedure for PAD. All patients were to be given their PAD immediately after surgery, the best plan for a PAD program. Despite ordering the PAD unit one month before surgery, significant anemia was noted. A 1.3 g/dL decrease was noted between pre-donation and pre-surgical testing (data on file). As documented by others as well [27, 28], the use of PAD resulted in anemia; patients did not return to their pre-donation hemoglobin and hematocrit values. Although the allogeneic transfusion rate was low, we feel that this reflects the acceptance of lower parameters for hemoglobin concentration and hematocrit rather than the efficacy of a one-unit PAD program. Had historical transfusion treatment been followed (hemoglobin concentration <10 g/dL), a transfusion rate of 38.1% would have been found. It should also be noted that 15% of patients were discharged with hemoglobin concentrations <9 g/dL. The acceptance of lower hemoglobin concentrations has a role in the lower allogeneic rates, not the efficacy of the one-unit PAD program.

The protocol was based on the work of Lotke et al. [30], showing fewer medical complications with the actual infusion of the donated unit. Our institution has since abandoned the above protocol based on its apparent lack of efficacy. This protocol may also place patients at risk, since a 100% autologous rate exposes them to donation error. Goldman et al. [31] revealed autologous error rates in Canada, and found an error rate of 6/149. Not all of these errors were related were to labeling (48%) or component preparation (25%). One patient received the wrong unit of donated blood, an event that is common. The College of American Pathologists noted 0.9% of 3,852 institutions studied, had at least one unit of PAD given to the wrong patient [32].

Cost is also an issue for PAD, for this is not an inexpensive procedure, with many costs related to procurement, as well as costs connected with the 50% to

75% autologous wastage noted. Billote et al. [28, 33] evaluated PAD in patients receiving total hip arthroplasty and found no benefit in PAD for non-anemic patients undergoing primary hip replacement. Each patient donated two units of autologous blood with an additional cost of US$ 758 per patient. Etchason et al. [34] evaluated the cost of a PAD program and concluded that the increased protection afforded by donating autologous blood is limited and may not justify the increased costs.

Use of erythropoietins

While the importance of pre-operative hemoglobin concentration has been discussed, the surgeon is often limited in his/her ability to return the patient to pre-donation hemoglobin values. Unhappy with the anemia caused by a PAD program, we have put our patients on another protocol. We obtain the hemoglobin concentrations at the time of surgical booking and based on the hemoglobin concentration, we identify those patients at high risk for a transfusion. Patients with hemoglobin concentrations >10 and <13 g/dL receive 40,000 U of recombinant human erythropoetin (rHuEPO) at three, two, and one week before surgery; they also receive iron supplementation. Nearly 75% of our patients avoid autologous donations and only 25% of those who are in need of transfusions have them. We recently compared 50 patients who received rHuEPO with 50 patients who were in an autologous program with automatic transfusion. Patients receiving rHuEPO had higher blood parameters, pre-operative, post-operative, and on discharge, compared with patients who participated in the autologous program. In addition, our overall cost was reduced because using a patient-specific approach only on the 25% of patients who received rHuEPO had transfusions.

In Europe, one brand of epoetin alfa (Eprex, Janssen-Cilag) and the brand epoetin beta (NeoRecormon, F Hoffmann-LaRoche) are licensed for use in patients with mild anemia, including those undergoing PAD. Epoetin alfa is licensed by most European countries for the treatment of patients with anemia who are scheduled to undergo orthopedic surgery.

In the United States, one brand of epoetin alfa (Procrit, Ortho Biotech) is licensed for use in PAD. Treatment with peri-operative epoetin alfa alone, without concomitant PAD, has been shown to reduce the need for transfusion in patients undergoing major orthopedic surgery by increasing the peri-operative hemoglobin concentration and the rate of erythropoietic recovery [19–21]. Two large, double-blind, placebo-controlled trials reported that the use of epoetin alfa significantly reduced the percentage of patients receiving allogeneic red blood cell transfusions compared with the patients who received placebo [19, 21]. A multicenter, double-blind, placebo-controlled, parallel-group study determined that epoetin alfa 300 U/kg for 15 consecutive days starting 10 days before surgery could reduce peri-operative transfusion requirements [20]. The mean number of units transfused was lower for patients receiving epoetin alfa

compared with patients receiving placebo. Figure 1 illustrates a typical protocol using epoetin alfa in PAD.

The rationale for the use of epoetin alfa is based on its pharmacologic properties, i.e., the increased rate of erythropoiesis. Because orthopedic surgery is usually an elective surgery, it is possible to administer a course of epoetin alfa to increase the hemoglobin concentration and hematocrit before surgery. Darbepoetin alfa is a new erythropoietic protein with a three-fold longer serum half-life than epoetin alfa that may also be useful in the surgical setting. The

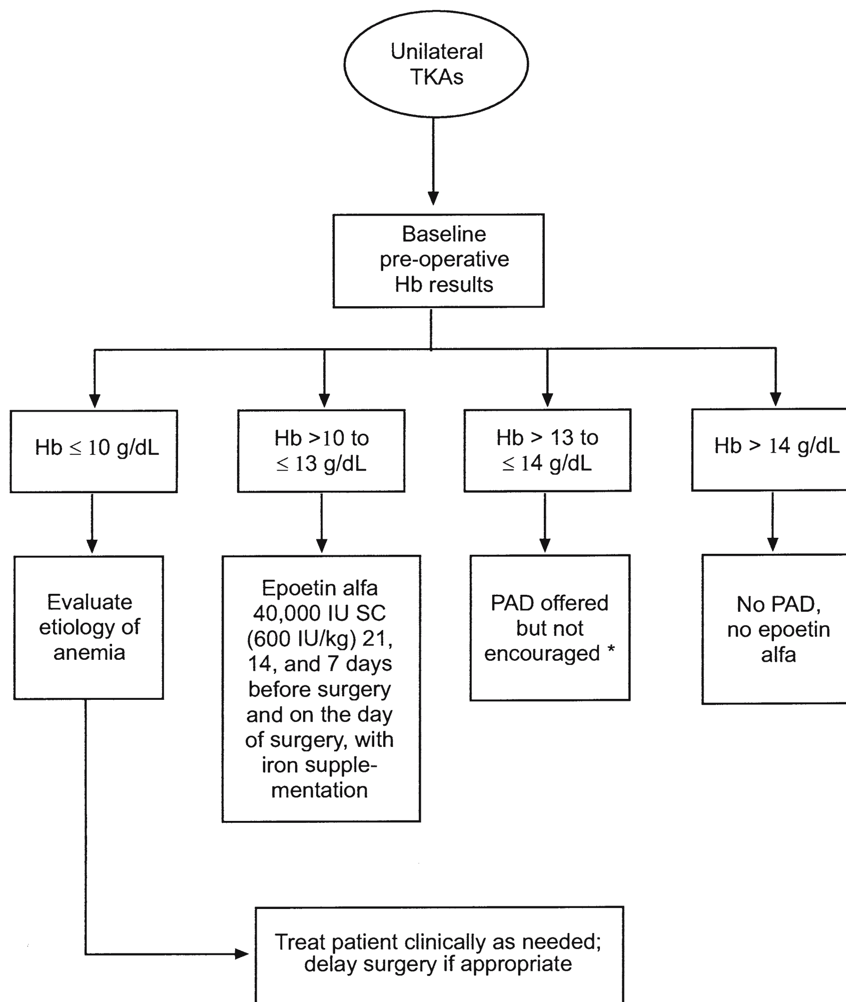

Figure 1. Our protocol for total knee athroplasty. * = if PAD obtained, the time between PAD and surgergy should be maximized. CBC = complete blood count; Hb = hemoglobin; PAD = pre-operative autologous donation; SC = subcutaneous; TKA = total knee arthroplasty.

longer half-life translates into less-frequent administration and may allow greater patient compliance and satisfaction.

Conclusions

Patients in a PAD program sustain costs not only associated with collecting the blood but also costs associated with its delivery (i.e., nursing care), with costs estimated anywhere from US$400 to US$600 per patient. The use of erythropoietic factors such as epoetin alfa, epoetin beta, and darbepoetin alfa, have the potential to increase important blood parameters such as hematocrit and hemoglobin concentration safely and reliably while reducing the cost and need for allogeneic blood transfusions in the orthopedic surgery setting.

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ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Abuse of recombinant erythropoietins by athletes

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History

History of doping

Athletes have always sought ways to enhance their performance. In ancient times, medicinal plants were used for doping, i.e., the artificial enhancement of athletic performance. According to Philostratus, a Greek physiologist in the third century AD, "[athletes should be]…freed from the use of clay and mud and irksome medicine" [1]. He described Olympians who enhanced their athletic abilities by eating bread soaked in opium.

Some of the earliest pharmaceuticals, amphetamine, strychnine, and ephedrine, rapidly became interesting to athletes in search of increased speed in athletic events. Canal swimmers in Amsterdam were reported to have used strychnine to win races. The first documented doping fatality at the Olympics shocked the 1960 Games [2] and it involved amphetamine abuse.

"There can be no doubt that stimulants are today widely used by athletes participating in competitions; the record-breaking craze and the desire to satisfy an exacting public play a more and more prominent role, and take higher rank than the health of the competitors itself."[3] Written in 1939 by a physiologist, this quote is still apropos today and it shows the length of time athletes have had the "win-at-all-cost" mentality.

Currently, sport is coping with both drugs developed with recombinant-DNA technology and genetic manipulation. In the 1970s, when the United States Congressional hearings documented significant abuse of stimulants and anabolic steroids [4], medical organizations took anti-doping stands and widespread amphetamine abuse was described in American professional football.

Characteristics of doping

Not all athletes dope – fortunately most do not – but those who do have a profound influence on sport. Ben Johnson set a world record in the 100-meter sprint at the 1988 Games of Seoul, but he was doped with stanozolol, an anabolic steroid. Until this Games-stopping event, the hope had been that doping was confined to strength sports. The Johnson affair led to vociferous cries from clean athletes for more testing and stricter penalties, and spurred sport to devote more resources to solving the problem. In retrospect, the resources were inadequate but served to heighten awareness and to begin to solve the problem.

An axiom of drugs and sport is that some athletes will try any new drug that has the potential to enhance performance. The trials are conducted secretly and are, of course, never published or presented. Sometimes information may be shared with close partners, but generally not. Often members of the athletes' entourage are deeply involved in doping.

Doping has occurred in nearly all sports, but certain ones have predilections [5, 6]. Endurance events, such as long-distance running, cycling, swimming, and skiing events, often have participants who have tried various types of blood doping including the use of erythropoietic proteins. Short-distance speed events are known for abuse of stimulants; and weightlifting, throwing events, and several other sports have been plagued with anabolic androgenic steroid doping. Participants in shooting events used beta-blockers to improve shooting scores [7].

Development of testing

By the mid-1960s, international sport officials were very concerned about drugs and were actively exploring several approaches to deal with the problem. Until the mid-1960s, sport had used education as its main approach, but elite athletes were now beginning to receive substantial financial rewards for success and education alone was not effective. In 1967, Prince de Merode presented a survey of the problem to the International Olympic Committee (IOC). Soon thereafter, he became chairman of the IOC Medical Commission and plans were made to implement urine testing to detect doping [8]. After experimenting with urine testing at the Winter Olympics of Grenoble in 1968, the IOC implemented widespread testing at the Summer Olympic Games of Munich in 1972. In 1984, only five IOC-accredited laboratories existed; now 25 laboratories operate in 23 countries. In the early years of the program, testing was confined to the Olympic Games. It has been adopted by other competitions, and now athletes may be tested at any time during the year. Out-of-competition testing was implemented when it became clear that the ergogenic effects of drugs, such as anabolic steroids and erythropoietic proteins, persisted for many days after administration was discontinued. Without random, out-of-competition testing, athletes could discontinue doping before announced in-

competition tests, thus evading detection. Approximately 10,000 samples are tested per year, and of these, approximately 50% are collected as out-of-competition tests.

The Court of Arbitration for Sport (CAS)

The Court of Arbitration for Sport (CAS) was created by the IOC in 1983 to resolve sport-specific disputes [9]. CAS provides a forum for judicial resolution of disputes among athletes, national and international sport federations, national Olympic Committees, games organizers, sponsors, and others. Disputes are presided over by a panel of three arbitrators selected from a pool of CAS arbitrators. All CAS decisions are final with nearly no possibility of appeal.

Doping cases are subject to the strict liability rule that states that: "doping is… the presence in the athlete's body of a Prohibited Substance…" [10].

Thus, if a prohibited substance is found in an athlete's body, a doping violation has occurred irrespective of any other factor [9]. At times this rule results in seemingly harsh consequences, as was demonstrated by the case of the Romanian gymnast who placed first in the Women's Individual All-Round Event at the Olympic Games of Sydney. The athlete tested positive for pseudoephedrine that was allegedly given to her by the team doctor.

The two most common subjects that are argued in CAS cases are the validity of the analytical methods used to establish that a prohibited substance has been detected in the athlete's body fluid and the chain-of-custody of the sample. The principles of both the analytical methods that may be applied and the chain-of-custody are specified in the Medical Code of the Olympic Movement [10]. During the adjudication procedure, the laboratory director typically defends the analytical results and the sample-collection officials defend the chain-of-custody issues.

The World Anti-Doping Agency

Faced with many positive test results, mounting numbers of drugs and methods to consider banning, laboratories to accredit and re-accredit, and complex issues involving new fields of science, the IOC fostered the development of a new agency, the World Anti-Doping Agency to supervise the drug-control effort. This move coincided with revelations in 1998 that many cyclists competing in the Tour de France were doped with recombinant human erythropoietin (rHuEPO). Since the IOC had been criticized for being slow to develop adequate anti-doping programs, the anti-doping agency was structured to operate outside the direct control of the IOC. It is expected that this agency, with its substantial resources, will have a major impact on the doping problem.

Doping by expanding the red cell mass

"Induced erythrocythemia" is a term used to describe expansion of the blood volume by transfusion of whole blood, transfusion of packed red cells, or by administration of erythropoietic proteins. The term "blood doping" has been used to describe any of these three methods, although obvious differences exist in the techniques and the infrastructure required to use them.

Autologous and homologous blood doping in sport

Athletes have used both homologous and autologous blood products. The typical technique for autologous transfusion involves phlebotomy, freezing the red blood cells under glycerol, allowing eight to 12 weeks for hemoglobin recovery while continuing training, and infusion of the red blood cells one to two days before the scheduled athletic event. The use of homologous blood products avoids the regeneration period, but is associated with multiple risks and adverse effects. The terms "blood packing" and "blood boosting" usually refer to doping with homologous and autologous blood products, respectively.

Rumors and anecdotes suggest that an airplane equipped with refrigeration devices was used to support doping at the 1976 Games in Montreal. The first documented case of blood doping occurred in the 1980 Olympics in Moscow when a Finnish distance runner freely admitted having received two units of blood shortly before he won medals in the 5- and 10-km races [11]. At the 1984 Games in Los Angeles, seven cyclists from the United States team were found to have blood doped [12]. One cyclist doped by autologous transfusion and had his personal-best time at the Olympic trials a month before the Games. By the time the news reached his team-mates, it was too late to use the autologous infusion paradigm, so with the aid of a physician, whole blood from relatives and unrelated donors was transfused to the cyclists in a motel room [13]. Four of the seven cyclists won medals. During the ensuing investigation, the athletes justified the act by pointing out that the IOC list of prohibited substances did not explicitly ban blood doping. Shortly thereafter, the IOC added doping with blood products to the list. The only other documented case is that of a United States skier who admitted to blood doping with autologous blood in 1987 [14].

Doping with rHuEPO in sport

Given that blood doping with transfusions is a complicated matter that requires substantial infrastructure, such as blood harvesting equipment, processing devices, and refrigeration, it is understandable that the availability of a drug, even one that required intravenous or subcutaneous injection, would be used. It is not known when doping with rHuEPO began. The secrecy and privacy that surrounds the topic precludes detailed knowledge, but it is understood that ath-

letes and their medical entourage follow pharmaceutical developments closely. Thus, it is not surprising that rumors of rHuEPO's potential for abuse by athletes first surfaced before rHuEPO was approved for marketing (1987), and that rumors of actual use circulated before the 1988 Winter Games of Calgary (Tab. 1).

In the late 1980s, a cluster of up to 20 deaths occurred among elite cyclists in the Netherlands and Belgium. The story was widely reported, followed by an official investigation, but no details emerged. It is speculated that rHuEPO may have been involved in these deaths [15, 16]. We were unsuccessful in obtaining the details through contacts in the sport community. Because the deaths occurred after rHuEPO received marketing approval, it was widely speculated that they were due to excessive doses of the drug. In 1990, "erythropoietin" and analogues were added to the IOC list of prohibited substances.

Although it was widely accepted that rHuEPO was being abused throughout the 1990s, it was not until the 1998 Tour de France that its use was documented. The discovery of rHuEPO and other drugs in the trunk of an automobile just before the race led to a widespread investigation that eventually resulted in recovery of many drug products, confessions from several athletes, and evidence of widespread involvement of cycling teams and coaches [17–19]. This exposé differed from other sensational drugs in sport stories (such as Ben Johnson, the track and field athlete, in 1988) in that most of the competitors were involved, not an individual athlete. This finding convinced cycling officials that rHuEPO doping was pervasive and that users and winners were highly correlated. Further, officials in other endurance sports understood that their sports were at risk. Some of the steps taken collectively by sport included further implementation of blood screening methods (discussed later in this chapter), monitoring athletes' red blood cell parameters, increased funding for research on detection, formation of expert committees, and more emphasis on the role of police authorities. A most unfortunate aspect of such developments is that success is equated with doping, thereby raising suspicions about any athlete who performs well. This belief has led drug-free athletes to speak out against doping and to pressure authorities for more effective action.

Prevalence of doping with erythropoietic proteins

It is nearly impossible to estimate the prevalence of blood doping of any kind. Only one survey has attempted to do so and commingled data on transfusion doping with rHuEPO doping. Scarpino et al. [20] interviewed 1,015 Italian athletes and reported that 7% regularly used red blood cell infusions or rHuEPO techniques and that 25% were "occasional" users. In the same study, coaches, managers, and team physicians also estimated that 7% of athletes were regular users of doping. Sport hot-lines are another source of information. Throughout the 1990s, the United States Olympic Committee operated an

Table 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.

anonymous hot-line for athletes to call to request information about drugs and sport. Committee reports indicate that the number of calls regarding rHuEPO increased during the 1990s.

The lay sport literature reports widespread doping with erythropoietic proteins in endurance sports such as track, cycling, and cross-country skiing. These reports are largely confined to elite athletes competing in major events. From the frequency of these sensational reports, one gains the impression that such doping is common, but prevalence cannot be estimated from such data. To date, neither college sport administrators nor the media have reported doping with erythropoietic proteins in the college sport population.

Now that a urine test for erythropoietic proteins (discussed later) is beginning to be deployed, some additional data will be forthcoming from sports organizations. The IOC, World Anti-Doping Agency, and international sport federations are likely to periodically make summary data available. The nature of these reports will vary, but in some cases, they will include the names of athletes and the specific events. Since late in 2000, the media have reported several cases of doping with erythropoietic proteins [21]. The media reports occur many months before the adjudication proceedings that could find that doping did not occur. By late 2002, CAS had heard two cases of alleged doping with rHuEPO. In one case, the Court upheld the laboratory result and determined that the athlete had doped with rHuEPO [22]. In the second case, CAS concluded that rHuEPO doping had not been proven due to a flaw in the analysis [23]. Although no one knows to what extent athletes use erythropoietic proteins, it is increasingly obvious from all sources of data that they are being used. Although the use currently appears to be confined to the highest echelons of endurance sport, if the history of doping with other agents (notably steroids) is any indication, their use will spread.

Does expanding the red cell mass enhance performance?

The underlying theory of doping is that increased oxygen-carrying capacity increases performance of muscle tissue by increasing oxygen supply. The ability to perform sustained aerobic exercise depends on both oxygen delivery to muscles and ability of the tissues to use it. Which of the two limits exercise capacity has been the subject of many investigations and debates [13, 24–26]. $VO_{2\,max}$, a widely accepted index of physical fitness [27], correlates with red cell mass [28], thereby supporting the hypothesis that an expanded red cell mass enhances performance by increasing the amount of oxygen delivered to muscle. The increase in blood viscosity due to increased concentrations of hemoglobin does not limit delivery, as long as the hemoglobin concentration is <20 g/L [29]. Other possible theories advanced to explain the increase exercise capacity are that the expanded blood volume leads to increased cardiac output, improved buffering capacity for the lactic acid accumulated during exercise, and enhanced heat dissipation.

A paradox exists in exercise physiology. Athlete fitness is negatively correlated with hematocrit, yet increasing the hematocrit by transfusion or by doping with erythropoietic proteins improves $VO_{2\,max}$ and performance [30, 31]. Exercise training expands plasma volume and decreases hematocrit and hemoglobin concentrations, thus causing a negative correlation between hematocrit and fitness [30–33]. Values for hematocrit, hemoglobin, and red blood cell count are in the lower range of normal for athletes [30, 34]. In one study, athletes with the lowest hematocrits had the highest aerobic working capacity and isometric adductor strength [31].

At first it was thought that the hemoconcentration that accompanies endurance competitions would lead to increased blood viscosity and that this would severely limit cardiac output and thereby limit the usefulness of rHuEPO. Hematocrit is highly correlated with blood viscosity [31]; however, because hematocrits of 50% and more are often recorded among competing athletes, other factors must be operating. In one study of athletes competing in an ultra-marathon, the immediate post-race hematocrits were not different from the baseline values, and on the day after the competition, the values were lower than baseline [30]. Apparently replenishing lost fluid and electrolytes during very long events is sufficient to avoid serious increases in viscosity.

The notion that dehydration during long endurance events will lead to hemoconcentration and increased blood viscosity and, therefore, result in athletes experiencing strokes and other complications of increased blood viscosity is difficult to confirm. No media reports exist of athletes experiencing such catastrophes during endurance events. The cluster of deaths in cyclists in the late 1980s [15] could be related to increased blood viscosity, but the athletes were not competing at the time of death.

Autologous blood infusion increases performance

Many clinical experiments have shown that transfusions increase human performance [24, 25, 35–37]. The most convincing study [25] used a double-blind, sham-infusion controlled, cross-over design. Highly trained elite athletes received 900 mL of autologous red blood cells collected and frozen approximately seven weeks earlier; 24 hours after the transfusions, the athletes experienced a 35% increase in run-time to exhaustion, a 5% increase in $VO_{2\,max}$, and a 7% increase in hemoglobin concentration. The authors concluded that the limit to aerobic activity was the transport of oxygen to muscle. In another study, this time using 10-km race-time as the outcome rather than physiological measurements, six highly trained but not elite athletes improved their mean run-times from 33.3 to 32.1 min and their hematocrits increased 5% shortly after receiving a 400 mL autologous infusion of red cells collected 11 weeks earlier [37]. An interesting aspect of autologous infusions is that the magnitude of the improvement is related to the baseline level of fitness. Individuals who are moderately fit experience the greatest improvement while

individuals who are poorly fit or extremely fit experience less improvement in maximal oxygen uptake [36]. Improvement in human performance is a consistent feature of blood transfusions, provided that the amount of red cells infused is sufficient to increase serum hemoglobin concentration and hematocrit and thereby deliver more oxygen to working muscles. The improvements can be shown in race-times, fixed-run times, $VO_{2\,max}$, run-times to exhaustion, and other measures of performance. Accordingly, sport officials search for ways to deter those athletes who are determined to dope with transfusions or any other means to increase the delivery of oxygen to muscles.

rHuEPO enhances performance in healthy subjects

It is well known that rHuEPO administered to patients with anemia significantly improves fatigue, physical symptoms, and physical performance [38]. (See Chapters 9 and 10 for further information.) In the present context, the question is whether or not rHuEPO enhances physical performance in healthy subjects and, particularly, in athletes. Since the release of rHuEPO almost 15 years ago, few studies on have been done with athletes and most of the studies that have been done have involved its effect on red cell indices and other markers.

Two placebo-controlled studies have shown that rHuEPO increased $VO_{2\,max}$ by 6.0% to 7.7% after three to four weeks of subcutaneously administered rHuEPO (150 U/kg/week) [39, 40]. Typically, the weekly dose is administered subcutaneously in doses of 150 U/kg/week. At a higher dose (180 to 210 U/kg/week, administered subcutaneously), the $VO_{2\,max}$ increase was similar (7%) [41]. These studies, which used recreational athletes, establish that doses of rHuEPO that are sufficient to increase the hematocrit to nearly 50% also increase the $VO_{2\,max}$ up to 7%. Anecdotal reports, however, indicate that after a few weeks of modest doses (150 U/kg/week), athletes lower the weekly dose to approximately 60 U/kg/week. To determine the effect of such a regimen, after three weeks at 150 U/kg/week, investigators [40] decreased the dose to 60 U/kg/week for an additional five weeks and found that the improvement in $VO_{2\,max}$ (4.7% to 9.7%) and increased hematocrits continued. This study and a similar low-dose study [42] provide support for the anecdotal reports from athletes, and confirm that maintenance doses of 60 U/kg/week are capable of enhancing aerobic performance for at least three weeks after discontinuation of drug. The question of how long the benefits last after rHuEPO is discontinued will be discussed.

Adverse effects of rHuEPO in athletes

Unlike studies in patients with anemia, no surveys have been done of the adverse effects of rHuEPO in athletes. Athletes tend to be healthy and that may

offer some protection against the known side-effects in patients with anemia; however, athletes are deliberately increasing their hematocrits to levels associated with thromboembolic and other complications. Despite the increases, only one case has been reported that appears to link rHuEPO to a cerebral thrombosis. The athlete admitted to using rHuEPO and other drugs [43]. Athletes who use rHuEPO are at risk of developing true iron deficiency, functional iron deficiency, or iron overload. Of course, careful professional medical management could avoid these complications. Cazzola reports that an investigation by Italian magistrates [44] reveals that some professional cyclists have evidence of iron overload with ferritin levels in excess of 1,000 ng/mL [45]. The investigation also provided data consistent with a risk of post-treatment blunted production of endogenous EPO [45]. In addition Berglund and Ekblom [42] have studied the effect of rHuEPO on the blood pressure of athletes. The systolic and diastolic blood pressure values at rest were unchanged after rHuEPO treatment; however, systolic blood pressure markedly increased during submaximal exercise. The initial and final values were 177 mmHg and 191 mmHg, respectively [42]. The propensity of athletes to titrate their hematocrits to high levels and to take rHuEPO without adequate medical supervision, together with their risk for iron disorders and exercise-induced increased systolic pressure, make it likely that more adverse effects are occurring than are reported in the medical literature. This under-reporting is inherent to the secretive nature of doping.

Detecting erythropoietic proteins in body fluids

Direct and indirect tests

Sport classifies tests for doping substances as direct or indirect. A direct test identifies the substance by an unambiguous method such as gas chromatography-mass spectrometry, whereas indirect tests measure, for example, the serum concentration of markers that correlate with the use of a prohibited substance, without directly identifying the substance. Direct tests sufficed for many years, but now that doping includes endogenous steroids and glycoproteins, a variety of new strategies and indirect tests have been developed [46]. Indirect tests have not been used to declare that an athlete has used a substance, but it is hoped that with sufficient validation, these tests could become definitive.

Detecting the use of pharmaceutical testosterone was a challenge with gas chromatography-mass spectrometry because pharmaceutical and endogenous testosterone could not be distinguished. The problem was partly solved by performing longitudinal tests of the urinary steroid profile and by determining the $^{13}C/^{12}C$ ratio of urinary testosterone by isotope ratio mass spectrometry [47]. The glycoproteins present a special challenge because, to date, it has not been practical to develop a mass spectrometry-based method that is sensitive

enough to detect either rHuEPO or recombinant human growth hormone (rHuGH) in human urine. Sonksen and colleagues attempted find a group of blood parameters that would indicate recent use of rHuGH. After collecting baseline blood samples, they administered rHuGH for several days and monitored serum concentrations of six substances known to be influenced by rHuGH [48] A composite score indicative of recent use of rHuGH was developed. The results showed good separation between the scores of the placebo-treated and rHuGH-treated subjects. The investigators intend to expand the studies to a much larger number of subjects and to determine the variability of the markers in various ethnic groups. The hope is to find a composite score that is so convincing that the indirect test is considered definitive. Until 2000, no practical direct test existed for rHuEPO; therefore, certain sports implemented indirect tests to identify potential users of rHuEPO.

Indirect tests for doping with erythropoietic proteins

The hematocrit 'health' test

The International Cycling Union, concerned that EPO had pervaded elite competition, declared that athletes could not compete with a hematocrit >50% and >47%, for men and women, respectively [49, 50]. The International Ski Federation implemented a similar rule based on hemoglobin values exceeding 185 g/L (men) or 165 g/L (women). One strategy was to determine the values immediately before an event and withhold the athletes from competition if the limits were exceeded. The hematocrit cut-off values have been changing, but generally they are 50% for men and 47% for women. The hematocrit test has been called a "health test" because it is considered dangerous for an athlete to compete if the hematocrit is greater than the cut-off. The term "health test" pre-empts legal action because the athlete is not declared a drug user, he/she is only unable to compete. The ban on competition is lifted after 15 days, provided that the hematocrit has decreased to acceptable values.

Aside from the health issue, the argument in favor of a hematocrit test is that by imposing an upper limit, the test prevents excessive use of rHuEPO. An argument against the hematocrit test is that it would discriminate against individuals who have naturally high hematocrit values [51, 52]. Partially countering this argument is the finding that of 334 hematocrits determined on 34 professional cyclists before rHuEPO was available, the values ranged from 39% to 48% (mean: 43%) [53]. In addition, a protocol was developed to determine if an athlete's hematocrit naturally exceeds 50%. Postural changes in hematocrit are avoided by taking samples after sitting for 15 min. Increased hematocrits due to dehydration have not been a significant problem [30]. Despite the controversial nature of the test, its use is having the intended effect: Hemoglobin values among elite cross-country skiers increased dramatically from 1994 to 1996 and declined after the test was implemented [54].

A disadvantage of the hematocrit test is that it draws attention to the benefits of an increased hematocrit, thus tempting athletes with a natural baseline <50% to find a way to increase it. Indeed, it is common lore that some athletes check their hematocrits using portable centrifuges and self-administer saline infusions and phlebotomy if the values are too high. They are also reported to take anticoagulants to prevent thromboembolic events.

Hypochromic macrocytes

Casoni et al. [55] studied red cell indices after administering rHuEPO every other day at an average dose of 15 U/kg/day for up to 45 days. The parameters that changed the most were mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). The authors defined red cells with MCV > 128 fL and MCH <28 pg as hypochromic macrocytes and proposed a cut-off of 0.6% to distinguish rHuEPO users from healthy controls. No further work on this particular index has been published.

Serum transferrin receptor (TfR) and Ferritin

Serum levels of serum transferrin receptor (TfR) and ferritin are regulated by cellular iron status, and cellular iron uptake is facilitated by TfR-mediated endocytosis. As a result of externalization of TfR during the endocytic cycle, a soluble form of TfR can be detected in serum. Thus, the major determinants of serum TfR concentration are cellular iron demands and red blood cell proliferation rate. Since rHuEPO expands the red cell mass, it was logical to determine if serum TfR and ferritin could serve as indirect markers of rHuEPO administration.

Several studies have shown that amounts of serum TfR increase, amounts of ferritin decrease, and that the ratio of serum TfR/ferritin increases when rHuEPO is administered [41, 56, 57]. Furthermore, these changes are detectable in most subjects for up to one week after discontinuing rHuEPO [41]. The change in serum TfR/ferritin ratio was less dramatic in subjects treated with supplemental iron [41] than in subjects who did not receive iron [56]. Thus, the absence of supplemental iron may exaggerate the sTfR/ferritin ratio. Most users of rHuEPO are likely to take supplemental iron. The specificity of the changes in serum TfR, ferritin, and sTfR/ferritin has not been evaluated in a large group of healthy normal controls of different ethnicities, subjects with disease, or under various conditions of iron supplementation, altitude, or training. Nevertheless, these markers are relatively simple to measure and they may be useful as indirect markers of the use of erythropoietic proteins, either alone or in combination with other markers.

Multiple markers of erythropoietic activity: the Australian studies

In the year before the Sydney Olympic Games of 2000, Australian scientists did an extensive series of studies designed to find a combination of blood markers that would indicate which athletes were using rHuEPO [39, 58]. The approach was to administer rHuEPO for three weeks, collect blood for analy-

ses before, during, and for two weeks after administration was discontinued, and measure a variety of potential markers in serum and blood. The data were analyzed by various statistical models that provided the optimum combination of variables and the weight to attach to each. The main study compared placebo-treated with rHuEPO-treated subjects who received 50 U/kg three times per week for 25 days [58]. The treatment group comprised 49 Australian subjects and 24 Chinese subjects.

The study found that five markers provided the most discrimination between the placebo-treated and rHuEPO-treated groups. These were hematocrit, reticulocyte hematocrit (MCV of reticulocytes multiplied by the number of reticulocytes), percent macrocytes, serum concentration of EPO, and serum concentration of serum TfR. The total score was referred to as the “on-score” [58]. By the end of the 3-week rHuEPO administration period, the “on-scores” of the placebo-treated and rHuEPO-treated groups differed significantly. Serum EPO and sTfR were particularly increased in the rHuEPO-treated group compared with the placebo group. No differences were noted between the Australian and the Chinese subjects or between men and women.

The Australian investigators also calculated the “on-score” on approximately 1,200 elite athletes from 12 countries [58]. Based on these data, they calculated cut-offs for the “on-score” values beyond which the risk of a false-positive was very low. At a meeting of experts three months before the Sydney Games [59], the “on-score” was not approved as a standalone index of use of erythropoietic proteins largely due to legal and medical concerns. It was approved, however, as a technique to indicate which urine samples should be tested by the recently developed, definitive urine test (discussed later). The rules stated that an athlete could not be declared “positive” for rHuEPO unless the “on-score” was greater than the cut-off score and the urine test showed rHuEPO. Samples could be declared “suspicious” if the “off-score” was increased or if only one of the blood or urine tests was positive. No athletes were declared positive during the Games of Sydney, but seven were reported as “suspicious” [60].

Index of recent use of erythropoietic proteins

The Australian researchers found that after rHuEPO was discontinued, the reticulocyte count and serum EPO concentrations were depressed while the hematocrit remained increased [58]. From these three parameters they calculated an “off-score” and proposed that an increased “off-score” be used to identify athletes who had recently discontinued rHuEPO. The “off-score” was increased from day three to day 13 post-administration of rHuEPO. Although the statistical certainty that the “off-score” indicated recent use was quite high, the IOC expert committee did not accept the test largely because of the legal difficulty of proving that an athlete used rHuEPO on the basis of an indirect test. The “off-score” is used today to indicate which athletes should be followed closely with other testing.

Urine EPO concentrations by immunoassay

Attempts to detect abuse of erythropoietic proteins by urine immunoassay have not been successful. Since rHuEPO and endogenous urinary EPO cannot be distinguished by the available antibodies [61], a diagnostic test would depend on urinary EPO concentrations that are far above the normal range. In addition, <5% of the dose is excreted in urine [62] and urinary EPO concentrations are affected by pH, specific gravity, and exercise [63, 64]. Small increases in urinary EPO were detected by a immunoradiometric assay after large doses (200 U/kg every other day for 10 days) [61].

Direct tests for doping with erythropoietic proteins

The isoelectric focusing test for urinary rHuEPO

A method for detecting rHuEPO in urine by electrophoresis was first described in 1995 [65]. Although this test had practical limitations, it demonstrated conclusively that the isoform pattern of urinary endogenous EPO differs from the pattern of urinary rHuEPO, and it was the first successful attempt to develop a direct test for urinary rHuEPO. Similarly in 2002, Skibeli et al. [66] isolated EPO from human serum and showed, using gel electrophoresis, that endogenous and recombinant EPO differed.

A significant improvement in practical detection occurred in when Lasne and de Ceaurriz [67] described a method for detecting rHuEPO in urine based on isoelectric focusing with immunoblotting plus one novel and critical step: a second blot ("double-blotting") [68]. After the isoforms of rHuEPO are separated by isoelectric focusing, the first blot is performed, then the membrane containing the transferred proteins is incubated with anti-EPO antibody. The second blot transfers only the anti-EPO antibodies to a second membrane, and the second membrane is incubated with a second antibody directed against the first antibody. This step markedly reduces non-specific binding and yields clear isoform patterns. After the second antibody is incubated with streptavidin-horseradish peroxidase and substrate, the emitted chemiluminescence is captured to produce an image of the gel [69].

This method shows that endogenous EPO is highly heterogeneous and is composed of a number of isoforms. Most of these isoforms are not present in the recombinant hormones. After administration of rHuEPO or darbepoetin alfa (a new erythropoeisis-stimulating protein with a longer half-life than rHuEPO), a striking change in urinary EPO pattern is observed with the appearance of new isoforms corresponding to the excretion of the injected drugs and, after sufficient doses, the disappearance of the endogenous isoforms. By analyzing the isoelectric pattern it is possible to determine if the excreted EPO is of natural or recombinant origin. Figure 1 is an electropherogram showing the band pattern of darbepoetin alfa in a urine obtained from a patient who was treated with darbepoetin alfa.

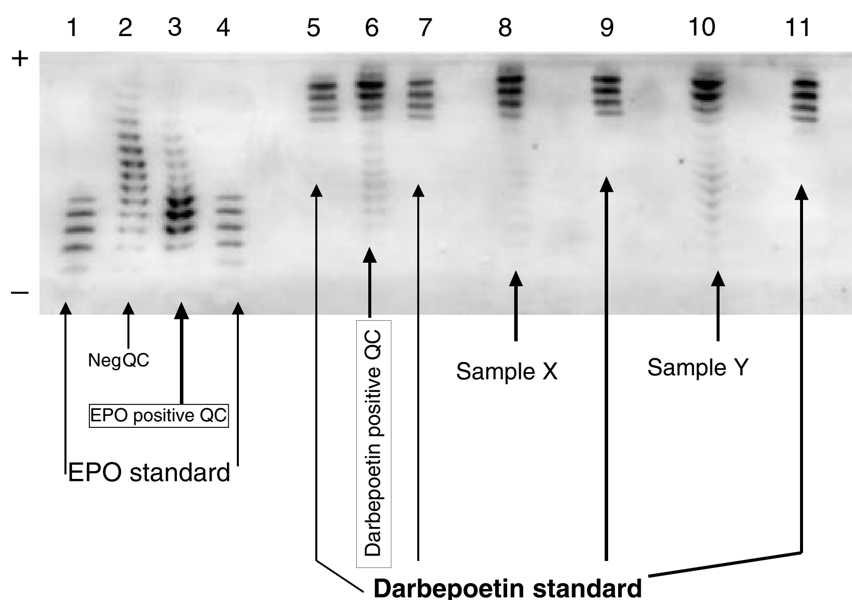

Figure 1. Electropherogram of rHuEPO and darbepoetin alfa standards; of extracts of urine obtained from drug-free control volunteers and patients treated with rHuEPO or darbepoetin alfa; and of extracts of urine from unknown individuals. The anode and cathode sides of the electropherogram are labeled + and –, respectively. The rHuEPO standard is in lane one and four and the darbepoetin standard is in lanes five, seven, nine, and 11. The negative quality control sample (lane two) is an extract of urine from drug-free volunteers showing the normal pattern of endogenous EPO. The EPO positive quality control sample (lane three) and darbepoetin positive quality control (lane six) are urine extracts obtained after the administration of rHuEPO or darbepoetin alfa, respectively. Samples X and Y are urine extracts from two unknown individuals (lanes eight and 10). The bands of endogenous urinary EPO are faint or absent in lanes six, eight, and 10.

Despite the fact that isoelectric focusing is technically demanding and may take up to three days to complete, it has generated a great deal of interest because it is a direct test that can yield conclusive results. Further, the results of the test and the underlying method have been accepted by CAS [22]. Presently, at least six IOC-accredited laboratories have implemented the test and several more are working on the method. Investigators are working on means to improve the test, to expand its use to all 25 IOC-accredited laboratories, and to further define criteria for determining if rHuEPO is present. In addition, investigators are working on ways to detect erythropoietic proteins with mass spectrometry.

Darbepoetin alfa detected at Winter Olympic Games of Salt Lake, 2002

As the IOC-accredited laboratory designated to perform the testing at the Games of Salt Lake, our laboratory began implementing the isoelectric-focusing technique for detecting erythropoietic proteins about eight months before

the Games. Since darbepoetin alfa was likely to be approved for marketing before the Games, we were aware that athletes might have access to it at the time of the Games. Accordingly, we obtained a urine sample from a patient being treated with darbepoetin alfa. As shown in Figure 1, the electropherogram of this urine sample confirmed [70] that the isoforms of darbepoetin alfa were more acidic than those of rHuEPO [71] because the former migrated to the more acidic region of the gel. This sample was important because IOC laboratory regulations require that analyses include a reference standard, a known positive quality control urine sample, and a known negative quality control urine sample. The details of the method and the main finding have been described [69, 70].

During the Games of Salt Lake, our laboratory analyzed 598 urine samples for the drugs on the IOC List of Prohibited Substances. In addition, 1,222 blood samples were obtained from athletes competing in the endurance sports of cross-country skiing, biathlon, Nordic combined, long-track speed skating, and short-track speed skating. These blood samples were tested at the venues by international sport federations before competition for hemoglobin concentrations and reticulocyte counts. If the reticulocyte count exceeded 2% or if the hemoglobin concentration exceeded 16.5 g% for women or 17.5 g% for men, the athlete returned after competition and an additional blood and urine samples were obtained for EPO analysis at the laboratory. Of the 1,222 blood samples, 133 (10.6%) had reticulocyte counts >2% and eight samples (0.6%) had increased hemoglobin concentrations [72]. In total, the laboratory received 77 combined blood and urine samples and of these, samples from three athletes met our criteria for darbepoetin alfa use [72]. All three athletes were cross-country skiers who had won a total of eight medals.

According to the procedure in effect at these Olympics, before announcing test results to the public, a committee reviews the laboratory findings and two hearings are held. The hearings, which are attended by the representatives of the athlete with or without the athlete, delves into the details of the testing. If the testing results are upheld, the IOC Executive Board reviews the case and makes the final decision to announce the findings to the public. In addition, the athlete has the right to appeal the IOC decision to CAS. As there is little to lose, most athletes do appeal to CAS. The darbepoetin alfa cases were be adjudicated by CAS in late 2002 and early 2003.

Epoetin alfa and darbepoetin alfa detection studies

Because athletes do not reveal their doses or dosing regimen, the only experimental approach to determine how to test for the occurrence of doping is to administer likely doses and analyze the urine at various times after the last dose. The early work of Wide et al. [65] established that their gel electrophoresis assay detected urinary rHuEPO for up to 24 hours after administering rHuEPO at 60 U/kg/week for seven to nine weeks.

We have used the isolectric focusing method [68–70] to determine the approximate time-course of detection of epoetin alfa in urine. We gave

150 U/kg/week to 15 healthy young volunteers for three weeks. If the hematocrit exceeded 48%, the next dose was withheld. After discontinuing epoetin alfa, all urine samples were positive by isoelectric focusing for two days and approximately 50% were positive for four days.

We have also determined that darbepoetin alfa is detectable in urine for up to 12 days. A modest single dose (0.40 µg/kg) was detected in eight of nine subjects for two to four days post-administration and in some subjects for up to 12 days post-administration. Thus, the detectability of darbepoetin alfa appears to be similar or longer than that of rHuEPO, which is consistent with darbepoetin alfa's longer serum half-life [73].

One limitation of detection-time investigations is that they do not necessarily mimic the dosage regimens used by athletes. Typically athletes become aware of the retrospectivity of a test and adjust their doping schedules so as to evade detection. In the case of a rapidly evolving new test, like isoelectric focusing for urinary rHuEPO, athletes are undoubtedly searching for ways to foil tests as diligently as laboratories are working on improvements to the tests. Perhaps the athletes who were found to be using darbepoetin alfa at the 2002 Games of Salt Lake did not expect the test to detect it.

Retrospectivity of the urinary isoelectric focusing test

The ability of isoelectric focusing to control the abuse of erythropoietic proteins among athletes is a complex function of dose, dosing regimen, detection times, urine collection time relative to last dose, and whether or not the test is announced in advance or a surprise (short-notice or out-of-competition testing). Sport administrators control the testing time and it is generally agreed that unannounced short-notice testing is the most effective deterrent. The athlete controls the dose and dosing interval, and the ability of the test to detect erythropoietic proteins is related to the urinary pharmacokinetics of the protein and the inherent sensitivity of the test. A good test is one that can detect the erythropoietic proteins for as long as the beneficial effects of the drug are present. The interplay of these factors is only beginning to be explored.

The pharmacodynamic effects of erythropoietic proteins that are particularly pertinent in the context of detecting users and that have been studied in healthy subjects, as opposed to patients with anemia, are the effects on hematocrit, hemoglobin concentration, and $VO_{2\,max}$. The effect on $VO_{2\,max}$ is the most relevant because it is a direct measure of performance. rHuEPO administered at doses of 150 to 230 U/kg/week for approximately three weeks produces an increase of $VO_{2\,max}$ of 6% to 8%, as expected [39–41]. Only two studies have monitored $VO_{2\,max}$ after rHuEPO was discontinued. In the first study, rHuEPO was administered for 25 days (150 U/kg/week), and four weeks after the last dose, the $VO_{2\,max}$ determined was not distinguishable from the $VO_{2\,max}$ of the placebo group [74]. It has been suggested that athletes may use a low-dose maintenance regimen that is sufficient to maintain the hematocrit just beneath the 50% health test threshold [40, 67]. To explore the effects of a low-dose regimen on $VO_{2\,max}$, Russell et al. [40] administered rHuEPO for three

weeks at a dose of 150 U/kg/week, then decreased the dose to 60 U/kg/week for an additional five weeks. They determined that the $VO_{2\,max}$ in week 12, four weeks after the last dose, was still increased (3.1% to 4.5%). The hematocrit remained increased for approximately 17 days after the last dose. Although these results need to be confirmed, the data on low doses of rHuEPO suggest that for a urine test for rHuEPO to be effective, it should be capable of detecting rHuEPO for three to four weeks after discontinuing a dose of 60 U/kg/week. Such data are not available.

Another approach to the retrospectivity issue is to assume that the effect on $VO_{2\,max}$ lasts as long as the hematocrit or hemoglobin concentration remain increased. While this assumption has not been directly investigated, it is reasonable to assume that the duration of action on $VO_{2\,max}$ will correlate with hematocrit and hemoglobin concentration. In the three studies [40, 41, 74] that measured hematocrits after rHuEPO was discontinued, a fixed dose of rHuEPO (80–230 U/kg/week) was administered for three to five weeks. During the rHuEPO administration phase, the hematocrit steadily increased until a plateau of approximately 50% was reached at 12 to 14 days. The hematocrit-time graphs of these studies show that the hematocrit is unchanged (approximately 50%) for 12 to 20 days after rHuEPO is discontinued. The hematocrit was last measured on days 24, 28, and 30 post-administration, and for each study the hematocrit was still greater than that of the placebo group. Thus performance as measured by $VO_{2\,max}$ and the hematocrit appear to follow more or less parallel time-courses, again consistent with three to four weeks of enhancement after the last dose. An athlete could be enhanced and yet have a negative urinary isoelectric focusing test.

Other erythropoietins

Athletes will experiment with other erythropoietic proteins as they become available. If laboratories are to keep up with this fast-moving field, they need detailed information on the new products, a reference standard for the product, and at a minimum, a urine sample collected from a subject known to be receiving the substance. Ideally, the laboratories would administer the substance to volunteers to obtain data on the isoform patterns and the pharmacokinetics of detection. These requirements are not easy to fulfill, particularly in the United States where ethics committees generally require that the drug be available as Food and Drug Administration (FDA)-approved product. If not, the investigator must file an application for a Investigational New Drug, a lengthy, complicated, and expensive process. Alternatively, the investigator may collaborate with colleagues who are working under an FDA-approved protocol.

Epoetin alfa, epoetin beta, and darbepoetin alfa

The current isoelectric focusing test [67] is able to detect administration of epoetin alfa, epoetin beta, and darbepoetin alfa [69, 70], allowing sport to con-

trol these erythropoietic proteins provided that the urine is collected when sufficient amounts of the material are present. More work is needed on the time-course of detection after various doses have been administered. A few studies are underway.

Epoetin omega

Epoetin omega has been administered to patients with anemia receiving hemodialysis [75] and some information is available on its structure [76]. Epoetin omega is produced in baby hamster kidney cell cultures and is less acidic than epoetin alfa or epoetin beta. In the current isoelectric focusing assay, a reference standard of epoetin omega migrates to the most basic area of the electropherogram. We recently analyzed a urine sample with isoforms consistent with epoetin omega, suggesting that it is being used by some athletes.

Epoetin delta

We are following the development of epoetin delta (dynepo), produced by human cells and being developed by Transkaryotic Therapies, Inc. Until we can obtain a reference standard of epoetin delta and a known positive urine sample, we will not know whether or not the existing isoelectric focusing assay will be able to detect it. Detection will depend in large part on how the manufacturer selects the final glycoprotein fractions. We anticipate that the band pattern will be basic, like epoetin alfa, and therefore detectable in the current assay.

Generics

Proprietary DNA vector technology has been used to develop a generic epoetin alfa [77]. Undoubtedly, new erythropoietic proteins are under development and products that are similar to epoetin alfa and epoetin beta are being produced outside the conventional pharmaceutical industry.

EPO *gene manipulation*

International sport organizations are mindful that gene manipulation might be used in the future to produce athletes with exceptional characteristics. At the first meeting of the IOC Gene Therapy Working Group, gene therapy was defined such that it would not include enhancement of athletes' performance ("…transfer of genetic material to human somatic cells for the treatment or prevention of disease or disorders") [78]. The group opined that gene manipulation is not on the immediate horizon, but that methods such as proteomic analysis might be developed to be prepared when gene-based doping becomes a reality.

Summary

The inherent complexity of the topic stems from interface between human behavior and the many disciplines that have something to offer, including chemistry, pharmacology, medicine, law, ethics, and sociology. Ultimately if sport continues to rely on testing as the solution, it is necessary to focus resources on physical and chemical methods for detecting drugs. At the same time, sport hopes to change its culture through education and espousing ethical positions.

Doping with erythropoietic proteins, the most recalcitrant and threatening problem for sport in recent years, is coming under control. The problem is threatening because the drugs are highly efficacious and they enhance performance in endurance sports and, perhaps, even sports that rely on short bursts of energy. The problem is recalcitrant because the methods to detect erythropoietic proteins in body fluids are complex, therefore global implementation is all the more difficult, and it is difficult to track athletes in their travels and implement collection procedures wherever they may be.

The current test is adequate from the perspective of unambiguous identification and legal defensibility, and it will no doubt improve; however, it is probably not sensitive enough to detect recombinant erythropoietic proteins for as long as performance enhancement will last. Fortunately such a degree of sensitivity is not required to contain the overall problem: The current test simply needs to be applied widely enough in short notice, out-of-competition testing.

Acknowledgements
We are grateful for financial support from the United States Anti-Doping Agency and the International Olympic Committee for studies on erythropoietic proteins, to Amgen Inc. for generously providing epoetin alfa and darbepoetin alfa, and to Steve Elliott, PhD, for his advice on the interpretation of isoelectric focusing analysis results and related matters. A. Breidbach led the research group that performed the isoelectric focusing analysis for the figure.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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Antibodies to endogenous and recombinant erythropoietin

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Introduction

Owing to the very few cases of anti-erythropoietin (EPO) antibodies arising in patients treated with recombinant human EPO (rHuEPO) before 1998, it was generally believed that EPO is weakly immunogenic. Several recent reports, however, suggest that this theory may be wrong. Indeed, low amounts of rHuEPO are immunogenic in animals such as the mouse, rat, rabbit, or horse, despite the high degree of sequence identity of the mammalian EPO molecules. Interestingly, in most cases injection of rHuEPO to these animals not only leads to production of antibodies that recognize the injected rHuEPO but also cross react with the endogenous EPO molecule and neutralize it, leading to pronounced anemia in rHuEPO-treated animals [1, and our own unpublished data]. A hybrid molecule linking recombinant human granulocyte-macrophage colony-stimulating factor (rHuGM-CSF) and EPO has been administrated to rhesus monkeys. This treatment induced a neutralizing immune response to EPO, whereas no antibodies against GM-CSF were detected. Again, the anti-EPO antibodies cross reacted with monkey EPO and induced a pronounced anemia [2]. Thus, the low incidence of neutralizing anti-EPO antibodies among rHuEPO-treated patients is most likely due to the good quality of the available rHuEPO preparations rather than to a low antigenic property of the molecule itself. rHuEPO appears to differ from endogenous human EPO only at the level of glycosylation. This high similarity explains why rHuEPO treatment exhibits a high degree of both efficiency and safety.

During the last four years, however, a large number of cases of pure red cell aplasia due to neutralizing anti-EPO antibodies have been reported in patients treated with rHuEPO for anemia associated with renal failure of various etiologies [3]. In this review, we address several questions:

- What is the incidence of anti-EPO antibodies?
- What are the characteristics of these antibodies?

- Does treatment with rHuEPO induce non-neutralizing anti-EPO antibodies?
- What are the most efficient assays to detect antibodies and when should a treating physician require such assays?
- What are the physician's options for patients found to have neutralizing anti-EPO antibodies?
- What could be the reasons for the sudden appearance of pure red cell aplasia due to anti-EPO antibodies?

Structure of EPO and rHuEPO

Endogenous EPO is a glycoprotein of 165 amino acids. The full molecular mass is approximately 30 Kd, with the average molecular mass of the protein moiety approximately 18 Kd and the remaining mass attributed to the carbohydrates. The carbohydrates are linked to three asparagine residues (Asn24, Asn38, Asn83) and one serine residue (Ser126). Although sugars are not involved in EPO receptor (EPOR) binding or activation and are not necessary for *in vitro* biologic activity [4, 5], the deglycosylated molecule is biologically inactive *in vivo* because of its rapid clearance. Moreover, the deglycosylated molecule exhibits a rather high tendency to aggregate. Solubility of unglycosylated EPO can be increased by changing the asparagine residues bearing carbohydrates to charged amino acids, such as lysine. Such a modified form of EPO has been co-crystallized with the extracellular domain of EPOR [6]. The EPO molecule stacks in four alpha helices connected by less structured loops. (See Chapter 3 for more information.) Two disulfide bridges stabilize this ternary structure. One disulfide bridge (Cys7-Cys161) connects the *N*- and *C*-terminal regions of the molecule and the other (Cys29-Cys33) forms a small bridge inside the loop connecting the two first helices. Both these disulfide bonds are absolutely required for biologic activity. Careful analysis of circulating endogenous EPO and of rHuEPO has shown that the protein part of the molecules was identical, but that the glycosylation pattern could be different. Indeed, all the currently commercially available forms of rHuEPO (epoetin alpha, epoetin beta, epoetin omega) have more branched oligosaccharides like tetra-antennary structures that seem to be lacking in endogenous EPO. Most of these structures are fully sialylated, and rHuEPO exhibits more acidic forms than endogenous EPO. A high proportion of these tetra-antennary structures is present on the epoetin beta molecule and to a less extent on the epoetin alpha and epoetin omega molecules. Since both epoetin alpha and epoetin beta are produced in Chinese hamster ovary (CHO) cells, the difference in carbohydrate composition between these two molecules is most likely due to differences in the purification process. Epoetin omega, which is produced in baby hamster kidney (BHK) cells, exhibits roughly the same glycosylation pattern as epoetin alpha [7].

Methods for the detection of anti-EPO antibodies

Detection of anti-EPO antibodies can be achieved using biological or biochemical tests. Biological tests detect only antibodies able to neutralize the activity of EPO. Normal erythroid progenitors or EPO-sensitive cell lines are cultured in the presence of serum to be tested and inhibition of cell proliferation is recorded [8]. Typical results using the UT-7 cell line are shown in Figure 1. The sensitivity of these tests is rather good, and antibodies able to neutralize less than 500 mU EPO/mL serum are easily detectable. Their specificity can be tested by increasing EPO concentrations that must relieve proliferation inhibition. Some EPO-sensitive cell lines, such as UT-7, are also sensitive to other growth factors like GM-CSF [9] that can be used to control the specificity of the inhibition. Anti-EPOR antibodies could also inhibit cell proliferation with the same characteristics, although such antibodies have not been reported to date. Other inhibitory molecules present in the serum to be tested could also inhibit cell proliferation. Although they can be easily distinguished from anti-EPO antibodies, they could strongly decrease the efficiency of these tests. This method presents other limitations: it requires a good expertise in the use of these growth factor-sensitive cell lines, requires several days (up to 14 days when using normal erythroid progenitors), and only neutralizing antibodies can be detected. For these reasons, biological tests are used to

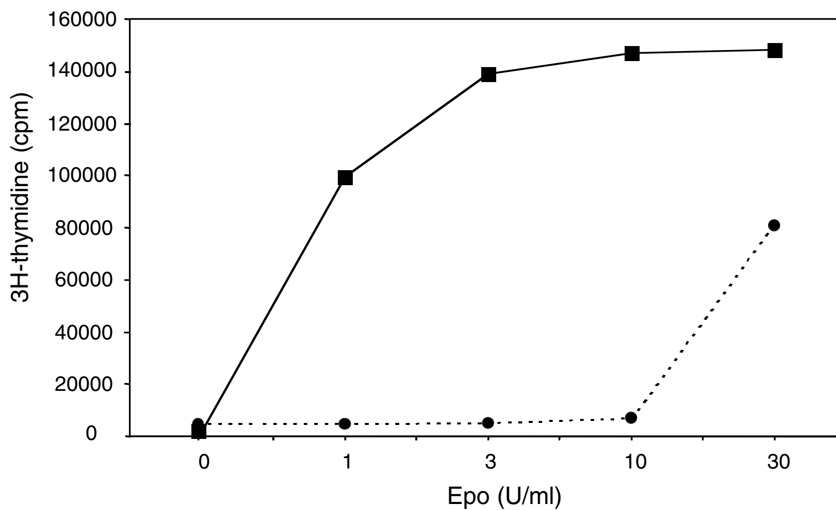

Figure 1. Biologic assay for the evidence of anti-EPO neutralizing antibodies. EPO-dependent UT-7 cells were incubated for 48 hours with increasing concentrations of EPO in the presence of 10% control serum (squares) or 10% serum from patient with pure red cell aplasia due to anti-EPO antibodies (circles). Incubation was done in 96 well plates (total volume 100 µL, 5,000 cells seeded per well). The cells were labeled with 1 µCi ^{3}H-thymidine during the last four hours of incubation.

characterize an essential property of anti-EPO antibodies – their ability to inhibit EPO biological activity, rather than to screen the presence of anti-EPO antibodies in blood samples. Such screenings are realized by biochemical methods that are based either on ELISA tests or on the precipitation of radiolabeled EPO. Several groups have developed anti-EPO enzyme-linked immunosorbent assay (ELISA) [10, 11]. In these assays, polystyrene microplates are coated with EPO. After incubation with the serum to be tested, bound IgG are detected by enzymes linked to anti-human immunoglobulins. The sensitivity of these assays has not been reported; however, the signal-to-noise ratio seems to never exceed five, suggesting that the presence of antibodies could sometimes be difficult to assess. The main limitation of these assays seems to be the non-specific binding of human immunoglobulins. Methods using radiolabeled EPO are highly sensitive [3, 8, 12]. In these assays, ^{125}I-EPO is incubated with serum to be tested, and IgG-bound ^{125}I-EPO is precipitated using protein A or protein G covalently bound to agarose beads (Fig. 2). Antibodies able to bind 50 mU EPO/mL serum are easily detectable.

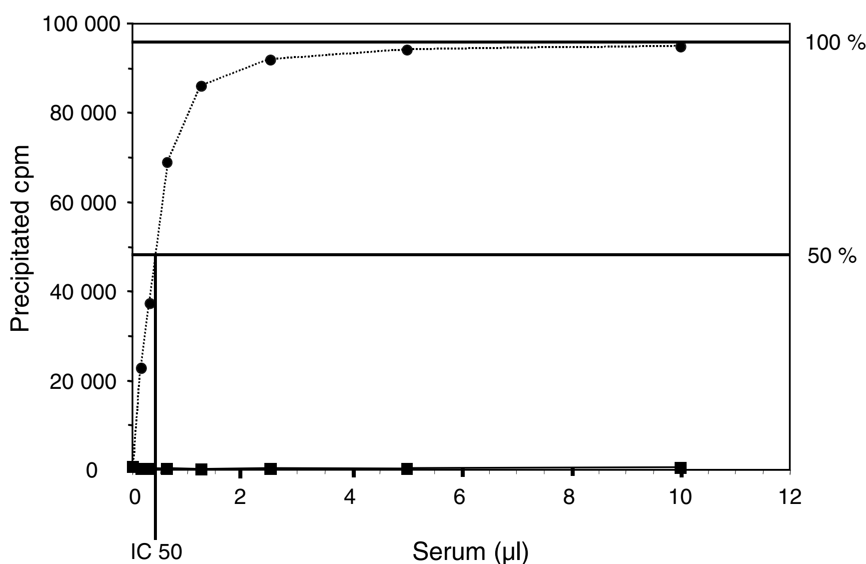

Figure 2. Precipitation of ^{125}I-EPO by serum from a patient with pure red cell aplasia due to anti-EPO neutralizing antibodies. ^{125}I-EPO (120,000 cpm corresponding to 1 ng) of EPO was incubated overnight with increasing amounts of serum from a patient with pure red cell aplasia (circles) or with a pool of serum from healthy volunteers (squares). Total incubation volume was 200 μL. 50 μL protein G Sepharose beads were added and the incubation was continued for two hours with shaking. The beads were washed three times with Tris buffer saline (10 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Tween 20, 0.02% sodium azide, and 0.1% bovine serum albumin. The radioactivity of the beads was then measured and the amount of serum required to bind 500 pg of EPO was determined (IC 50). This value was used to estimate the binding capacity of the patient serum (125 U/mL for this patient).

The assay is highly reproducible and it allows a quantitative measurement of the antibody concentration (Garaud et al., submitted). In its simple form, the assay essentially detects IgG class antibodies but other immunoglobulin classes can be detected either by adding IgG molecules that recognize other human immunoglobulin classes, such as IgM or IgA to insoluble protein A or protein G or by using specific antibodies against anti-human classes or sub-classes immunoglobulins covalently bound to agarose beads. The latter method allows the determination of class and sub-class of anti-EPO antibodies.

Presence of anti-EPO antibodies

Very few anti-EPO antibody cases have been reported in patients who have never been treated with rHuEPO. Linardaki et al. have shown the presence of anti-EPO antibodies in two patients with pure red cell aplasia as an initial manifestation of systemic lupus erythematosus [13]. Peschle et al. [14] and Casadevall et al. [8] reported the spontaneous appearance of anti-EPO antibodies in three patients. One of these patients also harbored a thymoma, but no pathologies were detectable in the other two patients. In each case, the antibodies induced pure red cell aplasia. The antibodies have been extensively characterized in the study of Casadevall et al. [8]. Their affinity was very high (Kd of 430 pM), similar to the affinity of EPO for EPOR. The EPO binding capacity of the patient serum was 2.7 U/mL serum. The high-affinity and the high-binding capacity allowed the neutralization of all circulating EPO. The antibodies were directed against a conformational epitope present in the protein part of the molecule.

The antibodies could not be thoroughly characterized in the study of Peschle et al. due to the lack of available pure EPO at the time. Anti-EPO antibodies disappared spontaneously [8] or as pure red cell aplasia resolved after immunosuppressive treatment [14, 15]. Nevertheless, the spontaneous appearance of such antibodies seems to be very rare and no other cases have been documented to date. Since these antibodies induce severe pure red cell aplasia, it is unlikely that other similar cases would have remained unrecognized. The appearance of non-neutralizing anti-EPO antibodies cannot be ruled out (see below), but seems impossible to be detected with current assays.

The induction of neutralizing anti-EPO antibodies in patients treated with rHuEPO was very low up to 1998. Indeed, during the first 10 years of rHuEPO use, only three cases of such antibodies were reported [12, 16, 17]. In each case, these antibodies induced profound anemia. The three patients were treated with rHuEPO for anemia associated with renal failure. Thus, it can be concluded that rHuEPO is extremely well tolerated and has induced only a very few cases of neutralizing antibodies causing PRCA. Some reports, however, suggest that rHuEPO could induce non-neutralizing anti-EPO antibodies with a high frequency since such antibodies were detected in up to 67% of rHuEPO-treated patients in one study. These antibodies have been detected by ELISA

using large volumes of serum because of their presumed low affinity for EPO. These antibodies have not been carefully characterized and neither their affinity for EPO nor the part of the molecule recognized by these antibodies have been reported. We have tested much serum from asymptomatic rHuEPO-treated patients by immunoprecipitation of ^{125}I-EPO and we have never detected such antibodies. Since this assay required extensive washing of the immunoprecipitates, we cannot exclude that low-affinity antibodies with a high dissociation rate could have been lost during the washing procedure in our assays [10]. Interestingly, Pazur et al. reported the induction of antibodies against the carbohydrate chains of rHuEPO in rHuEPO-immunized rabbits [18]. Neither the affinity of these antibodies for EPO nor their ability to neutralize the biological activity of EPO have been reported. Since glycosylated carbohydrates are not involved in the binding or activation of EPOR, however, it is likely that such antibodies did not inhibit EPO biological activity. Moreover, since rHuEPO and endogenous EPO differ at the level of glycosylation, it is tempting to speculate that the low-affinity antibodies detected with high frequency by Castelli et al. could be directed against the glycosylated carbohydrate moiety of rHuEPO. Whether these antibodies, if any, decrease the efficiency of rHuEPO treatment remains to be tested, but the low number of patients with neutralizing antibodies reported up to 1998 make rHuEPO one of the most successful therapeutic application of recombinant DNA technology.

The situation suddenly changed after 1998. At the time of writing this chapter, more than 160 cases of pure red cell aplasia have been reported in patients treated with rHuEPO for anemia associated with chronic renal failure. Moreover, a study from the Food and Drug Administration (FDA) revealed an exponential growth of these cases during the studied period (July 1997 to December 2001) [19]. We have had the opportunity to assay for the presence of anti-EPO antibodies in several of these cases of pure red cell aplasia, and we found that most were due to anti-EPO neutralizing antibodies [3]. Most of these antibodies were very similar to those that we have previously described in a patient who never received rHuEPO [8] with high titer (EPO-binding capacities ranging from three to 125 EPO units/mL serum) and high affinity (Kd ranging from 90 to 400 pM). The antibodies recognized the proteinic part of the molecule (Fig. 3). Except for one patient, denatured EPO molecules were no longer recognized by these antibodies, demonstrating that they were directed against conformational epitopes. Most of these antibodies were developed in patients treated with Eprex brand of epoetin alfa (see below), but the antibodies recognized all forms of rHuEPO, as well as the erythropoietic protein darbepoetin alfa, which is not a form of rHuEPO.

After evidence of neutralizing antibodies, rHuEPO was immediately withdrawn and the patients received red blood cell transfusions. Depending on their general status, most patients also received an immunosuppressive therapy (intravenous immunoglobulins, corticosteroids, cyclosporine, or cyclophosphamides). The antibody titers decreased at various rates. In some patients, the antibodies are no longer detectable and these patients have recovered some red

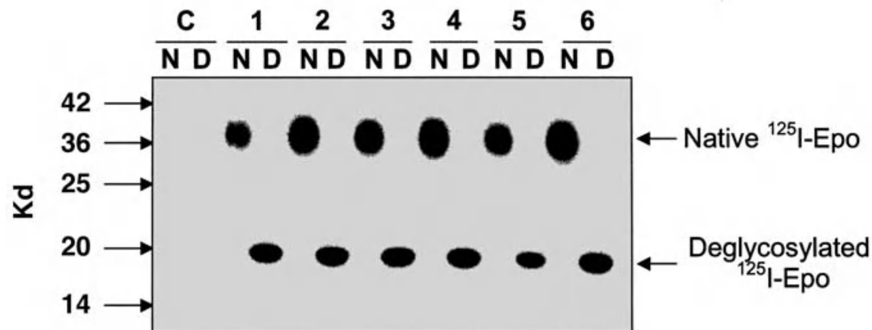

Figure 3. Anti-EPO neutralizing antibodies recognized the protein part of the EPO molecule. ^{125}I-EPO was sequentially deglycosylated by incubating for one hour at 37 °C with *Arthrobacter ureafaciens* neuraminidase and for 18 hours with a mixture of O-glycosidase, endoglycosidase F and N-glycosidase F (all glycohydrolases were from Roche, Mannheim, Germany). Native (N) and deglycosylated (D) ^{125}I-EPO were then immunoprecipitated with serum from six patients with pure red cell aplasia due to neutralizing anti-EPO antibodies or control serum (C). Immunoprecipitates were then analyzed by polyacrylamide gel electrophoresis and autoradiography.

blood cell production, limited by their disease status. None was treated with rHuEPO again. The number of studied cases is still too low to draw any reliable conclusions concerning the efficiency of the various immunosuppressive therapies. Clearly, it is of the utmost importance not to challenge the antibody-inducing rHuEPO by the use of another type of rHuEPO including darbepoetin alfa, since all antibodies that we have tested cross-reacted with all available erythropoietic proteins. Theoretically, all erythropoietic proteins should be able to boost antibody production in patients. An intriguing observation is that anti-EPO antibodies disappeared in several patients and that these patients then recovered red blood cell production, thus showing that these patients produced endogenous EPO. The disappearance of antibodies seems to be hastened in patients treated with immunosuppressive therapy, but antibodies also disappeared in some untreated patients. Thus, it appears that endogenous EPO production is unable to boost antibody production. Whether this is due to the properties of endogenous EPO or to the low amounts of endogenous molecules produced in patients with chronic renal failure is unknown. We did not succeed in measuring EPO concentration in serum samples containing anti-EPO antibodies. Nevertheless, we also observed the spontaneous disappearance of anti-EPO antibodies in the single patient who did not have chronic renal failure and was not treated with rHuEPO. In contrast to patients with chronic renal failure, patients without chronic renal failure should not have decreased production of endogenous EPO. EPO production was probably strongly increased due to anemia in this latter patient, suggesting that the spontaneous disappearance of anti-EPO antibodies is probably not due to the low concentrations of endogenous EPO produced in patients with chronic renal failure.

The reasons why such antibodies appeared remain unclear. Clearly, their appearance is due to treatment with rHuEPO. We obtained serum samples from five patients before the onset of anemia and we did not detect anti-EPO antibodies. Since, however, rHuEPO has been used safely during more than 10 years, these problems are not due to the molecule itself but to modifications concerning either the patient population, medical practice, or the commercial product. All patients developing anti-EPO neutralizing antibodies were treated with rHuEPO for anemia associated with chronic renal failure of various etiologies. They suddenly became refractory to rHuEPO treatment and developed anti-EPO antibodies after three to 63 months of rHuEPO treatment during which time they had a good response to the hormone. This pool of patients is heterogeneous for all the tested parameters, except that all were treated with rHuEPO for anemia associated with chronic renal failure. Careful examination of the patient's medical history did not find any peculiar new medication introduced before the appearance of the anti-EPO antibodies or a a viral infection. The patients did not have HLA A, B, or DR in common. Moreover, we could not find any evidence that this population of patients was different from those previously treated with rHuEPO, patients who did not develop such antibodies. Some of the patients who developed anti-EPO antibodies were previously treated successfully with rHuEPO for years before developing anti-EPO antibodies. The medical practices and especially those related to hemodialysis do not seem to be correlated with the appearance of these antibodies. Although all patients received rHuEPO subcutaneously, the route of injection, intravenous route *versus* subcutaneous route, cannot simply be given as an explanation. Indeed, the subcutaneous route introduced in 1991 has been widely used in France since that time without induction of anti-EPO antibodies before these cases were identified. We cannot, however, not exclude that the route of injection could favor the formation of antibodies. The most likely explanation would be that slight modifications of the commercial product is responsible for the induction of antibodies. Most patients (36/44 in the antibody cases detected in our laboratory and 78/82 patients with pure red cell aplasia in the FDA report) were treated with a brand of epoetin alfa (Eprex) when they developed antibodies. In our study, one patient received epoetin beta (NeoRecormon) only and seven patients received both Eprex and NeoRecormon. Thus, although we do not have definitive proof, we can hypothesize that subtle modifications concerning Eprex could be responsible for the appearance of antibodies. To date, we have no indication concerning the possible modifications of Eprex that could favor antibody development, but the appearance of antibody cases correlates with a modification of Eprex additives. Indeed, rHuEPO was formulated with human serum albumin before 1998. For public health reasons, human serum albumin was removed from Eprex preparations and replaced by other additives after 1998. The frequency of appearance of such antibodies remains relatively low compared with the number of patients treated with rHuEPO (one patient per 10,000 patients treated with rHuEPO develops pure red cell aplasia). Some characteristics of these patients combined

with Eprex modifications could possibly lead to the production of the antibodies. Lines of investigation such as linkage with a specific HLA subtype having been eliminated, and we must consider more subtle parameters such as possible polymorphisms of the EPO molecule itself. Theoretically, these polymorphisms could affect either the proteinic part of the EPO molecule itself (direct polymorphism of the *EPO* gene) or the glycohydrates of the EPO molecule (differences in the glycosylation process). The later, however, seems unlikely because antibodies are directed against the protein moiety of the molecule and because, as previously stated, Eprex seems to be more similar to endogenous EPO than NeoRecormon in terms of glycosylation. Clearly, the possible polymorphisms should be carefully examined since, if present, they could permit identification of a population that should not receive rHuEPO treatment.

rHuEPO is also used to correct anemia associated to other pathologies such as cancer, hematologic malignancy, or auto-immunodeficiency syndrome (AIDS), as well as for illegal blood doping in many sports. No anti-EPO antibodies have been detected to date in any patient or athlete treated with rHuEPO. A study on a limited number of patients with myelodysplatic syndromes, some treated and some not with rHuEPO, did not detect anti-EPO antibodies even in those with a low response to rHuEPO [20]. Two reasons could explain why these patients did not develop antibodies. First, in patients with cancer, hematologic malignancy, or AIDS, we can hypothesize that they have overall decreased immune responses with lower probability of developing antibodies. Moreover, these patients are usually treated for shorter periods with rHuEPO than are patients with chronic renal failure. The lack of a case of anti-EPO antibodies in athletes illegally using rHuEPO for blood doping is more of a problem of numbers of abusers, since the incidence of antibodies is low in the larger population of patients treated with rHuEPO. Nevertheless, the degree and duration of the anemia observed in patients developing antibodies, leading to long-lasting red blood cell transfusion, enforce the ban against the use of rHuEPO outside recognized medical indications.

When should a physician ask for an anti-EPO antibody test? Systematically testing all patients treated with rHuEPO is clearly unnecessary. Indeed, the sensitivity of the currently available tests is such that the patients should present objective symptoms before antibodies could be detected. The low percentage of patients developing anti-EPO antibodies would make this screening very expensive for a poor result, and except for the immediate halt to rHuEPO treatment, no clear therapy could be proposed to rapidly ameliorate antibody formation. Such testing, however, should be done rapidly in case of a sudden appearance of an aregenerative anemia without other classical cases of anemia (viral infection, bleeding, inflammation, iron deficiency). Moreover, antibody titer must be steadily determined to follow the efficiency of immunosuppressive treatment.

Conclusions

In conclusion, although antigenicity of EPO is probably high, the number of cases of rHuEPO-induced antibody formation remains very low compared with the very high number of patients treated with rHuEPO. Nevertheless, the sharp increase of such cases during the past few years shows that highly stringent quality control processes are absolutely required to keep this drug safe. Whether these cases will lead to the identification of a patient sub-population in which special care must be taken when prescribing rHuEPO is now the main question. If this question can be answered, significant good would have come from a regrettable situation.

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Erythropoietins and Erythropoiesis
ed. by G. Molineux, M.A. Foote, and S.G. Elliott
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New molecules and formulations of recombinant human erythropoietin

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Introduction

Recombinant human erythropoietin (rHuEPO), is a glycoprotein hormone commonly used for the treatment of anemia associated with chronic kidney disease [1–3]. It is also indicated for the treatment of anemia associated with cancer, human immunodeficiency syndrome virus (HIV) infection, and for use in surgical situations to reduce allogeneic blood transfusion requirements. (For further information see Chapters 9, 10, 11, and 12). A number of studies have shown that rHuEPO is well tolerated and effective at ameliorating anemia, restoring energy levels, and improving patient quality of life in these indications. The clinical benefits of rHuEPO are well understood and appreciated.

The endogenous EPO protein is naturally optimized for maintenance of hemostasis in the body where the protein can be produced on demand in the amounts needed. However, that which is optimal for natural *in vivo* production may be sub-optimal in the context of clinical intervention. For example, rHuEPO must be administered frequently by injection to be efficacious. The discomfort of injections and the inconvenience to the patient and healthcare provider burdens both groups.

Approaches to enhancing a drug's properties have included new formulations and delivery systems of the existing drugs whereby the circulating concentration of the drug is maintained for extended periods of time (sustained delivery). Such improvement can be accomplished in several ways, including use of pumps or slow-release formulations. Alternatively, proteins can be modified chemically with attached polymers that confer a longer half-life (sustained duration of action). Attempts to modify the protein itself by *in vitro* mutagenesis or through protein fusions to other peptides or protein hormones have been explored. Glycoengineering has been successfully applied to rHuEPO. With this process, new carbohydrate attachment points are introduced into the protein, increasing the amount of attached carbohydrate and increasing *in vivo* activity and serum half-life without substantially lowering *in vitro* activity. The new molecule can be administered with extended dosing intervals with no loss of efficacy.

The inherent limitations of rHuEPO due to its peptidic composition or mechanism of action can potentially be bypassed entirely with new molecular entities such as small molecules or antibodies (EPO mimetics). These compounds may have advantageous biological or chemical properties not present in rHuEPO, such as an oral route of delivery, (small molecule), or structural conformations that hold little in common with EPO, thus exploiting different routes of elimination. Finally, stimulation of erythropoiesis by mechanisms different from those of rHuEPO, such as those steps upstream or downstream from receptor activation step, have been attempted. This chapter discusses new molecules being considered or developed and their limitations, if any.

Considerations of new formulations and drug entities

The purpose of any therapeutic intervention is to treat the patient without causing harm, and rHuEPO has been particularly successful in this regard. The molecule is very effective at stimulating erythropoiesis with minimal side-effects. This excellent safety profile has created a high standard against which any new erythropoiesis-stimulating molecule will be measured. Not only should a new drug entity have improved properties, but it should also match the excellent safety profile of rHuEPO. The preferred properties of any new drug or formulation include retention or increase in efficacy and lack of new or unwanted side-effects or toxicities.

One particular concern is anti-EPO antibodies [4]. (See Chapter 14 for further information.) Antibody formation to new drug entities is important not only because the drug may lose efficacy, but also because such antibodies might cross-react with endogenously produced EPO resulting in pure red cell aplasia, a very serious and severe form of anemia. Potential causes for antibody formation not only include the structure of the molecule itself, but also the breakdown products or aggregates generated during manufacturing or storage. Accordingly, new drugs should be designed and manufacturing procedures put in place to minimize this risk.

EPO molecules with altered activity

One approach to increase activity of EPO is to alter the interaction with the EPO receptor (EPOR). EPO activates erythroid precursor cells by binding and activating EPOR on the surface of erythroid progenitor cells [5]. (See Chapters 3 and 5 for further discussion.) Receptor activation occurs through homodimerization, whereby the two EPOR binding sites on a single EPO molecule crosslink two EPOR [6]. The two binding sites on rHuEPO have different affinities, high (approximately 1 nM) and low (approximately 10 μM) [7]. Initial binding is to the high-affinity binding site, followed by homodimerization of the receptor by binding to the low-affinity binding site [8].

Molecules that alter or increase affinity at either of the two sites can have increased activity.

Despite numerous attempts to identify them, no erythropoietic molecules suitable for clinical development have been reported that increase biological effect as a consequence of increased receptor affinity. The reasons are severalfold. First is the theoretical concern that antibodies, were they to develop in patients, may be targeted to the changed region of the molecule and would be neutralizing because they would interfere with the EPO:EPOR interaction. Should these antibodies cross-react with endogenous EPO, pure red cell aplasia would likely result. A second reason is that increased affinity does not always translate into increased *in vivo* activity because there are additional requirements beyond receptor binding required to effect a biologic response *in vivo*. Each receptor-binding event is transient because EPO:EPOR complexes are rapidly internalized and degraded [9, 10]. In addition, the EPOR-signaling pathway is down-modulated shortly after activation [10–12]. Thus, *in vivo* erythropoiesis requires continuous stimulation of multiple EPOR through multiple binding events. As a consequence, molecules cleared quickly have low *in vivo* activity. For example, increased *in vitro* activity and increased receptor affinity have been observed with EPO analogs that remove sialic acid from carbohydrates or remove *N*-linked carbohydrates entirely [13, 14]; however, these molecules have reduced *in vivo* activity due to a more rapid clearance [13, 15]. Increased concentrations can partially compensate for the increased clearance; however, these compounds must be administered more frequently to be fully efficacious.

Changes in rHuEPO amino acid sequence can result in increased stability. These changes can include removal of amino acids that are unstable (tryptophan [16]); or are subject to oxidation (methionine), deamidation (asparagine), or changes that confer increased conformational stability, such as those that stabilize alpha helices or connecting loops. Such molecules may be more amenable to long-term storage or suitable for formulations where more stable EPO molecules are essential, such as in slow-release formulations or automated delivery systems. Removal of proteolytic cleavage sites by *in vitro* mutagenesis can enhance *in vivo* stability. It may be possible to remove antigenic sites, thereby reducing immunogenicity.

Compounds that bind through the high-affinity site but do not dimerize because of reduced binding at the low-affinity site can function as antagonists [8]. Such molecules may have some clinical utility for treatment of EPO responsive polycythemias, and several such molecules have been described [8, 17].

Molecules with increased serum half-life

A longer duration of action can allow for reduced frequency of administration. One approach that has been successfully applied to rHuEPO is glycoengineer-

ing [18]. Glycoengineered molecules bind and activate EPOR in the same manner as rHuEPO, resulting in similar biological responses while at the same time reducing clearance and enhancing activity [19]. Other strategies to increase duration of action of EPO included chemical modifications, such as the addition of a polyethylene glycol molecule (pegylation) or gene fusions between EPO and other proteins. In these cases, the goal is to reduce clearance rate by increasing hydrodynamic size.

Glycoengineering

rHuEPO is a glycoprotein hormone consisting of approximately 40% carbohydrate [20]. The carbohydrate component consists of three *N*-linked carbohydrates attached to Asp at amino acid positions 24, 38, and 83, and an *O*-linked carbohydrate attached to Ser at amino acid position 126 [20] (Fig. 1). Unlike the invariant protein sequence, the carbohydrate is variable in structure, resulting in glycoforms with modest differences in sizes, structures, and sugar content [22, 23]. A typical *N*-linked carbohydrate made by mammalian cells is

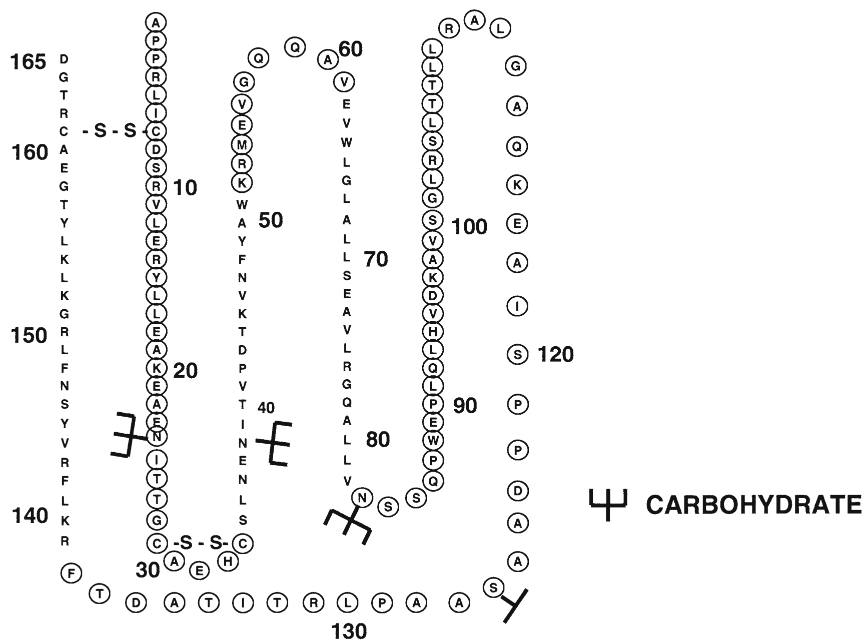

Figure 1. Amino acid sequence of human erythropoietin. Recombinant human erythropoietin (rHuEPO) is 165 amino acids in length [21]. Disulfide bonds (-S-S-) join Cys7 to Cys161 and Cys29 to Cys33. The 3 *N*-linked glycosylation attachment points are at Asn24, Asn38, and Asn83 and the *O*-linked carbohydrate is attached to Ser126. Forked structures schematically depict the attached carbohydrates.

branched with two to four arms. The end of each arm is usually capped by a sialic acid; sialic acid content exhibits microheterogeneity in the different glycoforms. The sialic acid is of importance because it is the only negatively-charged sugar on the carbohydrate. Variations in the amount of sialic acid can affect the electrostatic properties of the molecules to which it is attached.

The carbohydrate is essential for *in vivo* but not for *in vitro* biological activity [13, 24]. The sialic acid component of carbohydrate, in particular, plays a critical role in the *in vivo* biological activity of rHuEPO. Removal of sialic acid from the carbohydrate of EPO results in almost complete loss of *in vivo* activity [14, 25]. Studies on glycoforms of rHuEPO containing different sialic acid contents demonstrated a direct relationship between increased sialic acid content and increased *in vivo* activity [19]. The increased *in vivo* activity was due to an increased serum half-life of the molecule and not increased receptor affinity. The theoretical maximum number of sialic acids on rHuEPO is 14 (up to four sialic acids for each of the three *N*-linked carbohydrates and up to two sialic acids for the *O*-linked carbohydrate) [23]. It was hypothesized that *in vivo* activity may be increased beyond that observed with rHuEPO by adding new sialic acid containing *N*-linked carbohydrates. Each new *N*-linked chain could add up to four additional sialic acids.

To add more *N*-linked carbohydrate, new *N*-linked glycosylation sites were introduced into the amino acid sequence of EPO. *N*-linked carbohydrate is attached to an Asn present in the consensus sequence Asn-Xxx-Ser/Thr (where Xxx can be any amino acid except proline) [26]. This sequence is necessary but not sufficient for *N*-linked carbohydrate addition [27]. During synthesis of a glycoprotein, appropriate consensus sequences are recognized by oligotransferases in the cell, resulting in attachment of carbohydrate. This carbohydrate is subsequently modified by the action of additional intracellular enzymes. The protein is then secreted from the cell into the circulation [28].

For the purpose of potentially developing a new drug with properties superior to then available products, it became apparent that simply adding an *N*-linked consensus sequence to rHuEPO would not be sufficient. The changes needed to be introduced in such a way that the resultant molecule was efficiently glycosylated and retained activity, conformation, and stability. To increase the likelihood of success, the amino acid changes were introduced into a region of the molecule distal to the receptor-binding site to ensure that the molecule would efficiently activate EPOR. This effort was aided by structure-function studies that defined the active sites of rHuEPO and determination of amino acids important for maintenance of structure [17, 29, 30].

EPO glycosylation analogs with introduced *N*-linked glycosylation consensus sequences were constructed and tested [18]. Several candidates containing additional carbohydrate had acceptable activity and conformation characteristics. Two of these consensus sequences (Asn30-Thr32 and Val87-Asn88-Thr90) were combined to generate a new molecule with two additional *N*-linked carbohydrates. This molecule (darbepoetin alfa) had near-normal *in vitro* activity, was glycosylated efficiently, and had a similar conformation and

stability to rHuEPO. The carbohydrate content was increased from 40% to 51%, the size from approximately 30 Kd to approximately 37 Kd, and the maximum number of sialic acids was increased from 14 to 22.

Studies in mice administered darbepoetin alfa revealed that more rHuEPO was required to obtain a response similar to that of darbepoetin alfa [18, 19]. In pre-clinical studies, animals were administered rHuEPO or darbepoetin alfa at various dose intervals. The relative *in vivo* activity difference of rHuEPO and darbepoetin alfa increased as the dosing interval increased. Three-fold more rHuEPO than darbepoetin alfa was required to elicit a similar response when the drugs were administered three times per week. This difference increased to 13-fold when the molecules were administered at weekly intervals [19]. The increased *in vivo* activity and the differing potencies with changes in dose interval could be explained by an observed three-fold increase in serum half-life of darbepoetin alfa over rHuEPO [19, 31]. The observation that the serum half-life increased in proportion to the number of added carbohydrate chains indicated that the carbohydrate directly affected clearance. Testing in humans mirrored the results found in animals. The serum half-life of darbepoetin administered intravenously was increased approximately three-fold [31]. In clinical trials, patients were converted from rHuEPO administered two to three times per week to darbepoetin alfa administered once per week or from weekly rHuEPO to once-every-other-week darbepoetin alfa. The hemoglobin concentrations were successfully maintained with the less frequent dosing schedule [32, 33]. More recently, clinical results suggest that hemoglobin concentrations can be successfully maintained when darbepoetin alfa is administered at once every three to four weeks dosing intervals [34].

One theoretical concern with any alteration in a protein's amino acid sequence or structure is immunogenicity. Several characteristics of darbepoetin alfa and its methods of manufacture, minimize the potential for antibody formation. The particular amino acid substitutions in darbepoetin alfa had a minimal effect on structure and stability. The carbohydrate and sialic acid content of the material selected during the purification process was maximized for several reasons: first, the higher carbohydrate content enhances the *in vivo* activity. Secondly, carbohydrate can increase solubility and stability of proteins thereby inhibiting formation of aggregates and other byproducts [35–38]. Finally, *N*-linked carbohydrate is large relative to the peptide backbone giving the carbohydrate a "shielding" effect potentially inhibiting antibody formation. Antibody formation was monitored during clinical trials with darbepoetin alfa and in all patient samples examined, no neutralizing antibody was detected [33, 39].

Pegylation

Pegylation of proteins has been used successfully to increase serum half-life of proteins [40]. Pegylation involves chemical attachment of the polymer, polyethylene glycol (PEG), to reactive regions of proteins or carbohydrates.

Pegylated molecules have an increased hydrodynamic size because they create a "water shell" around the molecule. The attachment of PEG is thought to improve solubility and possibly reduce immunogenicity due to shielding by the conjugate. In addition, the increased hydrodynamic size can result in reduced clearance and thus increase *in vivo* activity.

Mixing activated polyethylene polymers with proteins under appropriate chemical reaction conditions produces pegylated proteins. The duration of the drug development process using this strategy is relatively short because existing starting materials can often be used for the chemical reaction. PEG is thought to be relatively inert and non-immunogenic by itself, so it is a suitable starting material for protein-conjugate therapeutics.

One issue with drugs made by solution or solid-phase chemistry can be poor specificity of conjugation in the chemical reaction or generation of undesirable by-products. Many pegylation chemistries have been tried to reduce undesirable by-products, improve the specificity and efficacy of PEG attachments, and minimize immunogenicity risk of the protein conjugate while maximizing the *in vitro* and *in vivo* activity of the resultant molecule [41]. The current chemistries typically target the reactive amino groups on Lys or the amino terminal amine. rHuEPO has eight Lys, some of which are part of the active site [17]. Therefore, some pegylated EPO molecules conjugated to Lys may have low activity because PEG may interfere with receptor binding and activation. Other pegylated EPO molecules may have low activity because attached polymer results in structural alterations that interfere with receptor interaction.

Analogs of proteins can be made to increase specificity. For example, Cys substitutions at targeted regions can allow addition of the conjugate with high specificity to the sulfhydryl group [42, 43]. Another strategy is to make pegylated EPO synthetically: During synthesis, a PEG-conjugated amino acid could be introduced instead of the unconjugated amino acid. This approach allows specific targeting of particular amino acid positions for PEG attachment, such as the glycosylation sites, and reduces heterogeneity and the potential for loss of *in vitro* activity. It is not clear that these molecules will retain the same stability, *in vivo* activity, and lack of immunogenicity as their glycosylated counterparts, however.

Fusion proteins

Several EPO gene fusion proteins have been reported, including EPO/interleukin-3 (IL-3) [44], and EPO/granulocyte-macrophage colony-stimulating factor (GM-CSF) [45]. The theory behind creation of such molecules is that they can impart to the fusion protein biologic properties of both molecules. One can imagine that co-administration of an early-acting growth factor such as rHuIL-3 with rHuEPO can increase efficacy of rHuEPO. The fusion protein being larger may impart increased activity for both partners because of reduced clearance. The fusion protein also ensures that both molecular entities

are simultaneously present. Simultaneous administration by fusing two drugs can simplify administration, especially when the two proteins have different pharmacokinetic parameters. The ability to independently adjust dosing of the fusion partners is lost, however. Furthermore, the difficulty in retaining a non-immunogenic structure has been a challenge and neither EPO/IL-3 nor EPO/GM-CSF fusion molecules have been approved for use in humans.

EPO dimer has been generated as a potential therapeutic [46, 47]. In general, the increased size can reduce clearance because of slowed transport out of the serum compartment [46]. EPO dimer may also have increased *in vitro* activity due to altered avidity to the receptor [48].

The two protein partners are typically joined by a linker peptide that included Gly, Ala, and Ser. These three amino acids are thought to result in linkers that are flexible and relatively inert, allowing independent folding of the two proteins into their appropriate three-dimensional structures. Full *in vitro* activity of both proteins in fusion proteins does not always occur [44]. The fusion proteins have been reported to have increased risk of immunogenicity [49], presumably because of altered folding or stability.

EPO mimetics

rHuEPO is currently administered by either subcutaneous or intravenous injection. Because of its large size and peptidic nature, delivery by other routes such as oral or transdermal or by inhalation can be quite challenging. The potential use of rHuEPO for treatment of neuronal trauma by promoting neuronal survival [50] is limited by its poor transport across the blood brain barrier [51]. One possible solution is a small molecule, an EPO mimetic, capable of stimulating EPOR. EPO mimetics are compounds that mimic the activity of EPO but bear no structural homology. EPO mimetics can have new biological or biophysical properties not present in EPO. Designed appropriately, such a compound has the potential to be delivered by routes that are more convenient than currently in use for rHuEPO.

Significant challenges are associated with the identification and development of a useful small molecule EPO mimetic. First, the need to be small to be delivered orally is confounded by the need to be large enough to have sufficient affinity for EPOR to be effective. The compound must have appropriate pharmacokinetic parameters so that it persists sufficiently long in the serum to be efficacious. Finally, the compound should not have unwanted side-effects due to either toxicities of the compound itself or breakdown products of it. In spite of these challenges, work has proceeded and progress has been made in attempts to identify lead compounds that may be amenable to oral delivery.

Several strategies have been used to identify EPO mimetics. The first is to screen peptide and small molecule libraries for those compounds that can stimulate erythropoiesis using *in vitro* bioassays as screens. According to this strategy, an understanding of the mechanism of activation is not necessary, and

compounds active in the assay may activate EPOR by a different mechanism than by rHuEPO. Another strategy is to identify molecules that directly bind to and agonize EPOR in a manner similar to that of rHuEPO. The latter strategy can be performed in two steps: the first step is to identify compounds that bind EPOR and the second step is to covalently link the compounds into bivalent dimers that can agonize the receptor by EPOR homodimerization.

The latter strategy takes advantage of the observation that an EPOR mutant containing an Arg129 to Cys129 mutation was constitutively active [52]. A disulfide bond formed between the Cys129 residues on the receptors resulted in homodimerization and receptor activation demonstrating that EPO was not essential for receptor activation (Fig. 2). X-ray crystallography results demonstrated that EPOR forms a 2:1 complex with EPO [57]. Each receptor uses the same region on its surface to bind to two surfaces on EPO, resulting in receptor homodimerization. Further confirmation of the homodimerization mechanism was the discovery of agonist monoclonal IgG antibodies that could homodimerize EPOR [53]. Anti-EPOR antibodies activated because they were bivalent, had two binding sites, and could simultaneously bind and cross-link two EPOR (Fig. 2). Monovalent, Fab fragments could bind but did not agonize

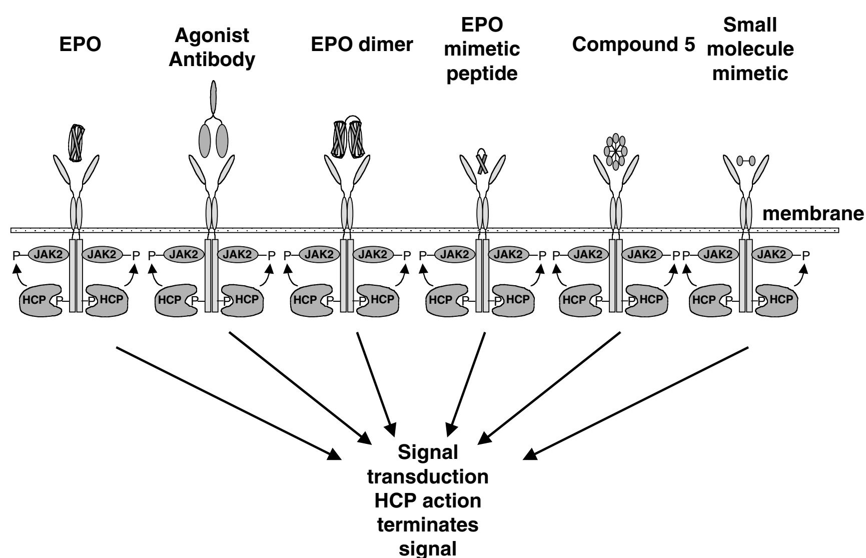

Figure 2. Mechanism of erythropoietin receptor (EPOR) activation. EPOR are homodimerized because of the two asymmetric receptor-binding sites on rHuEPO. EPO binding results in phosphorylation of EPOR, JAK-2 recruitment, and phosphorylation of JAK-2. The activation of JAK-2 results in downstream signaling events. Hematopoietic cell phosphatase (HCP) can bind the activated (phosphorylated) receptor resulting in dephosphorylation of JAK-2, thereby terminating signal transduction. The EPO mimetic compounds; agonist antibody [53], EPO dimer [46, 47], EPO mimetic peptide [54], compound 5 [55], and small-molecule mimetics [55, 56] can all homodimerize and activate EPOR in a manner similar to that of rHuEPO.

the receptor, confirming a requirement for bivalent binding. Wrighton and colleagues screened peptide phage libraries and one peptide was identified that could bind and agonize EPOR [54]. This peptide, AF11154, had no homology to EPO. It self associates into dimers to form a bivalent molecule that could homodimerize EPOR in a 2:2 mimetic:EPOR complex [58]. Additional sequence modification of this peptide resulted in EMP1, a 20-amino acid peptide with an approximate 50-fold increase in affinity over the starting peptide (Tab. 1). The affinity was increased more by covalent linkage of the peptide [59]. The activity of this peptide was still significantly lower (500-fold) than that of rHuEPO when tested in an *in vitro* bioassay. In addition, the *in vivo* activity was very low (25,000-fold less than rHuEPO). This work demonstrated, however, that a molecule smaller than rHuEPO could successfully dimerize and activate the receptor. An attempt to discover other EPO agonist peptides by another group was also successful [61]. The *in vitro* activity was not increased, however, and the size of this peptide was not decreased compared with EMP1.

One explanation for the low *in vivo* activity of mimetic peptides is their rapid clearance. One group addressed this problem by creating a fusion protein between EMP1 and a larger protein, plasminogen activator inhibitor (PAI1)[60], resulting in an increase in molecular weight from 4.8 Kd to 66 Kd. The *in vivo* activity was significantly increased (2500-fold); its *in vivo* activity, however, was still significantly less than that of rHuEPO (100-fold) and the ability to be delivered orally was compromised by the size increase.

The peptide mimetics described above are significantly larger (4.2 Kd) than the preferred size of an orally bioavailable compound (<0.6 Kd). These peptides may be used to design lead compounds of smaller size. Some small molecule agonists have been isolated based on the EMP1 structure [56]; however, their *in vitro* activities were low (Tab. 1). An independent approach was to directly screen for small-molecule EPO mimetics that could dimerize EPOR. Small molecule libraries containing compounds with two-fold symmetry were screened to find dimerizing compounds that agonize the receptor [55, 63]. This strategy did not result in discovery of agonist compounds, however. A small molecule (compound 1, approximately 5 Kd) that bound but did not agonize EPOR was discovered. Compound 1 was made active *in vitro* by oligomerizing it with a multivalent crosslinker resulting in a molecule (compound 5) containing eight compound-1 molecules joined together. Compound 5 binding to EPOR was increased somewhat (10-fold) over that of compound 1; however, the size (6.4 Kd) was greater than that required to be orally bioavailable. In addition, its *in vitro* activity was low relative to rHuEPO and the compound had toxicity. The feasibility of the small-molecule approach to discovery of small molecules that could agonize EPOR was demonstrated, however.

Further progress in development of small molecule EPO mimetics has been slow. Progress has been made with the development of small molecules that can agonize other cytokine receptors including granulocyte colony-stimulating factor receptor [64] and insulin receptor [65]. The insulin mimetic is noteworthy in that it is orally active in rodents [66]. This work demonstrates that small

Table 1. Activity of erythropoietin and mimetics

Compound	MW (Kd)	*In vitro* activity (EC50)	Binding (IC50)	*In vivo* activity (U/mg)	Relative difference in activity (molar)	Notes	Ref
rHuEPO	18,200	5–10 pM	100 pM	>100,000	1	peptide monomer	[20,53]
EMP1	4200	400 nM	200 nM	2	25,000	peptide dimer	[54, 58]
EMP dimer	4800	approx 20 nM	approx 2 nM	200	250	peptidedimer	[59]
EMP-PAI1	63,000	NA	NA	5000	100	peptide dimer	[60]
ERP	3085	100 nM	approx 1 nM	NA	NA	peptide dimer	[61]
ERB	2170	approx 3 nM	45 nM	NA	NA	peptide monomer	[62]
EPO Mab 71	150,000	200 pM	1 nM	NA	NA	peptide dimer	[53]
Compound 1	500	0	60 µM	NA	NA	nonpeptide monomer	[55, 63]
Compound 5	6400	1–5 nM	4 µM	NA	NA	nonpeptide dimer	[55]
A1B10C1	2100	1–10 nM	NA	NA	NA	nonpeptide dimer	[56]

EC, extracellular; IC, intracellular.

molecules can be identified that can activate cytokine receptors and retain properties suitable for oral delivery.

EPO mimetics without homodimerization

Difficulties with development of small molecules that activate EPOR by dimerization can be bypassed by targeting a different mechanism. One report describes a peptide that activates EPOR by binding to a domain on EPOR similar to major histocompatibility complex (MHC) peptides (Fig. 2). This 23-amino acid peptide is reported to have both *in vitro* and *in vivo* EPO activity [62]. The molecule appears to activate by binding EPOR at a region distal to its binding site (transmembrane domain), suggesting that it activates differently than does rHuEPO. The mechanism may be similar to that of the virus envelope protein, gp55, that also activates EPOR by an interaction with the EPOR transmembrane region [67, 68]. The nature of how activation EPOR occurs by gp55 is not understood.

Another approach to mimetic discovery is to modulate steps downstream from receptor activation such as by inhibiting hematopoietic cell phosphatase (HCP) [69, 70]. HCP is an enzyme that dephosphorylates JAK-2, a kinase that is part of the EPO signal transduction cascade [5]. JAK-2 is normally activated (phosphorylated) as a consequence of EPOR activation. HCP binds to activated (phosphorylated) EPOR and then dephosphorylates JAK-2, terminating signal transduction. Truncated EPOR lack the HCP binding site, and thus HCP cannot dephosporylate JAK-2, resulting in hypersensitivity of the receptor to EPO. EPOR truncations have been described in humans whereby the affected individuals have increased hemoglobin concentrations but very low EPO concentrations due to a hypersensitive EPOR [71, 72]. These observations suggest that small molecule antagonists of HCP may result in increased EPOR activity that increases erythropoiesis in the absence of added EPO.

One concern of HCP inhibitors relates to observations associated with HCP mutations in mice. These mice (motheaten) have a defective *HCP* gene [73] and have multiple hematopoietic abnormalities, including increases in macrophages, lymphocytes, and erythrocytes. HCP is a negative regulator for several different cytokine receptors besides EPOR [69, 70, 74, 75]. Although HCP inhibitors may be effective at increasing erythroid cell number, increases in cell number of other hematopoietic cells may limit the usefulness of these compounds.

Gene therapy

Controlled delivery of *EPO* genes to humans is another promising approach for EPO therapy. Early work in this field depended on direct injection of plasmid DNA containing constitutively active *EPO* genes into the muscles of mice

[76], resulting in a measurable increase in hematocrit. Several concerns became apparent from these studies, including inefficient and variable delivery of the *EPO* gene and subsequent variations in EPO concentration. EPO expression also decreased over time. Expression systems and gene delivery methods with improved efficiency have been reported [77–80]. Current *EPO* gene therapy protocols require repeated administration of EPO genes. In addition there is concern that the therapy may be irreversible or result in altered gene expression resulting in tumorigenicity. Over-expression of *EPO* genes could result in polycythemia with little ability to correct the condition.

The efficiency and irreversibility concerns have been addressed by developing implantable capsules containing EPO-expressing cells [81]. The capsules can be removed, halting EPO delivery. A further improvement would be to construct vectors whereby EPO expression is controlled by a small molecule such as tetracycline, enabling increased EPO expression in response to oral administration of the gene activator [82]. Controlled expression of the *EPO* gene has been demonstrated in mice using tetracycline [77, 78, 83], mifepristone [84], or rapamycin [85]. Additional advances are the development of vectors where EPO expression is controlled by oxygen tension [82], or methods that target the kidney for gene transfer [86]. Despite these advances, safe and controlled EPO delivery using gene therapy methods suitable for human use remains a distant but tantalizing opportunity.

New formulations and devices

Endogenous EPO concentration is exquisitely controlled in the body by rapid changes in expression. In contrast, protein therapeutics are placed in a non-physiologic environment for extended periods of time, which in some instances may be years. Safe storage in any formulation requires that conditions and formulations be designed to minimize formation and accumulation of unnatural breakdown products or alterations in EPO structure. Inappropriate formulations that do not maintain the integrity of the product can risk exposing the patient to an abnormal form of the protein.

Formulations containing rHuEPO have been successfully developed and used safely and effectively for more than a decade. Despite the success of current formulations, change is sometimes required to keep up with regulatory or safety concerns or to allow for new technologies, such as new devices or delivery systems. Such manufacturing and formulation changes included removal of excipients such as human serum albumin or bovine-derived products.

Prolonged stimulation of erythropoiesis is one desirable property that may be addressed by new delivery systems, including devices that allow controlled release of rHuEPO over a long time. This approach necessitates that the molecule remain stable in the device for a prolonged period of time. Another approach is to introduce rHuEPO into a biodegradable matrix that degrades slowly over time (slow release) [87, 88]. This strategy requires development of

methods to immobilize rHuEPO in a matrix, such as microparticles, that breakdown at predictable rates and release the product in a controlled manner.

The use of an appropriate slow-release process has not been successful for several reasons. The main one is a requirement that the protein remain intact and unchanged during both the processing of the material and during the prolonged exposure in the body. Protein integrity is a particularly difficult requirement in biodegradable matrices because the protein is in a concentrated hydrated state at physiologic temperatures for extended periods of time. Small amounts of contaminating rHuEPO aggregates, misfolded rHuEPO, or breakdown products may compromise not only efficacy but also safety (immunogenicity). Another concern is that too rapid breakdown of the matrix may result in excessive delivery resulting in an overdose.

Conclusions

Nearly two decades have passed from the heady days when the *EPO* gene was cloned and rHuEPO was first administered to a patient. Recombinant HuEPO has proven to be a safe and efficacious molecule for EPO replacement therapy, setting the bar high for any improvements that may follow. Nonetheless there remains a desire for better erythropoietic molecules, new formulations, or more useful delivery systems. The discovery, development and regulatory approval of darbepoetin alfa shows that it is possible to improve EPO replacement therapy in a safe and effective manner. In this case, darbepoetin alfa performs the same function as rHuEPO, but has increased *in vivo* activity and reduced serum clearance, and a similar safety profile. Further progress is anticipated as new devices that can simplify the administration of these drugs are developed. Additional changes in rHuEPO or in formulations are anticipated that may need to be developed to allow effective use of these delivery devices. The future will be exciting as small-molecule EPO mimetics are discovered, though matching the safety profile of rHuEPO presents a substantial hurdle to any small-molecule program. New erythropoietic agents may be administered orally. Permanent correction of anemia may occur through gene therapy, thereby allowing additional treatment opportunities. These developments may require extensive research and testing; however, many believe that these developments are not a question of if, but when.

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Glossary and abbreviations

AAV	adeno-associated virus
ACD	anemia of chronic disease
ACE	angiotensin-converting enzyme
AIDS	acquired immunodeficiency syndrome
ATII	angiotensin II
AT1R	ATII type-1 receptor
AUC	area under the serum concentration time curve, estimated by integration (generally by use of trapezoidal rule) from time zero to the last collection point ($AUC_{(0\text{-}t)}$) with extrapolation to infinity where possible ($AUC_{(0\text{-}inf)}$)
BFU-E	erythroid burst-forming unit
BHK	baby hamster kidney
BNDF	brain-derived neurotrophic factor
CAD	carboxyl-terminal transactivation domain
CAS	Court of Arbitration for Sport
CBER	Center for Biologics Evaluation and Research
CDER	Center for Drug Evaluation and Research
CDK	cyclin-dependent kinase
CFR	Code of Federal Regulations
CFU	colony-forming unit
CFU-E	erythroid colony-forming unit
CFU-GEMM	granulocyte-macrophase, erythrocyte, macrophage, megakaryocyte colony-forming unit
CGMP	Current Good Manufacturing Processes
CHO	Chinese hamster ovary
CL	clearance from the central compartment, estimated from $AUC_{(0\text{-}infinity)}$/Dose
C_{max}	maximum observed concentration
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMP	erythropoietin mimetic protein
EPO	erythropoietin
EPOR	receptor for erythropoietin

F	bioavailability – percentage of the dose that is measurable in the circulation after extravascular (e.g., SC) administration. True estimates are obtained in an IV/SC cross-over study using the same dose wherein $F = 100*(AUC_{SC}/AIC_{IV})$
FasL	Fas activity ligand
FDA	Food and Drug Administration
FH4	tetrahydrofolate
FIV	feline immunodeficiency virus
FmuLV	Friend murine leukemia virus
G0	period of cell cycle without perceived growth or replication
G1	period of cell cycle characterized by rapid protein synthesis
GATA-1	erythroid transcription factor
G-CSF	granulocyte colony-stimulating factor
GHR	growth hormone receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
HCP	hematopoietic cell phosphatase
HIF-1	hyporxia-inducible factor-1
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
IOC	International Olympic Committee
IP	intraperitoneal, intraperitoneally
IRS	insulin receptor substrate
IV	intravenous, intravenously
JAK	Janus kinase
K_m	plasma concentration at which half-maximal effect is observed
Kd	kilodalton
LN	natural logarithm
MAT	mean absorption time – average time a molecule remains at absorption site. Calculated from $1/K_a$
MCH	mean corpuscular hemoglobin
MCV	mean corpuscular volume
MDS	myelodysplastic syndromes
MHC	major histocapatability complex
MRT	mean residence time. After IV dosing, it is the average time a molecule remains in circulation. After SC or IP dosing, it is the sum of the mean absorption time (MAT) and the average time a molecule remains in the circulation. Calculated from moment theory as $AUMC^2/AUC$

n	population
NMR	nuclear magnetic resonance
ODD	oxygen-dependent degradation domain
PAD	predonated autologous blood
PAI	plasminogen activator inhibitor
PEG	polyethylene glycol
polyA	polyadenylation
PRCA	pure red cell aplasia
pVHL	vonHippel-Lindau protein
r	recombinant
rHuEPO	recombinant human erythropoietin
rHuGH	recombinant human growth hormone
rHuIFN	recombinant human interferon
RT-PCR	reverse transcription polymerase chain reaction
SC	subcutaneous, subcutaneously
SCF	stem cell factor
SCFR	receptor for stem cell factor
SDS-PAGE	sodium dodecyl sulfate-polyacrilamide gel electrophoresis
SFFV	spleen focus-forming virus
SHIP	SH2-containing inositol-5-phosphatase
SOCS-1	supressor of cytokine-signaling-1
STAT	signal transduction and activator of transcription
TfR	receptor for transferrin
TGF	transforming growth factor
T_{max}	time at which the maximum concentration is observed
TNF	tumor necrosis factor
V_d	volume of distribution (generic term)
V_{ss}	volume of distribution at steady state (estimated after single dose administration after distribution processes have been completed). In the case of a 2-compartment system, it is the sum of the central and peripheral volume s of distribution
V_z	volume of distribution associated with the terminal phase (not very practical)
V_{max}	theoretical maximum rate of elimination associated with the non-linear pathway
VEGF	vascular endothelial growth factor

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