

Review

Erythropoietin as an angiogenic factor

D. Ribatti*, A. Vacca*, A. M. Roccaro*, E. Crivellato† and M. Presta‡

*University of Bari Medical School, Bari, †University of Udine Medical School, Udine, ‡University of Brescia Medical School, Brescia, Italy

Abstract

Erythropoietin (Epo) is produced by the fetal liver and adult kidney and is an essential stimulator of erythropoiesis. It has, however, been shown to modulate host cellular signal transduction pathway to perform many other functions. New sites of Epo production have been found, such as the female reproductive organs and central nervous system. This review summarizes the involvement of Epo in the regulation of angiogenesis in both normal and pathological conditions.

Keywords Angiogenesis, central nervous system, erythropoietin, hypoxia, tumour.

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Introduction

Erythropoietin (Epo) is a low-molecular weight (30 kDa) glycoprotein hormone stimulator of erythropoiesis produced in the fetal liver and subsequently in the adult kidney [1]. Erythropoietin promotes the proliferation and differentiation of erythroid precursors and leads to an increased expression of the antiapoptotic proteins [2] and inhibition of apoptosis [3], by controlling the dynamic balance between erythropoiesis and erythrocyte loss in order to maintain red cell volume [4,5]. Erythropoietin is the best known hypoxia-regulated gene and this regulation occurs mainly at the mRNA level and is mediated by hypoxia-inducible factor-1 (HIF-1) [6].

Erythropoietin exerts its action through its specific receptor (EpoR), a member of the cytokine receptor superfamily, which is mainly expressed on erythroid colony-forming units (CFU-E) [4,5]. Major signal-transduction pathways activated by Epo include the JAK-2/signal transducer and activator of transduction (STAT-5) and the Ras/mitogen-activated protein kinase (MAPK) pathways involved in

the inhibition of apoptosis and the stimulation of cell proliferation in response to Epo [7].

Recently, the importance of the Epo-EpoR system in primary and definitive erythropoiesis has been determined by generating lines of mutant mice lacking either the Epo or the EpoR gene [8,9]. Both lines died of severe anaemia between embryonic days 13 and 15.

Erythropoiesis was considered to be the sole physiological action of Epo until Epo and EpoR were found to be expressed in other sites besides liver and kidney: bone marrow macrophages [10], neurones, astrocytes, brain endothelial cells, microglia and even oligodendrocytes [11]; trophoblast cells of the human placenta [12]. Considerable amounts of Epo are also present in human milk [13]. Erythropoietin R is expressed by a variety of nonhaematopoietic cell types, including endothelial cells [14], neurones [15] and trophoblast cells [16].

Although the specific functions of Epo/EpoR in these sites is not yet completely clarified, there is increasing evidence suggesting a wider biological role of Epo/EpoR not related to erythropoiesis. Among the extra-haematopoietic functions of Epo, angiogenesis, the process through which new blood vessels arise from preexisting ones, has been indicated. The potential role of Epo in angiogenesis may be considered as a subset of its possible function in improving overall tissue oxygenation and of its antiapoptotic role.

Department of Human Anatomy and Histology (D. Ribatti); Department of Biomedical Sciences and Human Oncology, University of Bari Medical School, Bari, Bari (A. Vacca, A. M. Roccaro); Section of Anatomy, Department of Medical and Morphological Research, University of Udine Medical School, Udine (E. Crivellato); Unit of General Pathology and Immunology, Department of Biomedical Sciences and Biotechnology, University of Brescia Medical School, Brescia (M. Presta), Italy

Correspondence to: Domenico Ribatti, Department of Human Anatomy and Histology, Policlinico, Piazza Giulio Cesare, 11, I-70124 Bari, Italy. Tel.: +39 080 5478240; fax: +39 080 5478310; e-mail: ribatti@anatomia.uniba.it

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Cross-talk between haematopoiesis and angiogenesis

The relationship between endothelial and haematopoietic cells has been seen as an indication that a common progenitor,

the haemagioblast, give rises to both cell types in the yolk sac, the initial site of haematopoiesis and blood vessel formation during mammalian development [17].

Haematopoiesis is regulated by several cytokines and interleukins (IL) including granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), IL-3, IL-4, IL-6 and IL-8 [18]. These bioactive proteins are also involved in key functions of the vascular endothelium [19]. Granulocyte-colony stimulating factor and GM-CSF receptors have been detected on the surface of endothelial cells [20]. These cytokines induce endothelial cells to migrate and proliferate and are angiogenic in the rabbit cornea [20] and in the chick chorioallantoic membrane (CAM) [21].

Accordingly, endothelial cells exposed to recombinant human vascular endothelial growth factor (VEGF) manifest increased mRNA for several haematopoietic growth factors, including G-CSF, GM-CSF, stem-cell factor and IL-6, which may act as growth factors for myeloid and lymphoid cells [22]. Vascular endothelial growth factor may thus play an important role in the growth of haematological neoplasms via a paracrine mechanism. Fibroblasts growth factors (FGFs) positively regulate haematopoiesis by acting on stromal cells, early and committed haematopoietic precursor cells and some mature blood cells, exerting both autocrine and paracrine functions in these biological processes [23].

Erythropoietin and angiogenesis

Angiogenesis is a complex process, where several cell types and mediators interact to establish a specific microenvironment suitable for the formation of new vessels. Angiogenesis takes place in various physiological and pathological conditions, such as embryonic development (where is associated with vasculogenesis, i.e. the formation of capillaries from endothelial cells differentiating *in situ* from groups of mesodermal cells), wound healing, the menstrual cycle and chronic inflammation and tumours [24,25].

The therapeutic use of recombinant human Epo (rHuEpo) in the treatment of anaemia and arteriovenous fistulae (AVF) in haemodialysis [26–28] is responsible for the appearance of vascular side-effects, including hypertension and thrombosis. In fact, the sustained dose-dependent rise in haematocrit that Epo produces effectively abolishes symptoms of anaemia, but at the cost of an increase in blood viscosity [26], while the role of haematocrit and Epo therapy in the pathogenesis of AVF thrombosis is still controversial, as although the Epo treatment is significantly correlated with the AVF failure, no such correlation was observed with the level of haematocrit [28]. These findings related to the relationship existing between Epo and the vascular system has prompted investigation of its interaction with endothelial cells.

Erythropoietin binding to its receptor in differentiating haematopoietic cells activates JAK/STAT and other signal transduction pathways to control cellular proliferation, survival and specific gene expression. Accordingly, endothelial

cells expressed EpoR that bound JAK2 and included its transient activation after rHuEpo exposure [29]. It is interesting to note that JAK2 is involved in the intracellular signalling of receptors for various cytokines, including the angiogenic G-CSF and GM-CSF [30]. It has been demonstrated that GM-CSF induces JAK2 activation in immortalized EA.hy 926 endothelial cells [31] and in human umbilical vein endothelial cells (HUVECs) [32]. Taken together, these findings suggest a role for the JAK2/STAT5 signalling pathway in cytokine-mediated angiogenesis. Moreover, Epo also stimulates JAK2 phosphorylation in cultured muscle cells and neurones [33,34].

Erythropoietin R mRNA is expressed in endothelial cells of HUVECs [14], bovine adrenal capillaries [35] and rat brain capillaries [36]. Erythropoietin stimulates the proliferation and migration of cultured HUVECs [29] and human and bovine endothelial cells *in vitro* [35,36], as well as microvascular endothelial cells isolated from rat mesentery [37] and in the rat aortic ring model [38]. Moreover, Epo induces endothelin-1 (ET-1) release and an increase in cytosolic-free calcium in endothelial cell cultures [39,40]. Recombinant human Epo induces a proangiogenic phenotype in human endothelial cells, including both early (i.e. increase in cell proliferation and matrix metalloproteinase-2 production) and late (differentiation into vascular tubes) angiogenic events [29]. *In vivo*, in the chick embryo CAM assay, the angiogenic activity of rHuEpo was similar to that exerted by FGF-2, a well-known angiogenic cytokine, and endothelial cells of the CAM expressed EpoR, which colocalized with factor VIII positivity [29]. More recently, Jaquet *et al.* [41] investigated the angiogenic potential of rHuEpo on endothelial cells derived from human adult myocardial tissue and compared the angiogenic potential of rHuEpo to that of VEGF. They found that rHuEpo stimulated capillary outgrowth up to 220%, compared with the nonstimulated physiological outgrowth. Erythropoietin therefore exhibited the same angiogenic potential as VEGF.

It has been demonstrated that Epo decreases the activity of endothelial isoforms of nitric oxide synthase (eNOS) in human coronary artery endothelial cells [42]. Experimental data indicate that NOS, depending on isoforms, the timing and the degree of activation, displays contradictory effects during physiological and pathological angiogenesis [43].

Finally, it is to note that in vascular smooth muscle cells, Epo can stimulate calcium influx, suggesting that calcium mobilization may contribute to the hypertension associated with Epo treatment, and act as a vascular growth factor [44]. Moreover, Epo acts as a viability factor during cardiac development and is necessary to prevent apoptosis and expansion or proliferation of myocardial and endocardial progenitor cells [34].

Erythropoietin and angiogenesis in female reproductive organs

In normal adult tissue, vascular growth is a relatively rare event, indicated by capillary endothelial cell proliferation

rates of only 0.01% to 0.14% in most tissues [45]. However, the female reproductive tract of primates undergoes substantial vascular growth and remodelling associated with the menstrual cycle and pregnancy [46].

A paracrine Epo/EpoR system in the uterus may play an important role in uterine angiogenesis through EpoR expressed by endometrial vascular endothelial cells [47]. Moreover, Epo mRNA is expressed in normal human endometrium and ovary, while JAK-2, EpoR-phosphotyrosine and STAT5 are expressed at their Epo-responsive sites [48]. Erythropoietin production in female reproductive organs is oestrogen-dependent. Administration of 17 α -estradiol (E2) leads to a rapid and transient increase in Epo mRNA in the uterus, oviduct and ovaries [47]. Finally, injection of Epo into the uterine cavity of ovariectomized mice leads to blood vessel formation in the endometrium [47].

Erythropoietin and angiogenesis in the nervous system

In the brain, there is a paracrine Epo/EpoR system that is independent of the endocrine system in adult erythropoiesis: neurones express EpoR and astrocytes produce Epo [44,49]. It has been demonstrated *in vitro* and *in vivo* that Epo is a potent inhibitor of neuronal apoptosis induced by ischaemia and hypoxia [50]. The induction of EpoR production in neuronal cultures exposed to low oxygen tension suggests that the Epo response may involve both an up-regulation of Epo production and an increase in neuronal sensitivity to Epo mediated by increasing EpoR production, suggesting that Epo may act as a survival factor for neurones and can play a role in the stress response to hypoxia or ischaemia [34]. A temporal and spatial cellular expression of Epo and EpoR has been described after focal permanent ischaemia in mice [51].

Angiogenesis generated in the vascular system may provide indirect neuroprotection in the central nervous system. Furthermore, angiogenesis in the brain may be closely related to neuronal survival in patients with ischaemic stroke [52]. A benefit of angiogenesis may result from the restoration of blood flow in the ischaemic border through arteriolar growth and capillary formation during cerebral ischaemia [53]. As new vessel formation occurs in the ischaemic border of the brain several days following a stroke [52], its induction by Epo provides indirect protection of the brain tissues and contribute to the functional recovery after Epo treatment in animal models and in human stroke [51,54,55], and demonstrates that the protective role of Epo is more general and extends beyond haematopoiesis.

Recently, Morita *et al.* [56] have demonstrated that, in addition to VEGF, Epo plays a key role in the development of the neovascularization associated with the retinopathy of prematurity (ROP), suggesting a therapeutic possibility of Epo and VEGF inhibitors in ROP treatment.

Erythropoietin, hypoxia and angiogenesis

Erythropoietin production is regulated by tumour oxygen supply. A deficiency in tissue oxygen results in Epo production in the kidney and liver [57], and also in the brain [58]. Erythropoietin production in the kidney appears to be transient, whereas it is more sustained in the brain [58].

Hypoxia induces cells to respond through multiple gene products such as Epo and VEGF that will improve oxygen delivery to the tissues, or enzymes of the glycolytic pathway that will adapt the cellular metabolism to decreased oxygen availability.

A specific survival role for Epo and induction of EpoR by hypoxia were demonstrated in cultures of neurones and astrocytes [34,59,60]. The induction of Epo and EpoR by hypoxia suggests that Epo administration has a therapeutic potential role for tissue damage from ischaemia or hypoxia in the central nervous system [51,55].

The primary *trans*-acting factor for Epo and VEGF is HIF-1. This consists of the regulatory subunit HIF-1 α and the constitutively expressed HIF-1 β subunit [61] and is overexpressed in the majority of human cancers, even more so in their metastases, and allows cancer cells to better adapt to hypoxia. Hypoxia rescues HIF-1 α from proteasomic degradation and leads to its nuclear translocation and heterodimerization, an activation of HIF-1-target genes, including those encoding Epo, VEGF, and other genes involved in erythropoiesis, angiogenesis, vasodilation and glucose metabolism [61].

Erythropoietin and tumour angiogenesis

Erythropoietin may be considered as an endogenous stimulator of vessel growth during tumour progression through an autocrine and/or a paracrine loop, as also postulated in renal carcinoma [62]. The high expression of Epo and EpoR in solid tumours could improve the hypoxic survival of cancer cells [63]. Hypoxia mediates the selection of neoplastic cells with diminished apoptotic potential by providing a growth advantage to cells with genetic alterations that impair apoptosis [64]. This hypoxia-mediated clonal selection has been suggested as an important biologic mechanism of tumour progression. Hypoxia also may be involved in the development of a more aggressive phenotype and may contribute to metastasis [65] and treatment resistance [66]. Hypoxia-inducible factor-1 regulates the expression of several genes known to confer a growth advantage on hypoxic cancers [61], and HIF-1-mediated Epo expression is thus unlikely to be an exclusive mechanism for hypoxic cell survival.

Erythropoietin may stimulate proliferation and inhibit apoptosis of EpoR-bearing tumour cells [63]. Thus, the negative effect of Epo on tumour growth may be further aggravated by its known angiogenic activity [64]. On the basis of these considerations, Epo administration to a patient with multiple myeloma or with myelodysplastic syndrome might promote an angiogenic response in their bone

marrow and cause further malignant transformation resulting in plasma cell leukaemia and, respectively, acute monoblastic leukaemia [67–69].

Erythropoietin and EpoR are known to be expressed in several anatomical sites and malignancies from these organs, including breast carcinoma, gastric cancer, malignant tumours of the female reproductive organs and of the central nervous system, and show up-regulation of Epo and EpoR on the neoplastic cells as well as on the tumour vasculature [64,70–72]. In detail, in haemangioblastoma, an highly vascularized tumour of the central nervous system, Epo mRNA was observed and *in situ* hybridization revealed neoplastic stromal cells as Epo-producing cells [73]. In gastric carcinoma EpoR level correlates with angiogenesis and progression [71]. Yasuda *et al.* [72] reported that Epo and EpoR are expressed in malignant tumours of the female reproductive organs, where tumour cells and capillary endothelium showed EpoR immunoreactivity, and that the injection of a monoclonal antibody against Epo or the soluble form of EpoR (sEpoR) into tumours reduces capillaries and causes tumour destruction. They also found that blockade of Epo signalling on xenografts of uterine and ovarian cancer leads to the destruction of tumours in nude mice.

Finally, it may be conceivable to hypothesize that tumour-derived Epo may influence also tumoural vascular lymphatic biology. Recently, Acs *et al.* [64] demonstrated that cells of squamous dysplasia and carcinoma of the uterine cervix show an increased expression of EpoR, detectable also in the endothelial cells of lymphatic vessels.

Concluding remarks

The multiple organ response to Epo that includes haematopoietic, neuronal, endothelia and muscle cells may be a consequence of the similarities of respective differentiating cells [74] and suggests that the protective role of Epo is more general and extends beyond haematopoiesis. In fact, accumulating evidence that Epo and EpoR are expressed in various nonhaematopoietic organs suggests that Epo signalling is involved in the regulation of other cell functions, including promotion of angiogenesis, prevention of cellular apoptosis, and protection against the effects of hypoxia. Erythropoietin produced by tumour cells may also stimulate indirect effects, such as the release of additional growth factors in the tumour microenvironment. Thus, deprivation of Epo signalling may be a useful therapy for Epo-producing malignant tumours. Otherwise, the parallel function of Epo, by promoting angiogenesis and acting as a survival factor on endothelial cells, makes it an interesting substance for the treatment of patients with ischaemic disease.

Nevertheless, some important questions regarding Epo biology still remain to be addressed: (a) the need to further understand EpoR signalling mechanisms in endothelial and vascular smooth muscle cells; (b) the mechanisms underlying vascular EpoR up-regulation by hypoxia; (c) the mechanisms upregulating EpoR expression in tumoural vs.

normal vasculature; and (d) the role of the Epo signalling pathway in lymphatic vessels.

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