REVIEW ARTICLE

Proteases and receptors in the recruitment of endothelial progenitor cells in neovascularization

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ABSTRACT. Since the initial discovery of endothelial progenitor cells (EPC), and their promise in increasing angiogenesis and vasculogenesis, a myriad of papers have highlighted their potential application in experimental and clinical neovascularization and in tissue engineering. However, promising reports are contrasted by other studies that could not find a role for EPC in neovascularization. Presently, two types of endothelial progenitor cell populations are recognized. The first population provides early-outgrowth $CD34^+/VEGFR\text{-}2^+$ cells, or colony-forming unit endothelial cells (CFU-EC), which represent myeloid cells with some endothelial properties, but no ability to form endothelial colonies. They can stimulate neovascularization by paracrine means, but are not incorporated in the endothelial lining themselves. The second population generates the late-outgrowth endothelial colony-forming cells (ECFC) from a very scant blood-derived cell population. ECFC have a very high proliferative potential, can insert into the endothelial lining of new blood vessels, and can also form endothelial tubes by themselves after stimulation with the proper angiogenic stimulus. This review surveys the mobilization of progenitor cells from the bone marrow, the homing of EPC (CFU-EC) to areas of neovascularization, and the participation of EPC (ECFC) in the endothelial lining of newly formed blood vessels. Specific emphasis has been placed on the role of proteases, which include serine proteases, including urokinase, L-cathepsin, and several ADAM- and matrix metalloproteinases. The specific properties of ECFC make them a potential source of cells for tissue engineering applications, but much has to be learned about their nature, origin and properties.

Keywords: endothelial colony forming cell, ECFC, EPC, angiogenesis, matrix metalloproteinase, urokinase

Once the vascular tree has grown to maturity, the endothelium remains in a rather quiescent state. However, during tissue repair after injury, in response to tissue growth or in pathological conditions, neovascularization occurs, often as sprouting from the existing microvessels. In addition to this angiogenic process, it is generally believed that vasculogenesis can also contribute to neovascularization in the adult. This process is driven by the recruitment of circulating endothelial progenitor cells (EPC), and the subsequent participation of these cells in the growing vessels [1, 2]. Asahara *et al.* [1] described for the first time the isolation and characterization of EPC and their contribution to therapeutic neovascularization in animals. This work shifted the existing view on neovascularization, since at that time the overall consensus was that new vessel formation, in adult life, was solely the result of proliferation and rearrangement of mature cells of the existing vasculature. The interest in these cells was further stimulated by the observation that alterations in the number or function of EPC have been associated with different manifestations of cardiovascular disorders and atherosclerosis [3, 4]. However, subsequent studies pointed to a limited participation of these circulating cells in the endothelial lining of newly grown vessels [5] and challenged the existence of EPC [6, 7].

At present, two different phenotypes of EPC have been recognized, with predominant characteristics of either monocytic or endothelial cells. Here, we will briefly describe the characteristics of the different cell types that have been indicated as EPC. Subsequently, we will discuss current insights into the molecular mechanisms that are involved in mobilization and recruitment of progenitor cells to areas of neovascularization, with specific emphasis on the role of proteases and receptors.

IDENTITY OF ENDOTHELIAL PROGENITOR CELLS

On the basis of different cell culture protocols, immunohistochemical characterization, and flow cytometric analysis, two different populations of EPC have been recognized. First, myeloid EPC are obtained when mononuclear cells from cord or peripheral blood are cultured for four to seven

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days in endothelial growth media. These cells that display both myeloid and endothelial cell markers most likely are derived from a subpopulation of monocytes and are also referred to as early-outgrowth cells. A second EPC population is contained in the $CD34^+/CD45^-$ cell fraction that circulates in low numbers. Upon culture in endothelial cell growth medium for about 14 days, a very small number of these cells can develop into colonies of so-called late-outgrowth cells, also referred to as endothelial colony-forming cells (ECFC) [8], or blood outgrowth endothelial cells (BOEC) [9]. These cells display the characteristics of mature endothelial cells, have a high proliferative capacity, and are able to form capillary-like structures. Both types of cells can contribute to neovascularization, but the nature of their contribution is different. The myeloid EPC predominantly support angiogenesis and arteriogenesis in a paracrine fashion, whereas the late-outgrowth cells can physically incorporate into the endothelial lining of the new vascular structures (see also Hirschi *et al.* [9] and Fadini *et al.* [4] for review).

Cell culture and characterization of EPC

Ideally, EPC should be characterized by a combination of a specific set of surface antigens and functional properties that confer specific endothelial functions to these cells. However, this goal has only partially been achieved, due to the limited number of cells initially available in the blood and the shift in (surface) protein expression during the maturation of these cells. In embryonic development, endothelial cells are derived from $CD34^+$, VEGFR-2^+, $CD133^+$-positive hemangioblasts of the blood island (VEGFR-2 stands for vascular endothelial growth factor [VEGF] receptor 2). When hemangioblasts are exposed to VEGF, they differentiate into $CD34^+$VEGFR-2^+ angioblasts that function as EPC in embryonic vasculogenesis. In addition, CD34 is also expressed on a wide range of mesoderm progeny including, blood, endothelial, fibroblast, epithelial and some cancer cell populations [10]. VEGFR-2 (KDR/Flk-1) was the first endothelial marker used in the isolation of EPC [1].

Assuming circulating EC would be similar to the embryonic angioblasts, Asahara *et al.* [1] plated cells enriched for $CD34^+$ and VEGFR-2^+ on fibronectin in an "endothelial medium". In co-culture with $CD34^+$-depleted mononuclear cells, clusters with round cells centrally and sprouts of spindle-shaped cells at the periphery appeared within three days. These attaching cells endocytosed acetylated-LDL, stained with *Ulex europaeus* lectin (UEA-1), and displayed surface expression of endothelial markers CD31, Tie-2, E-selectin and VEGFR-2. Later, the protocol was adopted by Ito *et al.* [11] and Hill *et al.* [12], who cultured unselected peripheral blood mononuclear cells (with additional re-plating selection) in endothelial medium. Similarly, round cells centrally, with spindle-shaped cells at the periphery appeared and were named colony-forming unit endothelial cells (CFU-EC, also known as CFU-Hill). This and comparable assays became very popular because of the correlation of CFU-EC with endothelial function and cumulative indexes of cardiovascular risk (Framingham risk factor score) [3, 12-14]. However, from subsequent studies it became clear that these colonies were in fact not endothelial cells, but consisted of a core of round, hematopoietic cells, including myeloid progenitor cells, monocytes and T lymphocytes, and spindle-shaped monocytes/macrophages that display some features of endothelial cells, such as CD31, CD105, CD144, CD146, vWF, VEGFR-2, and UEA-1 [15-19]. Furthermore, proteomic analysis showed that these colonies had been contaminated by platelet microparticles, explaining the presence of several endothelial markers [20].

Although the CFU-EC were not composed of endothelial progeny, it does not exclude CFU-EC from being involved in angiogenesis or serving as a biomarker for clinical outcome in cardiovascular diseases. Indeed, it has been shown that monocytes/macrophages are potent circulating regulators of angiogenesis and arteriogenesis, and play an important role in the initiation of angiogenesis during wound healing, tissue ischemia and tissue remodeling without actually being incorporated into the endothelial lining [21-27]. Thus, early-outgrowth cells can support angiogenesis in an indirect manner by producing essential growth factors and cytokines, similar to monocytes [21, 24, 27-31].

The late-outgrowth ECFC have the classical endothelial cobblestone phenotype and display a wide range of vascular endothelial markers, but do not express CD45, CD14, or CD115, nor do they ingest bacteria (a monocyte/macrophage characteristic). In comparison with the early-outgrowth CFU-EC, late-outgrowth ECFC show exponential growth and a high proliferative capacity [8, 15, 30, 32, 33]. Furthermore, in contrast to CFU-EC, the late-outgrowth ECFC spontaneously form blood vessels that associate with the nearby vessels and become a part of the systemic circulation in mice [15, 34, 35]. On the basis of these and other experiments, it has been suggested that the late-outgrowth ECFC are derived from circulating cells that best approximate the true "endothelial progenitor cell" definition of EPC [9, 15, 36].

Further characterization of different types of EPC

The antigen CD133 has been suggested as a marker of stem/progenitor cells. Case *et al.* [37] and Timmermans *et al.* [38] carefully studied the functional properties of isolated circulating $CD133^+CD34^+$VEGFR-2^+ cells in hematopoietic and endothelial assays [9]. They demonstrated that this selected population of circulating cells is highly enriched in hematopoietic progenitor activity, but, in contrast to what was expected at that time, does not give rise to any endothelial colonies *in vitro*. In addition, more than 99% of these cells also expressed the pan-leukocyte antigen CD45. Although the cells failed to give rise to endothelial colonies, cells with this marker profile (or any combination of these markers) appear to play a role in angiogenesis in various human disease states and are predictive biomarkers for cardiovascular disease [39-43].

In contrast to $CD34^+CD45^+$ cells, culturing $CD34^+CD45^-$ cells resulted in colonies of highly proliferative endothelial cells [37, 38]. These endothelial colonies expressed VEGFR-2, but not CD133. They manifested as blood-outgrowth endothelial cells (BOEC), and likely represent the same cell population as the late outgrowth ECFC mentioned above.

Additional evidence for the existence of two different EPC populations came from studies in patients with chronic myeloproliferative disorders (CMPD) [44]. ECFC derived from CML patients, or Ph-negative CMPD were not clonally related to the cells that gave rise to the hematopoietic disorder. However, the disease marker was present in all CFU-EC derived from the blood of these patients, thus confirming that CFU-EC are derived from myeloid cells.

From the foregoing, it is clear that a distinction between the two populations is needed for understanding the contribution of EPC in angiogenesis. However, as literature data on the recruitment of EPC from the bone marrow and their contribution to angiogenesis often fail to make this discrimination, we shall refer in the forthcoming discussion to CFU-EC for early-outgrowth myeloid cells that have acquired endothelial markers, and to ECFC for the few cases where late-outgrowth progenitor cells have been used, which have a true endothelial nature. Furthermore, we shall use EPC to indicate the overall populations of circulating or non-defined EPC, which *de facto* largely consist of CFU-EC progenitors.

MOBILIZATION OF ENDOTHELIAL PROGENITOR CELLS FROM THE BONE MARROW

The adult bone marrow is the principal reservoir of stem and progenitor cells, including hematopoietic and vascular precursors, such as endothelial progenitor cells. Anatomically, two distinct zones can be recognized in the bone marrow, the osteoblastic and vascular zones *(figure 1)*. In the hypoxic, osteoblastic zone, or stem cell niche, stem cells are in close contact with stromal cells (osteoblast, fibroblast, endothelial and reticular cells). The combined effect of a very low oxygen tension (hypoxia) and interaction with stromal cells preserves the maintenance and function *(stemness)* of hematopoietic and vascular stem/progenitor cells [45]. Migration of stem cells towards the vascular zone facilitates their proliferation and differentiation, followed by the disengagement from the bone marrow and entry into the circulation [46]. Bidirectional movement of stem cells between these two zones is regulated by multiple signaling and adhesion molecules, which contribute diverse characteristics to each niche's function. Well-studied signaling molecules involved in stem cell niche regulation include SCF/c-Kit, Jagged/Notch, SDF-1/CXCR4, and angiopoietin-1/Tie2 (Ang-1/Tie2) (see also Kiel *et al.* [47], Blank *et al.* [48], and Arai *et al.* [49] for review).

Proteolytic modification of receptors and cytokines in the mobilization of progenitor cells

Proteinases play an important role in the overall process of mobilization of progenitor cells [50]. They include serine proteases, cysteine cathepsins, matrix metalloproteinases (MMP) and metalloproteinases of the ADAM family. They act by receptor activation, generation of receptor ligands, cleavage of adhesion molecules and matrix proteins, and inactivation or modification of cytokines. For example, after binding an activating ligand, Notch is activated by two subsequent proteolytic events. The first, executed by a metalloproteinase (likely ADAM-10 or -17), releases the extracellular domain. Subsequently, at the inner side of the plasma membrane, another protease, γ-secretase, liberates the cytoplasmic tail of Notch, which induces further signaling in the cell.

Following the seminal observations of Heissig *et al.* [51], it became clear that MMP-9 plays a central role in the mobilization of EPC. It was demonstrated that, after fluorouracil-induced bone marrow ablation in mice, myelosuppression was accompanied by elevated plasma levels of stromal cell-derived factor-1 (SDF-1) and vascular endothelial cell growth factor (VEGF), which upregulated the expression of MMP-9 in bone marrow cells (both HSC and stromal cells). MMP-9 liberated sKitL (also known as stem cell factor [SCF]) from its membrane precursor KitL on bone marrow stromal cells. Signaling of sKitL, through c-Kit (SCF receptor) recruited c-Kit^{+} stem/progenitor cells to the circulation, including VEGFR-2^{+} EPC *(figure 1)*. These observations were underscored by experiments in MMP-9$^{-/-}$ mice, which demonstrated impaired release of sKitL and HSC motility. This deficiency resulted in impairment of hematopoietic recovery and increased mortality after bone marrow ablation, while exogenous sKitL restored hematopoiesis and survival. Of note, HSC, cardiac, epithelial and EPC all express the receptor for sKitL, c-Kit [52, 53].

Subsequent studies in endothelial nitric oxide synthase (eNOS)-knockout mice identified nitric oxide (NO) as a crucial cofactor in VEGF-induced MMP-9 activation [54]. Mice deficient in eNOS showed reduced VEGF-induced mobilization of EPC (CFU-EC) and increased mortality after myelosuppression. Mechanistically, MMP-9 activity was reduced in the bone marrow of eNOS $^{-/-}$ mice, because of reduced S-nitrosylation of MMP-9 [55]. The importance of NO in progenitor cell function was further highlighted by the work from Sasaki *et al.* [56], who treated dysfunctional bone marrow cells from ischemic cardiomyopathy (ICMP) patients with the novel eNOS transcription enhancer AVE9488. AVE9488 increased eNOS mRNA levels and eNOS activity, which at least partially reversed the impaired functional activity of BMC, improving the neovascularization capacity of infused BMC in an *in vivo*, ischemic hindlimb model.

Apart from VEGF and SDF-1, which are considered to be among the most effective mobilizers of EPC, a vast number of growth factors and cytokines have been described to modulate mobilization of EPC (CFU-EC) [57]. Granulocyte colony-stimulating factor (G-CSF), which has a strong stem/progenitor cell mobilizing potential, induces the release of elastase and cathepsin G from neutrophils, leading to the cleavage of adhesive bonds on bone marrow stromal cells [58, 59]. Moreover, these proteases can cleave and inactivate SDF-1, which is released by stromal cells and signals through its receptor CXCR4 on stem/progenitor cells [59, 60]. Similarly, G-CSF induced upregulation of the exopeptidase CD26 on CXCR4^{+} stem cells. CD26 is able to splice the N-terminal dipeptide from SDF-1, which causes inhibition of CXCR4 activation and reduced cell retention, resulting in an additional mechanism of mobilization [61, 62].

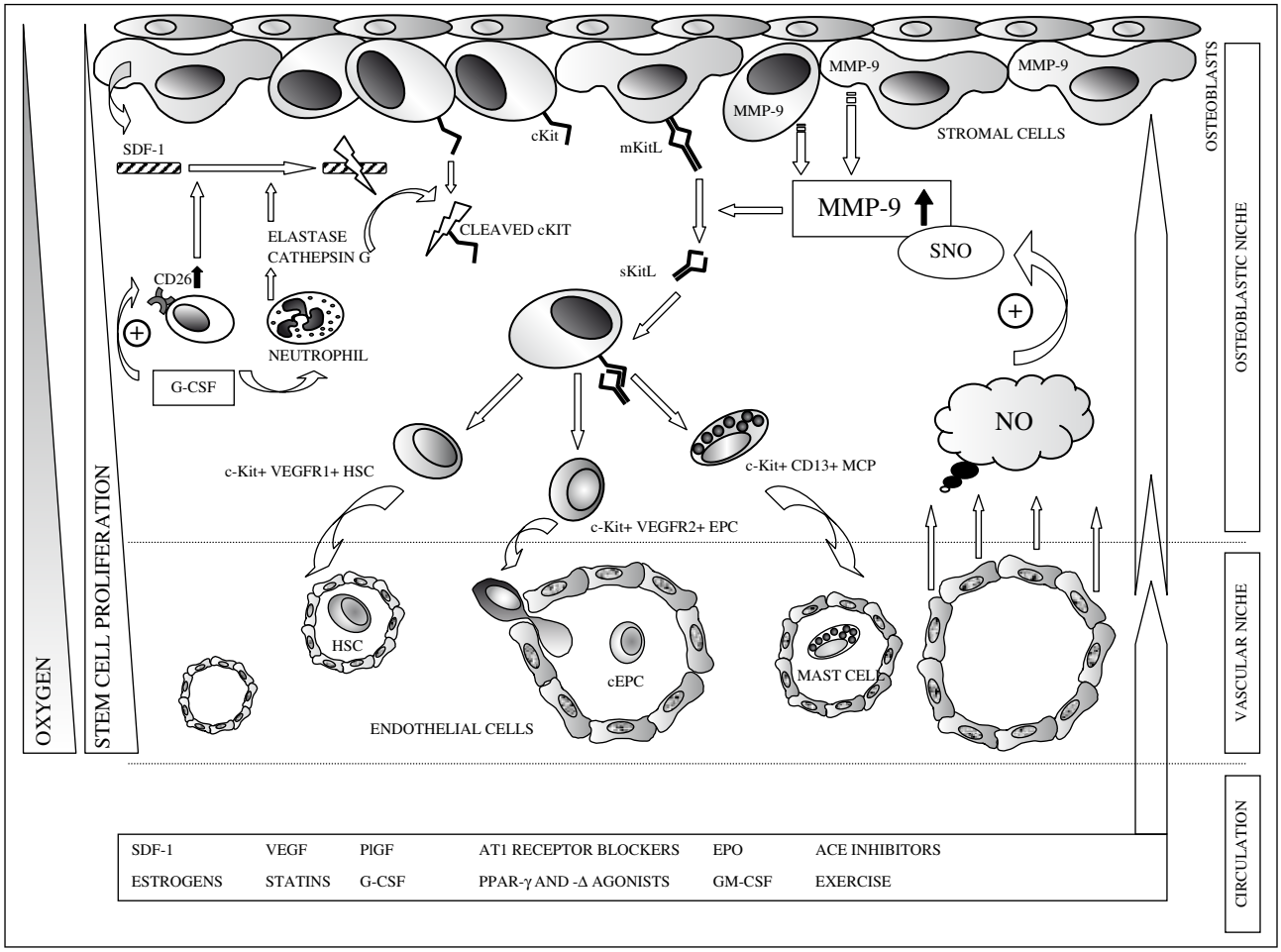

Figure 1
Progenitor cell mobilization. Under steady-state conditions c-Kit$^+$ HSC and EPC reside in the osteoblastic niche of the bone marrow. Low oxygen tension (hypoxia) and direct contact with stromal cells, including osteoblasts, maintains their quiescent state. Different factors (chemokines/cytokines/pharmacological substances) released in the circulation cause the upregulation of MMP-9 in stromal cells and HSC. Membrane-bound Kit ligand (mKitL) is processed into soluble Kit ligand (sKitL), which confers signals that enhance mobility of HSC and EPC to the vascular niche and promotes proliferation. The release of nitric oxide by bone marrow endothelial cells enhances the activity of MMP-9, which involves S-nitrosylation. In addition, the retention factor SDF-1, can be cleaved and inactivated by a G-CSF-induced upregulation of CD26 on HSC and release of elastase and cathepsin G from neutrophils.

A role for the plasminogen activator system in the recruitment and homing of CD34$^+$ progenitor cells has also been suggested. Selleri *et al.* [63] documented the co-mobilization of myeloid and monocytic cells with an increased uPAR expression, as well as the increase in serum levels of soluble uPAR and cleaved soluble uPAR after G-CSF-induced CD34+ HSC mobilization. Both fragments were able to attract CD34+ progenitor cells *via* activation of the high-affinity fMet-Leu-Phe (fMLP) receptor (FPR) and desensitization of the SDF-1 receptor CXCR4 (which facilitates bone marrow retention of HSC) both *in vitro* and *in vivo* [64]. Recently, compelling data from Tjwa *et al.* [65] demonstrated that membrane-anchored uPAR is present on a subpopulation of HSC, regulating stem cell-cycle status, adhesion to the bone marrow microenvironment, and homing and engraftment *in vivo*. During G-CSF treatment, plasmin is most likely to be responsible for cleavage of uPAR and increased plasma levels of soluble uPAR. The concomitant loss of the interaction of uPAR with $\alpha_4\beta_1$ integrin thereby promoted stem cell mobilization. Similarly, impaired stem cell mobilization as well as homing and engraftment of transplanted stem and progenitor cells occurred in uPAR-deficient mice [65].

Mobilization of ECFC progenitors from the bone marrow

Due to their low frequency and the incomplete characterization of the circulating precursors of ECFC, there is no information available at present on the origin and mobilization of cells that form the ECFC. It seems plausible these cells already develop into an endothelial cell lineage in the bone marrow, in a process that requires VEGF and Notch signaling [66, 67], and that their recruitment to the vascular niche of the bone marrow proceeds in a way comparable to that used by other progenitor cells.

HOMING OF EPC TO AREAS OF NEOVASCULARIZATION

Recruitment of EPC to sites of neovascularization or endothelial injury strongly resembles that of an inflammatory response. Once in the vicinity of an injured vessel, EPC can interact with activated platelets, the damaged endothelial monolayer and components of the subendothelial matrix. This process has many similarities

with the homing of leukocytes to the activated endothelium. This should not be surprising, given that all studies on the homing of EPC have been done with cells that are CFU-EC, and therefore largely, if not exclusively, myeloid in nature.

Homing and incorporation of EPC in the endothelial lining or sub-endothelial space is a multi-step process, which involves chemo-attraction, rolling and tethering of progenitor cells, subsequent firm adhesion and, finally, extravasation *(figure 2)*. Various degrees of blood vessel and tissue injury may result in the production and release of cytokines and chemokines creating a gradient within the vessel wall. A large number of cytokines and growth factors, including VEGF [24, 68, 69], insulin-like growth factor 2 (IGF2) [70], monocyte chemotactic protein 1 (MCP-1/CCL2) [71], interleukin-8, CXCL1 and CXCL7 [72, 73], bradykinin [74], macrophage migration inhibitory factor (MIF) [75], and SDF-1/CXCL12 have been described as participating in EPC (CFU-EC) recruitment.

The latter one, SDF-1, and its specific receptor CXCR4 play a major role in stem cell recruitment and retention to ischemic areas [76-78]. In physiological conditions, SDF-1 is constitutively expressed, but a range of stimuli such as inflammation [79], tissue damage and hypoxia rapidly increase SDF-1 levels. SDF-1 is expressed or is surface-bound at injured smooth muscle and endothelial cells, and is released by activated platelets [80-83]. Activated platelets secrete high levels of SDF-1. Platelets are probably the first responders to vascular trauma and, after their adherence to subendothelial matrix structures and subsequent activation, release the necessary factors for progenitor cell mobilization and homing to the damaged area [81]. Reduced tissue and plasma levels of SDF-1 have been correlated with unstable coronary artery disease [84] and impaired wound healing in diabetes [85]. The gene expression of SDF-1 is regulated by the transcription factor hypoxia-inducible factor-1α (HIF-1α) and is also induced by VEGF overexpression, thus mediating SDF-1-dependent recruitment of $CXCR4^+$ progenitor cells to injured arteries and hypoxic regions [24, 86, 87].

As with the impaired SDF-1/CXCR4 signaling in diabetic patients, Kränkel *et al.* [74], reported that circulating progenitor cells from cardiovascular disease patients showed low levels of the kinin B2 receptor (B2R) and decreased migratory capacity toward bradykinin (BK), suggesting that impaired homing and migration might contribute to impaired neovascularization after ischemic complications.

Retention of $CD34^+$ progenitor cells by developing endothelial tubes in vitro

The interaction of EPC with capillary-like endothelial tubes can be mimicked *in vitro* in a three-dimensional fibrin-based tube formation assay [88]. We applied time-lapse video microscopy to study the movement (chemokinesis) of peripheral and cord blood-derived $CD34^+$ progenitor cells and the selective homing towards sites of tube formation (chemotaxis). The formation of tubes by human microvascular endothelial cells in a fibrin matrix was induced by addition of

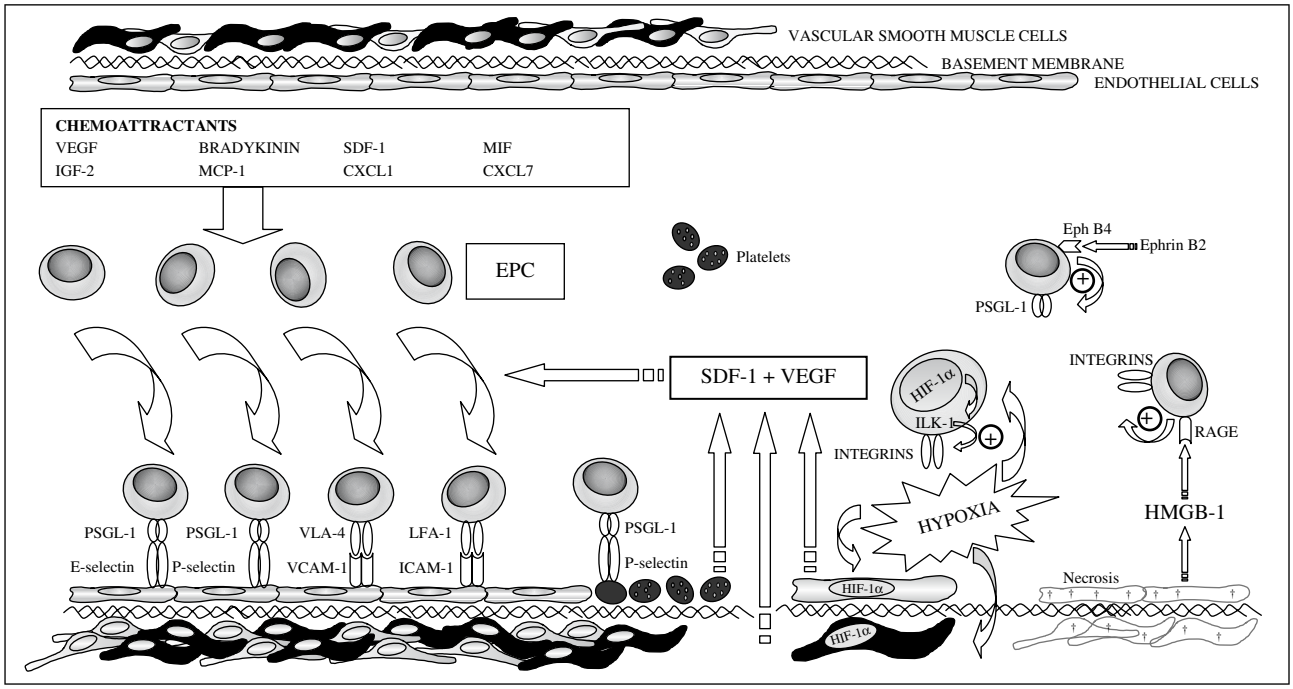

Figure 2

EPC homing, based on CFU-EC forming EPC. After vascular injury, activated endothelial cells present E- and P-selectin, ICAM-1 and VCAM-1 on their membrane. EPC tether, roll and firmly adhere to these adhesion molecules by their ligands PSGL-1, VLA-4 and LFA-1. In the case of endothelial denudation, platelets rapidly create a platform for EPC adhesion by adhering to the subendothelial matrix and exposing P-selectin. In addition, activated platelets release SDF-1 and VEGF, which attract EPC. Hypoxia is an important regulator in EPC homing. First, it stimulates vascular smooth muscle cells, adventitial fibroblast and endothelial cells to produce the chemoattractants SDF-1 and VEGF. Second, it increases the activity of integrins on EPC *via* integrin-linked kinase-1 (ILK-1). Necrotic endothelial cells release the nuclear factor HMGB1 that further enhances the interaction of integrins with their ligands. Regulation of PSGL-1 on EPC was also demonstrated for the ephrin B2-EphB4 axis.

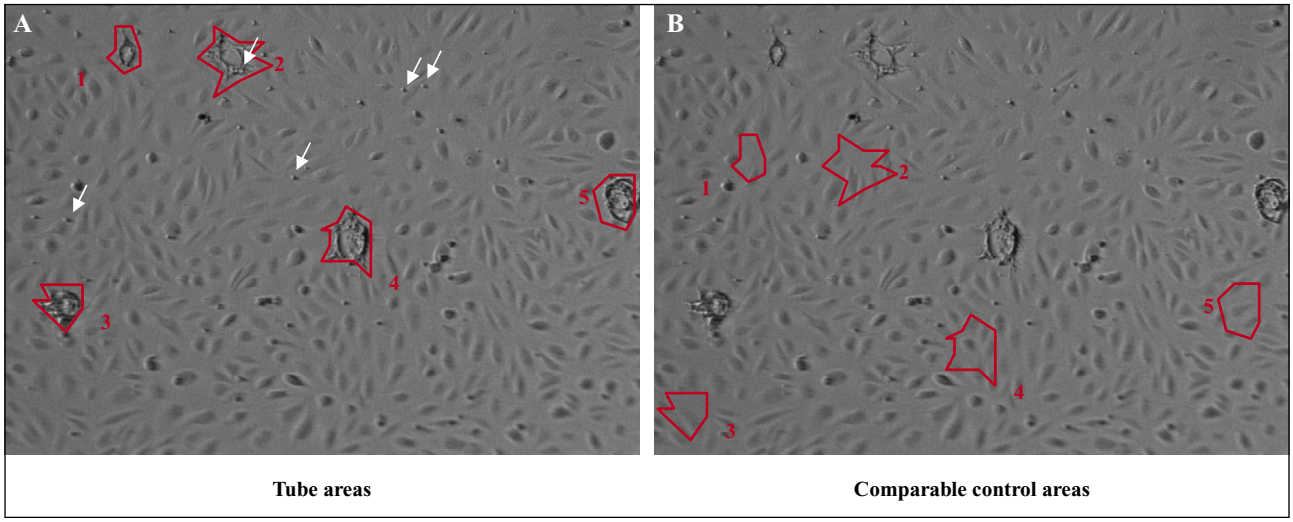

Figure 3
Movement of CD34$^+$ cells was recorded by time-lapse video microscopy over an 8-hour period. CD34$^+$ cells (10^4/cm^2) were added to tube-forming monolayers of human microvascular endothelial cells. The first two hours were excluded from further analysis because of non-specific turbulence in the medium caused by addition of the CD34$^+$ cells. Quantification was performed by counting the individual cells that entered and egressed the marked tubular areas, as defined for five tubular areas in panel **A**. As control, the marked areas were projected three times to other sites on the same monolayer, in which no tubular structures were present (indicated in panel **B**). Again, the number of cells entering and being retained in these marked areas was counted. Further details and data of the quantification are given in *table 1*.

VEGF/TNF-α or bFGF/TNF-α *(figure 3)*. Subsequently, the CD34$^+$ progenitor cells were placed on top of the fibrin matrix covered with endothelial cells, which had started to form tubular structures. The CD34$^+$ cells displayed a random movement over the stimulated endothelial monolayer during an 8-hour observation period. However, once the CD34$^+$ cells reached a tubular structure they remained associated with that structure, either moving around in the tubule, or silently trapped. Quantification of these data *(table 1)* showed that 80% of the cells that reached a tubular area were trapped, while in comparable control areas only 5% was present. Furthermore, the CD34$^+$ cells entered a tube area as frequently as control areas of the same size and shape, suggesting chemokinesis rather than chemotaxis. However, one should note that the presence of serum, which contains large amounts of SDF-1, likely interfered with SDF-1-induced chemotaxis.

When the CD34$^+$ cells were fixed with paraformaldehyde before adding them to the tube formation system, a complete arrest of the administered CD34$^+$ cells on the stimulated endothelial monolayer was observed, indicating that the movement was indeed an active process of the cells. In contrast, fixation of the endothelial monolayer and tubular structures interfered neither with the random movement of the CD34$^+$ cells, nor with the accumulation of CD34$^+$ cells at the tubular structures. The response to fixed tube structures and the absence of a chemotactic response suggest that adhesion receptors rather than cytokines were involved. Preincubation of the endothelial monolayer with a cocktail of blocking antibodies against E- and P-selectin, ICAM-1 and VCAM-1 to study this process was not sufficient to block the accumulation of CD34$^+$ cells at the tubular structures, suggesting that other adhesion factors may also be involved.

Receptors involved in EPC (CFU-EC) homing to areas of angiogenesis in vivo

The initial adhesion of EPC (CFU-EC) seems to be mediated by injury/ischemia-induced upregulation of E- and P-selectin. A number of reports describe the expression analysis and *in vivo* blocking of P- and E-selectin, thereby attenuating the interaction with P-selectin glycoprotein ligand-1 (PSGL-1) on EPC (CFU-EC) [89]. Treating E-selectin-deficient mice with soluble E-selectin (sE-selectin) enhanced the efficacy of EPC transplantation to induce neovascularization and salvage of the ischemic limb. Conversely, when E-selectin was knocked down by E-selectin small interfering RNA (siRNA), blood flow recovery after EPC transplantation was significantly impaired. The beneficial contribution of sE-selectin was further enhanced by stimulating ICAM-1 expression on endothelial cells [90]. In addition, Foubert *et al.* [91] demonstrated that PSGL-1 expression on EPC was regulated by ephrin receptors and their ligands, key regulators in vascular development. EphB4 receptor activation with an ephrin-B2-Fc chimeric protein increased the angiogenic potential of human EPC in a nude mouse model of hindlimb ischemia, by induction of PSGL-1 and adhesion to E-selectin and P-selectin. In the case of more severe injury, when subendothelial matrix components are exposed, activated platelets and coagulation factors (fibrin) rapidly produce a microenvironment facilitating PSGL-1-mediated adhesion of CD34$^+$ progenitor cells [73, 83, 92].

Integrins expressed on the surface of EPC mediate the firm adhesion and transmigration of EPC to the damaged endothelium. Again, the murine model of hindlimb ischemia was utilized to demonstrate that progenitor cells from β_2-integrin-deficient mice are less capable of homing to sites of ischemia and of improving neovascularization. Preactivation of the β_2-integrins expressed on EPC by activating antibodies augments the EPC-induced neovascularization.

Table 1
Migration and tubular homing of peripheral and cord blood $CD34^+$ cells

Source of CD34+	Stimulus for endothelial tube formation	Area studied	Entering/leaving CD34+ cells		$CD34^+$ retention (%)
			Donor 1	Donor 2	
PB-$CD34^+$	VEGF + TNF-α	Tubular structures	123/59	47/18	52.0-61.7%
		Control monolayer	83/71	47/42	10.6-14.5%
CB-$CD34^+$	bFGF + TNF-α	Tubular structures	21/5	47/17	63.8-76.2%
		Control monolayer	29/28	37/34	2.3-8.1%
CB-$CD34^+$	VEGF + TNF-α	Tubular structures	41/8	71/12	80.5-83.1%
		Control monolayer	51/48	45/39	5.9-13.3%
CB-$CD34^+$ on fixated tube assay	VEGF + TNF-α	Tubular structures		54/5	90.70%
		Control monolayer		21/19	9.50%
Fixated CB-CD34	VEGF + TNF-α	Tubular structures		0/0	0/0*
		Control monolayer		0/0	0/0*

Migration and tubular homing of peripheral and cord blood $CD34^+$ cells on monolayers of human microvascular endothelial cells, on a 3D-fibrin matrix that had been induced to generate tubular structures after exposure to either bFGF/TNF-α (10/10 ng/mL) or VEGF/TNF-α (25/10 ng/mL) as previously described [88]. Mononuclear cells were isolated from cord blood or peripheral blood, and subsequently $CD34^+$ cells were selected using magnetic antibody cell separation (MACS). Flow cytometry demonstrating a purity of $90.1 \pm 1.8\%$ of cells positive for $CD34^+$. The movement of CD34+ cells on the endothelial monolayer was recorded by time-lapse video phase-contrast microscopy for eight hours (recording window: 1.28 mm x 0.96 mm). During analysis of the data, the areas with tubular structures were marked and the number of cells entering and leaving the areas was counted. As control, the same surface areas were projected to areas of the same endothelial culture, but devoid of tubes. The data show that the entrance into tubular areas and control areas are comparable. Most cells remained associated with the tubular areas (retention 80%), while little retention occurred in the control area. Data are given for two independent experiments with cells from different donors (5-12 tube areas evaluated per experiment. Protocols on the use and collection of blood samples were approved by the VU University Medical Center ethics committee.
* No movement of fixated CB-CD34+ cells.

zation *in vivo* [93]. This work was supported by the fact that not only VLA-4 ($\alpha_4\beta_1$ integrin) and LFA-1 ($\alpha_L\beta_2$ integrin), but also their counterparts ICAM-1 and VCAM-1, were upregulated in ischemic tissue [94-96]. Neutralization of one of these factors reduced adhesion and migration of EPC *in vitro* and reduced recovery of hindlimb blood flow, capillary density and incorporation of EPC into ischemic tissues *in vivo* [96-98]. Mechanistically, cell adhesion molecule (CAM)-integrin-mediated firm adhesion was reported as being regulated by hypoxia as well as necrosis. High-mobility group box 1 (HMGB1) is a nuclear protein that is released extracellularly upon cell necrosis and tissue damage. Binding of HMGB1 to its receptor RAGE (receptor for advanced glycation end products) on EPC resulted in rapidly increased integrin affinity and induced integrin polarization, enhancing the *in vitro* adhesion and migration and *in vivo* homing and incorporation of EPC in the tumor vasculature [99].

Exposure of endothelial cells to hypoxia increased the endogenous amount and kinase activity of the protein integrin-linked kinase (ILK-1) in a NFκB- and HIF-1α-dependent manner. Overexpression of ILK-1 resulted in ICAM-1 upregulation, whereas blocking ILK-1 abrogated the expression of ICAM-1 under hypoxia, with co-committed reduction in EPC homing and poor neovascularization *in vivo* [100, 101]. In addition, the adipokine leptin, a published modulator of vascular remodeling and neointima formation, has recently been described as increasing the expression of $\alpha_V\beta_5$- and α_4-integrins in EPC [102].

Proteases involved in homing of EPC (CFU-EC)

Transmigration of EPC into the injured or hypoxic tissue is the last step of homing and recruitment of progenitor cells to the area of angiogenesis. The invasive capacity is crucial for tissue repair and restoration of organ function [103], stressing the importance of protease production and release by EPC. EPC and EPC-derived cells express various cysteine cathepsins, MMP, and u-PA and the receptor uPAR [63, 104-106].

Urbich *et al.* [104] demonstrated the crucial role of cathepsin L in EPC-mediated neovascularization. Cathepsin L was highly expressed in EPC and was essential for matrix degradation and invasion by EPC *in vitro*. Cathepsin L-deficient mice showed impaired functional recovery following hindlimb ischemia. Infused cathepsin L-deficient progenitor cells neither homed to sites of ischemia nor augmented neovascularization. This could be reversed by forced expression of cathepsin L in mature endothelial cells. Recently, the same group revealed a decreased cathepsin L expression and activity in EPC from patients with type 2 diabetes, suggesting a novel mechanism for diabetes-related impairment of neovascularization [107].

Membrane-type-(MT-)1-MMP (MMP-14) is another powerful protease involved in migration and invasion of cells including $CD34^+$ progenitor cells [106]. Recently, it was shown that the balance between the expression of MT1-MMP and its membrane-anchored inhibitor RECK [108], is involved in the regulation of homing, retention, egression, and mobilization of immature human $CD34^+$ progenitor cells [109]. Steady-state egression of human $CD34^+$ cells and, to a greater extent, their G-CSF-induced mobilization, is accompanied by an increase in MT1-MMP and a simultaneous reduction in RECK expression, which facilitate MT1-MMP-mediated CD44 cleavage and progenitor cell motility and mobilization. Furthermore, MMP-2 was found to affect the invasive properties of EPC. MMP-$2^{-/-}$ mice responded poorly to hindlimb ischemia because of reduced neovascularization. Transplantation of MMP-$2^{+/+}$ bone marrow cells dramatically improved the recovery of these mice [110]. Stimulation with TNFα, IL-8 or SDF-1 resulted in increased MMP levels, facilitating the migration of EPC into Matrigel plugs or transwell systems [36, 111-113].

Finally, a role for the plasminogen system in EPC homing and recruitment was anticipated. Xiang *et al.* [114] investi-

gated the potential of the plasminogen system *in vivo*. Blocking the protease inhibitor plasminogen-activator inhibitor-1 (PAI-1) with a sequence-specific DNA enzyme at the time of myocardial infarction, resulted in increased engraftment of exogenously delivered $CD34^+$ progenitors in the infarct zone. Abrogation of the natural inhibition of PAI-1 on u-PA was most likely responsible for the observed effect [114].

Homing of ECFC progenitors

The circulating progenitors of ECFC are probably cells that can be recruited to areas of neovascularization and tissue repair. Their endothelial nature makes direct interaction and incorporation into the endothelial lining possible. However, one would expect that the circulating counterpart of ECFC must be able to recognize an area in need of (neo)vascularisation support, and to home to and migrate into it. Recent studies have suggested that the combination of CFU-EC and ECFC was more effective in stimulating neovascularization and tissue repair, and also pointed to a directed influx and action of the ECFC [36, 115], although this issue remains controversial.

While no information is presently available on homing receptors, it is likely that these cells use similar proteases as other endothelial cells, such as MT1-MMP, MMP-2, MMP-9 and the urokinase/plasmin system [116]. These proteases are indeed expressed in ECFC. Cultured ECFC displayed relatively high u-PA and MMP-2 levels compared to normal endothelial cells [105, 117]. Inhibition of ECFC-associated u-PA by monoclonal antibodies that block u-PA activity or binding to its receptor significantly reduced proliferation, migration and capillary-like tube formation *in vitro* [105, 117].

PARTICIPATION OF EPC IN THE NEWLY FORMED VESSELS

A number of studies using various animal models have suggested that bone marrow-derived and circulating progenitor cells are capable of repopulating within damaged organs, possibly contributing to angiogenesis (see Tanaka *et al.* [118], and Zampetaki *et al.* [103] for review). However, the contribution of bone marrow-derived EPC to neovascularization has been seriously challenged by other groups, who found no evidence for such a process in tumor vascularization in mice [6, 7]. Differences in the evaluation at early and late time points, reflecting a contribution in the initial phase only, and the fact that the ECFC are so few that they are not easily encountered in mice, may, in part, explain these differences [119]. If EPC and ECFC contribute to angiogenesis and/or vasculogenesis, they might do so at three levels. Firstly, they can provide growth factors or other signals that stimulate and orchestrate the angiogenesis process. Secondly, ECFC can enforce the endothelial lining of preexisting and newly formed vessels by being incorporated in the endothelial lining (intussusception). Finally, ECFC can organize themselves into new vascular structures that connect to the resident circulation.

Intussusception of circulating EPC in healing human blood vessels

The extent to which endothelial progenitors contribute to *human* angiogenesis and vascular maintenance is still a matter of controversy [120, 121]. The identification of gender- or HLA-mismatched cells in (injured) tissue after transplantation of solid organs or bone marrow has been used in various studies as an indicator of the intussusception of circulating cells in a vascular bed. In an early study, Hruban *et al.* [122] detected cells of recipient origin in allografted hearts following cardiac transplantation. At that time, endothelial cells of recipient origin had not been identified in allografted hearts, apart from a rare number of flattened cells lining the vascular lumens. Additional immunohistochemical analysis revealed that these cells were in fact macrophages [122]. Indeed, after damage of the vasculature, monocytes can spread over the exposed vessel matrix, an initial coverage that later can be replaced by endothelial cells. More decisive was a small but significant number of reports based on sex-mismatched bone marrow transplantations, which suggested that human bone marrow-derived EPC incorporate into tumor vessels [5, 123], skin [124], endometrium [125] and transplanted hearts and livers [126-130]. In all these studies, only very low numbers of cells (usually less than 1-5%) were encountered in the endothelial lining of healthy, small blood vessels. Vascular injury, as occurs at sites of atherosclerotic plaque development, or after myocardial infarction, increased the number of incorporated bone marrow-derived cells to a various degree [127, 130]. A time-course of endocardial biopsies demonstrated that there was neither an increase nor a decrease in the number of incorporated cells over a 10-year period [129].

Contribution of late-outgrowth ECFC, but not early EPC to endothelial tubular structures

In vitro studies on endothelial tube formation have shown that the contribution of classical EPC, the early-outgrowth cells that mainly reflect myeloid cells, and late-outgrowth ECFC, are different. The classical early EPC (CFU-EC) associate with the newly forming microvessels, but show little incorporation into the endothelial lining, nor are they able to form tubes by themselves [15, 30-32, 131]. Instead, they can stimulate and possibly shape the growth of new vessels. CFU-EC act by releasing angiogenic growth factors, and possibly by cellular contact. Therefore, they can be considered as angiogenic accessory cells.

In contrast, the ECFC incorporate into existing differentiated endothelial tubule structures *in vitro*, and are also able to form tubules readily, by themselves [15, 34, 117, 131]. This process proceeds similarly as the tube formation by microvascular endothelial cells, and depends on proper integrin-matrix interactions and the pericellular recruitment of proteases, including u-PA, the metalloproteinases MT1-MMP and MMP-2, and cathepsin L [104, 105, 117].

More convincingly as regards clinical applications, several reports have also shown that peripheral blood- and cord blood-derived ECFC can form functional blood

vessels when implanted *in vivo* [15, 34, 132]. This opens avenues for the engineering of new vasculature in tissues with limited blood supply. However, Au *et al.* [133] reported that umbilical cord blood-derived ECFC, when co-transplanted with mesenchymal cells in mice, formed stable vascular structures, while similar cells obtained from peripheral blood formed only a limited number of unstable vascular structures. Thus, ECFC are able to participate in blood vessels as true endothelial cells, but much has still to be learned about their prerequisites and functional properties.

CONCLUSION

A number of clinical conditions requires an enforcement of the endothelial lining of blood vessels, either because cell replacement is needed due to endothelial injury or apoptosis, or to keep up with the expansion of the endothelial lining during neovascularization. The concept of an EPC that can differentiate into a true endothelial cell with high proliferation potential is attractive, but also hotly debated and more complex than originally anticipated. As discussed above, EPC can be recruited, in particular by stimuli induced by hypoxia, to areas of tissue repair and neovascularization, and participate directly or indirectly in the growth of the new vasculature. However, our understanding of the true nature of these progenitor cells is rather fragmented. Different types of cells, which were originally recognized as EPC, reflect neovascularization-supporting cells with different functions and background. Two populations have currently been identified, but the existence and the contribution of other small populations within the blood-derived inoculum, and additional functions, cannot be excluded.

The first population involves the early-outgrowth EPC (CFU-EC), which have a myeloid origin. They represent monocytes with endothelial properties and a strong ability to provide factors to stimulate angiogenesis, but little capacity to incorporate persistently into the endothelial lining. The decrease in circulating EPC observed in diabetes and cardiovascular disease may reflect a contribution to vascular maintenance and repair in patients, but alternatively may merely reflect increased oxidative stress [134]. Future studies will clarify whether these cells reflect the course of the disease, making them suitable as a prognostic marker, or whether their reduction also contributes to the etiology of the vascular complications (causal), thus providing a perspective on new therapies.

The second population (ECFC or late-outgrowth EPC) grows out as colonies of real endothelial cells. Better identification of these cells in the blood by specific markers would help to improve the isolation, separation and amplification of these cells, and the possible use of these cells for autologous and possibly heterologous transplantation. Much has still to be learned; it may well be that these cells perform best in collaboration with other mesenchymal cells. Furthermore, the differences between ECFC obtained from cord blood and those from peripheral blood require further attention.

The mobilization and recruitment of the progenitor cells that develop into the myeloid CFU-EC and endothelial ECFC is determined in the bone marrow and formation of similar new interactions at the sites of homing and neovascularization. Proteases, such as MMP-9, play an important role in mobilization from the bone marrow by disintegration of existing cell adhesions and chemokine-receptor interactions. Once liberated, the circulating progenitor cells home *via* receptors that act as cell adhesion molecules and recognize cytokine gradients, such as SDF-1 in hypoxic areas. The subsequent invasion process requires proteolytic activity to squeeze the cells through the collagen-rich extracellular matrix and/or the fibrin matrix of a fibrinous exudate. In this latter process, MT1-MMP, MMP-2 and u-PA acting on a cellular uPA receptor are involved. Indeed, cultured ECFC have a high expression of u-PA, which appears to be involved in migration and tubule formation. Similarly roles for MT1-MMP, MMP-2 and cathepsin L are likely. Furthermore, these and other proteases also activate and modify cytokines, angiogenic growth factors and their receptors, thus fine-tuning the regulation of the neovascularization process.

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