

● INVITED REVIEW

# Growth factor- and cytokine-stimulated endothelial progenitor cells in post-ischemic cerebral neovascularization

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## Abstract

Endothelial progenitor cells are resident in the bone marrow blood sinusoids and circulate in the peripheral circulation. They mobilize from the bone marrow after vascular injury and home to the site of injury where they differentiate into endothelial cells. Activation and mobilization of endothelial progenitor cells from the bone marrow is induced *via* the production and release of endothelial progenitor cell-activating factors and includes specific growth factors and cytokines in response to peripheral tissue hypoxia such as after acute ischemic stroke or trauma. Endothelial progenitor cells migrate and home to specific sites following ischemic stroke *via* growth factor/cytokine gradients. Some growth factors are less stable under acidic conditions of tissue ischemia, and synthetic analogues that are stable at low pH may provide a more effective therapeutic approach for inducing endothelial progenitor cell mobilization and promoting cerebral neovascularization following ischemic stroke.

**Key Words:** endothelial progenitor cells; mobilization; growth factor; cytokine; neovascularization; ischemic stroke

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## Introduction

Worldwide, stroke is the second leading cause of death responsible for 4.4 million (9%) of the total 50.5 million deaths each year. In the USA stroke is the third leading cause of death, behind heart disease (with which it is closely linked) and cancer (Stroke Statistics. [www.uhnj.org/stroke/stats.htm](http://www.uhnj.org/stroke/stats.htm)). Of all strokes, 87% are ischemic and 10% are intracerebral hemorrhagic strokes, while 3% are subarachnoid hemorrhagic strokes (Go et al., 2013).

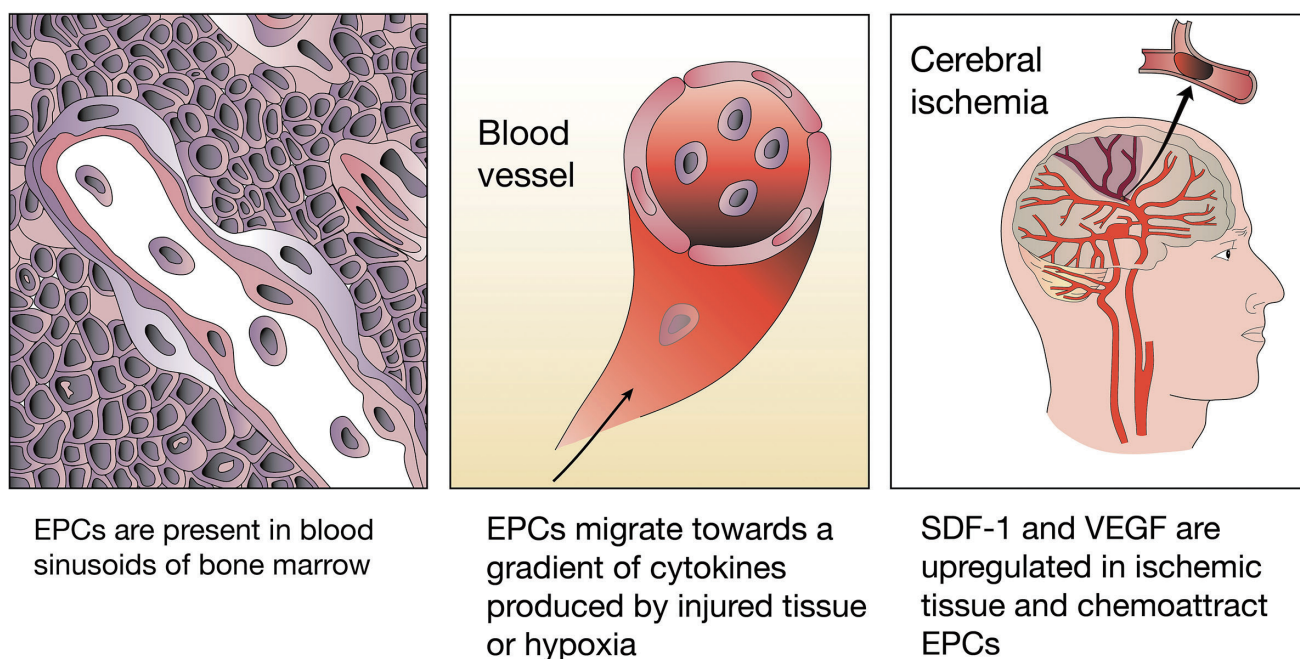
Regulation of cerebral blood flow is critical for the maintenance of neural function (Pratt et al., 2004). On exposure to hypoxic conditions, blood vessel networks of the cerebral microvasculature expand to meet the growing oxygen demands and brain capillary density increases over a 2-week period (Xu and LaManna, 2006). This neovascularization response requires new vessel formation as well as the remodeling of existing vasculature to form new collaterals. Growth factor-driven angiogenesis leads to increased capillary density, restoring tissue oxygen levels. After transient middle cerebral artery occlusion in rats followed by reperfusion, microvessel density increased especially within the inner margin of the cystic infarct, and was accompanied by increased leakiness to immunoglobulin (IgG) and fluorescein-dextran (Yu et al., 2007). Magnetic resonance imaging demonstrated increased angiogenesis 4 weeks after initiation of embolic stroke in rat brain (Ding et al., 2008).

Neovascularization (new blood vessel formation) occurs

through vasculogenesis, angiogenesis, and/or arteriogenesis; all three can occur in response to tissue hypoxia and injury. Vasculogenesis and angiogenesis are the fundamental processes during formation of new blood vessels after vascular injury *e.g.*, following ischemic stroke. Angiogenesis is defined as the growth of new vessels from preexisting ones, whereas vasculogenesis is the formation of new blood vessels by '*de novo*' production of endothelial cells *e.g.*, differentiation of precursor cells into endothelial cells (Risau, 1997; Eguchi et al., 2007). Bone marrow-derived endothelial progenitor cells (EPCs) are considered to play an important role in endogenous vascular repair after vascular injury and in the maintenance of endothelial integrity.

## Activation and mobilization of endothelial progenitor cells

EPCs are immature endothelial cells that share common stem/progenitor cells and hematopoietic characteristics and circulate in the peripheral blood. The discovery of EPCs in the peripheral blood was first shown in 1997 (Asahara et al., 1997). These cells are mobilized from the bone marrow after vascular injury and home to the site of injury where they differentiate into endothelial cells (**Figure 1**). EPCs possess functional and structural characteristics of stem cells as well as of mature endothelial cells. They coexpress the surface markers CD34, CD133 and vascular endothelial growth factor receptor 2 (VEGFR2). The coexpression of specific



**Figure 1 Homing and functional role of endothelial progenitor cells (EPCs) in post-cerebral ischemia.**

EPCs migrate from the bone marrow *via* blood vessels to sites of tissue injury and ischemia. Stromal cell-derived factor 1 (SDF-1) and its receptor CXCR4 are critical mediators for ischemia-specific recruitment of circulating EPCs. Hypoxia-induced vascular endothelial growth factor (VEGF) expression precedes neovascularization after cerebral ischemia. Early EPCs secrete mainly proangiogenic cytokines including VEGF, placental growth factor, transforming growth factor- $\beta$ , thrombopoietin, hepatocyte growth factor, fibroblast growth factor, macrophage migration inhibitory factor, macrophage colony stimulating factor, interleukin-8, as well as a few antiangiogenic cytokines, and also neurotrophic and neuroregulatory cytokines including brain-derived neurotrophic factor.

surface markers like CD34, CD133, and VEGFR2 characterizes a specific progenitor cell subset at a specific maturation stage. The expression of CD34 decreases over time as EPCs differentiate towards endothelial cells (Asahara et al., 1997). Other surface markers that have been described to define subpopulations of EPCs are von Willebrand factor (vWF), CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1), CD144 (vascular endothelial cadherin, VE-cadherin) and CXC chemokine receptor type 4 (CXCR4) (Sabatier et al., 2009).

EPCs are obtained from isolated peripheral mononuclear cells (MNCs) in culture, and can be divided into two morphologically and functionally different populations, namely the 'early outgrowth EPCs' or circulating angiogenic cells (CACs), and the 'late outgrowth EPCs' or endothelial colony-forming cells (ECFCs). The first type are largely quiescent cells, while the second type form highly proliferative endothelial colonies derived from single cells and spontaneously display capillary tube-like formation in Matrigel. Early EPCs are derived from a myeloid lineage and only early EPCs express the myeloid markers CD45 and CD14 (Yoder et al., 2007; Zhang et al., 2009). Early EPCs secrete predominantly proangiogenic cytokines including vascular endothelial growth factor (VEGF), placental growth factor (PIGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), thrombopoietin (TPO), hepatocyte growth factor (HGF), fibroblast growth factors (FGFs), macrophage migration inhibitory factor (MIF), macrophage colony stimulating factor (MCSF), interleukin-8 (IL-8), as well as a few antiangiogenic cyto-

kines, and also neurotrophic and neuroregulatory cytokines including brain-derived neurotrophic factor (BDNF) which may enable EPCs to exert an important trophic influence on neuronal tissue (He et al., 2004).

Activation and mobilization of EPCs from the bone marrow is induced *via* the production and release of EPC-activating factors *e.g.* hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), VEGF or erythropoietin in response to peripheral tissue hypoxia such as after acute ischemic stroke or trauma (Hoenig et al., 2008). In the periphery, stromal cell-derived factor 1 (SDF-1) mediates migration and homing of EPCs to the vascular endothelium through a CXCR4 dependent mechanism (**Figure 1**) (Zheng et al., 2007; Tilling et al., 2009).

HIF-1 $\alpha$  expression in hypoxic tissue is upregulated in a time-related manner around the ischemic boundary zone and induces various signaling pathways, one of which involves upregulation of VEGF (Marti et al., 2000; Althaus et al., 2006). VEGF mRNA expression is evident 1 hour after reperfusion following middle cerebral artery occlusion in rat and peaks at 3 to 24 hours thereafter (Hayashi et al., 1997; Plate et al., 1999). VEGF mediates endothelial nitric oxide synthase (eNOS) expression of nitric oxide (NO) following cerebral ischemia (Chen et al., 2005a). eNOS regulates BDNF expression in the ischemic brain and influences neuroprogenitor cell proliferation, neuronal migration, and neurite outgrowth and affects functional recovery after stroke in mice (Chen et al., 2005a).

Mobilization of EPCs from the bone marrow is also dependent on the production of NO and the local activity of

matrix metalloproteinases (Heissig et al., 2002). EPCs are considered to migrate and home to affected sites like the penumbra following ischemic stroke *via* cytokine gradients, where they act in a paracrine fashion leading to endothelial cell proliferation and stabilization or through differentiation into endothelial cells. Several EPC-activating factors such as VEGF, SDF-1, monocyte chemoattractant protein-1 (MCP-1) are also involved in the neovascularization process of the damaged tissue.

### Angiogenic potential of endothelial progenitor cells influenced by growth factors and cytokines

The influence of growth factors and cytokines on angiogenic potential of human EPCs *in vitro* studies has been recently reviewed (Peplow, 2014). Indices of angiogenic potential are chemotactic migration, capillary tube-like formation, proliferation, and apoptosis. Early EPCs are taken to be the cell type obtained by short-term culture of MNCs on fibronectin-coated dishes for up to 7 days, with a spindle-shaped morphology and double positive for DiI-acLDL uptake and Ulex europaeus lectin-1 (UEA-1) binding, and do not spontaneously form capillary tube-like structures in Matrigel. Late EPCs are taken to be the cell type obtained by longer-term culture for 2 to 4 weeks of MNCs on fibronectin or collagen, give a cobblestone-like appearance to the monolayer, are double positive for DiI-acLDL uptake and lectin binding, and spontaneously display capillary-like tube formation when placed on Matrigel coated dishes (Goretta et al., 2013). Members of various families of growth factors and cytokines were tested for effect. These included the pro-angiogenic factors VEGF and PlGF of VEGF family, FGF-2 of FGF family, monocyte chemoattractant protein-1 (MCP-1) of C-C chemokine family, and SDF-1 which belongs to the intercrine family and is upregulated by factors such as stem cell factor (SCF), IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and downregulated by interferon- $\beta$  (IFN- $\beta$ ) (Peled et al., 1999; Tran, 2006); nerve growth factor (NGF) and BDNF of the nerve growth factor family; other growth factors such as SCF, TGF- $\beta$ 1, macrophage stimulating protein (MSP), TPO, and the proinflammatory cytokines IL-1 $\beta$ , IL-3, and TNF- $\alpha$ .

In the chemotactic assays, SDF-1, VEGF, IL-1 $\beta$ , MIF, PlGF and TPO when tested alone increased the migration of early EPCs. A much greater migration occurred to MIF at 10 ng/mL (0.8 nmol/L) than to SDF-1 at 200 ng/mL (22 nmol/L), indicating that MIF can influence cell types other than macrophages. Moreover, increased migration was found for SDF-1 and VEGF in combination. Pretreatment of early EPCs with TNF- $\alpha$  at 10–100  $\mu$ g/mL decreased migration towards VEGF. Increased migration of early CD34<sup>+</sup> cells occurred with SDF-1, VEGF and MCP-1 when tested alone. For the assays of capillary tube-like formation, early EPCs were stimulated to form tube-like structures by VEGF, as also were early CD34<sup>+</sup> cells by SDF-1 and FGF-2. VEGF increased tube-like formation by late EPCs and late CD34<sup>+</sup> cells. TGF- $\beta$ 1 did not modify the capacity for tube-like formation by late EPCs.

Pretreatment with TNF- $\alpha$  had the potential to decrease tube-like formation of early and late EPCs. The proliferation of early EPCs was increased by SDF-1 and VEGF, while that of CD34<sup>+</sup> cells was increased by NGF, SCF, IL-3, TPO, and granulocyte macrophage colony-stimulating factor (GM-CSF). TNF- $\alpha$  decreased proliferation of early and late EPCs. Apoptosis of early EPCs was reduced by BDNF. Moreover, SDF-1, VEGF2 and TPO, when tested separately, reduced apoptosis of serum-starved early EPCs; SDF-1 and VEGF in combination may exert a synergistic effect on cell survival. All these findings were from studies performed under neutral conditions (pH 7.4).

Extracellular acidosis is a common feature of injured tissues and tumor microenvironment and is an important regulator of cell survival and activation. It is a typical feature of the inflammatory microenvironment and present during the processes of wound healing, tumor growth, hypoxia or ischemia (Trevani et al., 1999; Kumar et al., 2007; Chiche et al., 2010). Transplanted EPCs in animal studies and clinical trials are likely to be exposed to an acidic inflammatory milieu, and it would be pertinent to determine the effect of acidosis on functional features of EPCs including angiogenic potential. The proliferation of CD34<sup>+</sup> cells induced by TPO, SCF or IL-3 alone was completely inhibited when the cells were treated for 1 minute prior to acidic exposure (pH 6.5), while that brought about by GM-CSF was markedly reduced when the cells were exposed to acidic conditions for 15 minutes. It was hypothesized that the biological activity of these factors was impaired at low pH values, with the proliferation of CD34<sup>+</sup> cells being completely abolished by acidic treatment of VEGF and GM-CSF. A combination of TPO, SCF and IL-3 supported CD34<sup>+</sup> proliferation in acidic medium, suggesting that these factors acted synergistically with each other (D'Atri et al., 2011). In addition, VEGF and GM-CSF alone did not reduce apoptosis of CD34<sup>+</sup> cells exposed to acidic conditions (pH 6.5). Pretreatment of cells with TPO, SCF or IL-3 resulted in a significant decrease in apoptosis at pH 6.5, with IL-3 having the greatest effect. A combination of TPO, SCF and IL-3 prevented CD34<sup>+</sup> cell death. That each growth factor and cytokine alone did not preserve cell functionality indicated that prevention of CD34<sup>+</sup> cell death by acidosis required the synergistic action of several overlapping signaling cascades triggered by TPO, SCF and IL-3 (D'Atri et al., 2011).

### Regulation of cerebral blood flow in the ischemic brain

Stroke patients with greater cerebral blood vessel density appear to make better progress and survive longer than patients with lower vascular density (Krupinski et al., 1994). Physical activity improves long-term stroke outcome by eNOS-dependent mechanisms that increase angiogenesis and cerebral blood flow (Gertz et al., 2006). As mentioned earlier, VEGF mediates eNOS expression of nitric oxide (NO) following cerebral ischemia (Chen et al., 2005a). eNOS regulates BDNF expression in the ischemic brain and influenc-

es neuroprogenitor cell proliferation, neuronal migration, and neurite outgrowth and affects functional recovery after stroke in mice (Chen et al., 2005a). VEGF and angiopoietin-1 (Ang-1) with its Tie receptor (Tie-2) are important angiogenic factors that control angiogenesis to form large and small vessels in the mature vascular system (Marti and Risau, 1999). However, VEGF also causes blood-brain barrier leakage, inflammation and brain edema. A combination of submaximal doses of VEGF and Ang-1 enhances angiogenesis and is more effective than the maximal dose of either alone (Chae et al., 2000; Valable et al., 2005; Zhu et al., 2006). Coadministration of VEGF with Ang-1 also reduces blood-brain barrier leakage compared to VEGF alone (Valable et al., 2005; Zhu et al., 2006).

Stem cells from multiple sources such as bone marrow cells, embryonic, and peripheral blood stem cells can promote angiogenesis within ischemic tissue (Jackson et al., 2001; Kocher et al., 2001). Post-ischemic angiogenesis requires participation of bone-marrow derived EPCs (Zhang et al., 2002), perivascular microglia, and pericytes (Hill et al., 2004; Kokovay et al., 2006). Stem cell therapeutic approaches have the potential to be the most promising future therapies for poststroke neovascularization and regeneration.

## Future perspectives

Despite these recent advances, there remains several challenges facing the translation of cellular therapies for stroke from *in vitro* and animal studies to clinical trials. Some growth factors may be less stable under the acidic conditions of tissue ischemia and the biological activity of these factors impaired, thereby limiting their usefulness as exogenous mediators. Growth factor analogues that are stable at low pH may provide a better therapeutic strategy. A synthetic SDF polypeptide analog has been engineered that is more effective in inducing EPC migration, including greater stability and function, compared with exogenous administered SDF-1 $\alpha$  (Hiesinger et al., 2012). Statins are widely used for preventing stroke and in rodents have been shown to induce the expression of VEGF and BDNF and promote mobilization and proliferation of EPCs (Zhang et al., 2003; Chen et al., 2005b). Circulating EPC levels are increased in patients with ischemic stroke treated with statins during the acute phase (Sobrino et al., 2012). Low circulating EPC level was independently predictive of severe neurological impairment and major adverse clinical outcomes in patients after acute ischemic stroke (Yip et al., 2008). Administration of an inhibitor of TNF- $\alpha$  production up to 6 hours after ischemia reduces brain edema in rats in which the middle cerebral artery has been occluded (Vakili et al., 2011). New possibilities for combinatorial therapies using administered growth factor analogues or inhibitors/antagonists, statins, and EPCs are opened up for the treatment of patients with ischemic stroke.

**Conflicts of interest:** None declared.

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