Chapter 4
Erythropoiesis-Stimulating Agents

Steve Elliott

Abstract Erythropoiesis is the process whereby erythroid progenitor cells differentiate and divide, resulting in increased numbers of red blood cells (RBCs). RBCs contain hemoglobin, the main oxygen carrying component in blood. The large number of RBCs found in blood is required to support the prodigious consumption of oxygen by tissues as they undergo oxygen-dependent processes. Erythropoietin is a hormone that when it binds and activates Epo receptors resident on the surface of cells results in stimulation of erythropoiesis. Successful cloning of the *EPO* gene allowed for the first time production of recombinant human erythropoietin and other erythropoiesis stimulating agents (ESAs), which are used to treat anemia in patients. In this chapter, the control of Epo levels and erythropoiesis, the various forms of ESAs used commercially, and their physical and biological properties are discussed.

Introduction

Erythropoietin (Epo) is a late-acting growth factor, so named because of early studies suggesting it had a singular effect on stimulation of red blood cell formation (erythropoiesis). Epo functions by binding and activating an Epo receptor expressed on the surface of committed erythroid progenitor cells resulting in their proliferation and differentiation. This elegant process results in formation of enucleated hemoglobin-containing red blood cells (RBCs) that are released into the circulation where they can bind oxygen in the lungs and deliver it to tissues with a high oxygen demand, e.g., brain or working muscles.

Successful cloning of the *EPO* gene in the 1980s [1, 2] allowed the commercial production of recombinant human erythropoietin (rHuEpo). The utility of rHuEpo in treating anemia (low hemoglobin [Hb] levels) was explored in patients with chronic

S. Elliott (✉)
Department of Hematology, Amgen, Inc., Thousand Oaks, CA 91320, USA
e-mail: selliott@amgen.com

G.H. Lyman, D.C. Dale (eds.), *Hematopoietic Growth Factors in Oncology*, Cancer Treatment and Research 157, DOI 10.1007/978-1-4419-7073-2_4,

kidney disease (CKD), anemia of cancer (AoC), chemotherapy-induced anemia (CIA), and anemia of inflammation such as rheumatoid arthritis. It was also studied in patients scheduled for major surgery who were expected to require blood transfusions. rHuEpo (e.g., epoetin alfa) has been approved for use in humans and is currently used primarily to treat anemia associated with CKD and CIA.

This review will examine the structure, function, and regulation of Epo production, mechanisms affecting clearance of Epo, erythropoiesis, various forms of ESAs, and the data suggesting Epo has effects on other biological processes beyond erythropoiesis.

Hemoglobin, Erythropoietin, and Erythropoiesis

Red Blood Cells and Hemoglobin

RBCs in humans represent 40–45% of total blood volume and 99% of all circulating cells. In a healthy person with roughly 5 L of blood, this represents approximately 2.5×10^{13} cells. RBCs are long-lived with a lifespan of approximately 100–120 days [3]. The daily loss of RBCs, approximately 0.8–1.0% of the total, is matched by a prodigious production capacity of $\sim 2.5 \times 10^{11}$ cells/day [4]. Hb residing within mature RBCs is the primary oxygen-binding component and constitutes 99% of the cytosolic protein [5]. Iron residing within hemoglobin (2.5 g) represents the major component of total body iron (3–4 g) and levels are tightly regulated [6]. These high levels of iron, hemoglobin, and RBCs are consistent with the importance of maintaining oxygen homeostasis in the body.

Hb is a tetrameric protein with each subunit containing a tightly associated nonprotein heme iron. In human adults the major form of Hb is hemoglobin A, consisting of two α and two β (beta) subunits. The affinity of O_2 for Hb in RBCs is increased with low temperature, low CO_2, and low 2,3 DPG as occurring in lungs. Affinity is reduced by increases in body temperature, hydrogen ion, 2,3-diphosphoglycerate, or carbon dioxide concentration (the Bohr effect) under conditions where O_2 levels are low, such as working muscles. Therefore, in muscles that are consuming O_2 and generating CO_2 and lactic acid, O_2 is released from Hb [7].

Erythropoietin

As O_2 levels decrease with exercise, blood loss, anemia, or change in concentration of oxygen in breathed air, the breathing rate, heart rate, and adjustments in O_2-carrying capacity are made to meet the demand. One mechanism to increase O_2-carrying capacity is to increase hemoglobin levels by stimulating formation of increased numbers of Hb-containing RBCs through increases in erythropoietin

(Epo) levels which stimulates erythropoiesis. Epo is a circulating glycosylated protein hormone that is the primary regulator of RBC formation. Endogenous Epo (eEpo) is synthesized primarily in the kidney, although it is also made at lower levels in other tissues such as liver and brain [8–12].

Endogenous Epo in humans is transcribed as a 1.6–2-kb mRNA [2] and translated into a 193 amino acid precursor [1, 13]. During transit through the secretory apparatus, the 27 amino acid signal peptide and C-terminal arginine are removed and carbohydrate chains are added to three N-linked glycosylation sites and the one O-linked glycosylation site [13, 14]. The secreted protein contains 165 amino acids with approximately 40% of the mass composed of carbohydrate. The structure of rHuEpo is a compact globular bundle, which contains four alfa-helices in a characteristic 4-helix bundle, a topology shared with other growth factors [15, 16] (Fig. 4.1).

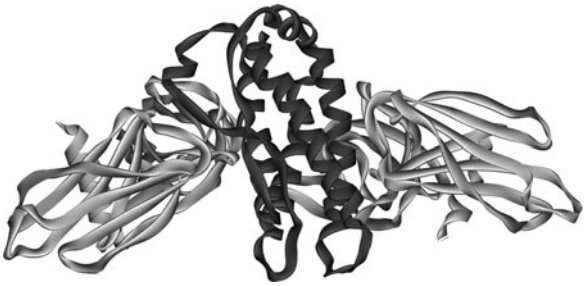

Fig. 4.1 Structure of an Epo:EpoR complex. The *top view* (*ribbon diagram*) of the crystal structure shows Epo (*gray*) bound to the extracellular domains of two EpoRs (*dark gray*). Two different surfaces of Epo bind to similar regions of EpoR resulting in high- and low-affinity binding sites. The "cross-linked" EpoR is activated resulting in downstream signaling

Erythropoiesis

RBC production (erythropoiesis) is a result of an elegant proliferation and differentiation pathway (Fig. 4.2). Early hematopoietic progenitor cells residing primarily in the bone marrow differentiate in the presence of an early growth factor such as SCF, IL-3, or GM-CSF [17] into burst-forming unit erythroid (BFUe) and then into colony-forming unit (CFUe) cells [18, 19]. BFUe cells acquire responsiveness to and become dependent on Epo as they differentiate into the CFUe stage [17, 20]. Further differentiation of CFUe cells results in molecular and physical changes and the immature "blast" cells become proerythroblasts, and finally, erythroblasts. These cells begin to take up iron and show increased synthesis and accumulation of Hb. The late-stage erythroblasts discharge their nucleus (enucleation), resulting in reticulocytes that are released into the circulation. After several days, reticulin (ribosomal RNA and associated material) declines resulting in mature, circulating RBCs.

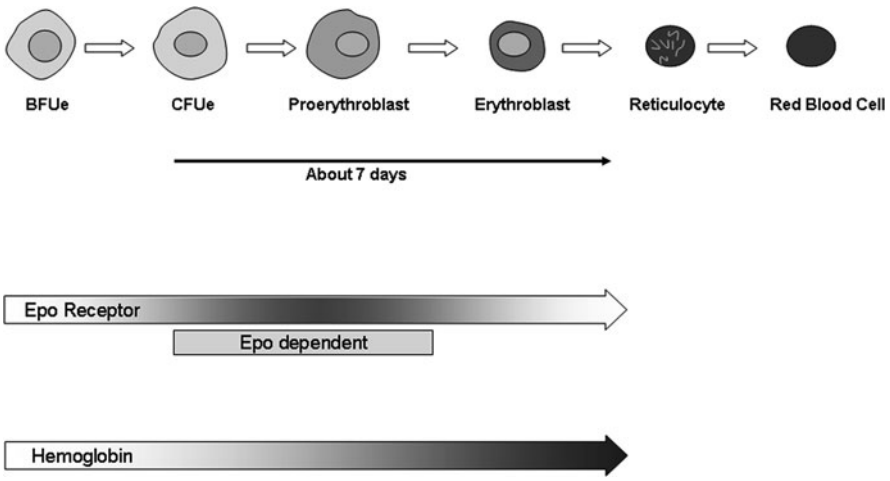

Fig. 4.2 Erythropoiesis. The schematic diagram shows the differentiation of erythroid progenitor cells. BFUe (burst-forming unit erythroid) cells differentiate in blood islands in the bone marrow into CFUe (colony-forming unit erythroid) and ultimately into reticulocytes that are released into the circulation. The process involves increased synthesis of EpoR and hemoglobin

Epo Receptor and Its Activation

The mechanism by which Epo stimulates erythropoiesis has been the subject of considerable investigation. Radiolabeled Epo was shown to bind to a "receptor" expressed on the surface of erythroid cells. The gene encoding the Epo receptor was identified by expression cloning and found to be a single gene with no apparent homologs [21, 22]. The human *EPOR* gene encodes a 508 aa protein which following removal of the 24 aa signal peptide results in a 484 aa protein with a molecular weight of approximately 52.7 kDa [23]. Addition of an N-linked carbohydrate chain results in a secreted protein with a calculated size of 56–57 kDa which is comparable to the size of mature EpoR determined by Western immunoblotting (~59 kDa) [23].

In early studies, EpoR transcripts were detected by Northern analysis in bone marrow or spleen with none detected in heart, kidney, liver, or brain [24]. This is consistent with [^{125}I]Epo-binding studies which also suggested that high-level expression of EpoR is normally restricted to cells of the erythroid lineage [24–28]. Reticulocytes and circulating RBCs do not express EpoR [29–31].

While other components may mediate affinity to Epo or aid in signal transduction, current evidence suggests that the activation of EpoR is initiated by a direct interaction of a single Epo molecule with two EpoRs via high- and low-affinity binding sites effectively "cross-linking" them (Fig. 4.1). The binding and cross-linking by Epo induces a conformational change thereby bringing together two separate regions on the transmembrane and intracellular regions of the receptor. Activation results in cross-phosphorylation of EpoR and Jak2 which is followed by activation of the downstream STAT5, MAP kinase, and PI3 kinase/Akt pathways [32].

Following stimulation of signal transduction, negative regulators of EpoR, including Src homology region 2 domain-containing phosphatase 1 (SHP-1) and suppressor of cytokine signaling proteins SOCS-1 and SOCS-2, downmodulate responses [33, 34].

Normal circulating levels of Epo in humans are approximately 5 pM, substantially below the kDa of the Epo:EpoR interaction indicating that only a fraction of the EpoR is Epo bound but this level of binding is sufficient to maintain erythropoiesis at a rate that will maintain RBC levels. Lower Epo receptor occupancy results in apoptosis of precursor cells (post-CFUe stage) [35].

Epo responsive erythroid cells express 300–1,100 high-affinity (~100 pM) surface EpoR/cell [1, 2, 34]. The half-maximal response to Epo of CFUe cells is achieved when approximately 7% of their cell surface-expressed EpoR are bound by Epo suggesting approximately 70 surface EpoR are bound by Epo at this Epo concentration [9]. These observations indicate that a single Epo–EpoR binding event is insufficient for stimulation of complete differentiation of erythroid precursors. Instead, adequate concentrations of Epo must be present during the entire process to ensure survival and proliferation of the cells.

Acquired Epo responsiveness appears to require a threshold amount of EpoR expression and Epo binding. This notion is consistent with a number of observations. EpoR mRNA and surface EpoR protein expression increase up to the normoblast stage of cell differentiation followed by a rapid decline thereafter (Fig. 4.2) and this timing is associated with acquired Epo responsiveness [29, 31, 36]. Mice with haploinsufficiency containing only one functional allele of EpoR have lower EpoR levels and decreased hematocrit. Colony formation using cells recovered from these animals requires higher Epo concentrations compared to control animals [37]. BFUe cells which show minimal response to Epo [17, 38, 39] acquire Epo-dependent growth following forced over-expression of EpoR [26]. Similarly forced over-expression of EpoR in various leukemic cell lines will make them dependent on Epo for growth or survival [40–43]. HEL cells express low but detectable levels of EpoR on their cell surface [31] but these cells show increased STAT5 phosphorylation if EpoR levels are increased by forced over-expression of EpoR [44].

Expression of EpoR is necessary but not sufficient for Epo responsiveness. OCIM1 cells are an immortalized cell line derived from a patient with erythroleukemia [45]. These cells express EpoR on their surface at levels comparable to that found on Epo responsive erythroid precursor cells but they do not respond to Epo. Functional EpoR was cloned from OCIM1 cells [22], indicating the lack of Epo responsiveness was not due to mutations in EpoR. While forced expression of EpoR in some cell types (32D, FDCP1, and BaF3) results in acquisition of Epo responsiveness [40–43], this was not true of forced over-expression in other cell types such as NIH-3T3 or CTLL2 suggesting they lack positive factors or over-express negative factors [46–48]. A subline of EpoR CTLL2-transfected cells was initially nonresponsive but became dependent on Epo following selection for growth on Epo. Both the subline and the original transfected cells expressed similar levels of surface EpoR and both expressed Jak2, but Epo induced Jak2 phosphorylation

only in the subline [48–51] indicating the JAK-2 to STAT5 pathway was present but could not be activated.

Erythropoiesis-Stimulating Agents (ESAs)

Endogenous Epo and rHuEpo

Recombinant HuEpo was initially used for the treatment of anemia associated with chronic kidney disease. It is also indicated for cancer patients who have CIA. According to a number of studies rHuEpo is well tolerated, can correct and maintain hemoglobin levels, and reduce the likelihood of transfusions when used according to guidelines [52–58].

Epoetin alfa, the first commercialized rHuEpo (Epogen®, Procrit®, Eprex®), is a 165 amino acid glycoprotein with an average molecular weight of 30,400 Da. As is eEpo, epoetin alfa is a heavily glycosylated protein containing N-linked and O-linked complex carbohydrates with over >50 different forms described [59–61]. There is also a natural variation in charge due to the presence of a variable number of sialic acids (up to four) on each of the three N-linked carbohydrate chains and up to two sialic acids on the single O-linked carbohydrate chain.

Endogenous Epo and rHuEpo have the same amino acid sequence but are not identical to each other [62, 63]. This is because eEpo is a complex biological produced by specialized cells that are impacted by forces unique to the body, including a complex interaction of circulating growth factors, nutrients, and particular cell–cell interactions. The circulating eEpo is then subject to differential clearance of the various glycoforms. It is not possible to duplicate these processes in a manufacturing setting. Some of the resulting differences have been described. For example, endogenous Epo has sialic acid attached to galactose in NeuAcα2→6 or 2→3 linkages. Chinese hamster ovary (CHO) cells are frequently used to manufacture rHuEpo and because these cells lack a sialotransferase with NeuAcα2→6 activity only NeuAcα2→3 linkages are present in rHuEpo made from these cells [64]. rHuEpo made in CHO cells but not eEpo can contain traces of *N*-glycolylneuraminic acid in addition to the typical Neu5Ac found in urinary Epo [65, 66]. rHuEpo can also be made in other cell types where NGNA was also found. Endogenous Epo has a substantially higher content of sulfate on the attached carbohydrate compared to rHuEpo. Thus eEpo can be considerably more negative (acidic) than rHuEpo [67, 68] and this difference has been exploited to detect abuse of rHuEpo in athletes [63].

Follow-on Biologics

Glycosylation of proteins may impact properties such as binding affinity for cognate ligands, resistance to proteolytic degradation, and physical properties such as stability and solubility, and these can affect product quality as well as

clearance, efficacy, and safety [69–73]. Changes in cell lines, minor changes in growth conditions or manufacturing processes, can affect the final ESA product characteristics including the microheterogeneity of glycoforms. It is not possible for another manufacturer to match exactly the product profile of the innovator. Thus, the term "generic" is not used to describe rHuEpo molecules made by different manufacturers. Instead, the descriptors "follow-on biologics" or FOBS, "biosimilars" or "biosimilar biopharmaceuticals" are used [74].

The inherent difficulties in matching product characteristics and the demand that biosimilars have similar efficacy and safety to the innovator products [75, 76] have slowed approval of biosimilar rHuEpos because they require more oversight in the drug approval process [75–77]. In contrast to vaccines, whose efficacy depends on the ability to stimulate protective immunity in hosts, the safety and efficacy of recombinant biopharmaceutical proteins depends in part on their biochemical and immunochemical similarity to the corresponding endogenous proteins. These features help to optimize therapeutic activity of the recombinant proteins while minimizing their potential immunogenicity [78–81]. Neutralizing Abs should they form may cause or contribute to certain immunopathologies, such as Ab-mediated pure red cell aplasia (PRCA) in patients treated with ESAs [82, 83]. Antibody-mediated PRCA is a serious hematologic condition characterized by the onset, following a period of successful therapy, of severe ESA-resistant anemia that most often resolves only after cessation of ESA therapy and intervention with immunosuppressive agents [84]. The sudden appearance in 1999 of increased number of patients who presented with antibody-mediated PRCA was associated with changes in manufacturing processes of Eprex thereby highlighting the potential adverse consequence of manufacturing differences on product safety of FOBs [81, 82].

rHuEpo FOBs are also manufactured and distributed in countries where less oversight on drug manufacturing results in significant product quality and in structural differences from epoetins distributed in the United States and EU [85–87]. These ESAs can vary considerably from each other and from marketed epoetins manufactured in the United States and EU in labeling, drug content, and specific activity. There may also be considerable lot-to-lot variability. There have been reports that some patients administered with these ESAs have presented with antibody-mediated PRCA [88].

In addition to differences in product characteristics, there is also confusion about naming conventions of newer rHuEpo molecules. Following the introduction of the first epoetin alfa a second was approved in the EU (Recormon/Neorecormon Roche Diagnostics GmbH, Mannheim, Germany) and was given the name epoetin beta. It has the same amino acid sequence as epoetin alfa manufactured by Amgen, Inc. with some minor differences in the microheterogeneity in the attached carbohydrate. Other epoetins followed with different Greek names. Epoetin omega, produced in baby hamster kidney cells, differed somewhat from epoetin alfa and beta in the glycosylation profile [89]. However, this agent is distributed only in South Africa. Dynepo (epoetin delta) was manufactured by Shire Pharmaceutical Contracts Ltd, Hampshire, UK and was approved in Europe for the treatment of anemia in adult patients with chronic renal failure, but as of December 2008 was withdrawn from

the market. Other ESA biosimilars are being introduced in the EU: Silapro/Retacrit (epoetin zeta). However, Abseamed/Binocrit/Hexal is marketed as an "epoetin alfa" as are some epoetins manufactured in countries outside the United States and EU.

Darbepoetin Alfa

Aranesp® (darbepoetin alfa; Amgen, Inc.) is manufactured in the United States and marketed in Europe, the United States, Australia, Canada, and others. Aranesp® is a novel hyperglycosylated rHuEpo analog with five amino acid changes that compared to epoetin alfa contain two additional N-linked glycosylation sites resulting in the attachment of two additional carbohydrate chains for a total of five [90]. Aranesp® has the same mechanism of action as rHuEpo, stimulating of erythropoiesis through activation of the Epo receptor (EpoR). However, Darbepoetin alfa has increased in vivo potency [69, 90, 91] due to a threefold longer serum half-life and mean residence time than epoetin alfa [91, 92]. The longer serum half-life allows for more convenient modes of administration, including extended dosing intervals with a similar efficacy and safety profile to epoetin alfa.

Other ESAs

Mircera® (methoxy polyethylene glycol-epoetin beta) is epoetin beta with a covalent attachment to the peptide backbone of a linear methoxy polyethylene glycol (PEG), resulting in approximately a doubling in size compared to epoetin alfa (30.4 vs 60 kDa) [13, 93]. PEG-epoetin beta has a prolonged elimination half-life in patients with CKD, approximately 134 h [94]. Mircera® is approved for the treatment of anemia associated with CKD in Europe.

Hematide is a nonnaturally occurring Epo dipeptide mimetic that binds to the Epo receptor in a manner similar to rHuEpo, thereby activating it [95, 96]. The dipeptide was pegylated resulting in a longer serum half-life and it is currently in clinical development.

Control of Circulating Epo Levels

Synthesis of eEpo

In adult mammals, Epo is expressed in liver hepatocytes [9] and in interstitial fibroblast-like cells in the kidney which are uniquely located adjacent to kidney tubular cells where they can sense changes in oxygen levels and rapidly and robustly respond [8, 97]. In anemic subjects, circulating Epo levels were inversely proportional to Hb levels which in severe cases of anemia were increased up to 1,000-fold [98]. In isolated nuclei from kidneys from hypoxic vs normal animals, Epo mRNA

levels increased substantially with hypoxia (reduced O_2) in a short time-frame (2–4 h) [99] and rapidly declined following introduction of normal O_2 levels. The increased Epo production was due to a logarithmic increase in the number of cells producing Epo in the renal cortex of bled mice as the degree of hypoxia increased and not to the rate/cell in these cells which appeared constant [10]. The increased response to hypoxia is blunted if Epo-producing cells are lost (e.g., due to kidney disease) or have compromised ability to sense reduced O_2 levels explaining the anemia associated with progressive kidney disease.

The mechanism by which hypoxia upregulates Epo production is now partially understood. Hypoxia inducible factor (HIF) is a transcription factor that binds to a hypoxia response element (HRE) found in certain genes thereby stimulating mRNA synthesis from them [100, 101]. Homologs of HIF (HIF-1α, HIF-2α, HIF-3α) each form a heterodimer composed of an oxygen-sensitive HIFα and a constitutive HIF-1β [102]. HIF-2α is thought to play the dominant role in regulation of *EPO* gene transcription. HIF is expressed constitutively but its levels are controlled by rapid O_2-dependent degradation. Degradation of HIF-α is triggered when it is hydroxylated at specific prolines within an oxygen degradation domain by an O_2-dependent enzyme, HIF-prolyl hydroxylase; HIF-PH [103–106]. Such hydroxylation triggers an association of the hydroxylated HIF with the von Hippel-Lindau protein (VHL), which promotes ligation with ubiquitin and subsequent degradation by the proteasome [106–108]. Thus at low levels of O_2, HIF accumulates due to reduced activity of HIF-PH, resulting in increased eEpo synthesis. At increased O_2 tension, however, HIF levels decline rapidly due to increased HIF-PH activity (within minutes) resulting in a rapid halt in Epo synthesis.

Control of Epo Levels – Clearance

In adult and pediatric patients with CKD, the elimination half-life of epoetin alfa administered intravenously (IV) ranged from 4 to 13 h [109], similar to that reported for eEpo (5 h) [110]. Subcutaneous (SC) administration resulted in slower absorption, with peak plasma levels achieved after 5–24 h. Peak plasma levels were lower than that observed with IV administration (5–10%) with an apparent extended $t_{1/2}$ (~20–25 h) [111, 112]. This clearance profile is known as "flip–flop pharmacokinetics" [113], where the rate of absorption is slower than the rate of elimination. In the case of rHuEpo, absorption is rate limiting and the increased apparent half-life after SC dosing reflects the absorption rather than elimination rate.

Bioavailability estimates for SC rHuEpo range from about 20 to 40%, suggesting a substantial loss of material during transport from the interstitial space to the lymphatic system and blood [109]. The pharmacokinetic (pk) characteristics of rHuEpo in healthy volunteers appear similar or comparable to those in several other populations, including chronic kidney disease, liver cirrhosis, and myelodysplastic syndrome patients.

In contrast to rHuEpo, darbepoetin alfa has an extended serum half-life [91]. Following IV administration in hemodialysis patients, the serum half-life of darbepoetin alfa was roughly threefold longer than that observed with epoetin alfa (25 vs 8.5 h) [92]. With SC administration, darbepoetin alfa concentrations peaked at 34–58 h post-dose and the serum half-life was extended approximately twofold compared to IV administration. A later study that examined pk parameters in CKD patients following SC administration at extended times reported a mean terminal serum half-life of 70 h [114]. The mean terminal half-life of darbepoetin alfa administered SC in cancer patients treated with chemotherapy was also approximately 70 h [115] and was comparable in pediatric patients with CIA [116].

The mean $t_{1/2}$ of PEG-epoetin beta was 134 h in patients receiving peritoneal dialysis when administered IV [94]. Unlike rHuEpo and darbepoetin alfa, the terminal half-life when administered SC was similar; PEG-epoetin beta did not display "flip–flop" pharmacokinetics. Bioavailability of PEG-epoetin beta was 52% suggesting that IV administration may be the more efficient route.

The mechanism by which ESAs are cleared has been studied. Clearance of ESAs was first thought to be mediated primarily by liver hepatocytes through an asialoglycoprotein receptor (ASGR) [117, 118] or through the kidney [63, 119]. But these conclusions were not supported by other studies [120–122]. Binding of Epo to EpoR can lead to cellular internalization and degradation [123, 124] suggesting that Epo receptor-mediated uptake and metabolism may affect clearance. An engineered rHuEpo analog (NM385) that was devoid of detectable receptor binding but retained similar structure and carbohydrate content to rHuEpo was constructed and its clearance properties were examined [125]. In rodents NM385 had a slightly longer terminal half-life but similar clearance compared with rHuEpo, suggesting that ESAs may be cleared to some degree through this pathway but this may not be the only or dominant one.

Other studies suggested clearance may be via metabolism in tissue. Indeed, the lymphatic system is thought to play an important role in the reduced bioavailability after subcutaneous administration of proteins [126]. Degraded, but little intact, darbepoetin alfa was found in tissues following administration to rats suggesting that darbepoetin distributed to tissue where it was degraded [127, 128]. Thus clearance of Epo may occur by diffusion from the blood to the interstitium where metabolism occurs, such as by cells involved in the reticuloendothelial scavenging pathway or lymphatic system.

PEGylated epoetin beta and darbepoetin alfa have reduced receptor-binding activity suggesting that the effect of PEG and additional carbohydrate on clearance may be due to reduced Epo receptor-mediated endocytosis and degradation. However, PEGylated rHuEpo and PEGylated NM385 (that lacks receptor-binding activity) both had similar clearance properties [125], suggesting that EpoR-mediated clearance is minimally impacted by hyperglycosylation or PEGylation. Hyperglycosylated and PEGylated ESAs have other biophysical characteristics such as increased hydrodynamic size. Thus a more likely possibility is that these ESAs have larger hydrodynamic size that reduces transport from the blood to the interstitial fluid where degradation takes place.

Pharmacodynamics

In cell culture with hematopoietic cells obtained from bone marrow or peripheral blood, there is an increase in number of colonies that grow in semisolid medium with increasing concentration of Epo. The rate of growth of individual cells appears relatively constant indicating that increasing concentrations of Epo support growth and survival of increasing numbers of progenitor cells. At concentrations above approximately 1 unit/mL, no additional colony growth occurs [69]. Normal circulating levels of Epo are ~10–30 mU/mL [129]. In healthy subjects, this level is sufficient to produce enough RBCs to maintain a normal hematocrit and increased Epo concentrations result in an increase in rate of erythropoiesis [69, 130, 131].

In patients with CKD, production of erythropoietin is impaired, and this is the primary cause of their anemia [52, 130]. "Replacement therapy" by administration of ESAs can stimulate erythropoiesis and raise hemoglobin levels in these patients. The rate of hematocrit increase varies among patients and is dependent upon the dose of epoetin alfa, within a therapeutic range of approximately 50–300 U/kg 3 times weekly. A greater biologic response is not typically observed at higher doses [130]. Other factors affecting the rate and extent of response include availability of iron stores, the baseline hematocrit, and the presence of concurrent medical problems.

Patients with cancer also can have anemia either due to the disease itself or due to the effects of chemotherapy. In these patients, the level of circulating Epo is insufficient for the degree of anemia. The response in these patients to rHuEpo varies and incomplete response is attributed to insufficient iron levels or to the inhibition of erythropoiesis caused by elevated levels of inflammatory cytokines that inhibit erythropoiesis [132, 133].

RHuEpo administered to CKD patients showed a modest increase in the rate of erythropoiesis with increasing doses of Epo (~fourfold) [130]. However, this change was relatively small compared to the changes in circulating Epo concentrations that occur with severe anemia (~1,000-fold increase) [129]. Indeed, administration of rHuEpo to animals can result in peak serum concentrations that are substantially above that associated with maximal rates of erythropoiesis, yet at high doses there is a dose-dependent increase in final Hb levels. These observations indicate that Epo concentration level per se is not the primary driver of enhanced erythropoiesis in this setting. Instead, the magnitude of increase in RBC concentration is primarily controlled by the length of time Epo concentrations are maintained at higher levels, and increased starting concentrations of Epo result in a prolonged time that concentration is above a threshold. Darbepoetin alfa, a novel ESA with a prolonged serum half-life that has increased in vivo potency compared to faster clearing ESAs, exploits this association [69, 90].

The effect on hematocrit of rHuEpo administration is prolonged compared to the exposure time of ESAs due to the disproportionate relationship between rHuEpo serum half-life and RBC lifespan. Administration of rHuEpo results in circulating levels above baseline that can last for 2–4 days. This time period is comparable to the lifespan of reticulocytes ($t_{1/2}$=1–5 days) [134, 135] but short compared to the

lifespan of RBCs (100–120 days) [3]. Thus, a short duration of increased rHuEpo exposure results in a prolonged increase in RBC concentration. This observation is exploited in clinical settings where intermittent dosing schedules can increase or maintain Hb levels for extended time periods. Dose and dosing schedule are determined according to both pk–PD parameters as well as patient and physician practice patterns. For example, patients on hemodialysis present to the clinic 2–3 times/week and may be dosed on each visit with an ESA.

In patients with CIA, administration of rHuEpo is typically 2–3 times/week or 1 time/week according to pk and PD parameters. However in this setting, patients typically present to the clinic on a 3–4 week schedule which is the time between chemotherapy sessions. Darbepoetin alfa with an extended serum half-life is indicated for reduced frequency of administration compared to epoetin alfa allowing ESA administration to more closely match office visits.

Nonhematopoietic Effects of Epo

ESAs were reported to promote proliferation or survival of nonhematopoietic cells and show benefit to various ischemic insults in organs such as heart, kidney, and brain. The effect was reportedly due to an interaction of Epo with Epo receptors expressed on those cells [136]. The possibility that nonhematopoietic tumor cells expressed EpoR and responded to Epo raised concerns about the use of ESAs in patients with cancer [137]. While these proposals have its proponents, the hypothesis is controversial.

EpoR mRNA was detected in nonerythroid tissues using sensitive RT-PCR methodologies. The significance of this observation is unclear because most RT-PCR experiments were nonquantitative, and those that were quantitative showed that EpoR expression in nonhematopoietic cells ranged from 10-fold to 1,000-fold lower than in cells known to bind or respond to Epo [24, 28, 138–141]. The studies that reportedly detected EpoR protein in nonhematopoietic cells were based largely on antibodies that were shown to be nonspecific; they did not distinguish between EpoR positive and negative cells in IHC experiments and the EpoR protein in Western immunoblotting experiments was often misidentified [23, 138, 142–144].

The possibility that Epo affected growth or survival of nonerythroid hematopoietic cells is inconsistent with the minimal change in circulating nonerythroid cell counts when Epo was administered to normal animals and humans [145, 146]. Furthermore, mice that were engineered to express EpoR exclusively in hematopoietic cells showed normal size and development [147]. The notion that rHuEpo promoted survival of ischemic insult in the brain was not fully supported by pk studies showing that rHuEpo was poorly transported into the brain [148, 149]. Some studies using in vitro cell culture systems showed putative activation of signaling, stimulation of in vitro proliferation, or increased survival of tumor cells or cell lines following ESA addition. However, the effects were generally modest, were compromised by methodological issues, and many studies from other investigators demonstrated no effect of ESAs [138, 150, 151]. Studies examining

growth-promoting activity of tumor cells by ESAs in animal models almost universally saw either no effect or reported a decrease in tumor cell growth in the ESA arm of the studies [151].

The tissue protective effects reportedly due to ESA administration may be indirect. For example, Katavetin argued that the beneficial effect of ESA administration was not explained by a reduction in apoptosis due to EpoR activation by ESAs but alternatively due to a decrease in oxidative stress [152]. Thus the enhanced erythropoiesis due to ESA administration may reduce oxidative stress through increased mobilization of iron from tissues into the erythron [153]. Additional work is necessary in this area to test and confirm the various hypotheses.

Conclusion

Before the availability of rHuEpo, the methods to treat anemia were limited to transfusion with its associated problems, or administered iron or steroids which were largely ineffective by themselves. The cloning of the Epo gene allowed commercial production of rHuEpo and new ways to treat patients with anemia. Second generation molecules with a longer serum half-life, such as darbepoetin alfa, were discovered and developed providing additional physician and patient convenience. Experiments with rHuEpo also allowed an enhanced understanding of anemia, the mechanisms by which Epo functions and how Epo levels are controlled through both synthesis of Epo and its clearance. Scientists continue to provide advances which should provide increased understanding of these processes and further advance our ability to treat patients.

References

1. Lin FK, Suggs S, Lin CH, et al. Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci USA. 1985;82:7580–4.
2. Jacobs K, Shoemaker C, Rudersdorf R, et al. Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature. 1985;313:806–10.
3. Smith JA. Exercise, training and red blood cell turnover. Sports Med. 1995;19:9–31.
4. Elliott S, Pham E, Macdougall IC. Erythropoietins: a common mechanism of action. Exp Hematol. 2008;36:1573–84.
5. Hebbel RP, Eaton JW. Pathobiology of heme interaction with the erythrocyte membrane. Semin Hematol. 1989;26:136–49.
6. Ganz T. Iron homeostasis: fitting the puzzle pieces together. Cell Metab. 2008;7:288–90.
7. Elliott S. Erythropoiesis-stimulating agents and other methods to enhance oxygen transport. Br J Pharmacol. 2008;154:529–41.
8. Koury ST, Bondurant MC, Koury MJ. Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. Blood. 1988;71:524–7.
9. Koury ST, Bondurant MC, Koury MJ, Semenza GL. Localization of cells producing erythropoietin in murine liver by in situ hybridization. Blood. 1991;77:2497–503.
10. Koury ST, Koury MJ, Bondurant MC, Caro J, Graber SE. Quantitation of erythropoietin-producing cells in kidneys of mice by in situ hybridization: correlation with hematocrit, renal erythropoietin mRNA, and serum erythropoietin concentration. Blood. 1989;74:645–51.

11. Lacombe C, Da Silva JL, Bruneval P, et al. Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. J Clin Invest. 1988;81:620–3.
12. Maxwell PH, Osmond MK, Pugh CW, et al. Identification of the renal erythropoietin-producing cells using transgenic mice. Kidney Int. 1993;44:1149–62.
13. Lai PH, Everett R, Wang FF, Arakawa T, Goldwasser E. Structural characterization of human erythropoietin. J Biol Chem. 1986;261:3116–21.
14. Browne JK, Cohen AM, Egrie JC, et al. Erythropoietin: gene cloning, protein structure, and biological properties. Cold Spring Harb Symp Quant Biol. 1986;51:693–702.
15. Syed RS, Reid SW, Li C, et al. Efficiency of signalling through cytokine receptors depends critically on receptor orientation. Nature. 1998;395:511–16.
16. Cheetham JC, Smith DM, Aoki KH, et al. NMR structure of human erythropoietin and a comparison with its receptor bound conformation. Nat Struct Biol. 1998;5:861–6.
17. Mitjavila MT, Natazawa M, Brignaschi P, et al. Effects of five recombinant hematopoietic growth factors on enriched human erythroid progenitors in serum-replaced cultures. J Cell Physiol. 1989;138:617–23.
18. Uoshima N, Ozawa M, Kimura S, et al. Changes in c-Kit expression and effects of SCF during differentiation of human erythroid progenitor cells. Br J Haematol. 1995;91:30–6.
19. Broudy VC, Lin N, Zsebo KM, et al. Isolation and characterization of a monoclonal antibody that recognizes the human c-kit receptor. Blood. 1992;79:338–46.
20. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995;83: 59–67.
21. Winkelmann JC, Penny LA, Deaven LL, Forget BG, Jenkins RB. The gene for the human erythropoietin receptor: analysis of the coding sequence and assignment to chromosome 19p. Blood. 1990;76:24–30.
22. Jones SS, D'Andrea AD, Haines LL, Wong GG. Human erythropoietin receptor: cloning, expression, and biologic characterization. Blood. 1990;76:31–5.
23. Elliott S, Busse L, Bass MB, et al. Anti-Epo receptor antibodies do not predict Epo receptor expression. Blood. 2006;107:1892–5.
24. Liu ZY, Chin K, Noguchi CT. Tissue specific expression of human erythropoietin receptor in transgenic mice. Dev Biol. 1994;166:159–69.
25. Wognum AW, Lansdorp PM, Humphries RK, Krystal G. Detection and isolation of the erythropoietin receptor using biotinylated erythropoietin. Blood. 1990;76:697–705.
26. McArthur GA, Longmore GD, Klingler K, Johnson GR. Lineage-restricted recruitment of immature hematopoietic progenitor cells in response to Epo after normal hematopoietic cell transfection with EpoR. Exp Hematol. 1995;23:645–54.
27. Ashihara E, Vannucchi AM, Migliaccio G, Migliaccio AR. Growth factor receptor expression during in vitro differentiation of partially purified populations containing murine stem cells. J Cell Physiol. 1997;171:343–56.
28. Billia F, Barbara M, McEwen J, Trevisan M, Iscove NN. Resolution of pluripotential intermediates in murine hematopoietic differentiation by global complementary DNA amplification from single cells: confirmation of assignments by expression profiling of cytokine receptor transcripts. Blood. 2001;97:2257–68.
29. Broudy VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T. Erythropoietin receptor characteristics on primary human erythroid cells. Blood. 1991;77:2583–90.
30. Sawada K, Krantz SB, Kans JS, et al. Purification of human erythroid colony-forming units and demonstration of specific binding of erythropoietin. J Clin Invest. 1987;80:357–66.
31. Fraser JK, Lin FK, Berridge MV. Expression of high affinity receptors for erythropoietin on human bone marrow cells and on the human erythroleukemic cell line, HEL. Exp Hematol. 1988;16:836–42.
32. Wojchowski DM, Gregory RC, Miller CP, Pandit AK, Pircher TJ. Signal transduction in the erythropoietin receptor system. Exp Cell Res. 1999;253:143–56.
33. Jegalian AG, Wu H. Differential roles of SOCS family members in EpoR signal transduction. J Interferon Cytokine Res. 2002;22:853–60.

34. Minoo P, Zadeh MM, Rottapel R, Lebrun JJ, Ali S. A novel SHP-1/Grb2-dependent mechanism of negative regulation of cytokine-receptor signaling: contribution of SHP-1 C-terminal tyrosines in cytokine signaling. Blood. 2004;103:1398–407.
35. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science. 1990;248:378–81.
36. Mayeux P, Billat C, Jacquot R. The erythropoietin receptor of rat erythroid progenitor cells – characterization and affinity cross-linkage. J Biol Chem. 1987;262:13985–90.
37. Jegalian AG, Acurio A, Dranoff G, Wu H. Erythropoietin receptor haploinsufficiency and in vivo interplay with granulocyte–macrophage colony-stimulating factor and interleukin 3. Blood. 2002;99:2603–5.
38. Migliaccio AR, Jiang Y, Migliaccio G, et al. Transcriptional and posttranscriptional regulation of the expression of the erythropoietin receptor gene in human erythropoietin-responsive cell lines. Blood. 1993;82:3760–9.
39. Migliaccio G, Migliaccio AR, Visser JW. Synergism between erythropoietin and interleukin-3 in the induction of hematopoietic stem cell proliferation and erythroid burst colony formation. Blood. 1988;72:944–51.
40. Migliaccio AR, Migliaccio G, D'Andrea A, et al. Response to erythropoietin in erythroid subclones of the factor-dependent cell line 32D is determined by translocation of the erythropoietin receptor to the cell surface. Proc Natl Acad Sci USA. 1991;88:11086–90.
41. Santucci MA, Pierce JH, Zannini S, et al. Erythropoietin increases the radioresistance of a clonal hematopoietic progenitor cell line expressing a transgene for the erythropoietin receptor. Stem Cells. 1994;12:506–13.
42. Wang Y, Kayman SC, Li JP, Pinter A. Erythropoietin receptor (EpoR)-dependent mitogenicity of spleen focus-forming virus correlates with viral pathogenicity and processing of env protein but not with formation of gp52–EpoR complexes in the endoplasmic reticulum. J Virol. 1993;67:1322–7.
43. Gobert S, Chretien S, Gouilleux F, et al. Identification of tyrosine residues within the intracellular domain of the erythropoietin receptor crucial for STAT5 activation. EMBO J. 1996;15:2434–41.
44. Binder C, Lafayette A, Archibeque I, et al. Optimization and utilization of the SureFire phospho-STAT5 assay for a cell-based screening campaign. Assay Drug Dev Technol. 2008;6:27–37.
45. Broudy VC, Lin N, Egrie J, et al. Identification of the receptor for erythropoietin on human and murine erythroleukemia cells and modulation by phorbol ester and dimethyl sulfoxide. Proc Natl Acad Sci USA. 1988;85:6513–17.
46. Longmore GD, Lodish HF. An activating mutation in the murine erythropoietin receptor induces erythroleukemia in mice: a cytokine receptor superfamily oncogene. Cell. 1991;67:1089–102.
47. Sakamoto H, Kitamura T, Yoshimura A. Mitogen-activated protein kinase plays an essential role in the erythropoietin-dependent proliferation of CTLL-2 cells. J Biol Chem. 2000;275:35857–62.
48. Yamamura Y, Noda M, Ikawa Y. Erythropoietin receptor and interleukin-2 receptor use different downstream signaling pathways for proliferation and apoptosis-block. Leukemia. 1994;8:s107–10.
49. Wakao H, Harada N, Kitamura T, Mui AL, Miyajima A. Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways. EMBO J. 1995;14:2527–35.
50. Yamamura Y, Kageyama Y, Matuzaki T, Noda M, Ikawa Y. Distinct downstream signaling mechanism between erythropoietin receptor and interleukin-2 receptor. EMBO J. 1992;11:4909–15.
51. Minamoto S, Treisman J, Hankins WD, Sugamura K, Rosenberg SA. Acquired erythropoietin responsiveness of interleukin-2-dependent T lymphocytes retrovirally transduced with genes encoding chimeric erythropoietin/interleukin-2 receptors. Blood. 1995;86:2281–7.

52. Eschbach JW, Kelly MR, Haley NR, Abels RI, Adamson JW. Treatment of the anemia of progressive renal failure with recombinant human erythropoietin. N Engl J Med. 1989;321:158–63.
53. Guthrie M, Cardenas D, Eschbach JW, et al. Effects of erythropoietin on strength and functional status of patients on hemodialysis. Clin Nephrol. 1993;39:97–102.
54. Locatelli F, Baldamus CA, Villa G, Ganea A, Martin de Francisco AL. Once-weekly compared with three-times-weekly subcutaneous epoetin beta: results from a randomized, multicenter, therapeutic-equivalence study. Am J Kidney Dis. 2002;40:119–25.
55. Macdougall IC. An overview of the efficacy and safety of novel erythropoiesis stimulating protein (NESP). Nephrol Dial Transplant. 2001;16:14–21.
56. Glaspy JA. Hematopoietic management in oncology practice. Part 2: Erythropoietic factors. Oncology (Huntington). 2003;17:1724–30.
57. Singh AK, Szczech L, Tang KL, et al. Correction of anemia with epoetin alfa in chronic kidney disease. N Engl J Med. 2006;355:2085–98.
58. Nowrousian MR, Dunst J, Vaupel P. Erythropoiesis-stimulating agents: favorable safety profile when used as indicated. Strahlenther Onkol. 2008;184:121–36.
59. Rush RS, Derby PL, Smith DM, et al. Microheterogeneity of erythropoietin carbohydrate structure. Anal Chem. 1995;67:1442–52.
60. Sasaki H, Bothner B, Dell A, Fukuda M. Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. J Biol Chem. 1987;262:12059–76.
61. Takeuchi M, Kobata A. Structures and functional roles of the sugar chains of human erythropoietins. Glycobiology. 1991;1:337–46.
62. Wide L, Bengtsson C. Molecular charge heterogeneity of human serum erythropoietin. Br J Haematol. 1990;76:121–7.
63. Lasne F, Martin L, Crepin N, De Ceaurriz J. Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones. Anal Biochem. 2002;311:119–26.
64. Takeuchi M, Takasaki S, Miyazaki H, et al. Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. J Biol Chem. 1988;263:3657–63.
65. Noguchi A, Mukuria CJ, Suzuki E, Naiki M. Failure of human immunoresponse to *N*-glycolylneuraminic acid epitope contained in recombinant human erythropoietin. Nephron. 1996;72:599–603.
66. Hokke CH, Bergwerff AA, Van Dedem GW, Kamerling JP, Vliegenthart JF. Structural analysis of the sialylated N- and O-linked carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. Sialylation patterns and branch location of dimeric *N*-acetyllactosamine units. Eur J Biochem. 1995;228:981–1008.
67. Kawasaki N, Haishima Y, Ohta M, et al. Structural analysis of sulfated N-linked oligosaccharides in erythropoietin. Glycobiology. 2001;11:1043–9.
68. Strickland T, Adler B, Aoki K, et al. Occurrence of sulfate on the N-linked oligosaccharides of human erythropoietin. J Cell Biochem. 1992;Suppl 16D:167.
69. Elliott S, Egrie J, Browne J, et al. Control of rHuEPO biological activity: the role of carbohydrate. Exp Hematol. 2004;32:1146–55.
70. Sinclair AM, Elliott S. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. J Pharm Sci. 2005;94:1626–35.
71. Bernard BA, Yamada KM, Olden K. Carbohydrates selectively protect a specific domain of fibronectin against proteases. J Biol Chem. 1982;257:8549–54.
72. Runkel L, Meier W, Pepinsky RB, et al. Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-beta (IFN-beta). Pharm Res. 1998;15:641–9.
73. Sola RJ, Griebenow K. Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci. 2009;98:1223–45.

74. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. Crit Rev Ther Drug Carrier Syst. 1993;10:307–77.
75. Covic A, Kuhlmann MK. Biosimilars: recent developments. Int Urol Nephrol. 2007;39: 261–6.
76. Roger SD, Goldsmith D. Biosimilars: it's not as simple as cost alone. J Clin Pharm Ther. 2008;33:459–64.
77. Bouchet JL, Brunet P, Canaud B, et al. Position statements regarding usage of biosimilars of Epoetins. Position paper of the Societe de nephrologie, Societe francophone de dialyse, and Societe de nephrologie pediatrique. Nephrol Ther. 2009;5:61–6.
78. Antonetti F, Finocchiaro O, Mascia M, Terlizzese MG, Jaber A. A comparison of the biologic activity of two recombinant IFN-beta preparations used in the treatment of relapsing–remitting multiple sclerosis. J Interferon Cytokine Res. 2002;22:1181–4.
79. Crommelin DJ, Storm G, Verrijk R, et al. Shifting paradigms: biopharmaceuticals versus low molecular weight drugs. Int J Pharm. 2003;266:3–16.
80. Rosenberg AS. Immunogenicity of biological therapeutics: a hierarchy of concerns. Dev Biol. 2003;112:15–21.
81. Schellekens H. Recombinant human erythropoietins, biosimilars and immunogenicity. J Nephrol. 2008;21:497–502.
82. Smalling R, Foote M, Molineux G, Swanson SJ, Elliott S. Drug-induced and antibody-mediated pure red cell aplasia: a review of literature and current knowledge. Biotechnol Annu Rev. 2004;10:237–49.
83. Casadevall N, Nataf J, Viron B, et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. N Engl J Med. 2002;346:469–75.
84. Verhelst D, Rossert J, Casadevall N, et al. Treatment of erythropoietin-induced pure red cell aplasia: a retrospective study. Lancet. 2004;363:1768–71.
85. Schellekens H. Bioequivalence and the immunogenicity of biopharmaceuticals. Nat Rev Drug Discov. 2002;1:457–62.
86. Schellekens H. Follow-on biologics: challenges of the 'next generation'. Nephrol Dial Transplant. 2005;20:iv31–36.
87. Park SS, Park J, Ko J, et al. Biochemical assessment of erythropoietin products from Asia versus US Epoetin alfa manufactured by Amgen. J Pharm Sci. 2009;98:1688–99.
88. Keithi-Reddy SR, Kandasamy S, Singh AK. Pure red cell aplasia due to follow-on epoetin. Kidney Int. 2008;74:1617–22.
89. Belalcazar V, Ventura R, Segura J, Pascual JA. Clarification on the detection of epoetin delta and epoetin omega using isoelectric focusing. Am J Hematol. 2008;83:754–5.
90. Elliott S, Lorenzini T, Asher S, et al. Enhancement of therapeutic protein in vivo activities through glycoengineering. Nat Biotechnol. 2003;21:414–21.
91. Egrie JC, Dwyer E, Browne JK, Hitz A, Lykos MA. Darbepoetin alfa has a longer circulating half-life and greater in vivo potency than recombinant human erythropoietin. Exp Hematol. 2003;31:290–9.
92. Macdougall IC, Gray SJ, Elston O, et al. Pharmacokinetics of novel erythropoiesis stimulating protein compared with epoetin alfa in dialysis patients. J Am Soc Nephrol. 1999;10:2392–5.
93. Jarsch M, Brandt M, Lanzendorfer M, Haselbeck A. Comparative erythropoietin receptor binding kinetics of C.E.R.A. and epoetin-beta determined by surface plasmon resonance and competition binding assay. Pharmacology. 2008;81:63–9.
94. Macdougall IC, et al. Pharmacokinetics and pharmacodynamics of intravenous and subcutaneous continuous erythropoietin receptor activator (C.E.R.A.) in patients with chronic kidney disease. Clin J Am Soc Nephrol. 2006;1:1211–15.
95. Fan Q, Leuther KK, Holmes CP, et al. Preclinical evaluation of hematide, a novel erythropoiesis stimulating agent, for the treatment of anemia. Exp Hematol. 2006;34:1303–11.
96. Connolly PJ, Wetter SK, Murray WV, et al. Synthesis and erythropoietin receptor binding affinities of *N*, *N*-disubstituted amino acids. Bioorg Med Chem Lett. 2000;10:1995–9.

97. Suzuki N, Obara N, Yamamoto M. Use of gene-manipulated mice in the study of erythropoietin gene expression. Methods Enzymol. 2007;435:157–77.
98. Erslev AJ. Clinical erythrokinetics: a critical review. Blood Rev. 1997;11:160–7.
99. Schuster SJ, Badiavas EV, Costa-Giomi P, et al. Stimulation of erythropoietin gene transcription during hypoxia and cobalt exposure. Blood. 1989;73:13–16.
100. Jiang BH, Rue E, Wang GL, Roe R, Semenza GL. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem. 1996;271:17771–8.
101. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA. 1995;92:5510–14.
102. Kaelin WG Jr, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell. 2008;30:393–402.
103. Epstein AC, Gleadle JM, McNeill LA, et al. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 2001;107:43–54.
104. Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J. 2001;20:5197–206.
105. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science. 2001;294:1337–40.
106. Ivan M, Kondo K, Yang H, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science. 2001;292:464–8.
107. Hon WC, Wilson MI, Harlos K, et al. Structural basis for the recognition of hydroxyproline in HIF-1 alpha by pVHL. Nature. 2002;417:975–8.
108. Maxwell PH, Pugh CW, Ratcliffe PJ. Insights into the role of the von Hippel–Lindau gene product. A key player in hypoxic regulation. Exp Nephrol. 2001;9:235–40.
109. Heatherington AC. Clinical pharmacokinetic properties of rHuEpo: a review. In: Molineux G, Foot MA, Elliott SG, editors. Erythropoietins and erythropoiesis: molecular, cellular, preclinical and clinical biology. Basel: Birkhauser; 2003. pp. 87–112.
110. Eckardt KU, Boutellier U, Kurtz A, et al. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. J Appl Physiol. 1989; 66: 1785–8.
111. Cheung WK, Goon BL, Guilfoyle MC, Wacholtz MC. Pharmacokinetics and pharmacodynamics of recombinant human erythropoietin after single and multiple subcutaneous doses to healthy subjects. Clin Pharmacol Ther. 1998;64:412–23.
112. McMahon FG, Vargas R, Ryan M, et al. Pharmacokinetics and effects of recombinant human erythropoietin after intravenous and subcutaneous injections in healthy volunteers. Blood. 1990;76:1718–22.
113. Boxenbaum H. Pharmacokinetics tricks and traps: flip–flop models. J Pharm Pharm Sci. 1998;1:90–1.
114. Padhi D, Ni L, Cooke B, Marino R, Jang G. An extended terminal half-life for darbepoetin alfa: results from a single-dose pharmacokinetic study in patients with chronic kidney disease not receiving dialysis. Clin Pharmacokinet. 2006;45:503–10.
115. Glaspy J, Henry D, Patel R, et al. Effects of chemotherapy on endogenous erythropoietin levels and the pharmacokinetics and erythropoietic response of darbepoetin alfa: a randomised clinical trial of synchronous versus asynchronous dosing of darbepoetin alfa. Eur J Cancer. 2005;41:1140–9.
116. Blumer J, Berg S, Adamson PC, et al. Pharmacokinetic evaluation of darbepoetin alfa for the treatment of pediatric patients with chemotherapy-induced anemia. Pediatr Blood Cancer. 2007;49:687–93.
117. Fukuda MN, Sasaki H, Lopez L, Fukuda M. Survival of recombinant erythropoietin in the circulation: the role of carbohydrates. Blood. 1989;73:84–9.
118. Spivak JL, Hogans BB. The in vivo metabolism of recombinant human erythropoietin in the rat. Blood. 1989;73:90–9.

119. Fu J-S, Lertora JJL, Brookins J, Rice JC, Fisher JW. Pharmacokinetics of erythropoietin in intact and anephric dogs. J Lab Clin Med. 1988;111:669–76.
120. Widness JA, Veng-Pedersen P, Schmidt RL, et al. In vivo 125I-erythropoietin pharmacokinetics are unchanged after anesthesia, nephrectomy and hepatectomy in sheep. J Pharmacol Exp Ther. 1996;279:1205–10.
121. Kindler J, Eckardt KU, Ehmer B, et al. Single-dose pharmacokinetics of recombinant human erythropoietin in patients with various degrees of renal failure. Nephrol Dial Transplant. 1989;4:345–9.
122. Macdougall IC, Roberts DE, Coles GA, Williams JD. Clinical pharmacokinetics of epoetin (recombinant human erythropoietin). Clin Pharmacokinet. 1991;20:99–113.
123. Sawyer ST, Krantz SB, Goldwasser E. Binding and receptor-mediated endocytosis of erythropoietin in Friend virus-infected erythroid cells. J Biol Chem. 1987;262:5554–62.
124. Gross AW, Lodish HF. Cellular trafficking and degradation of erythropoietin and novel erythropoiesis stimulating protein (NESP). J Biol Chem. 2006;281:2024–32.
125. Agoram B, Aoki K, Doshi S, et al. Investigation of the effects of altered receptor binding activity on the clearance of erythropoiesis-stimulating proteins: Nonerythropoietin receptor-mediated pathways may play a major role. J Pharm Sci. 2009;98(6):2198–211.
126. Porter CJ, Charman SA. Lymphatic transport of proteins after subcutaneous administration. J Pharm Sci. 2000;89:297–310.
127. Yoshioka E, Kato K, Shindo H, et al. Pharmacokinetic study of darbepoetin alfa: absorption, distribution, and excretion after a single intravenous and subcutaneous administration to rats. Xenobiotica. 2007;37:74–90.
128. Agoram B, Sutjandra L, Molineux G, Jang G, Elliott S. Tissue distribution and excretion of 125I darbepoetin alfa in Sprague Dawley rats following a single subcutaneous or IV administration [abstract]. Nephrol Dial Transplant. 2006;21:304.
129. Erslev AJ. Erythropoietin titers in health and disease. Semin Hematol. 1991;28:2–7.
130. Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. N Engl J Med. 1987;316:73–8.
131. Egrie JC, Strickland TW, Lane J, et al. Characterization and biological effects of recombinant human erythropoietin. Immunobiology. 1986;172:213–24.
132. Hodges VM, Rainey S, Lappin TR, Maxwell AP. Pathophysiology of anemia and erythrocytosis. Crit Rev Oncol Hematol. 2007;64:139–58.
133. Bokemeyer C, Oechsle K, Hartmann JT. Anaemia in cancer patients: pathophysiology, incidence and treatment. Eur J Clin Invest. 2005;35:26–31.
134. Finch CA, Harker LA, Cook JD. Kinetics of the formed elements of human blood. Blood. 1977;50:699–707.
135. Hillman RS, Finch CA. Erythropoiesis: normal and abnormal. Semin Hematol. 1967;4: 327–36.
136. Brines M, Cerami A. Discovering erythropoietin's extra-hematopoietic functions: biology and clinical promise. Kidney Int. 2006;70:246–50.
137. Hardee ME, Arcasoy MO, Blackwell KL, Kirkpatrick JP, Dewhirst MW. Erythropoietin biology in cancer. Clin Cancer Res. 2006;12:332–9.
138. Laugsch M, Metzen E, Svensson T, Depping R, Jelkmann W. Lack of functional erythropoietin receptors of cancer cell lines. Int J Cancer. 2008;122:1005–11.
139. Sinclair AM, Rogers N, Busse L, et al. Erythropoietin receptor transcription is neither elevated nor predictive of surface expression in human tumour cells. Br J Cancer. 2008;98:1059–67.
140. Keller MA, Addya S, Vadigepalli R, et al. Transcriptional regulatory network analysis of developing human erythroid progenitors reveals patterns of coregulation and potential transcriptional regulators. Physiol Genomics. 2006;28:114–28.
141. Jeong JY, Feldman L, Solar P, Szenajch J, Sytkowski AJ. Characterization of erythropoietin receptor and erythropoietin expression and function in human ovarian cancer cells. Int J Cancer. 2008;122:274–80.

142. Brown WM, Maxwell P, Graham AN, et al. Erythropoietin receptor expression in non-small cell lung carcinoma: a question of antibody specificity. Stem Cells. 2007;25:718–22.
143. Della Ragione F, Cucciolla V, Borriello A, Oliva A, Perrotta S. Erythropoietin receptors on cancer cells: a still open question. J Clin Oncol. 2007;25:1812–13.
144. Kirkeby A, van Beek J, Nielsen J, Leist M, Helboe L. Functional and immunochemical characterisation of different antibodies against the erythropoietin receptor. J Neurosci Methods. 2007;164:50–8.
145. Ulich TR, del Castillo J, Yin SM, Egrie JC. The erythropoietic effects of interleukin 6 and erythropoietin in vivo. Exp Hematol. 1991;19:29–34.
146. Grossi A, Vannucchi AM, Rafanelli D, Rossi FP. Recombinant human erythropoietin has little influence on megakaryocytopoiesis in mice. Br J Haematol. 1989;71:463–8.
147. Suzuki N, Ohneda O, Takahashi S, et al. Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. Blood. 2002;100:2279–88.
148. Banks WA, Jumbe NL, Farrell CL, Niehoff ML, Heatherington AC. Passage of erythropoietic agents across the blood–brain barrier: a comparison of human and murine erythropoietin and the analog darbepoetin alfa. Eur J Pharmacol. 2004;505:93–101.
149. Buemi M, Allegra A, Corica F, et al. Intravenous recombinant erythropoietin does not lead to an increase in cerebrospinal fluid erythropoietin concentration. Nephrol Dial Transplant. 2000;15:422–3.
150. Hardee ME, Kirkpatrick JP, Shan S, et al. Human recombinant erythropoietin (rEpo) has no effect on tumour growth or angiogenesis. Br J Cancer. 2005;93:1350–5.
151. Sinclair AM, Todd MD, Forsythe K, et al. Expression and function of erythropoietin receptors in tumors: implications for the use of erythropoiesis-stimulating agents in cancer patients. Cancer. 2007;110:477–88.
152. Katavetin P, Tungsanga K, Eiam-Ong S, Nangaku M. Antioxidative effects of erythropoietin. Kidney Int. 2007;72:S10–S15.
153. Yazihan N, Uzuner K, Salman B, et al. Erythropoietin improves oxidative stress following spinal cord trauma in rats. Injury. 2008;39:1408–13.