

The Erythropoietin Receptor and Its Expression in Tumor Cells and Other Tissues

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Key Words. *EPO-R structure · EPO-R activation · Angiogenesis · CNS · Anemia · Tumors · Epoetin alfa*

LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Describe the structure of the erythropoietin receptor.
2. Describe the function of the erythropoietin receptor.
3. Describe the distributions of erythropoietin receptors in normal and tumor tissues.

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ABSTRACT

Erythropoietin (EPO) is the primary regulator of erythropoiesis, stimulating growth, preventing apoptosis, and promoting differentiation of red blood cell progenitors. The EPO receptor belongs to the cytokine receptor superfamily. Although the primary role of EPO is the regulation of red blood cell production, EPO and its receptor have been localized to several nonhematopoietic tissues and cells, including the central nervous system (CNS), endothelial cells, solid tumors, the liver, and the uterus. The presence of EPO receptors and the possibility of EPO signaling in these tissues and cells have led to numerous studies of the effects of EPO at these sites. In particular, expression of EPO and the EPO receptor in cancer cells has generated much interest because of concern that administration of recombinant human erythropoietin (rHuEPO) to patients with breast and other cancer cells expressing the EPO receptor may promote tumor growth via the induction of cell proliferation or angiogenesis. However, evidence supporting a growth-promoting effect has been inconclusive. Moreover, several preclinical studies have shown a beneficial effect of

EPO on delaying tumor growth. Further, it is conceivable that increased expression of EPO could reduce tumor hypoxia and ameliorate the deleterious effects of hypoxia on tumor growth, metastasis, and treatment resistance. On the other hand, EPO has also been shown to produce an angiogenic effect in vascular endothelial cells in vitro. However, there is no evidence that these effects occur in vivo to promote tumor growth. EPO and EPO receptors are expressed in neural tissue, and they are upregulated there by hypoxia. Animal studies have shown that administration of epoetin alfa (an rHuEPO) reduces tissue injury due to ischemic stroke, blunt trauma, and experimental autoimmune encephalomyelitis. These findings suggest that epoetin alfa may provide a therapeutic benefit in patients with stroke, trauma, epilepsy, and other CNS-related disorders. Clearly, further study of EPO and the EPO receptor in nonhematopoietic tissue is warranted to determine the potential therapeutic usefulness of rHuEPO as well as to determine the signaling pathway responsible for its effect in vivo. *The Oncologist* 2004;9(suppl 5):18-30

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INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that serves as the primary regulator of erythropoiesis by stimulating growth, preventing apoptosis, and inducing differentiation of red blood cell precursors [1]. Clinically, these actions translate into increased levels of hemoglobin, which has led to the widespread use of recombinant human EPO (rHuEPO, epoetin alfa) in the treatment of patients with anemia due to renal failure, cancer or cancer therapy, and azidothymidine therapy. Recently, EPO and its receptor have been localized in several nonhematopoietic tissues and cells, including the liver, the uterus, the central nervous system (CNS), vascular endothelial cells, and solid tumors. These findings have led researchers to explore the role of EPO in nonhematopoietic tissues and its potential use outside erythropoiesis, including its use in CNS disorders [2, 3].

STRUCTURE OF EPO AND THE EPO RECEPTOR

EPO

In humans, EPO mRNA encodes a protein with 193 amino acids [4, 5]. However, cleavage of 27 N-terminal amino acids (signal peptide) and loss of the C-terminal arginine during post-translation modification result in a 165-amino acid structure that comprises the mature protein (Fig. 1). The EPO molecule contains two structure-stabilizing disulfide bonds between amino acids 7 and 161 and 29 and 33, the reduction of which results in loss of bioactivity. Additionally, the EPO molecule possesses three N-linked sugars, at positions 24, 38, and 83, and one O-linked sugar at position 126 [4]. The O-linked sugar has no important function, but the N-linked sugars are necessary for stability of the EPO molecule in the circulation [4, 5]. Deglycosylated EPO is biologically active but is extremely short lived [6]. Importantly, the amino acid sequence of rHuEPO is identical to that of the endogenous form.

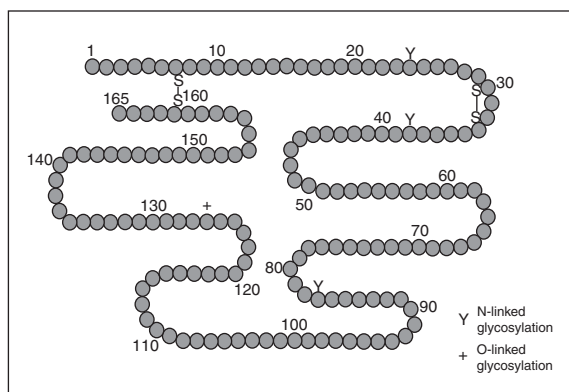


Figure 1. Structure of erythropoietin. Reprinted with permission from Mulcahy [5].

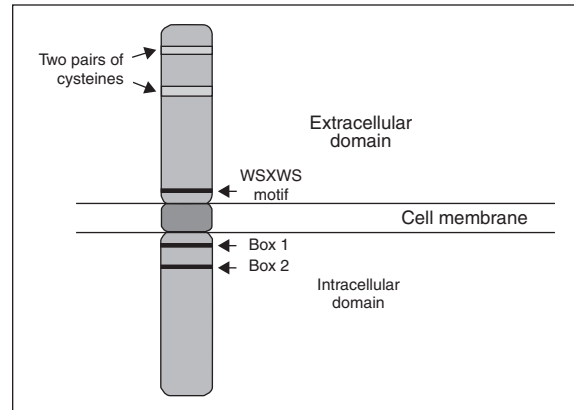


Figure 2. Schematic representation of the EPO receptor. The extracellular and intracellular domains are anchored in the cell membrane. Reprinted with permission from Mulcahy [5].

The EPO Receptor

The EPO receptor belongs to the cytokine receptor superfamily [7]. Included in this family are receptors for other hematopoietic growth factors, including growth hormone, prolactin, G-CSF, GM-CSF, thrombopoietin, oncostatin M, and several interleukins. Receptors in this family share several distinct features, including an extracellular ligand-binding domain with two pairs of conserved cysteine residues and a conserved motif, WSXWS, located close to the transmembrane domain; a single transmembrane domain; and an intracellular domain lacking catalytic activity (Fig. 2).

EPO exerts its effects by inducing homodimerization of two molecules of the EPO receptors on the cell surface, which initiates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction cascade that regulates cell proliferation and differentiation [8, 9]. Much of the current knowledge on the mechanism of EPO binding is based on the 1996 discovery of a family of peptides that exhibit EPO-mimetic activity [10]. One member of the family (EPO-mimetic peptide 1 [EMP-1]), a 20-amino acid peptide with no sequence homology to EPO, was used to determine the structure of the EPO/EPO receptor complex. X-ray crystallography revealed the EMP-1 peptide/EPO receptor complex to be a dimer of two EPO receptor molecules bound to a dimer of two EMP-1 peptides, analogous to the EPO/EPO receptor structure reported subsequently (Fig. 3 and Fig. 4) [10, 11].

MECHANISM FOR EPO RECEPTOR SIGNALING

Upon dimerization of the EPO receptor by EPO binding, an intracellular signaling pathway is established to enable erythroid differentiation [4, 5, 7]. Unlike many other receptors, the EPO receptor has no intrinsic tyrosine kinase activity to activate receptor signaling. Rather, signaling appears to

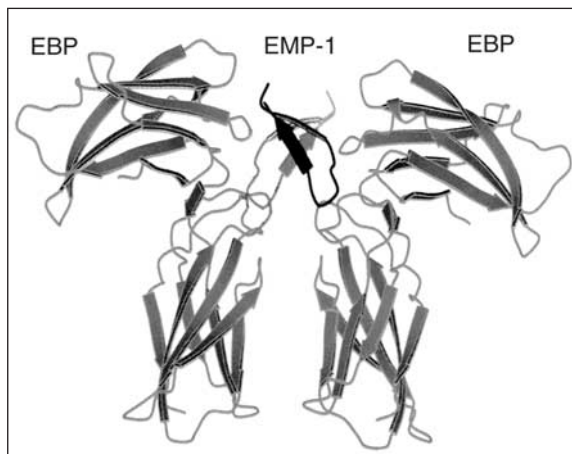


Figure 3. Crystal structure of the extracellular ligand-binding fragment (EBP)2/EMP-1 complex. Reprinted with permission from Livnah et al. [10].

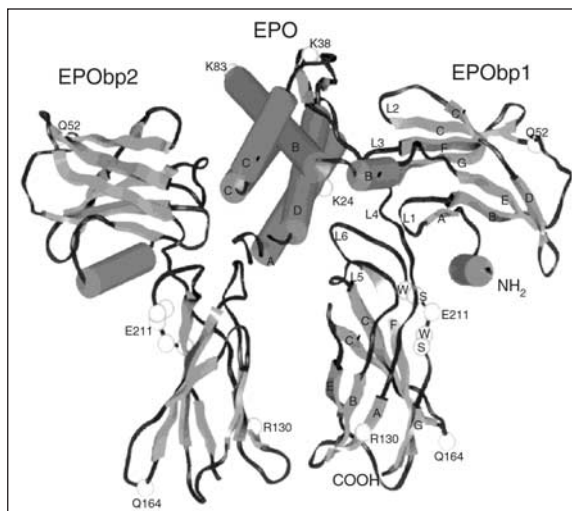


Figure 4. Crystal structure of the extracellular EPO-binding fragment (EBP)2/EPO complex. Reprinted with permission from Syed et al. [11].

be mediated by JAK2, a cytoplasmic tyrosine kinase constitutively associated with the intracellular domain of the EPO receptor. JAK2 is one of four JAKs (JAK1, JAK2, JAK3, and TYK2) known to exist in mammals and to participate in signaling from a range of cell-surface receptors, especially those of the cytokine receptor superfamily. Following homodimerization of the EPO receptor, JAK2 molecules associated with each of the individual EPO receptors are brought into close proximity, inducing their transphosphorylation and subsequent activation (Fig. 5). Activated JAK2 then phosphorylates several intracellular proteins, including the EPO receptor itself. The phosphorylated tyrosines act as docking sites for various intracellular proteins containing Src (tyrosine kinase) homology 2 domains, for example, Src-homology tyrosine

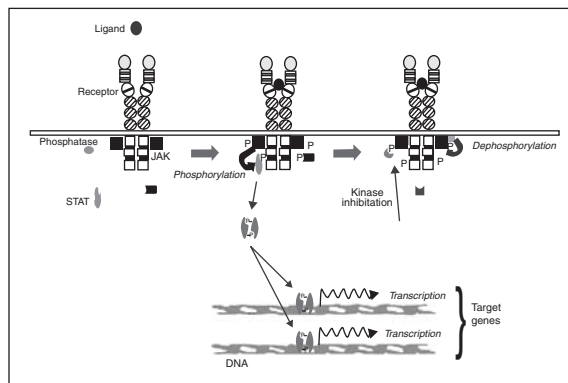


Figure 5. Activation and deactivation of the JAK/STAT pathway. Reprinted with permission from Ward et al. [72].

phosphatase (SHP)1, SHP2, phosphatidylinositol 3' kinase (PI3K), and one of the STATs, STAT5. On docking with the EPO receptor, tyrosines within these proteins undergo phosphorylation by JAK2, leading to their activation and downstream signal transduction [12]. One pathway activated is the JAK2/STAT5 pathway. Although the precise role of STAT5 in erythroid differentiation is not yet fully understood, JAK2-mediated STAT phosphorylation results in the formation of stable STAT dimers, which in turn translocate to the nucleus where they bind to specific regulatory sequences and activate the transcription of target genes resulting in erythroid differentiation [5, 7].

In addition to the STAT5 pathway, other signaling pathways, including RAS and PI3K, can be activated by EPO [7]. PI3K signaling is believed to result in the activation of AKT and p70^{S6K}, which play a key role in transcription and cell-cycle progression. EPO signaling may also activate many nonreceptor tyrosine kinases (e.g., c-fos/fes, p72^{Syk}, and hematopoietic progenitor kinase-1), as well as proliferation-stimulating tyrosine phosphatases SHP2, SH2 inositol 5'-phosphatase, and other signal transducers.

Termination of Signaling

In contrast to phosphorylation that activates EPO receptor signaling, dephosphorylation downregulates this activity. SHP1 (hematopoietic cell phosphatase), for example, acts as a negative regulator of signaling activity. SHP1 is activated by binding to phosphotyrosine 429 of the EPO receptor and subsequently dephosphorylates JAK2, thereby inactivating the kinase and downregulating the signaling cascade [5, 8, 13]. Cells bearing EPO receptors that lack the portion of the intracellular domain to which SHP1 docks are unable to recruit the phosphatase, and thus are hypersensitive to EPO. This truncated form of the EPO receptor, with its associated inability to recruit SHP1 and deactivate JAK2, is manifested clinically in a form of polycythemia [5, 14].

Additionally, it has been suggested that the protein factor Cis1 may inhibit proliferative signaling of the EPO receptor, possibly by attenuating STAT5 [15, 16], and further, that Cis1 may promote EPO receptor degradation [16, 17].

NEW DEVELOPMENTS

Darbepoetin alfa (novel erythropoiesis-stimulating protein [NESP]) is a newly introduced recombinant erythropoietic protein currently approved for the treatment of anemia associated with chronic renal failure and anemia in cancer patients with solid tumors. Like epoetin alfa and endogenous EPO, darbepoetin alfa promotes the proliferation, maturation, and differentiation of red blood cells. However, darbepoetin alfa differs from epoetin alfa in containing five N-linked carbohydrate chains (compared with three such chains in the epoetin alfa molecule) and up to 22 sialic acid residues (compared with 14 in epoetin alfa) [18]. As a result of its higher sialic acid content, darbepoetin alfa has a slower serum clearance and a longer half-life than epoetin alfa. On the other hand, the increased carbohydrate content results in a decrease in the binding affinity of darbepoetin alfa for the EPO receptor. In contrast to the situation with epoetin alfa, the nonhematopoietic effects of darbepoetin alfa in vitro and in vivo are largely unknown.

HYPOXIA AND INCREASED EXPRESSION OF THE EPO RECEPTOR

It has become increasingly apparent over the past decade that expression of the EPO receptor may be enhanced in a variety of nonhematopoietic cell types by the presence of hypoxia. Although the specific function of the EPO receptor at these sites is not fully understood, the presence of the receptor suggests a wider biologic role for EPO than previously believed. In the CNS setting, upregulation of both EPO and the EPO receptor has been demonstrated after ischemia in the rat brain [19]. Also, administration of rHuEPO in animal models has been shown to reduce injury after focal brain ischemia, to reduce the extent of concussive brain injury and immune damage in experimental encephalomyelitis, and to ameliorate the severity of kainate-induced seizures [20]. The presence of EPO in the brain appears to protect neurons from ischemic insult by inhibiting their apoptosis [21, 22]. Hypoxia-associated upregulation of EPO and EPO receptor expression has also been shown in human breast tumors [23-25] and other tumor types [26, 27]. Expression of EPO is known to be mediated by hypoxia-inducible factor 1 (HIF-1) transcription, which additionally targets angiogenic factors, such as vascular endothelial growth factor (VEGF), and a variety of genes encoding factors essential for systemic, local, and intracellular homeostasis and cell survival in a hypoxic environment (e.g., inducible

nitric oxide synthetase, transferrin, endothelin-1, and several glycolytic enzymes) [28]. Thus, HIF-1-mediated EPO expression is only one of a plethora of genetically controlled functions impacted by hypoxia. On the other hand, HIF-1 has not been identified as a regulator of EPO receptor gene expression, and the mechanisms underlying increased EPO receptor expression remain unknown [24]. Increased EPO receptor expression potentially may increase the sensitivity of the expressing cells to available EPO, leading researchers to explore the functionality of the EPO receptor outside erythropoiesis. Depending on the cellular context, one could hypothesize that increased EPO receptor expression may constitute a normal physiological response or a dysfunctional response [29]. For example, increased EPO and EPO receptor expression in the brain may protect brain cells from ischemic injury, while increased expression of the EPO receptor in tumor cells may lead to EPO-mediated cellular proliferation, antiapoptosis, and angiogenesis. While the functionality of the EPO receptor in neuronal tissue has been demonstrated, including EPO binding and signaling, no data to support a function in tumor cells exist to date. Although tumor cells express the EPO receptor, and reports of cellular proliferation have been shown at suprapharmacologic concentrations, evidence that EPO binds tumor cells or induces cellular signaling has not been shown. Moreover, systematically administered rHuEPO did not lead to tumor progression in several in vivo tumor studies. Alternatively, increased expression of EPO may have a protective effect by ameliorating hypoxia and thereby decreasing HIF-1 expression and the subsequent expression of VEGF and other factors that facilitate adaptation of tumor cells to hypoxia. Given the potentially wide range of functions of EPO and the EPO receptor, the mechanisms underlying these functions must be determined. Interestingly, *Lappin* and colleagues, repeating some work done by *Acs et al.* [23], found that EPO receptors were present in tumor cells but absent from surrounding normal breast tissue (*Maxwell*, unpublished data). This, *Lappin* noted, is significant, as it suggests the potential use of the EPO receptors of a tumor to target an EPO-attached drug to the tumor and not damage surrounding healthy tissue [2].

EPO-RECEPTOR EXPRESSION IN CANCER CELLS AND IMPACT OF EPO ON EPO-RECEPTOR-BEARING CANCER TISSUE

Recent data have shown that the EPO receptor is present not only in many normal nonerythroid cell types, such as endothelial cells [30, 31], neuronal cells [32], Leydig cells [33], myeloblasts [34], and megakaryocytes [35], but also in nonerythroid tumor cells and tumor cell lines. Expression of the EPO receptor has been reported, for example, in human

Table 1. Effect of rHuEPO on the clonogenic growth of human cell lines* (reprinted with permission from *Rosti et al.* [37])

Cell line	EPO (IU/ml)				
	0	0.5	1.0	2.0	10.0
K-562	186 ± 12	193 ± 15	181 ± 17	187 ± 16	184 ± 15
HEL	62 ± 5	67 ± 5	66 ± 6	71 ± 8	76 ± 4
HL-60	786 ± 16	791 ± 10	787 ± 15	797 ± 15	796 ± 21
PLB 985	9 ± 2	8 ± 1	8 ± 2	8 ± 2	9 ± 2
KG-1	155 ± 14	161 ± 15	149 ± 12	163 ± 15	154 ± 12
H69	257 ± 21	268 ± 18	270 ± 25	245 ± 27	253 ± 20
N417	368 ± 28	346 ± 30	387 ± 27	355 ± 31	367 ± 24
OCUM-1	68 ± 11	71 ± 9	66 ± 12	70 ± 8	64 ± 7

*Colony numbers = mean ± 1 standard deviation of three to four experiments

renal cell carcinoma [27] and breast carcinoma [23] cells, as well as in melanoma [36], renal carcinoma [27], breast carcinoma, HEP3B hepatoma, HeLa cervical carcinoma, SHSY5Y neuroblastoma, U87 glioblastoma, and U251 and U373 glioma cell lines [23]. The observation of EPO-receptor expression in cancer cells, coupled with the reported cell-growth-supporting ability of EPO, has raised concern that administration of rHuEPO to cancer patients with anemia may enhance the proliferation or survival of the cancer cells or stimulate angiogenesis and thus promote tumor growth. Several researchers have, therefore, examined the role of EPO and EPO receptor expression in cancer patients.

Three studies have shown that EPO does not influence the proliferation of cancer cell lines. *Rosti et al.* [37] investigated the proliferative potential of rHuEPO by testing the effects of this factor on clonogenic growth and DNA synthesis in 10 different cell lines derived from hematologic malignancies and solid tumors. Included in the cell lines were K-562 and HEL, both of which express EPO receptors. Results of that study showed that rHuEPO, even at high concentrations, had no effect on either colony growth or DNA synthesis in the cell lines tested, including K-562 and HEL (Table 1 and Table 2). More recently, *Selzer et al.* [36] showed that the EPO receptor is expressed in transformed human cell lines derived from normal melanocytes and in human melanoma cell lines derived from melanoma metastases, but that it is not expressed in normal primary human melanocytes. An analysis of subclones from one transformed cell line showed that not all subclones expressed the EPO receptor and that expression of the receptor did not affect cell growth. Thus, those investigators concluded that expression of the EPO receptor appears to be associated with, but is not absolutely required for, transformation of melanocytes to malignant melanoma cells, and further, that the EPO receptor may be a progression marker

for melanoma. In another study, *Westphal et al.* [38] investigated the effects of EPO on more than 25 different benign and malignant human cell lines. Expression of EPO receptor mRNA and protein was analyzed with reverse transcription polymerase chain reaction, Western blot, and immunocytochemistry, and the cellular responses to various concentrations of EPO were evaluated using tritiated thymidine uptake, Northern blot of *c-fos* expression, and tyrosine-kinase activity assay. EPO receptor mRNA and protein were identified in the majority of the tumor cell lines evaluated, including lines of nonhematopoietic origin. However, treatment with rHuEPO did not influence the proliferation rate of EPO-receptor-positive tumor cell lines; moreover, treatment with EPO neither affected the gene *c-fos* mRNA of those cell lines nor stimulated tyrosine-kinase

Table 2. Effect of rHuEPO on the percentage of cells in S phase in human cell lines* (reprinted with permission from *Rosti et al.* [37])

Cell line	EPO (IU/ml)		
	0	1.0	5.0
K-562	37.0 ± 2.0	37.1 ± 2.1	36.8 ± 1.7
HEL	27.3 ± 1.9	26.2 ± 1.3	25.8 ± 1.4
HL-60	26.4 ± 1.8	24.8 ± 2.1	25.6 ± 2.0
PLB 985	30.0 ± 1.7	27.8 ± 2.3	28.2 ± 2.5
KG-1	14.2 ± 1.3	14.0 ± 1.7	15.5 ± 1.8
H69	15.3 ± 1.5	15.8 ± 1.3	14.9 ± 1.6
N417	16.6 ± 1.8	17.0 ± 1.4	16.3 ± 2.2
MCF-7	20.0 ± 0.9	21.1 ± 1.2	19.7 ± 1.0
OCUM-1	16.1 ± 2.1	17.3 ± 2.4	15.3 ± 2.3
GBL-HU12	19.2 ± 1.5	20.9 ± 1.6	19.1 ± 2.0

*Colony percentages = mean ± 1 standard deviation of three to four experiments

activation. The investigators noted that the lack of expression of the proto-oncogene *c-fos* and lack of tyrosine-kinase activity despite EPO-receptor expression indicated that the EPO signal was probably not transduced into the cells, since both effects can be shown after successful signal transduction. Based on their findings, the investigators concluded that expression of the EPO receptor in tumor cells does not appear essential for their growth and, consequently, does not appear to have a deleterious effect in cancer patients.

In contrast, *Westenfelder* and *Baranowski* [27], in a study of nephrectomy samples from patients with renal cell carcinoma as well as cell lines of human and mouse renal adenocarcinomas, found that human renal cell carcinomas and cell lines expressed EPO receptor mRNA and protein, and that receptor activation by EPO stimulated the proliferation of human and mouse renal adenocarcinoma cell lines. According to the investigators, these findings suggested that rHuEPO administration to renal cell carcinoma patients has the potential to modify tumor growth by stimulating cell proliferation, and possibly by supporting angiogenesis. *Acs et al.* [23], on the basis of Western blot analysis and immunohistochemistry findings, reported high levels of expression of both EPO and its receptor in human breast cancer cells. Additionally, the investigators reported the occurrence of rHuEPO-stimulated tyrosine phosphorylation of MCF-7 cells and the proliferation of MCF-7 and BT549 cell cultures after exposure to rHuEPO. These findings led the investigators to propose that rHuEPO may be detrimental to breast cancer patients due to promotion of proliferation and survival of EPO-receptor-expressing breast cancer cells. However, the data supporting this hypothesis are not convincing. Demonstration of the EPO receptor in human breast cancer cells by Western blot is not unexpected, as EPO receptor expression has been found in several non-hematopoietic tissues including neuronal, intestinal, hepatic, and endothelial cells. Also, there are reports in the literature of hematopoietic and nonhematopoietic malignant cell lines that possess the EPO receptor but do not proliferate in response to EPO [37, 38]. Further, Western blot does not discriminate between EPO-receptor expression localized to the cell surface and expression localized to the cytoplasmic region of the cell, and the immunohistochemical analysis revealed extensive cytoplasmic localization. *Acs et al.* [23] indicated that EPO and EMP-1 peptide receptor elicited tyrosine phosphorylation, a hallmark of EPO signaling. However, the data presented do not demonstrate that EPO elicits a signaling cascade through the JAK/STAT pathway. A reactive band at 66 kDa (the molecular weight of EPO receptor) is not tyrosine phosphorylated, and no reactive band indicative of STAT phosphorylation is observed at 95 kDa. This would suggest that EPO is not initiating a signaling

cascade. Moreover, the amount of cell proliferation attributed to EPO stimulation is only 125%, which, although significant, is minimal compared with that seen in other cell types (UT-7 cells, for example, have induced a 300%-500% greater cell number versus control at low EPO concentrations). Thus, these findings do not adequately demonstrate that the existence of the EPO receptor in human breast cancer tissue or cell lines has an effect.

In another recent paper, *Arcasoy et al.* [25] also presented evidence to support a role for EPO receptors in breast cancer. EPO expression, EPO-receptor expression, and the presence of hypoxia were evaluated in 26 biopsy specimens from 10 breast cancer patients. Results of immunohistochemical staining indicated that EPO and EPO-receptor expression were upregulated in breast cancer tissue. Hypoxia was identified in approximately 60% of the biopsy specimens; however, the expression patterns of EPO and the EPO receptor relative to hypoxia were variable, and the concomitant occurrence of EPO and EPO-receptor expression and hypoxia in contiguous tissue sections was not established. This suggested that factors other than hypoxia, for example, dysregulated or aberrant gene expression, may regulate EPO or EPO-receptor expression in breast cancer cells. *Arcasoy et al.* [25] additionally examined the functional significance of the EPO receptor in breast cancer tissue by implanting rat adenocarcinoma cells into rats and observing the effects of EPO/EPO-receptor antagonists on tumor progression. Analysis of tumor depth 7 days after implantation showed that administration of soluble EPO receptor, anti-EPO antibody, and a JAK inhibitor decreased tumor depth in a dose-dependent manner, suggesting the involvement of the EPO signaling pathway in tumor progression. The significance of this finding is uncertain, as each of the test compounds was administered only once and the duration of the effect beyond 7 days was not determined.

Thews et al. [39] showed that the cytotoxic efficacy of cyclophosphamide was lower in rats with carboplatin (Paraplatin®; Bristol-Myers Squibb; Princeton, NJ)-induced anemia than in rats protected from carboplatin-induced anemia with rHuEPO and nonanemic control animals. The results of another experimental study showed that multidose administration of rHuEPO, rather than promoting tumor growth, actually induced tumor regression, prolonged survival, and reduced mortality in murine myeloma models [40]. Interestingly, these rHuEPO-triggered effects were found to involve immunomodulation [41]. Results of a study with anemic nude mice engrafted with human glioblastoma tumors indicated that administration of rHuEPO improved tumor radiosensitivity, presumably by correcting anemia and subsequently improving intratumoral oxygenation [42]. The growth curves of nonirradiated tumors in rHuEPO-treated

animals in that study were nearly identical to those of both nonanemic and anemic animals, leading the investigators to conclude that EPO per se had no effect on the growth rate of the tumors. In another similar study, prevention of anemia with rHuEPO partially restored the radiosensitivity of xenografted glioblastomas to fractionated irradiation [43]. *Kelleher et al.* [44] demonstrated, in a murine model, that tumor growth was not affected by rHuEPO in either anemic or normal animals. Tumor blood flow and oxygenation in the anemic animals were lower than in the normal controls. The administration of rHuEPO, therefore, may increase the efficacy of standard radiotherapy by improving tumor oxygenation.

A recently published study on the effects of rHuEPO in patients receiving radiotherapy for head and neck cancers has questioned the role of rHuEPO in improving disease control and survival [45]. In that placebo-controlled study, patients who received rHuEPO (epoetin beta) ($n = 180$) had significantly ($p = 0.0008$) poorer locoregional progression-free survival than patients who received placebo ($n = 171$). While these results may raise concern, several factors including an imbalance in groups (i.e., higher proportions of current smokers, relapsed disease, and underlying vascular disorders in the epoetin beta group) warrant further study. Moreover, the dosing regimen used in those studies raised hemoglobin levels beyond that which is done in normal clinical practice.

THE EPO RECEPTOR AND ENDOTHELIAL CELLS: IMPACT ON ANGIOGENESIS

Recent studies have indicated that EPO, once considered to act specifically on erythroid progenitors, can also affect nonhematopoietic cells, including vascular endothelial cells. Moreover, the EPO receptor has been demonstrated to be present on endothelial cells in vivo and in vitro [31]. EPO/EPO-receptor interactions have been reported to induce a range of cellular responses in endothelial cell cultures, including mitogenesis, chemotaxis, endothelin-1 release, and an increase in intracellular calcium [2, 30, 46-49]. *Anagnostou et al.* [30] demonstrated dose-dependent increases in cell proliferation following addition of rHuEPO to cultures of human umbilical vein endothelial cells (HUVECs) and bovine adrenal capillary endothelial cells (BACECs). For one rHuEPO preparation tested, the optimal dose for stimulating proliferation of HUVECs was 5 U/ml, which provided a 53% greater mean cell number compared with the control value at 2 days and a 256% greater mean cell number at 7 days ($p < 0.001$). Administration of two other rHuEPO preparations also resulted in consistent proliferation effects on HUVECs, although the maximal effects were smaller. For BACECs, the mean cell number was greater than the control value by 35% at 7 days with rHuEPO at doses of 0.5 U/ml or 5 U/ml, respectively, for two different

preparations ($p < 0.005$). Recombinant human EPO also enhanced the migration of HUVECs and BACECs. The mean HUVEC migration rates with two different preparations were 69% ($p < 0.001$) and 49% ($p < 0.005$) greater than the control values, respectively; for BACECs, the values were 36% ($p < 0.005$) and 41% ($p < 0.005$), respectively. Binding studies with radioiodinated rHuEPO identified an endothelial cell protein of 45 kDa as the principle receptor associated with the mitogenic effect of EPO.

Endothelial Cells and Angiogenesis

Tumor growth and expansion are often characterized by the inability of the local vasculature to supply sufficient oxygen and nutrients to the rapidly dividing neoplastic cells. The resulting hypoxic state causes changes in the tumor cells that can lead to cell stasis or death (apoptosis) or, alternatively, to responses aimed at improving tissue oxygenation and increasing the chance of cell survival and propagation. Among these responses are a switch to anaerobic metabolism for energy production and upregulation of EPO, which results in the higher oxygen-carrying capacity of the blood. Additionally, hypoxia may upregulate angiogenic factors that promote development of new blood vessels from pre-existing vessels in the hypoxic area. Neovascularization is vital for continued tumor growth, and the development of angiogenic characteristics appears to be a key event in tumor formation [50]. During neoangiogenesis, endothelial cells change their genetic program and express angiogenic characteristics that include cell migration, proliferation, and differentiation into vascular tubes, resulting in the formation of new blood vessels [51].

Angiogenesis is mediated mainly by VEGF. As in the case of EPO, expression of the VEGF gene and subsequent increased VEGF production is controlled by the transcription factor HIF-1 [52]. Following its secretion, VEGF binds to receptor tyrosine kinases (VEGF1 and VEGF2) on the surface of the vascular endothelial cells. Receptor kinases then trigger a cascade of intracellular signaling pathways that initiate angiogenesis. However, *Ribatti* and colleagues [46] recently provided evidence that EPO can also elicit an angiogenic response in endothelial cells in vitro and in vivo, and thus, like VEGF, is an effective angiogenic factor. In their study, *Ribatti et al.* [46] demonstrated that human EA.hy926 endothelial cells express an EPO receptor that binds to JAK2 and induces its transient activation after rHuEPO exposure. In agreement with the previous studies in HUVECs and BACECs, rHuEPO substantially increased EA.hy926 cell proliferation (Fig. 6). Furthermore, rHuEPO exposure resulted in a threefold greater matrix metalloproteinase 2 (MMP-2) activity in treated EA.hy926 cultures compared with cell cultures grown in untreated media.

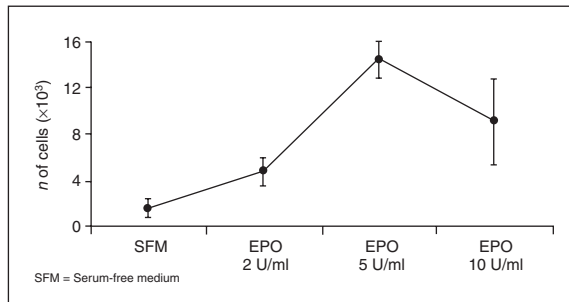


Figure 6. Effects of rHuEPO on EA.hy926 cell proliferation. The initial medium was removed after 24 hours and replaced every other day with fresh medium containing increasing concentrations of rHuEPO. Cell numbers were determined at day 6 of growth [47]. Reprinted with permission from Ribatti et al. [46].

MMP-2 is a major extracellular matrix proteolytic enzyme that degrades various components of the interstitial stroma and basement membrane and is secreted when endothelial sprouting takes place, thus promoting endothelial cell migration across the matrix. Thus, the ability of rHuEPO to substantially increase MMP-2 activity released by EA.hy926 cells represents a potential mechanism by which EPO promotes angiogenesis. Further, an *in vivo* chick embryo chorioallantoic membrane (CAM) assay showed that rHuEPO stimulates both the early invasive phase of the angiogenic process that leads to endothelial sprouting (characterized by an increase in cell motility, matrix degradation, and cell proliferation) as well as the late differentiation phase (characterized by the formation of hollow, branching, and anastomosing vascular tubes) (Fig. 7).

Therapeutic Implications of EPO Stimulation of Angiogenesis

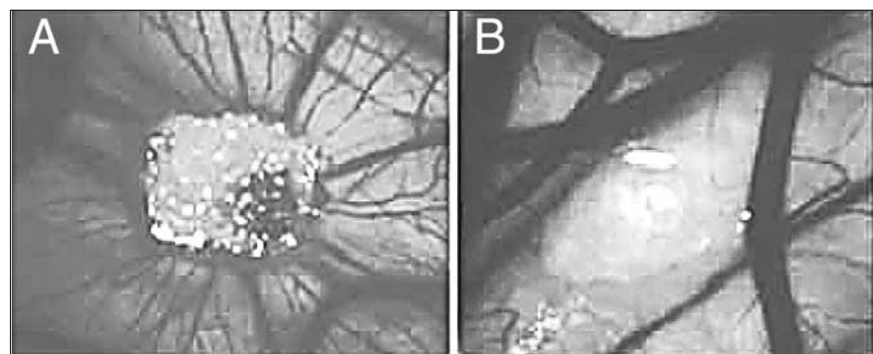
Because angiogenesis promotes tumor growth, some concern has been expressed regarding the angiogenic potential of EPO. However, EPO is a far less potent stimulator of angiogenesis than VEGF. Further, the angiogenic effect of EPO may be neutralized by its ameliorating effect on anemia. The possibility also exists that EPO may have a

protective effect with respect to fibrin deposition. Hypoxia can concomitantly trigger procoagulant mechanisms and mechanisms that suppress fibrinolysis. This leads to accumulation of fibrin inside the vascular lumen. Amelioration of anemia and correction of hypoxia with EPO may result in a decrease of fibrin deposition within the vascular lumen and a subsequent decrease in neovascularization [29]. Overall, understanding of the relationship among EPO, VEGF, and endothelial cell reactions to hypoxia is important for the development of new strategies to modify the endothelial response for therapeutic purposes [29].

THE EPO RECEPTOR AND CNS EFFECTS: NEUROPROTECTION, COGNITIVE BENEFITS

Tan et al. were the first to demonstrate that EPO is produced in rat organs other than the kidney and liver [53, 54]. In unstimulated animals, EPO mRNA was detected in the spleen, lungs, testes, brain, and several other organs. Moreover, in animals subjected to severe hypoxia, EPO mRNA was increased in the spleen, testes, and brain [53, 54]. These findings raised two important questions: first, whether oxygen sensing is a general phenomenon and found in organs other than the kidney and liver; and second, because of the limited diffusion of blood-borne EPO into the brain, whether EPO expression in the brain serves as a local physiological function [54], possibly providing protection against the deleterious effects of hypoxia. The first question was answered with the discovery in 1992 of HIF-1 [55], which is expressed in all cells of the body so far evaluated. As indicated elsewhere in this supplement [56, 57], HIF-1 is the master regulator for the expression of a large number of genes involved in maintaining oxygen homeostasis, including those for erythropoiesis, angiogenesis, and anaerobic metabolism. The second question constitutes a major area of investigation today, namely, the potential neuroprotective effects of EPO and its potential therapeutic value in the treatment of some CNS disorders.

Figure 7. CAM of a 12-day-old chick embryo incubated for 4 days with an implanted gelatin sponge containing 10 U rHuEPO. The embryo is characterized by an increased number of blood vessels with a radially arranged spoked-wheel pattern around the implant (A). In contrast, CAM of a 12-day-old chick embryo incubated for 4 days with an implanted sponge containing vehicle only (phosphate-buffered saline, negative control) shows no vascular response surrounding the sponge (B). Reprinted with permission from Ribatti et al. [46].



Expression of EPO and the EPO Receptor in the CNS

Following the findings of *Tan et al.* in rats, the EPO receptor was demonstrated in the brains of other species, including primates with increased expression occurring during hypoxia [53, 54, 58]. In addition, EPO was found in the cerebrospinal fluid of human patients [54], and EPO-receptor expression was demonstrated in several neuronal cell lines, including NT2, PC12, SN6, and SK-N-MC cells [32, 59]. In SK-N-MC cells, immunofluorescence studies showed that the EPO receptor is localized in the cell cytoplasm as well as in the plasma membrane, a finding considered important to understanding the mechanisms involved in any neuroprotective effects of EPO [59, 60].

Neuroprotective Function of EPO

Results of both in vitro and in vivo studies examining the function of EPO in the CNS have shown that EPO does in fact protect neurons from ischemic insult.

In Vitro Studies

It is well established that hypoxia/ischemia of the brain causes greatly enhanced release of the excitatory amino acid glutamate and that the neuronal damage produced by hypoxia/ischemia is mediated by glutamate [54]. In turn, glutamate-induced neurotoxicity is primarily mediated by the N-methyl-D-aspartate (NMDA) receptor. Binding of glutamate to the NMDA receptor leads to extensive influx of calcium, sodium, and water into neurons, resulting in impairment of mitochondrial function, excessive free radical production, cell swelling, and ultimately, cell death. In an important study, *Morishita et al.* [21] found that pretreatment with EPO protected cultured rat hippocampal and cerebral cortical neuron cells from glutamate toxicity. This protection was completely reversed by the concomitant application of a soluble EPO receptor, which is capable of binding with EPO. In similar experiments, pretreatment with EPO protected primary mouse neocortical neurons from injury by subsequently applied NMDA. On the other hand, concurrent application of EPO and NMDA resulted in cell damage and death, suggesting a preconditioning effect of EPO in vitro [19]. Additionally, EPO has been shown to rescue neuroblastoma cells from apoptosis induced by exposure to hypoxia [61] and to protect neuronal cultures from nitric-oxide-induced death [22].

In Vivo Studies

Findings of increased expression of EPO and its receptor in the brain during hypoxia/ischemia [19, 53, 58, 62, 63] led to and supported the idea that administration of exogenous EPO might be an effective therapeutic strategy in stroke and other hypoxia-related CNS disorders. Thus, several

groups have investigated intracerebral administration of EPO, with encouraging results. In a rat model, *Sadamoto et al.* [63] demonstrated that EPO infusion into the brain ventricles of stroke-prone spontaneously hypertensive rats prevented ischemia-induced place-navigation disability as well as cortical infarction and supported neuron survival in the ventroposterior thalamic nucleus. The same group, using a gerbil model of global cerebral ischemia, showed that infusion of EPO into the lateral ventricles prevented ischemia-induced learning disabilities, rescued hippocampal CA1 neurons from lethal insult, and increased the number of intact synapses in this area [22]. Further, infusion of soluble EPO receptors into animals given a mild ischemic insult insufficient to produce damage caused neuronal degeneration and impaired learning ability, confirming that EPO is crucial for neuronal survival following an ischemic event.

Possible Mechanisms of EPO Neuroprotection

It appears that brain-derived EPO can protect neurons by both direct and indirect mechanisms. One proposed mechanism for direct intervention is the repression of apoptosis by EPO, either by maintenance of Bcl-2 and Bcl_{xL} expression, as in the case of erythroid precursor cells [54], or by inactivation of caspases [64, 65] via an EPO-induced increase in intracellular Ca²⁺. The increase in calcium leads to a sustained increase in neuronal nitric oxide, which was recently shown to inhibit caspase function [65]. Other possible direct-acting mechanisms by which EPO may protect neurons are inhibition of glutamate release, increase of glutamate uptake, or desensitization of glutamate receptors and upregulation of enzymes that scavenge oxygen radicals [55]. Indirectly, EPO may afford neuroprotection by stimulating angiogenesis in the brain, which would increase the transport of oxygen-carrying blood to this organ and thus counteract the effect of ischemia on neurons [54].

USE OF rHUEPO IN CNS DISORDERS

Despite the demonstrated benefit of direct intraventricular injection of EPO in preventing ischemic neuronal tissue, this method of treatment is not practical in the clinical setting. Also, until very recently, systemic delivery was not considered a viable therapeutic approach, as it was believed that EPO could not cross the blood-brain barrier (BBB). However, evidence had appeared suggesting that large molecules, including insulin-like growth factor [66], leptin [67], and transferrin [68], could cross the BBB provided that receptors were present on the luminal surface of endothelial cells to allow translocation to occur [7]. Therefore, *Brines et al.* [20], in a series of studies using rodent models, examined expression of EPO and the EPO receptor in normal brain tissue. Additionally, they evaluated the ability of epoetin alfa to

cross the BBB and affect cognitive function and the outcome of neuronal injury. Results of immunological staining demonstrated the presence of EPO-receptor expression in neurons (restricted to the somata and dendrites) as well as within the astrocytic foot processes surrounding the capillaries and within or on the surface of capillary endothelial cells (Fig. 8). Follow-up studies with i.p.-injected biotinylated epoetin alfa revealed the presence of epoetin alfa around the neurons after 17 hours, confirming that circulating EPO can enter the CNS. Subsequent studies by this group indicated that systemic administration of epoetin alfa before or up to 6 hours after induction of focal ischemia in a rat model reduced injury by about 50%-75% (Fig. 9). Also, epoetin alfa was shown to reduce the extent of blunt-force brain injury and the severity of experimental autoimmune encephalomyelitis symptoms and to delay the onset and reduce the severity of kainate-induced seizures in rodent models.

Given the promising results regarding the prevention or treatment of various hypoxia-related CNS conditions in animal models, several small-scale trials of rHuEPO have been undertaken in humans. Breast cancer patients, particularly those receiving high-dose chemotherapy, frequently experience changes in cognitive function, including short- and long-term memory loss, decreased attention span and concentration, and impaired language skills. Thus, in a pilot study conducted in the United States, the effects of rHuEPO on cognitive function, asthenia, and quality of life (QOL) were evaluated in anemic stage I-III breast cancer patients receiving anthracycline-based adjuvant or neoadjuvant chemotherapy [69]. Cognitive function was assessed by Executive Interview (EXIT25), asthenia by the Functional Assessment of Cancer Therapy-Anemia (FACT-An) subscale, and QOL by the Linear Analog Scale Assessment (LASA, also referred to as the Cancer Linear Analog Scale or CLAS). Results of an analysis of 83 patients (44 epoetin alfa, 39 placebo) during chemotherapy and after a 6-month follow-up period showed that, for EXIT25, more patients treated with epoetin alfa than with placebo experienced improvements in cognitive function during chemotherapy; also, fatigue and QOL worsened to a lesser extent in the epoetin alfa-treated group. At follow-up, both groups showed restoration of cognitive function and QOL, although the improvements were generally greater in the epoetin alfa-treated group.

In a pilot study conducted in Germany, 20 ischemic stroke patients were given i.v. injections of rHuEPO within 5 hours of onset of symptoms [70, 71]. Cerebrospinal fluid EPO was 60-100 times greater than that of a control group of patients who had not received the hormone, demonstrating that i.v.-administered EPO reaches the brain. The treated patients subsequently had greater and earlier improvements in follow-up and outcome scores than the control patients;

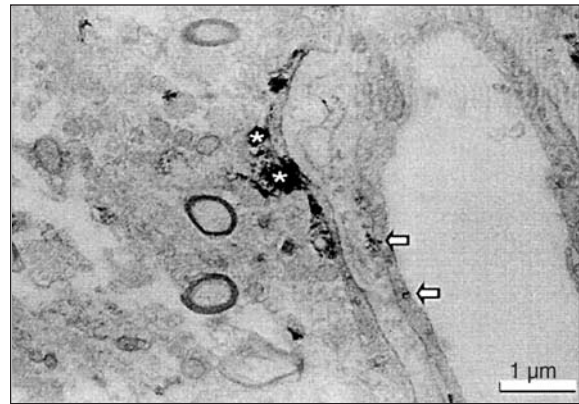


Figure 8. Transmission electron micrograph demonstrating prominent EPO receptor immunoreactivity within the astrocyte foot processes (asterisks). Additionally, EPO-receptor reactivity, although of lesser intensity, was observed within or on the surface of capillary endothelial cells (arrows). Reprinted with permission from Brines et al. [20].

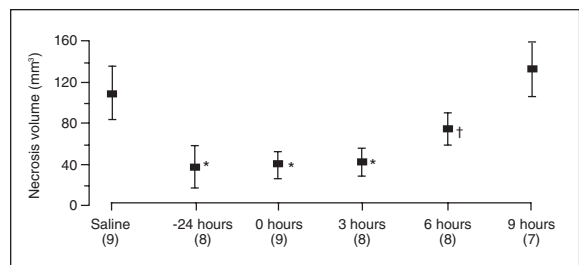


Figure 9. Intraperitoneal injection of epoetin alfa reduced infarct volume after cerebral artery occlusion in rats when administered 24 hours before occlusion ($p < 0.01$) and up to 6 hours after occlusion ($p < 0.05$). Reprinted with permission from Brines et al. [20].

also, they tended to have smaller areas of damaged brain tissue as measured by magnetic resonance imaging 1 month after their strokes.

SUMMARY

Until recently, EPO had been considered to act solely on hematopoietic cells. However, emerging data have shown that EPO is expressed in a variety of tissue and cell types, including cancer, vascular endothelial, and neuronal cells. Expression of EPO is induced by HIF-1, which is itself induced by hypoxia. EPO exerts its biological effects by binding to its cell surface receptor, resulting in tyrosine phosphorylation of the receptor and other intracellular proteins, including JAK2 and STAT5. The JAK/STAT pathway is utilized both in hematopoietic and nonhematopoietic cells (including brain cells) following binding of EPO to the EPO receptor. The recent findings of EPO-receptor expression in human breast and renal cancer cells, as well as in several tumor cell lines, have raised important questions in the

oncology setting about a possible tumor-growth-promoting effect of rHuEPO on EPO-receptor-bearing tumors. Overall, there has been no definitive evidence of such an effect, but the subject warrants further study. The demonstration of protective effects of rHuEPO in animal models exposed to a variety of neuronal insults has been especially intriguing, as

translation of such effects to the clinical setting could potentially offer therapeutic benefits to patients with ischemic stroke, trauma, epilepsy, neurodegenerative diseases, and cognitive dysfunction. Clearly, further study is needed to more thoroughly examine the mechanisms of EPO action and the role that EPO can play beyond hematopoiesis.

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**The Erythropoietin Receptor and Its Expression in Tumor Cells and Other
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