Molecular Biology of Erythropoietin

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Abstract

The glycoprotein hormone erythropoietin (EPO) is an essential viability and growth factor for the erythrocytic progenitors. EPO is mainly produced in the kidneys. EPO gene expression is induced by hypoxia-inducible transcription factors (HIF). The principal representative of the HIF-family (HIF-1, -2 and -3) is HIF-1, which is composed of an O_2-labile α-subunit and a constant nuclear β-subunit. In normoxia, the α-subunit of HIF is inactivated following prolyl- and asparaginyl-hydroxylation by means of α-oxoglutarate and Fe^{2+}-dependent HIF specific dioxygenases. While HIF-1 and HIF-2 activate the EPO gene, HIF-3, GATA-2 and NFκB are likely inhibitors of EPO gene transcription. EPO signalling involves tyrosine phosphorylation of the homodimeric EPO receptor and subsequent activation of intracellular anti-apoptotic proteins, kinases and transcription factors. Lack of EPO leads to anemia. Treatment with recombinant human EPO (rHuEPO) is efficient and safe in improving the management of the anemia associated with chronic renal failure. RHuEPO analogues with prolonged survival in circulation have been developed. Whether the recent demonstration of EPO receptors in various non-hemopoietic tissues, including tumor cells, is welcome or ominous still needs to be clarified. Evidence suggests that rHuEPO may be a useful neuroprotective agent.
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Key words: erythropoietin, red blood cells, recombinant drugs, anemia, kidneys, hypoxia-inducible factor

Introduction

Modern recombinant DNA technology enables the manufacture of human proteins in cultured animal cells, yeast and bacteria for use as drugs. More than 20 different blood cell-modulating proteins are currently produced. Some of these act as specific growth factors (Table 1) in the bone marrow and other hemopoietic tissues, where they inhibit the programmed cell death (apoptosis) of the hematopoietic stem and progenitor cells to maintain the growth of young blood cells. Red blood cell production requires the hormone erythropoietin (EPO), a glycoprotein, that is mainly of renal origin. Lack of EPO is the primary cause of the anemia associated with chronic renal failure. Before recombinant human EPO (rHuEPO) became available 15 years ago, about 25% of renal patients on dialysis needed regular transfusions of red cells. Treatment with rHuEPO has proved most useful to increase the quality of life of otherwise anemic patients, and the drug is amongst the top selling pharmaceutical products worldwide. With respect to this success, credit is due to the pioneering work of Miyake et al, who collected and concentrated 2550 l EPO—containing urine from patients with aplastic anemia in Kumamoto City. The material was used to purify the hormone (1) and to partially identify its amino acid sequence. Based on this work the human EPO gene could be characterized and be expressed in host cells (2, 3). Details of the industrial large-scale production of rHuEPO are described elsewhere (4, 5). The present review provides information on the structure of endogenous EPO and the recombinant products, the sites and molecular mechanisms of the hypoxic induction of the EPO gene, the effects of EPO on hematopoietic and non-hematopoietic tissues, and the therapeutic impact of rHuEPO and its analogues.

Structure of Human EPO

EPO is a member of the family of class I cytokines which fold into a compact globular structure consisting of 4 α-helical bundles (6, 7). Its molecular mass is 30.4 kDa (8), although it migrates with an apparent size of 34–38 kDa on SDS-polyacrylamide gels. The peptide core of 165 amino acids (9) suffices for receptor-binding and *in vitro* stimulation of erythropoiesis, while the carbohydrate portion (40% of the total molecule) is required for the *in vivo* survival of the hormone (10). The 4 carbohydrate chains of EPO have been analyzed in detail (11–13). The 3 complex-type N-linked oligosaccharides at asparagines 24, 38 and 83 are important in stabilizing EPO in circulation (14, 15). In contrast, the small O-linked oligosaccharide at serine 126 appears to

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Table 1. Hematopoietic Growth Factors

Factor	Size (kDa)	Sites of formation	Target cells
Erythropoietin*	30	Renal fibroblasts, hepatocytes, neuronal cells	BFU-E, CFU-E
Thrombopoietin	60	Hepatocytes, renal proximal tubular cells	Megakaryocytes
GM-CSF*	14–35	T-lymphocytes, monocytes, endothelial cells, fibroblasts	CFU-GM
G-CSF*	20	Monocytes, endothelial cells, fibroblasts	CFU-G
M-CSF	45–90	Monocytes	CFU-M

*Routinely used as recombinant drugs. BFU: burst-forming unit, CFU: colony-forming unit, CSF: colony-stimulating factor, E: erythroid, G: granulocyte, M: monocyte.

lack functional importance (16, 17).

Human urinary EPO and rHuEPO are identical with respect to their primary and secondary structure. Note, however, that there are minor quantitative differences in the composition of the N- and O-glycans of human urinary EPO (18) and human serum EPO (19), compared to those of rHuEPO. The differences in electrophoretic mobility enable anti-doping laboratories to detect rHuEPO in urinary samples of athletes misusing the drug to enhance their endurance capacity (20). In addition to the approved rHuEPO preparations (Epoetin alfa and Epoetin beta), which are expressed from genetically engineered Chinese hamster ovary (CHO) cells (3, 11, 14, 16), rHuEPO (Epoetin omega) from baby hamster kidney (BHK) cell cultures (21–23) has been applied in clinical trials (24–26). The N-glycans of rHuEPO produced in BHK cells appear to be sulfated to a higher degree (27) which may be relevant regarding the *in vivo* survival of the drug. Other cell lines successfully transfected with the human EPO gene to produce glycosylated rHuEPO included RPMI 1,788 human lymphoblastoid (28), COS African green monkey kidney (2, 29, 30), MDCK canine kidney (31), L929 mouse fibroblast (32) and C127 mouse mammary (33) cells. In view of the relationship between the multiantennary sialic acid—containing carbohydrate chains and the *in vivo* stability of the hormone (34), recently a CHO-cell derived hyperglycosylated rHuEPO analogue (Darbepoetin alfa) has been developed. This compound possesses 2 extra N-linked carbohydrate chains based on site-directed mutagenesis for exchange of 5 amino acids. Compared to the Epoetins, which have a plasma half-life of 6–8 hours, Darbepoetin alfa has a 3- to 4-fold longer plasma half-life (35).

EPO amounts are traditionally expressed in units (U), with 1 U of EPO producing the same erythropoiesis-stimulating response in experimental animals as 5 μmol cobaltous chloride. International reference preparations of human urinary EPO [2nd IRP (36)] and purified DNA-derived human EPO [IS 87/684, rDNA derived (37)] have been established. The specific activity of pure rHuEPO is 130,000 U/mg fully glycosylated protein. Darbepoetin alfa is expressed in gram with the biological activity of 1 μg Darbepoetin alfa peptide core weight corresponding to that of 200 U rHuEPO peptide core weight, both on theoretical grounds (38) and clinical experience (39, 40).

Sites and Mechanisms of EPO Production and Degradation

EPO is mainly produced by hepatocytes during the fetal stage. After birth, almost all circulating EPO originates from peritubular fibroblast-like cells located in the cortex of the kidneys (41–43). Transcription factors of the GATA-family may be important in the control of the time- and tissue-specific expression of the EPO gene (44). In adults, minor amounts of EPO mRNA are expressed in liver parenchyma, spleen, lung, testis and brain (45, 46). In brain, EPO exerts neurotrophic and neuroprotective effects, which are separate from the action of circulating EPO on erythropoietic tissues (47, 48). Tissue hypoxia is the main stimulus of EPO production [for references, see (49)]. In persons with intact renal function plasma EPO levels increase exponentially with decreasing blood hemoglobin (Hb) concentration. Values may rise to 10,000 U/*l* compared to the normal value of about 15 U/*l* [for references, see (50)]. EPO gene expression is not only stimulated when the O_2 capacity (corresponding to the Hb concentration) of the blood decreases, but also when the arterial pO_2 decreases (f.e. at high altitude residence) or when the O_2 affinity of the blood increases. Like other plasma glycoproteins EPO circulates as a pool of isoforms that differ in glycosylation, molecular mass, biological activity and immunoreactivity (51, 52). There is a diurnal fluctuation of the concentration of circulating EPO, with values being about 40% higher at midnight than in the morning (53).

The mechanisms of the degradation of circulating EPO are still incompletely understood. To a minor degree, EPO may be cleared by the liver and the kidneys. However, there is evidence to assume that EPO is mainly removed from circulation by uptake into erythrocytic and other cells possessing the EPO receptor (38). Accordingly, new rHuEPO formulations are presently tested which contain methoxy-polyethylene glycol to prevent internalization of the drug, thus resulting in prolonged biological half-life (54).

Control of EPO Gene Expression

Based on experiments with human EPO-producing hepatoma cell cultures major progress has been made in

understanding the nature of the O_2 sensor controlling the rate of the expression of the EPO gene and other hypoxia-inducible genes (55–59). There are several regulatory DNA sequences in the neighborhood of the EPO gene. The key sequence is located within the so-called hypoxia response element (HRE). It is composed of the nucleotides [A/G] CGTG, to which the hypoxia-inducible transcription factors (HIF) can bind. HIF are dimers composed of an α- and a β-subunit, the latter of which is identical with aryl-hydrocarbon nuclear translocator (ARNT). They belong to the family of basic helix-loop-helix (bHLH) proteins with a PAS domain (according to its presence in the Drosophila Period, ARNT and the Drosophila Single-minded proteins). There are at least three subtypes of the HIF-α subunit (-1α, -2α, -3α). Of these, only HIF-1α and HIF-2α , but not HIF-3α possess a C-terminal transactivation domain (C-TAD). HIF-1α/β is generally considered the primary mediator of hypoxia-induced gene expression (60, 61). The role of HIF-2α/β is only beginning to be understood (62) and HIF-3α/β may actually be a suppressor of hypoxic gene induction (63).

Both the 100–120 kDa HIF-1α and the 91–94 kDa HIF-1 β are continuously produced, with their mRNA levels being essentially unaltered by the induction of hypoxia (64, 65). However, HIF-1α is usually not detectable in normoxic cells (66), while HIF-1β is constantly present in the nucleus. HIF-1α possesses two oxygen-dependent proteolytic degradation domains (ODD) and two TADs. In the presence of O_2, HIF-1α is hydroxylated at the proline residues 402 (67) and 564 (68, 69) in the ODDs. This reaction is catalyzed by specific HIF-1α prolyl-hydroxylase domain (PHD) containing enzymes that belong to the group of α-oxoglutarate- and Fe (II)-dependent dioxygenases (70–72). On binding to the Fe^{2+} (a non-heme iron), O_2 molecules are split with one atom being transferred to the proline residues and the other forming CO_2 (and succinate) with α-oxoglutarate. Ascorbate prevents the inactivation of the PHDs due to oxidation of Fe^{2+} (73). The K_m values of the three PHDs for O_2 are essentially identical and close to atmospheric O_2 concentrations (74). Prolyl-hydroxylated HIF-1α is tagged by the von-Hippel-Lindau gene product pVHL which forms a complex with the E3 ubiquitin ligase (75, 76). Polyubiquitinated HIF-1α is immediately degraded by the proteasome (77, 78). Only under hypoxic conditions, HIF-1α is enabled to enter the nucleus and to heterodimerize with HIF-1β. The binding of pVHL is a prerequisite for the degradation of HIF-1α. Thus, the mutation of pVHL in patients suffering from congenital Chuvash polycythemia is associated with increased transcription of the EPO gene (79).

In addition, HIF-1α is hydroxylated at asparagine in position 803 in the C-TAD in the presence of O_2 which results in a reduced ability of HIF-1α to bind the transcriptional coactivator p300/CBP (80–82). Thus, there are at least 4 specific hydroxylases which function as cellular O_2 sensors (Fig. 1). Interestingly, studies utilizing transfected human cells overexpressing these hydroxylases have revealed differences in their subcellular localization. PHD-1 is exclusively present in the nucleus, PHD-3 occurs in both nucleus and cytoplasm, while PHD-2 and the asparaginyl-hydroxylase (also called FIH, factor inhibiting HIF) are mainly located in the cytoplasm (83, 84).

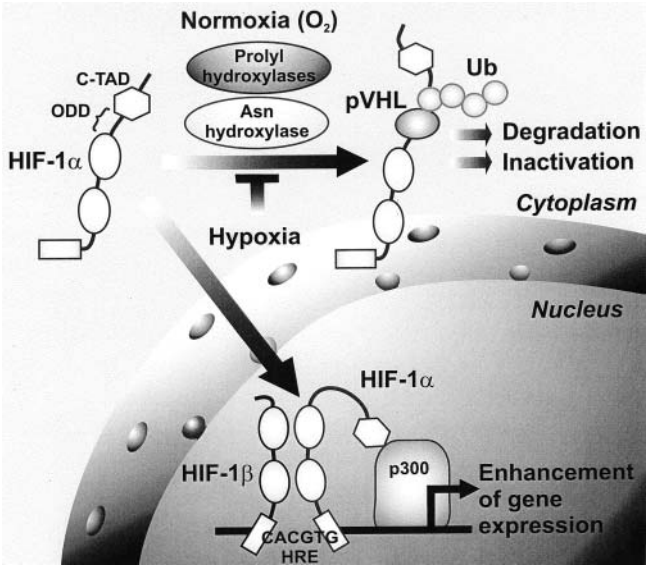

Figure 1. HIF-1α prolyl- and asparaginyl-hydroxylases as cellular O_2 sensors. In normoxia, (a) prolyl hydroxylation in the O_2-dependent degradation domain (ODD) results in binding of the von-Hippel Lindau protein (pVHL) and the ubiquitin ligase complex (Ub) with subsequent proteasomal degradation and (b) asparaginyl hydroxylation in the C-terminal transactivation domain (C-TAD) prevents binding of the p300/CBP transcriptional coactivator. In hypoxia, HIF-1α enters the nucleus to form the active transcription complex with p300/CREB and HIF-1β. Modified from Ref. (197).

The HIFs are not only the key regulators of EPO production. They are involved in most pathways of the adaptation of genes to hypoxia (85) and therefore considered attractive candidates for pharmacologic manipulation (86). For example, the earlier observation that Fe-chelating agents such as desferrioxamine increase EPO production (87–89) may be explained by inhibition of the activity of the Fe (II)-requiring HIF prolyl- and asparaginyl-hydroxylases (Fig. 2). On the other hand, since HIF-1 is associated with angiogenesis and tumor progression, oncologic investigations aim at identifying compounds that specifically inhibit HIF-1 driven gene expression (90).

Truly, however, with respect to the renal production of EPO it must be conceded that the role of HIF-1 has not been well explored. EPO mRNA expression in renal cells has been reported to follow an all-or-nothing fashion rather than to be a graded process (91). In addition, attempts have failed to establish renal cell cultures for study of O_2-dependent EPO production. Studies in EPO transgenic mice (92) have shown that the HREs are located at opposite sites of the gene in the kidney (between 9.5 and 14 kb 5´ to the gene) and the

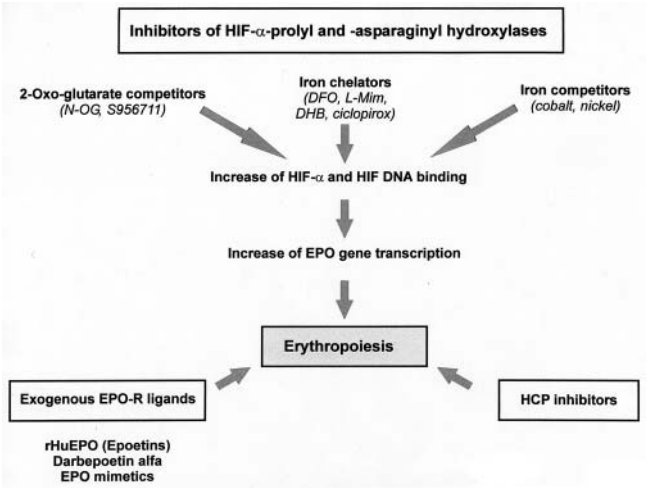

Figure 2. Novel pharmacological approaches to increase erythropoiesis. (a) Endogenous EPO production may be stimulated by compounds stabilizing HIF-α and enhancing HIF transcriptional activity. (b) Exogenous ligands of the EPO receptor (EPO-R) can be administered (clinically approved drugs are the Epoetins and Darbepoetin alfa). (c) Inhibitors of the hemopoietic cell phosphatase (HCP) may prolong the action of EPO. N-OG: N-oxalylglycine, S956711: 6-chloro-3-hydroxychinoline-2-carbonic acid-N-carboxymethylamide, DFO: desferrioxamine, L-Mim: L-Mimosine, DHB: 3, 4-dihydroxybenzoate.

liver (within 0.7 kb 3′ to the gene). Instead of HIF-1α the closely related HIF-2α may control EPO gene expression in the kidney, because HIF-2α was detected in the EPO producing renal fibroblasts of hypoxic rats (93). In contrast to HIF-1 and -2, HIF containing the 3α-subunit is thought to suppress the expression of hypoxia-responsive genes (63).

Finally, it has to be remembered that there are other transcription factors which can modulate EPO gene transcription. Imagawa et al (94, 95) have demonstrated that GATA-2 inhibits EPO gene transcription by binding to the EPO promoter under normoxic conditions. For example, the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) lowers EPO production by increasing GATA-2 DNA-binding (96). L-NMMA is considered one of the candidate substances that suppress EPO synthesis in patients with chronic renal failure (97). Furthermore, the EPO promoter and the 5′ flanking region contain binding sites for nuclear factor κB (NFκB) (98). Evidence suggests that both GATA-2 and NFκB are involved in the inhibition of EPO gene expression in inflammatory diseases. The pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) activate GATA-2 (99) and NFκB (99, 100). IL-1 and TNF-α are thought to contribute to the anemia of chronic disease partly by suppressing EPO production (101). Recent studies have shown that the GATA-specific inhibitor K-7174 restores EPO production in IL-1, TNF-α or L-NMMA treated human hepatoma cell cultures and experimental mice (102). Cyclic AMP has also been shown to antagonize the inhibition of EPO production by IL-1 and TNF-α (103), but the precise role of protein kinase A in the control of EPO mRNA expression still needs to be elucidated (104).

Mechanism of Action of EPO

Erythrocytic progenitors in the bone marrow are the principal targets of EPO. The normally low concentration of the hormone enables only a small percentage of progenitors to survive and to proliferate while the remaining progenitors undergo apoptosis. Thus, the primary mechanism by which EPO maintains erythropoiesis is the prevention of programmed cell death (105, 106). The most primitive EPO-responsive progenitor is the burst-forming unit-erythroid (BFU-E), which gives rise to several colony-forming units-erythroid (CFU-E). BFU-Es are devoid of transferrin receptor and express little GATA-1, whereas CFU-Es possess transferrin receptors and exhibit abundant levels of GATA-1 (107). GATA-1 is an important transcription factor in erythrocytic development (108). The balance between GATA-1 and caspase activity largely determines the rate of proliferation and differentiation of erythrocytic progenitors (106). GATA-1 induces the anti-apoptotic protein bcl-x_L (109). Erythrocytic progenitors may have the potential to produce small amounts of EPO to maintain basal rates of erythropoiesis (110). However, when the concentration of EPO rises in blood, either endogenously or following the administration of rHuEPO, many more BFU-Es and CFU-Es escape from apoptosis and proliferate to result in the growth and maturation of morphologically identifiable proerythroblasts and normoblasts. At the stage of the polychromatic normoblast hemoglobin is accumulated. Subsequently, the nucleus becomes pyknotic and is excluded from the cell. The time from the CFU-E to the reticulocyte is about 7 days and involves 4–6 cell divisions. Significant reticulocytosis becomes apparent about 3–4 days after an acute increase in plasma EPO.

CFU-Es are the most EPO-sensitive cells with the highest density of EPO receptors on their surfaces. The mature EPO receptor is a 484 aminoacid glycoprotein which is a member of the cytokine class I receptor superfamily (111, 112). It possesses a single hydrophobic transmembrane sequence, a variable cytoplasmic domain and an extracellular domain with conserved cysteines and a WSXWS-motif (112). Two of the membrane-spanning EPO receptor molecules form a dimer to which one EPO molecule binds. By means of light scattering, sedimentation equilibrium and titration calorimetry it has been shown that the EPO dissociation constants (K_d) are 1 nM and 1 μM for the two EPO receptor binding sites (113). Crystal structure analysis of the EPO receptor binding residues has already been carried out (114). With respect to novel pharmacological compounds used for therapy it is noteworthy that the degree of receptor binding depends on the carbohydrate content of EPO. Affinity for the receptor

decreases with glycosylation (35). Apparently, the carbohydrate portion of a glycoprotein ligand prevents receptor binding through electrostatic forces (115). Reduced receptor binding and internalization may provide an explanation for the prolonged *in vivo* half-life of hyperglycosylated EPO analogues such as Darbepoetin alfa (38) which has a 4-fold reduction in EPO receptor binding affinity compared to rHuEPO (35).

Figure 3 shows a scheme of EPO signalling. Ligand binding induces a conformational change and a more tighter connection of the two receptor molecules (116–118). As a result, two Janus kinase 2 (JAK2) tyrosine kinase molecules, which are in contact with the cytoplasmic region of the EPO receptor molecules, are activated (118, 119). Thereupon, several tyrosine residues of the EPO receptor are phosphorylated and exhibit docking sites for signalling proteins containing SRC homology 2 (SH2) domains (120, 121). As a result, several signal transduction pathways are channeled, including phosphatidyl-inositol 3-kinase (PI-3K/Akt), JAK2, STAT5, MAP kinase and protein kinase C (122–124). However, the specific roles of the various enzymes and transcriptional cofactors is only beginning to be understood with respect to the fate of the different erythrocytic progenitors in terms of survival, proliferation and differentiation (125–127). In addition, many observations have been derived from studies with cell lines, which may differ in response from primary EPO-responsive cells (128). Interestingly, EPO receptor signalling is inhibited by the cytokine-inducible SH2 protein 3 (CIS3; also known as SOCS-3, for suppressor of cytokine signalling), which can bind to phosphorylated EPO receptor and JAK2 (129). The effect of EPO is terminated by the action of the hemopoietic cell phosphatase (HCP) which catalyses JAK2 de-phosphorylation (130, 131). *In vitro* studies have shown that the EPO-induced signalling pathways return to nearly basal levels after 30–60 minutes (132). Apparently, the EPO/EPO-receptor complex is internalized following dephosphorylation of the receptor. The proteasome controls the duration of EPO signalling by inhibiting the renewal of cell surface receptor molecules (132, 133). Mutations of the cytoplasmic C-terminal regions of the EPO receptor and functional deficiencies of HCP may lead to familial erythrocytosis (134). On the other hand, inhibitors of HCP have been developed to prevent JAK2 de-phosphorylation and to prolong the action of EPO (135).

EPO was earlier thought to act exclusively on erythrocytic progenitors. However, recent studies have shown that EPO is a more pleiotropic hormone [for references see (136–138)]. For example, EPO receptor mRNA and/or protein have been shown to be present in endothelial cells (139, 140), epicardium and pericardium (141), renal mesangial and epithelial cells (142), pancreatic islets (143), placenta (144), and defined areas of brain (145–148). Based on these findings it has been proposed that EPO fullfills angiogenic and neurotrophic functions (48, 137, 138, 149). However, the physiological role of the EPO/EPO receptor system in non-erythrocytic tissues requires further clarification. Transgenic

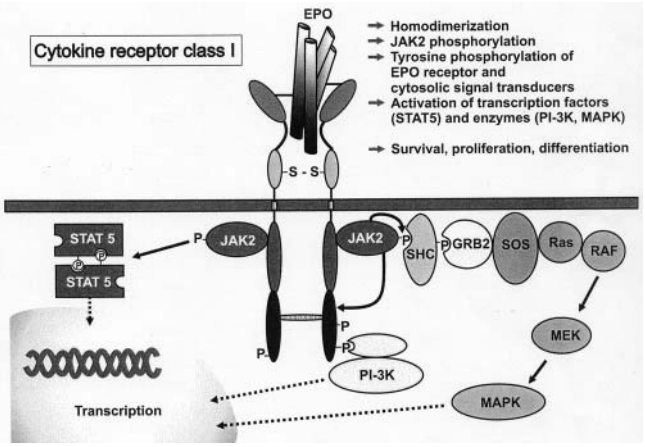

Figure 3. Simplified scheme of EPO signalling, involving autophosphorylation of JAK2 (Januse kinase 2), phosphorylation of the EPO receptor, homodimerization of STAT5 (signal transducer and activator of transcription 5), activation of PI-3K (phosphatidyl-inositol-3-kinase), phosphorylation of the adapter protein SHC (SrC-homology and collagen) to form a complex with GRB (growth factor receptor binding protein), SOS (son of sevenless) and the G-protein Ras, and the sequential activation of the serine-kinase RAF, MEK (syn. MAPKK) and MAPK (mitogen activated protein kinase). The signalling cascade results in survival, proliferation and differentiation of erythrocytic progenitors. The EPO/EPO-receptor complex is internalized and degraded. In addition, the action of EPO is terminated by HCP (hemopoietic cell phosphatase) which catalizes the dephosphorylation of JAK2.

mice expressing EPO receptor exclusively in hematopoietic cells develop normally, are healthy and fertile. They do not display neurologic disturbances (150).

An intriguing question is whether or not tumor cells express the EPO receptor and whether EPO promotes tumor growth. Initial studies utilizing a number of different tumor cell lines *in vitro* failed to show growth-modulating effects of rHuEPO when tested over a wide dose range (151–153), even when EPO receptor-positive cell lines were tested (154). Neither was rHuEPO found to stimulate the growth of freshly explanted human cancers in primary culture (155). On the other hand, EPO-binding sites and EPO receptor protein, respectively, were detected in biopsies of human lung carcinoma (156), human breast carcinoma (157–159), and human uterus cervical carcinomas (160). Furthermore, EPO receptor mRNA and/or protein have been shown in Hep3B human hepatocarcinoma (161), renal carcinoma (162), various breast carcinoma cell lines (157) and malignant tumors of female reproductive organs (163). Moreover, these recent studies indicate that EPO can indeed stimulate the proliferation of the tumor cells *in vitro* (157, 162) and in nude mice *in vivo* (164). Tumor regression can be induced by inhibition of EPO signalling produced by the local injection of anti-EPO antibody or soluble forms of the EPO receptor into

tumor tissue (163, 165). It will be an important issue to clarify the reasons between the earlier negative and the more recent positive results regarding the potential of EPO to stimulate the growth of tumor cells.

Clinical Implications and Future Directions

Therapy with recombinant human EPO has become a standard for correction of renal and non-renal anemias. Manufacturers of biogenerics may immediately step into the market when the patents of the original products expire. Current pharmaceutical attempts aim at developing follow-on biologics with an increased *in-vivo* survival. Thus, in addition to the conventional Epoetins, which have a plasma half-life of 6–8 hours, the hyperglycosylated Darbepoetin alfa has been approved, which has a plasma half-life of 24–26 hours (35). The most novel agent CERA which contains a polyethylene glycol polymer is still under investigation (54). While EPO is normally internalized and degraded following receptor-binding, evidence suggests that CERA may escape degradation by dissociating from the receptor. The development of erythropoietic drugs with a sustained efficacy compared with current therapies may allow less frequent clinical dosing. An alternate possibility of increasing the potency of rHuEPO could be the use of dimers or trimers of the protein (166). Another approach, which has apparently not yet been tested clinically, may be to administer EPO mimicking cyclic peptides that show no sequence homology to EPO but bind to the EPO receptor and enhance erythropoiesis in experimental animals (167). Non-peptidic ligands of the EPO receptor have also been described (168, 169). In addition, inhibitors of the hemopoietic cell phosphatase (HCP) may prove useful to prolong the action of EPO (135). EPO gene transfer is another alternative to the administration of rHuEPO (170). However, there is still lack of knowledge of the efficacy, stability and tissue-specificity of such transgenes. Present investigations focus on the effects of inhibitors of HIF-α prolyl- and asparaginyl-hydroxylases. Iron chelators or competitors such as desferrioxamine and cobalt have already been shown to stimulate EPO gene expression *in vivo* (87–89). In addition, HIF-dependent EPO gene expression may be enhanced by competitive inhibitors of prolyl- and asparaginyl-hydroxylases with respect to 2-oxoglutarate (74, 171).

Given intravenously or subcutaneously the Epoetins or Darbepoetin alfa are routinely administered to patients on hemodialysis or continous ambulatory peritoneal dialysis as well as to many predialysis patients (172–174). Overall, there seems to be an underutilization of rHuEPO during the predialysis period, although the correction of anemia allows the patients to enter dialysis later than without rHuEPO therapy and prevents left ventricular hypertrophy and congestive heart failure (175–177). RHuEPO can correct the anemia in practically all patients with renal failure. Reasons for rHuEPO resistance may be iron deficiency, inflammatory of infectious disease, aluminium overload, hyperparathyroidism and osteitis fibrosa. Iron deficiency is reflected by a proportion of hypochromic red cells >10%, a transferrin saturation <20% and a serum ferritin concentration <100 μg/*l* (178). A recent report indicates that the transferrin saturation is a better clinical marker for iron supplementation, although the reticulocyte hemoglobin content reflects the iron status more accurately (179). Most nephrologists set the target hematocrit value at 0.33–0.36 (hemoglobin 110–120 g/*l*). This level of anemia correction leads to an acceptably restored quality of life, exercise capacity, cardiac performance and cognitive function. The question is whether increasing the doses of rHuEPO to attain normal hematocrit values is beneficial. Unfortunately, a major randomized prospective long-term multicenter study on 1,233 patients with cardiac disease showed that the mortality rates were somewhat higher in the normal-hematocrit (0.42) than in the low-hematocrit (0.30) group (180).

Potential non-renal indications for rHuEPO administration include the anemias associated with cancer (primarily chemotherapy-associated anemia), autoimmune diseases, AIDS, bone marrow transplantation and myelodysplastic syndromes [for references see (181)]. In contrast with the high response rate in renal anemia, rHuEPO resistance (hemoglobin increase <10 g/*l* in 4 weeks) is often seen in patients with non-renal anemias. In tumor patients rHuEPO therapy aims at maintaining the patients' hemoglobin values above the transfusion trigger, increasing the exercise tolerance and improving quality of life parameters. A recent review has noted methological deficiencies in most reports claiming improved quality of life in rHuEPO treated patients (182). Clearly, hemoglobin concentrations in cancer patients should not be raised into the normal range. Such overtreatment caused a poorer survival rate in randomized, double-blind placebo-controlled trials on patients with metastatic breast cancer (183) and head and neck cancer patients under radiotherapy (184). Evidence-based clinical practice guidelines have been provided by the American Society of Clinical Oncology and the American Society of Hematology (185). Accordingly, the use of Epoetin is recommended as a treatment option for patients with chemotherapy-associated anemia with a hemoglobin concentration below 100 g/*l*. However, the dosage of Epoetin should be titrated to maintain a hemoglobin concentration of 120 g/*l* to avoid cardiovascular disorders. During over 15 years of the use of recombinant EPO in renal patients, no clinical evidence has been provided to assume that exogenous EPO induces or promotes tumor growth.

A fascinating finding of potential clinical relevance has been the demonstration of functional EPO receptors by neuronal cells (186, 187). Both EPO receptor mRNA and protein are expressed in defined areas of the mammalian brain, primarily in the hippocampus, capsula interna, cortex and midbrain (145, 146, 148). EPO exerts neuroprotective effects *in vivo* as first demonstrated in 1998 (188, 189), when rHuEPO was infused in the lateral ventricles of Mongolian gerbils with experimental cerebral ischemia. Similar to its effects on erythrocytic progenitors, EPO up-regulates the ex-

pression of bcl-x_L, an anti-apoptotic protein, in neuronal cells (190). Brines et al (191) first investigated the efficacy of systemically administered rHuEPO in rodent models of focal brain ischemia, concussive brain injury, experimental autoimmune encephalomyelitis and cainate-induced seizures. Presumedly mediated by EPO receptor-mediated transfer across the blood brain barrier, about 1% of systemically administered rHuEPO became detectable in the cerebrospinal fluid in rats after 3–4 hours (192). Details of the experimental studies on the neuronal effects of EPO have been summarized elsewhere (137, 149, 193–195). In view of the neuroprotective action of EPO in animal studies, Ehrenreich et al (196) recently performed a clinical trial with rHuEPO in patients suffering from acute stroke. In a double-blind randomized proof-of-concept study, 40 patients received either rHuEPO or saline. The trial resulted in a strong trend for reduction in infarct size in the rHuEPO treated patients, compared to the untreated controls as assessed by magnetic resonance imaging. This reduction was associated with a markedly improved neurological recovery and clinical outcome as determined one month after stroke. Progress is expected in understanding the value of rHuEPO for use as a neuroprotective drug in cerebral ischemia, brain trauma, inflammatory diseases and neural degenerative disorders.

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