Pleiotrophic Actions of Erythropoietin

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Abstract

Erythropoietin is the prime regulator of red blood cell production. However, recent evidence suggests that the hormone has multiple effects outside the hematopoietic system. Functional receptors have been identified on a wide variety of normal and malignant cell types, and numerous biologic effects of the hormone on these cells have been observed both in vitro and in vivo. These findings are causing a reassessment of the understanding of erythropoietin physiology. Moreover, there are important implications for the use of recombinant erythropoietin in the clinical setting.

Key words: cancer, angiogenesis, brain, gut, reproductive system

Brain and central nervous system

Erythropoietin (Epo) is a 34–39 kDa glycoprotein whose primary function is to regulate the growth, maturation and survival of erythroid progenitor cells (Table 1). Since the cloning of the Epo gene and the commercial availability of highly purified recombinant forms of the growth factor, Epo has gained widespread clinical utility in treating various anemias, such as those associated with renal failure, cancer, and AIDS, as an adjunct to chemotherapy or following bone marrow transplantation; and to boost hematocrits prior to pre-surgical blood donations for autologous transfusion. Epo usage also has developed a darker side, as a “performance enhancer” in competitive endurance sports such as cycling and Nordic skiing. Recently, Epo has received newfound attention for its role(s) outside of hematopoiesis. Epo receptors have been identified on a variety of different cell types, as summarized in Table 2, and Epo has been shown to have a survival and growth-enhancing effect on the receptor-bearing cells, both in vivo and in vitro. It is these nonhematopoietic targets of Epo that are the subject of this review.

Table 1 Actions of erythropoietin on hematopoietic cells

Survival (anti-apoptosis)
Growth and proliferation
Maturation and differentiation

Much evidence has been presented in recent years concerning the role of Epo in the brain and central nervous system, both during development and in the adult animal. Epo receptors have been localized to the embryonic germinal zone during normal neurogenesis (1) and to the adult subventricular zone, which continues to generate neurons throughout adulthood (1). Both Epo and its receptor have been localized in the human brain by immunohistochemistry from as early as 5 weeks post-conception through adulthood, although the specific spatial distribution patterns appear to change throughout development (2, 3). The available findings suggest that Epo is important in both neurodevelopment and brain homeostasis later in life, functioning in both an autocrine and paracrine fashion.

Epo and its receptor are expressed in capillaries at the brain-periphery interface and evidence exists from animal studies that systemically administered Epo can cross the blood-brain barrier (4, 5). Taken together with the known roles of Epo as both an anti-apoptotic and a proliferation factor in hematopoietic cells, and coupled with the fact that both Epo production and neurogenesis itself (1) are known to be hypoxia-inducible, it has been suggested that Epo also may play similar roles as a neuroprotectant and survival factor in the brain (1, 6, 7). Cell culture models of central nervous system precursors grown at normoxic (20% O₂) vs. hypoxic (3% O₂) conditions revealed greater cell proliferation, less apoptotic cell death and altered differentiation patterns in the cells grown hypoxically (8). Concomitantly, the cells grown hypoxically had increased Epo mRNA expression. Supplementation of normoxic cultures with Epo mimicked the altered cell growth conditions observed during hypoxic cell growth. In cultured neuronal cells, hypoxia both induces Epo receptor expression and also increases the cells' sensitivity to Epo (9). In animal models, rabbits treated with Epo either immediately prior to or up

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Table 2 Nonhematopoietic sites of erythropoietin action

Brain and central nervous system	neurogenesis anti-apoptotic factor protection against hypoxic damage
Gastrointestinal system	trophic factor for enterocytes cell growth and maturation anti-apoptotic factor
Female reproductive tract	estrogen-dependent expression role in cyclic angiogenesis other cyclic cell changes cell survival, proliferation and differentiation
Endothelial cells	cell proliferation and migration angiogenesis
Others (kidney, muscle, male reproductive tract)	cell survival and proliferation

to 24 hours following experimental ischemic brain injury had increased levels of Epo in their cerebrospinal fluid and decreased necrosis of cortical neurons (10). Both primary and cultured rat cortical neurons respond to Epo with approximately 10-fold greater sensitivity and with altered signaling pathway compared with erythroid progenitors (11), although the significance of this difference is not yet clear.

Gastrointestinal system

Epo has been measured in human milk and its role in development of the gastrointestinal tract has been investigated (12, 13, 14). Epo receptors are present on enterocytes of rat fetal and neonatal small bowel, although the role of Epo in development of the intestine is not entirely known. While enterally-fed Epo is not absorbed and, therefore, has no direct erythropoietic effect (12), when the hormone was delivered either enterally or parenterally to rats, it served as a trophic factor in the developing small bowel, resulting in greater bowel length and increased villous surface. In studies of cultured human enterocytes exposed to Epo, the cells migrated faster and exhibited less apoptotic damage in response to injury than did enterocytes not exposed to Epo (13). In very low birth weight infants given Epo for prevention or treatment of the anemia of prematurity, the incidence of necrotizing enterocolitis was lower than in similar birth weight infants not given Epo (14), further supporting the role of Epo in the growth and development of the intestinal tract. Both in vitro and in vivo, the effect of Epo on rat enterocytes was dose-dependent and could be neutralized by anti-Epo antibodies. Therefore, it is suggested that Epo has a direct effect on the development, maturation and function of the neonatal intestine and that the presence of Epo in human milk is likely related to this role.

Reproductive tract

Epo and its receptor have been implicated in maintenance and normal cyclic changes of the female reproductive tract. Epo mRNA has been identified in the oviduct and in the ovary; its expression in the oviduct is inducible by hypoxia and is rapidly and markedly downregulated by estrogen (15). Epo (both mRNA and protein) also is found in the murine uterus (16), where its production is estrogen-inducible. In ovariectomized mice, the injection of either Epo or estrogen results in a rapid rise in uterine Epo mRNA expression. Injection of Epo directly into the uterus of

ovariectomized mice promotes blood vessel formation by the endometrium, and this angiogenesis is inhibited by co-injection of soluble Epo receptor (16). Uterine endometrium expresses Epo in an estrogen-dependent fashion and Epo contributes to uterine angiogenesis (17). Endometrial vessels have been shown by immunocytochemistry to express not only Epo receptors but also the Epo signal transduction intermediates JAK2, STAT3 and STAT5 (17). The female reproductive system both produces and responds to Epo, and Epo signal transduction appears to contribute to normal cyclic changes in the female. During pregnancy, both Epo and its receptor have been co-localized to cytotrophoblastic villi (18) and in syncytiotrophoblasts (19) in the human placenta, and functional Epo receptors have been identified in endothelial cells from fetoplacental blood vessels (19). An autocrine role for Epo was proposed for the survival, proliferation and differentiation of placental trophoblastic cells. In addition to its function in the maintenance of normal cyclic changes in the female reproductive tract, increased levels of Epo were found in the peritoneal fluid of women with endometriosis, particularly those with earlier stage disease (18), suggesting a possible role of Epo in the pathology of this condition.

In the male rat, Epo mRNA has been identified in whole testes, in Sertoli and in peritubular myoid cells. Epo mRNA levels are stimulated by FSH and are decreased by testosterone (20). It is not known whether Epo can cross the blood-testis barrier; however, these preliminary findings suggest an as-yet undefined role for Epo in the male reproductive system.

Endothelial cells

The first non-erythroid cells on which Epo receptors were demonstrated were endothelial cells (21, 22). Since hematopoietic and endothelial cells are derived from common progenitors (23, 24) it is not entirely surprising that functional Epo receptors have been demonstrated on both cell types. The action of Epo on endothelial cells appears to be two-fold, promoting cell survival and growth as it does in erythroid cells, and directly promoting angiogenesis, a function unique to endothelial cells. Epo has been shown to increase the proliferation and migration of endothelial cells in culture and to stimulate the release of endothelin-1 (25). Other studies, however, using a variety of primary human endothelial cells indicate that Epo mediates release and increased activity of endothelial cell-derived nitric oxide synthase (NOS) but does not affect endothelin-1 release in these cells (26). Epo protects

bovine pulmonary aortic endothelial cells against apoptotic damage/death in response to toxic stimuli (27). Phosphorylation of the Epo signal transduction intermediate JAK2 and increased synthesis of matrix metalloproteinase-2 (MMP2) were also observed in response to Epo treatment of a variety of cultured human endothelial cells (28). Differential display has been used to identify and elucidate downstream events following Epo treatment of vascular endothelial cells. Among the genes regulated by Epo, several related to endothelial cell functions, energy transfer and subsequent signal transduction have been enumerated (29). Epo also has been shown to act directly as a potent angiogenic factor for endothelial cells. It provoked an angiogenic response comparable to that of the known angiogenic stimulus, basic fibroblast growth factor, when injected into the chick chorioallantoic membrane (28) and increased microvessel density in granulation tissue during wound healing (30). Further examination of the role of Epo on promoting angiogenesis is discussed below.

Other cell types

Epo receptors are expressed in certain cells of the melanocyte lineage, in transformed cell lines derived from melanocytes (although not on primary melanocytes), and in human melanoma cell lines derived from metastases (31). Greater than 50% of spontaneously transformed melanocytes have Epo receptors on their surface, although there is no evidence that Epo plays a significant role in affecting cell growth. Rather, since normal primary melanocytes lack receptors while their transformed counterparts do express receptors, it is suggested that Epo receptor expression may somehow be involved with the acquisition/generation of the transformed phenotype. It is possible, therefore, that receptor expression may be a marker for disease progression.

Epo receptor transcripts are readily detected in the cortex, medulla and papilla of human and rat kidney, as well as in the proximal tubular (mouse, human) and medullary (human) collecting duct cells (32). The demonstration of specific binding of ^{125}I -labeled Epo to the receptor-bearing cells and direct mitogenic activity of Epo on the cells (32) suggests a previously unrecognized function of Epo in the kidney. While the mitogenic effect of Epo on kidney cells, if it occurs in vivo, might be beneficial in the repair/recovery of kidney injury, it could be equally detrimental in cases of renal carcinoma.

Finally, Epo can stimulate myoblast proliferation, helping to expand the progenitor cell population during muscle differentiation. It has been suggested (33) that Epo may play a biological role during normal muscle development and repair.

Epo and angiogenesis

Angiogenesis, or the formation of new blood vessels from pre-existing ones, occurs sparingly under normal physiological conditions in the adult. Some notable exceptions are in the normal female reproductive cycle and in wound healing, as discussed briefly above. In addition, an adequate blood supply and the presence of endothelial cells is required for adequate hematopoiesis in the bone marrow (34), and an important role for the interaction/proximity of endothelial cells and hematopoietic progenitor cells in the marrow has been established (35). In vitro models of human bone marrow endothelial cell culture have demonstrated that the hematopoietic cytokines, particularly the combination of granulo-

cyte-macrophage colony stimulating factor and Epo, result in an increase in vascular tube formation in culture that is greater than the maximum increase achieved in the presence of vascular endothelial growth factor (VEGF) (36).

Pathological angiogenesis has been implicated in diseases including, but not limited to, diabetic retinopathy, renal disease, rheumatoid arthritis, inflammatory bowel disease, and cancer, including both leukemias and solid tumors (37, 38, 39, 40). One of the key elements involved with a tumor's switch to the angiogenic phenotype is hypoxia, and hypoxic up-regulation of the key angiogenic growth factors, VEGF and bFGF, as well others, occurs by a mechanism similar to the hypoxia induced up-regulation of Epo synthesis (41, 42). Hypoxia, thus, can serve both directly and indirectly as a trigger for tumor growth, by upregulating angiogenic cytokines and increasing angiogenesis to feed the developing tumor. Therefore, Epo upregulation by tumor hypoxia may lead to increased angiogenesis both directly, via Epo's mitogenic effect on endothelial cells (25, 28) in concert with the hypoxic upregulation of other angiogenic cytokines. In contrast, the therapeutic administration of Epo to cancer patients to relieve disease- or treatment-related anemia may lead to decreased angiogenesis by relieving local hypoxia and, thus, downregulating the production of VEGF (43, 44). Since high tumor VEGF levels have been correlated with poor treatment outcome and survival (45, 46), the Epo-dependent decrease in VEGF in the tumor microenvironment may, therefore, actually be predicted to decrease tumor angiogenesis, even in the presence of additional angiogenic stimuli, although potential mechanisms by which this may occur are not clear.

Epo and cancer

Epo is well established for its ability to alleviate the anemia associated with advanced cancer and with the myelosuppressive effects of various chemotherapy drugs, particularly platinum-based therapies (47, 48, 49, 50). The quality of life improvements for patients appear to be independent of tumor type or treatment protocol (51, 52). Epo also is used in the setting of bone marrow and peripheral blood stem cell transplantation (53).

Correction of anemia, especially via the clinical use of Epo, also appears to be important in the outcome of radiotherapy treatments for cancer (54, 55, 56, 57). Low hemoglobin levels, and associated low tissue oxygenation, causes a decrease in radiosensitivity of tumor cells (54, 58, 59, 60). Hypoxic regions of tumors are less sensitive to radiation damage than are well-oxygenated tumors and require higher doses of radiation to achieve oxidative damage and subsequent cell death (55). Further, patients with hypoxic solid tumors have been found to have shorter periods of disease-free survival following radiation treatment than those having well-oxygenated tumors (53). Studies of patients with head and neck cancers (55, 61, 62, 63), lung cancer (64), and pelvic malignancies (63, 64, 65, 66) whose anemias have been corrected - or prevented - through the clinical administration of Epo have improved local/regional tumor control, quality of life and survival in comparison with patients undergoing radiotherapy in the presence of anemia or whose anemia is addressed through the use of blood transfusions.

Animal studies suggesting further indications for the use of Epo in cancer treatment also have been published. Chemotherapeutic drugs may disrupt endothelial cell structure and, therefore, blood vessel integrity, negatively impacting (among other things)

the successful delivery of chemotherapeutic agents to their targets. Drugs such as bleomycin can cause pulmonary toxicity, including pulmonary fibrosis and decreased oxygen-carrying capacity in the circulation. The co-administration of Epo to animals receiving bleomycin has been shown to decrease alveolar thickening, pulmonary fibrosis and endothelial cell function by up to 60% and to partially restore pulmonary function resulting from bleomycin treatment (67).

Daily injections of Epo into MOPC-315 (myeloma) tumor bearing mice induced complete tumor regression in 30–60% of the mice. When rechallenged with the same tumor line, the mice rejected the tumor. In that study, the effect of Epo was attributed to a T-cell mediated mechanism (68).

In a phase II clinical trial of patients whose metastatic renal cell carcinoma was progressing on interleukin-2 (IL-2) alone, co-administration of Epo with IL-2 resulted in patients who had a partial response (i.e. tumor regression) or stable disease. Study results suggested that Epo decreased treatment toxicity, prevented drops in hemoglobin levels, and appeared to counteract the IL-2 driven rise in VEGF levels, thereby inhibiting angiogenesis (69).

On the other hand, there have been several studies demonstrating that functional Epo receptors are found on tumor cells (while, in some cases, NOT in the adjacent normal tissue), and the findings provide evidence that Epo can directly stimulate the survival, growth and proliferation of malignant cells. Table 3 summarizes some of the tumor cells that have been shown to express Epo receptors.

A recent study reported a patient with myelodysplastic syndrome (refractory anemia with ringed sideroblasts) who began treatment with Epo to reduce transfusion requirements and improve quality of life (70). Within six weeks of starting Epo therapy the patient developed acute myelogenous leukemia (AML). Interestingly, all signs of AML disappeared from the patient's blood and bone marrow following discontinuation of the Epo therapy.

Screening of primary renal carcinoma cells, in addition to renal carcinoma cell lines, indicated the expression of both transcript and mature Epo receptor protein on the cells. The cells bound ¹²⁵I-labeled Epo and exhibited a mitogenic response to Epo, suggesting that the receptors are functional (71). These findings suggest that delivery of Epo to renal carcinoma patients to alleviate their anemia might well hasten the proliferation of their tumors.

In numerous studies, Epo was cited as "safe and effective" for treating the anemia associated with multiple myeloma. However, myeloma cells and cell lines also have been demonstrated to express Epo receptors (72, 73, 74). Epo was reported to directly stimulate its receptor on myeloma cells and to result in phosphorylation of MAPK and other downstream signaling intermediates, some of which are essential for mitogenesis. Following initiation of Epo therapy as a "quality of life" decision in a patient undergoing no other treatment for her disease (72), the patient's condition worsened markedly, with an increase in serum IgA level and or myeloma M protein. The patient's condition improved (and both IgA and M protein levels decreased to pre-treatment levels) following discontinuation of Epo. The authors suggested the need for caution when prescribing Epo for patients with multiple myeloma.

Table 3 Tumor cells that have been shown to express erythropoietin receptors

Breast cancer cells (but not normal breast tissue, except in lactating women)
Melanoma cells (but not normal melanocytes)
Myeloma cells
Prostate cancer cells
Renal carcinoma cells
Tumors of the uterus or ovaries

We have discussed above the positive role of Epo in normal cyclic angiogenesis associated with the female reproductive cycle. Malignant tumors of the ovaries and uterus also express both Epo and Epo receptors (75), and their growth may be dependent on an Epo-receptor signal. Deprivation of Epo signaling (such as by local injection of anti-Epo antibodies or of soluble Epo receptor) induces apoptotic cell death of both tumor cells and of capillary endothelial cells. In addition, when xenotransplanted human ovarian tumors in nude mice were injected locally either with the anti-Epo antibodies or with soluble Epo receptors, the extent of tumor regression was found to be related to the frequency and dose of the injections (75).

Large numbers of clinical biopsy samples taken from patients with breast cancer (76, 77) have demonstrated expression of both Epo and Epo receptor, and frequently their expression was co-localized (77). Epo and Epo receptor expression was found in tumor tissue and its associated vasculature but not in adjacent normal tissue (38), although the exception to an absence of Epo expression in normal breast tissue is the lactating female, where mammary duct epithelial cells have been shown to express Epo and to provide the source of Epo that is secreted into breast milk (78), as previously discussed. In one study of 50 such clinical samples, expression levels were highest in hypoxic regions of tumors, suggesting that increased Epo signaling may promote breast cancer growth by hypoxia (76). In an experimental model of in vivo breast cancer tumor growth, local injection of antibodies to Epo, of soluble Epo receptor, or of antibodies to the Epo signaling intermediate JAK2 kinase resulted in a significant decrease in tumor growth (77).

Recent evidence also supports a role for Epo in the growth of human prostate cancer cells (79). Transformed but nontumorigenic prostate epithelial cells, in addition to established prostate cancer cell lines, express Epo receptors; and mature immunohistochemically identifiable Epo receptor protein also was identified on the cell surfaces. All of the cell lines tested exhibit a dose-dependent proliferative response to added Epo.

Taken together, these findings suggest that Epo has a wide variety of functions in several tissues and organs in addition to the bone marrow. Further studies will help to clarify this multiplicity of actions with respect to angiogenesis and cancer.

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