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Mini-review

# Hematopoietic growth factors and tumor angiogenesis

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ABSTRACT

Angiogenesis is regulated by numerous "classic" factors such as vascular endothelial growth factor (VEGF) and many other endogenous "non-classic"peptides, including erythropoietin (Epo), and granulocyte-/granulocyte macrophage colony stimulating factor (G-/GM-CSF). The latter play an important regulatory role in angiogenesis, especially under pathological conditions and constitute a crosslink between angiogenesis and hematopoiesis. This article reviews studies on the ability of hematopoietic cytokines to affect several endothelial cell functions in tumor angiogenesis. These findings in all these studies support the hypothesis formulated at the beginning of this century that a common ancestral cell, the hemangioblast, gives rise to cells of both the endothelial and the hematopoietic lineages.

## 1. Introduction

The relationship between endothelial and hematopoietic cells is considered as an indication that a common progenitor, the hemangioblast, gives rise to both cell types in the yolk sac, the initial site of hematopoiesis and blood. Hematopoiesis is regulated by several cytokines and interleukins (IL) with pleiotropic activity, including erythropoietin (Epo) granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3, IL-4, IL-6 and IL-8 [1]. Several evidences have clearly demonstrated that these molecules, formerly regarded as specific for the hematopoietic system, also affect certain endothelial functions and that hematopoietic factors clearly influence angiogenesis [2]. G-CSF and GM-CSF receptors have been detected on the surface of endothelial cells [3]. Accordingly, endothelial cells exposed to recombinant human vascular endothelial growth factor (VEGF) manifest increased mRNA for several hematopoietic growth factors, including G-CSF, GM-CSF, stem cell factor and IL-6, which may act as growth factors for myeloid and lymphoid cells [4]. VEGF may thus play an important role in the growth of hematological neoplasms via a paracrine mechanism. Fibroblast growth factors (FGFs) positively regulate hematopoiesis by acting on stromal cells, early and committed hematopoietic precursor cells and some mature blood cells, exerting both autocrine and paracrine functions in these biological processes [5].

## 2. Erythropoietin

In 1977, Epo purification was achieved using urine of patients suffering from aplastic anemia as a source material [6]. The pure human urinary Epo enabled the partial identification of its amino acid sequence, which, in turn, allowed the deduction of the nucleotide sequences required for probes used in the attempts to clone the Epo gene. In 1985, Lin et al. [7] and Jacobs et al. [8] cloned and transfected in mammalian cells the Epo gene in Chinese hamster ovary cells and, respectively, in African green monkey kidney cells. In 1986, Winearls et al. [9] and in 1987 Eschbach et al. [10] used successfully the DNA-derived recombinant human Epo (rHuEpo) to treat the anemia of chronic renal disease in clinical trials.

Epo binds to a transmembrane receptor, a member of the cytokine receptor superfamily, which is mainly expressed on erythroid colony-forming units (CFU-E) and consists of an extracellular domain, a transmembrane domain and an intracellular domain [11,12]. In 1989, D'Andrea and co-workers [13] cloned and characterized the murine Epo receptor (EpoR). A single Epo molecule binds to two adjacent EpoR on the target cell membrane and begins an intracellular signaling cascade.

Epo is essential for the survival, proliferation and differentiation of erythroid precursors in the bone marrow and leads to an increased expression of the anti-apoptotic proteins [14] and inhibition of apoptosis [15], by controlling the dynamic balance between erythropoiesis and erythrocyte loss in order to maintain red cell volume [11,12].

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## 3. The role of Epo in tumor angiogenesis

Epo stimulates both proliferation and migration of human and bovine endothelial cells *in vitro* and of endothelial cells isolated from rat mesentery [16–19], as well as in the rat aortic ring model [20]. Moreover, Epo induces endothelin-1 expression in endothelial cell cultures [20,21]. EpoR mRNA is expressed in human umbilical vein endothelial cells (HUVEC) [22], bovine adrenal capillaries [23] and rat brain capillaries [24]. A differential display analysis of HUVEC extracts has revealed four groups of genes that are upregulated by rHuEpo, including those encoding proteins involved in the control of vascular function (e.g. thrombospondin 1), gene transcription (e.g. c-myc), mitochondrial function (e.g. cytochrome C oxidase subunit 1), and regulators of signal transduction [25]. Epo favors tumor progression through effects on angiogenesis and tumor cells can directly release increasing amounts of VEGF and placental growth factor (PlGF) in response to Epo [26].

The presence of a paracrine or autocrine Epo/EpoR loop in tumorigenesis in female reproductive organs was suggested based on the mitogenic action of Epo as well as on the finding that injection of soluble EpoR (sEpoR) or Epo-monoclonal antibody into tumors was followed by apoptosis of tumor cells and endothelial cells [27].

Hardee et al. [28] demonstrated that local rhEpo administration induces a significant pro-angiogenic effect and a transient stimulation of tumor growth in window chambers. Moreover, three different inhibition strategies targeting endogenous Epo/EpoR, including the administration of sEpoR and anti-Epo monoclonal antibody proteins as well as tumor cell R103A-Epo antagonist expression, confirm that Epo blockade can effectively inhibit the host angiogenic response and thereby impair primary tumor growth [28].

## 4. Personal experience

In 1999, we have demonstrated for the first time that rHuEpo induces an increased endothelial cell proliferation, matrix metalloproteinase-2 (MMP-2) expression and differentiation into vascular tubes of human endothelial cells *in vitro* [18]. *In vivo*, in the chick embryo chorioallantoic membrane (CAM) assay, rHuEpo exerted an angiogenic activity comparable to that of FGF-2, and CAM's endothelial cells expressed both EpoR and factor VIII related antigen [18].

We have established a correlation between Epo/EpoR expression, angiogenesis, and tumor progression in human gastric cancer, hepatocellular carcinoma, neuroblastoma, melanoma, glioma [29–33]. In neuroblastoma, EpoR is diffusely and strongly expressed on endothelial cells within neoplastic nodules, whereas Epo shows a weak and focal cytoplasmic granular pattern in a few neuroblastoma cells in stages I and III, while this immunoreactivity increases in stage IVs. In human primary melanoma, Epo shows a weak and focal cytoplasmic pattern of expression in tumor tissue, where positive tumor cells are frequently associated in clusters, EpoR is diffusely and strongly expressed on endothelial cells within neoplastic nodules, and Epo/EpoR expression correlate with angiogenesis and tumor thickness. In human glioma, Epo and EpoR immunoreactivity of tumor cells and, respectively, endothelial cells, increases in IV malignancy grade tumor specimens, when compared with II malignancy grade. Moreover, when tested in the CAM assay, a grade IV glioma bioptic specimens induces an angiogenic response comparable to that induced by VEGF, which is significantly inhibited by the co-administration of an anti-Epo blocking antibody.

More recently, we found that Epo-R is over-expressed in bone marrow macrophages from multiple myeloma (MM) patients with active disease compared to benign monoclonal gammopathy of undetermined significance (MGUS) patients (Fig.1). The treatment of bone marrow macrophages with rHuEpo significantly increased the expression and secretion of key pro-angiogenic mediators, such as VEGF, hepatocyte growth factor (HGF) and monocyte chemotactic protein (MCP-1/CCL-2), through activation of JAK2/STAT5 and PI3K/Akt pathways. In addition, the conditioned media harvested from rHuEpo-treated bone marrow macrophages enhanced bone marrow-derived endothelial cell migration and capillary morphogenesis *in vitro,* and induced angiogenesis in the CAM assay *in vivo* (Fig. 2). Furthermore, we found an increase in the circulating levels of several pro-angiogenic cytokines in serum of MM patients with anemia under treatment with Epo. Overall, our findings highlight the direct effect of rHuEpo on macrophage-mediated production of pro-angiogenic factors, suggesting that Epo/Epo-R pathway may be involved in the regulation of angiogenic response occurring in MM [34].

In a second study on MM, we detected the expression of EpoR in bone marrow-derived endothelial cells from MGUS and MM patients (MGECs and MMECs, respectively) and assessed whether Epo plays a role in MGECs- and MMECs-mediated angiogenesis. We found that EpoR is overexpressed by both MGECs and MMECs even though at a lower level in the latter ones. Both endothelial cell types respond to rHuEpo in terms of cell proliferation, whereas activation of JAK2/STAT5 and PI3K/Akt pathways, cell migration and morphogenesis are enhanced by Epo in MGECs, but not in MMECs. In addition, the conditioned medium of both Epo-treated cells induce a strong angiogenic response *in vivo* in the CAM assay, comparable to that of VEGF (Fig. 3). Overall, these data highlight the effect of Epo on MGECs- and MMECs-mediated angiogenesis [35].

## 5. Granulocyte and granulocyte-macrophage colony stimulating factors

The purification and molecular cloning of granulocyte colony stimulating factor (G-CSF) was performed between 1984 and 1986 [36,37] and clinical development commenced in 1986 with approval for clinical use in cancer patients treated with chemotherapy [38].

G-CSF is a 30 k-Da glycoprotein produced constitutively by many tissues, particularly by monocytes and macrophages, endothelial cells and fibroblasts. G-CSF stimulates selectively *in vitro* committed progenitors to form neutrophil granulocyte colonies and *in vivo* administration of G-CSF causes an increase in neutrophil granulocyte production [39].

Purified human granulocyte macrophage colony stimulating factor (GM-CSF) was achieved in 1984 [40] and cloned in 1985 [41]. GM-CSF is a 22 k-Da glycoprotein, an inducible product of activated T and B cells and activated macrophages [42,43], expressed by T and B cells, macrophages, mast cells, endothelial cells, fibroblasts and adipocytes [44].

GM-CSF stimulates proliferation and differentiation of myeloid-committed progenitors (CFU-GM) into neutrophilic granulocytes and monocyte-macrophages. GM-CSF promotes proliferation and differentiation of eosinophils, basophils, megakaryocytes, and erythroid and dendritic cells in synergy with other factors. Activated G-/GM-CSF induce phosphorylation of JAK kinases and recruitment of STAT5 transcription factor to induce cellular differentiation [45].

G-/GM-CSF are used in acute leukemia to accelerate bone marrow recovery, as well as leukocyte proliferation and maturation in solid organ transplantation, aplastic anemia e neutropenia secondary to acquired immune deficiency syndromes [46].

## 6. The role of G-CSF and GM-CSF in tumor angiogenesis

Specific receptors for G-CSF and GM-CSF have been detected on the surface of endothelial cells [3,47–49]. Endothelial cells express the α and β subunits of GM−CSF−receptor and GM-CSF is able to activate JAK2. The effect is specific as inferred by the lack of effect of heat-inactivated GM-CSF or neutralized by specific antibodies [50].

These cytokines induce endothelial cells to migrate, proliferate and release plasminogen activator and are angiogenic *in vivo* in the rabbit cornea [3,51]. Subnanomolar concentrations of GM-CSF and G-CSF

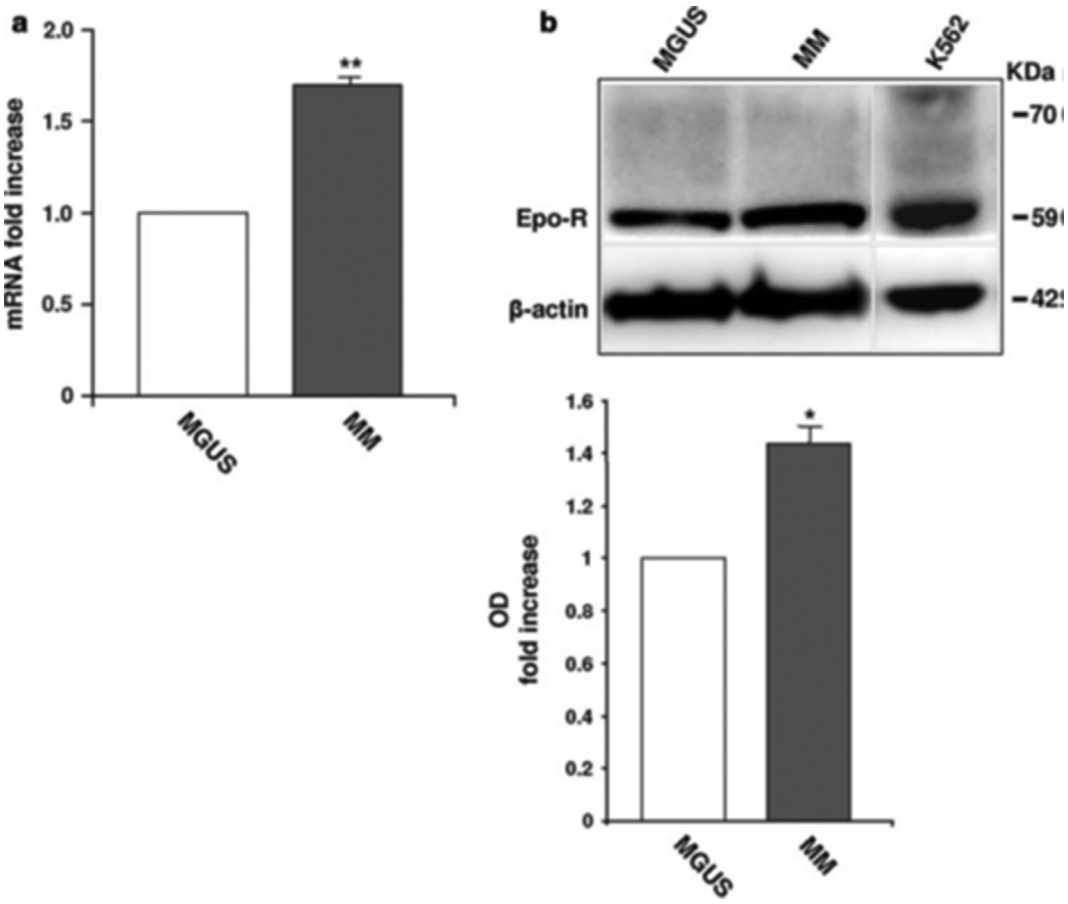


**Fig. 1.** Epo-R expression in bone-marrow derived macrophages from monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM ) patients. a Real-Time reverse transcription-PCR (normalized to GAPDH) as mean ± SD of 18 MM and 9 MGUS patients. b Western blot of representative MM and MGUS patients, and K562 control lysate. Fold increase of optical density (OD) in MM (grey bar) versus MGUS (empty bar) BMMAs as mean ± SD of 17 MM and 8 MGUS patients. Significances * P\0.03 and **P\0.003 by Wilcoxon signed-rank test (Reproduced from Ref. [34]).

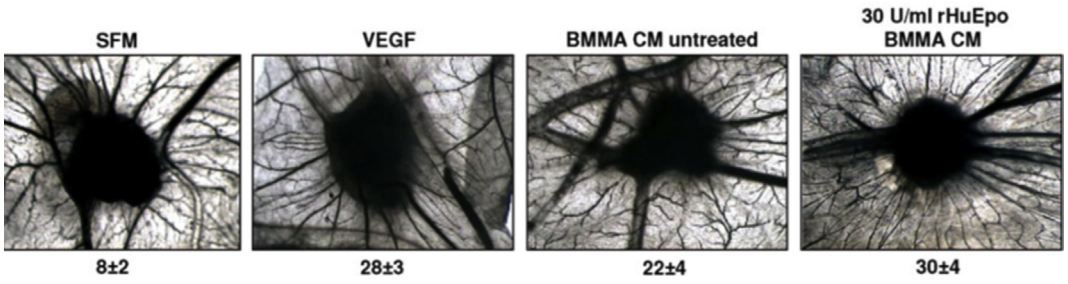


**Fig. 2.** RHuEpo-treated BMMAs CM enhance angiogenesis *in vivo*. Macroscopic pictures of gelatin sponges soaked with SFM alone or supplemented with VEGF or with the CM of BMMAs untreated and treated with 30 U/ml rHuEpo, implanted on the chick embryo CAM. Note numerous allantoic vessels developing radially towards the implants with the exception of the sponge adsorbed with SFM alone. Vessel counts are indicated. BMMA, bone marrow macrophage, SFM serum free medium, CM conditioned media (Reproduced from Ref. [34]).

induce the proliferation of endothelial cells derived from human vessels [3,51,52] and from murine microvascular capillaries [53,54].

GM-CSF induces angiogenesis in rat connective tissue by a direct effect on endothelial cells or by the recruitment and activation of macrophages that release their own angiogenic factors or by both effects [55] and also stimulate the repair of mechanically wounded endothelial cell monolayers [52]. Endothelial cells from large vessels or from microvasculature produces constitutively little or undetectable amount of GM-CSF and G-CSF. Several stimuli, including IL-1, tumor necrosis factor alpha (TNFα), lipopolysaccharide, acetylated low density lipoproteins, viruses, and phorbol esters, are potent inducers of GM-CSF and G-CSF [56,57]. In addition to regulating the expression of VEGF in the wound, GM-CSF can also mediate the Ang-1/Ang-2 expression ratio and the phosphorylation of Tie-2 [58]. In this context, GM-CSF not only initiates the sprouting phase of angiogenesis but can also promotes the maturation and stabilization of new microvessels.

Constitutive activation of IKKβ in esophageal epithelial cells leads to GM-CSF production, resulting in increased neovascularization, triggering an epithelial-mesenchymal signaling network mediated by cytokine secretion from esophageal epithelial cells [59].

G-CSF strongly induced the expression of Bv8 protein [prokineticin-2, a homolog of endocrine gland-derived VEGF (EG-VEGF)] in $CD11b^+$ $Gr^+$ myeloid derived suppressor cells (MDSCs) [60]. $CD11b^+$ $Gr^+$ cells are mixed populations of cells not only consisting primarily of neutrophils, but also including macrophages and dendritic cells [60]. BV8 blockade using neutralizing antibodies induced suppression of tumor

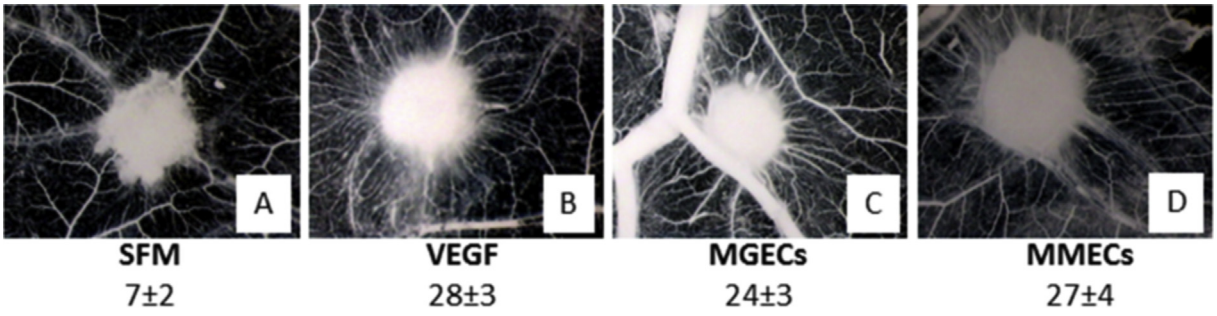


**Fig. 3.** RHuEpo stimulates angiogenesis *in vivo*. Macroscopic pictures of gelatin sponges soaked with serum free medium (SFM) alone (A) or supplemented with VEGF (B), or with 30 U/ml rHuEpo (C), or with the CM of MGUS endothelial cells (MGECs) and MM endothelial cells (MMECs) pre-treated with 30 U/ml rHuEpo (C, D), implanted on the chick embryo CAM. Note numerous allantoic vessels developing radially towards the implants with the exception of the sponge adsorbed with SFM alone. Vessel count are indicated. (Reproduced from Ref. [35]).

angiogenesis and growth and exerted additive effects with anti-VEGF antibodies in slowing growth of human and murine tumor cell lines [61]. Treatment with the combination of anti-VEGF and anti-G-CSF antibodies reduced growth of refractory tumors compared with anti-VEGF monotherapy [62]. Moreover, anti-G-CSF treatment suppressed circulating or tumor-associated $CD11b^+$ $Gr^+$ cells or Bv8 levels [62]. On the contrary, G-CSF delivery to animals bearing an anti-VEGF sensitive tumor resulted in reduced responsiveness to anti-VEGF treatment through induction of Bv8-dependent angiogenesis [62].

Roda et al. [63] examined melanoma growth and response to GM-CSF therapy in mice with hypoxia inducible factor 1 or 2 alpha (HIF-1α– or HIF-2α)–deficient tumor macrophages. GM-CSF treatment of control mice induced a low amount of VEGF production and a much greater production of sVEGFR-1, resulting in a decrease in tumor growth and angiogenesis. Deletion of HIF-1α from tumor-associated macrophages inhibited VEGF production in response to GM-CSF with no effect on sVEGFR-1 production, resulting in an even greater reduction in tumor growth and angiogenesis. On the contrary, deletion of HIF-2α from tumor-associated macrophages inhibited sVEGFR-1 production in response to GM-CSF with no effect on VEGF production and abrogated the antitumor response to GM-CSF [63].

By recruiting MDSCs, G-CSF induces a VEGF-independent angiogenesis in addition to increasing resistance to anti-VEGF drugs [64]. Intratumoral administration of GM-CSF stimulates macrophages in low $O_2$ environment to secrete high levels of a soluble form of VEGF receptor-1 (VEGFR-1) which neutralized VEGF and inhibited its biological activity, resulting in reduction of tumor growth and angiogenesis [65].

Melanoma exosomes induce GM-CSF expression by endothelial cells *in vitro* and HIF-1a expression in pre-metastatic lymph nodes *in vivo*, suggesting a relationship between melanoma exosome induced endothelial GM-CSF and macrophage mediated angiogenesis in lymph nodes [66].

A cell population with progenitor-like phenotype resident in human white adipose tissue (WAT) promotes the progression of local and metastatic breast cancer and angiogenesis. Reggiani et al. [67] identified two proteins that were significantly upregulated in WAT-derived progenitors after co-culture with breast cancer: GM-CSF and MMP-9. Combined GM-CSF neutralization and MMP-9 inhibition synergistically reduced angiogenesis and tumor progression.

## 7. Personal experience

We have demonstrated that GM-CSF triggers angiogenesis in the chick embryo CAM (Fig. 4) and induces vessel sprouting from chicken aorta; activation of JAK2, evaluated by the autophophorylation in tyrosine residues, is significantly enhanced in the CAM after GM-CSF stimulation at the same concentration (sub-nanomolar amounts) able to activate angiogenesis and vessel sprouting; STAT-3, which is a cytosolic effector of GM−CSF−dependent cell activation, is also tyrosine phosphorylated *in vivo*; AG-490, an inhibitor of JAK2, inhibited the angiogenesis induced by GM-CSF, but is ineffective when VEGF is used as a stimulus [68].

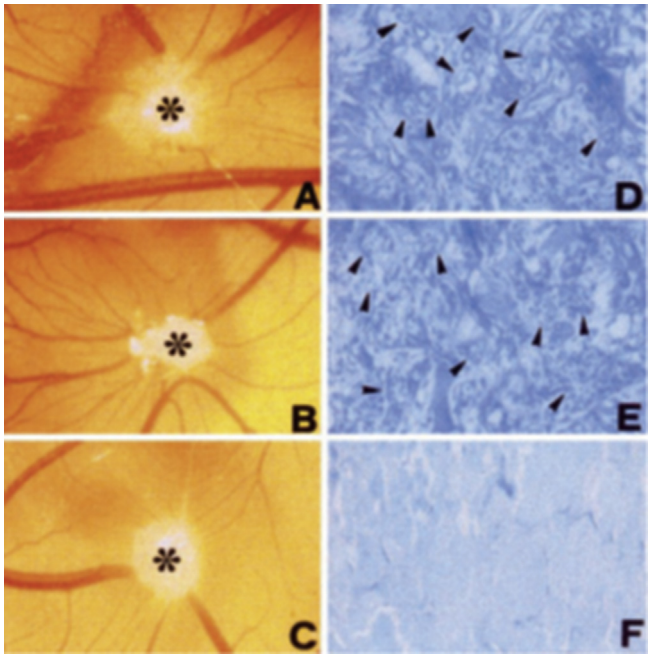


**Fig. 4.** CAMs recorded on day 12 of incubation 96 h after the implant of a gelatin sponge (asterisk) soaked with 400 ng of GM-CSF (A) or 500 ng of VEGF-A165 (B). Numerous blood vessels converge toward the sponge. No vascular reaction is detectable around a sponge treated with PBS only (C). D–F) Histological analysis of CAMs treated with GM-CSF (D),VEGF-A165 (E), and PBS (F). Note in panels D and E, numerous newly formed blood vessels (arrowheads) within an abundant network of collagen fibers. In contrast, blood vessels are absent in the implants treated with PBS (F). (Reproduced from Ref. [69]).

## 8. Concluding remarks and current scenario

Angiogenesis is regulated, under both physiological and pathological conditions, by numerous “classic” factors, including FGF-2, VEGF, angiopoietins (Angs), tranforming growth factor beta (TGF-β), and platelet derived growth factor (PDGF). Evidence has been accumulated that, in addition to the “classic” factors, many other endogenous “non-classic”peptides, including Epo, and G-/GM-CSF play an important regulatory role in angiogenesis, especially under pathological conditions (Table 1, Fig. 5) [69]. Several evidence have clearly demonstrated an involvement of Epo and C−/GM-CSF in tumor angiogenesis in both solid and hematological human tumors (Table 2).

**Table 1**
"Classic" and "Non classic" pro-angiogenic factors.

| "Classic" | "Non classic" |
|---|---|
| Vascular endothelial growth factor (VEGF) | Granulocyte- Granulocyte-Colony stimulating factor (GM-CSF) |
| Placental growth factor (PlGF) | Erythropoietin (Epo) |
| Fibroblast growth factor-2 (FGF-2) | Endothelins (ETs) |
| Transforming growth factor beta (TGF-β) | Adrenomedullin (AM) |
| Platelet derived growth factor (PDGF) | Leptin |
| Angiopoietins (Angs) | Nerve growth factor (NGF) |
| | Adiponectin |
| | Urotensin II |

**Table 2**
Solid and hematological human tumors in which Epo and C−/GM-CSF play a pro-angiogenic role.

| Epo | Ref |
|---|---|
| Female reproductive system | [27] |
| Breast carcinoma | [74] |
| Oral squamous cell carcinoma | [75] |
| Gastric carcinoma | [29] [76], |
| Colo-rectal cancer | [77] |
| Hepatocellular carcinoma | [30] [78] [79], |
| Neuroblastoma | [31] |
| Melanoma | [32] |
| Glioma | [33] |
| Non small cell lung cancer | [80] |
| Multiple myeloma | [34] [35], |
| **C−/GM-CSF** | **Ref** |
| Melanoma | [66] |
| Breast cancer | [81] |
| Head and neck squamous cell carcinoma | [82] |
| Colo-rectal cancer | [83] |
| Non small cell lung cancer | [84] |
| Baldder cancer | [85] |

As concerns Epo, contrary to reports indicating beneficial effects of Epo in patients with cancer, some of the available data do not support such an action of Epo. The use of Epo to treat anemia in patients with proliferative lesions, besides having positive effects on hematological parameters, has the serious adverse effect of promoting the neoplastic progression, stimulating angiogenesis. In this context, tumor growth-promoting effects and even enhanced metastasis observed in response to Epo-treatment could in some studies be attributed to stimulation of angiogenesis.

To act as a hematopoietic growth factor, GM-CSF is administered to mobilize monocytes and granulocytes from bone marrow, which leads to both systemic and local increases in the numbers of myeloid cells. Thus its pro-angiogenic effects may lead to both systemic and local increases in the numbers of myeloid cells (Fig. 5) [70]. On one hand, GM−CSF−induced enhancement of tumorigenesis was mediated by bone marrow derived cells (BMDCs), while on the other hand, GM-CSF acted synergistically with BMDCs to stimulate the function of endothelial cells. Local injection of low doses rhG-CSF augmented ischemia-induced angiogenesis *in vivo*, and this treatment regimen may be a safe modality for therapeutic angiogenesis [71].

GM-CSF is able to shorten the duration of chemotherapy induced neutropenia, but a meta-analysis found that GM-CSF does not prolong survival [72]. This has raised questions not only with respect to the clinical benefit of GM-CSF as an adjuvant therapy but also about its role in promoting tumor angiogenesis and progression. By recruiting MDSC, G-CSF induces a VEGF-independent angiogenesis in addition to increasing resistance to anti-VEGF drugs [73]. In this context, correction of cancer therapy-related neutropenia using recombinant G-/GM-CSF carries risks of disease recurrence.

It is well established that the angiogenic phenotype results from the imbalance between positive and negative regulator factors, so that the contribution of each "classic" and/or "non-classic" angiogenic factor may play a different role in the definition of the angiogenic phenotype.

A better knowledge of the mechanism of action and expression as well as the interactions of the new "non-classic" endogenous regulators

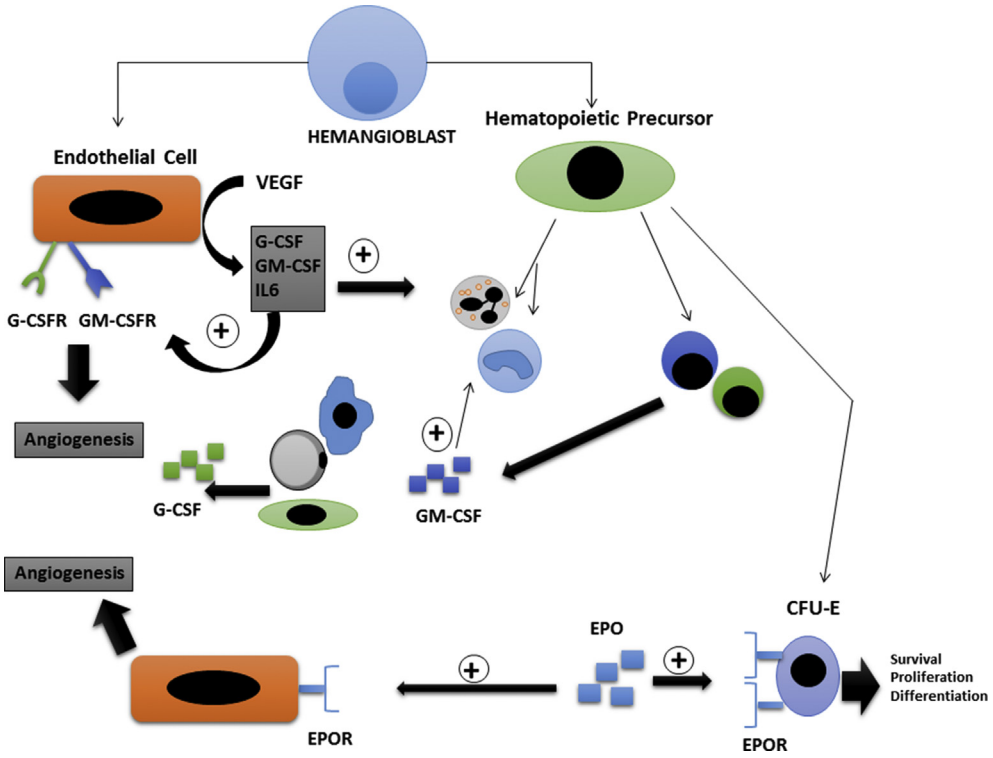


**Fig. 5.** Angiogenesis is regulated by numerous "classic" factors such as VEGF and many other endogenous "non-classic"peptides, including Epo, and G-/GM-CSF. The latter play an important regulatory role in angiogenesis, especially under pathological conditions and constitute a cross-link between angiogenesis and hematopoiesis.

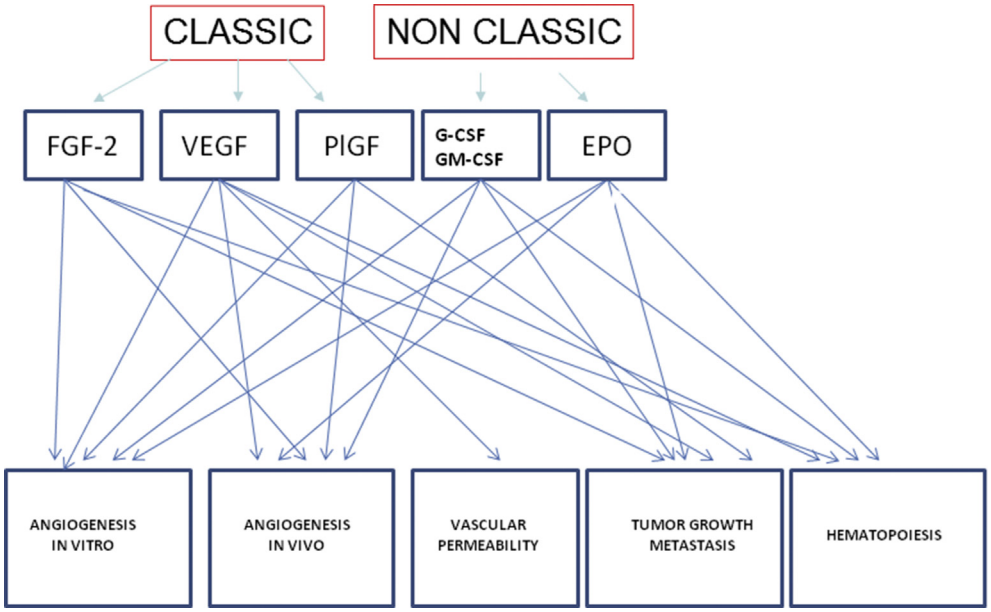


**Fig. 6.** Cross-talk between "classic" and "non classic" pro-angiogenic factors in the regulation of angiogenesis, tumor growth, metastasis and hematopoiesis.

of angiogenesis with their receptors (Fig. 6) will provide new insights that are essential for the development of chemical compounds that can modulate the activity of these new "non-classic" endogenous regulators and may have potential for antitumor activity.

## Conflicts of interest

The Authors declare that there are not conflict of interest.

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