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Endothelial Progenitor Cells and Neovasculogenesis

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Abstract—It is has been established that endothelial progenitor cells EPC are of great interest in the maintenance of endothelial integrity and postnatal neovascularization of the adult organism's tissues. It is shown that the formation of new blood vessels can be carried out both by angiogenesis and vasculogenesis from EPC. In this review, data are presented on the heterogeneity of the EPC in population. These cells are indentified by different markers and have different proliferative and functional capacities. They not only activate the neovascularization of tissues after damage, but also they are potential candidates for therapeutic angiogenesis.

Keywords: angiogenesis, vasculogenesis, vascular endothelium growth factors, homing

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INTRODUCTION

Endothelial progenitor cells are a unique cell fraction that are similar to embryonic angioblasts and promote postnatal angiogenesis. The process of the differentiation of endothelial precursors to endothelial cells and the formation of a primitive capillary network is called "vasculogenesis."

Until recently, it was considered that, in the adult organism, neovasculogenesis is carried out through two processes, i.e., arteriogenesis, which is the development of collateral vessels, and angiogenesis, which is the development of new capillaries due to the proliferation and migration of precursor differentiated endothelial cells [8]. However, in 1997, Asahara [3] showed that the population of CD34+ hemopoiesis cells of bone-marrow origin extracted from human peripheral blood is able to differentiate in vitro into cells with the phenotype of mature endothelial cells and leads to revascularization in vivo in response to acute tissue ischemia. Thus, in the adult organism, the formation of new vessels may occur, not only through angiogenesis, but also through vasculogenesis. For the first time in 1998, Shi extracted the population of immature endothelial cells form circulating mononuclear cells [50]. The precursor cells that take part in adult vasculogenesis were called "endothelial progenitor cells" (EPCs). To identify EPCs from peripheral blood, Asahara applied two markers, i.e., for hemopoietic precursors of CD34, and for mature endothelial cells of vascular endothelial growth factor-2 (VEGFR/kinase insert domain containing receptor—KDR).

The study and determination of the role of circulating EPCs in the recovery of damaged vascular endothelium and angiogenesis during tumor growth began with these initial works. In experimental models of the myocardium and lower-limb ischemia in rodents, it was shown that EPCs promote the formation of vascular network in damaged tissues [14, 44, 67]. Clinical studies have shown the effectiveness of bone-marrow cells and EPCs of peripheral blood in ischemic diseases [31, 35, 46, 47].

In addition, circulating EPCs are of great interest as diagnostic and prognostic markers of cardiovascular diseases. The decreased level of circulating EPC correlates with increased risk of coronary heart disease and myocardium infarction [10, 15, 63]. It was shown that, in patients with hypertension disease, the level of systolic blood pressure negatively correlates with the amount of EPCs [62]; in patients suffering from diabetes and stroke, the amount of EPCs is also decreased [6].

CHARACTERISTICS AND SOURCES OF EPCs

For the first time, EPCs were extracted from bone marrow and peripheral blood and were characterized by expression of superficial markers CD34 and/or CD133, and VEGFR-2. CD34, the marker of hemopoiesis cells, is also used to identify endothelial progenitor cells because both types of cells originate from the same precursor-hemangioblast [42]. CD34 is also expressed in an insignificant amount on mature endothelial cells. CD133, which is the opposite of CD34, is only expressed on immature cell types and is another, more primitive marker of hemopoiesis cells [16]. VEGFR-2 is a marker of mature endothelial cells and mediates the signal of vascular endothelial growth factor. Only 0.1–0.4% of CD34+ cells of bone marrow and peripheral blood express VEGFR-2 marker after mobilization by granulocyte colony stimulating factor (G-CSF) [71]. Certain authors allocate two EPC subpopulations, i.e., more primi-

tive $CD133^+CD34^+VEGFR\text{-}2^+$ and more mature $CD133^-CD34^+VEGFR\text{-}2^+$ [16].

Thus, endothelial precursors of bone marrow origin are the main candidates for maintenance and recovery of vessels and are characterized by a triad of markers, i.e., CD34, CD133, and VEGFR-2. However peripheral blood also contains endothelial progenitor cells of myeloid (monocyte) origin that do not express the marker of hemopoiesis cells CD34; they have phenotype $CD14^+CD34^-$ and are also able to differentiate into endothelial cells in vitro and form a vascular network in vivo [5].

$CD14^+$ as $CD14^-$ cells (known also as early- and late-growing EPCs) are extracted from peripheral blood and cultivated in vitro during differentiation into mature endothelial cells that express the same markers, have identical functional characteristics, and promote neovascularization in models in vivo [2, 56]. Thus, EPCs are not restricted by monocyte CD14 marker, but the expression of endothelial marker VEGFR-2 is a necessary condition for the functional activity of both $CD14^+$ and $CD14^-$ cells.

Early and late-growing EPCs differ in their proliferation capacities. Early EPCs obtained from monocyte cells have low proliferation activity and express markers typical of mature endothelial cells. Although these cells can be introduced into the endothelium monolayer, they insufficiently promote perfusion in vivo. Late EPCs are an alternative, since they have greater proliferating capacity and can be maintained in culture to a great degree. They play an integral role in neoangiogenesis in vivo and are vascular generating EPCs [66]. Recent studies have identified these cells as $CD34^+CD45^-$ progenitors [54]. CD^+ cells give a rise to early EPCs, whereas late EPCs only develop from the $CD14^-$ subpopulation [20, 73].

The two pathways of extracting early and late EPCs from peripheral blood were described [66]. At the first pathway, isolated mononuclear cells are cultivated in dishes processed with fibronectin with growth factors, which leads to the formation of endothelial colonies (colony-forming unit of endothelial cells) after 5–7 days. In the other case, the formation of a colony on plastic the processed with collagen after 14–21 days of cultivation is evaluated. Both $CD14^+$ and $CD14^-$ cells of peripheral blood upon cultivation in vitro in medium saturated with growth factors express the markers of mature endothelial cells (VEGFR-2), von Willebrand factor (vWF), endothelial nitric oxide synthase (eNOS). The functional ability of EPCs, along with the expression of superficial markers and the ability to form a vascular network, are estimated by the clonogenic potential.

The introduction of EPCs in vivo into immunocompromised mice can significantly improve perfusion; however, as was shown, only a small number of EPCs can be introduced into tissues and form new capillaries [19, 70]. As a consequence, EPCs are able to secrete pro-angiogenic factors and have a paracrine effect. This subsidiary EPC function can be integral in providing the survival of tissue resident cells and enhancing the formation of vascular network and tissue reparation. Early EPC produce more growth factors in comparison with late, that also indicates the different role of these two cell phenotypes in neovascularization [55]. As a result, although early EPCs have low levels of proliferation, they influence angiogenesis by the secretion of angiogenic growth factors that stimulate the proliferation of late EPCs or resident mature endothelial cells.

There are objectionable reports that the $CD34^+CD133^+VEGFR\text{-}2^+$ population is not made up of EPCs, but rather primitive hemopoiesis progenitors [9].

Thus, it is currently known that the bone marrow is a source of progenitor cells that can differentiate into EPCs. There are data on the fact that the majority of EPCs that circulate in blood flow (about 70%) are not of bone-marrow origin [26, 28, 71]. Mesenchymal stromal cells and tissue resident cells are alternatives to EPCs. It was shown that mononuclear cells of the spleen selected in growth medium with endothelial growth factors obtain an endothelial phenotype, form a primary vascular network in vitro, and increase reendothelialization in the experiment [60, 61]. The intravenous transfusion of spleen EPCs into animals after a splenectomy causes these cells to migrate toward the area of alteration. In the colon and liver, there is also a certain amount of progenitor cells [2]. In addition, in adipose tissues, there are stem cells that secrete a wide spectrum of pro-angiogenic growth factors and are able to differentiate into endothelial cells in vitro, as well as promote neovascularization in vivo [22, 49, 74]. EPCs were also found in adventitia of the vessel walls of an adult organism [30, 69].

Thus, EPCs are a heterogenic population. Either their phenotype of the function may change depending on the source of the origin; immature EPCs have high proliferative activity, determined EPCs form a new endothelium, and subsidiary EPCs produce growth factors that promote endothelium reparation (Fig. 1) [68]. Many organs and tissues contain endothelial progenitors, which together make up the circulating pool of EPCs.

MOBILIZATION AND MIGRATION OF EPC

Despite that EPCs of non-bone-marrow origin can be part of the circulating EPC pool, it is unknown how these progenitor cells migrate into blood. More is known about the mechanisms of EPC production from bone marrow. The niche of stem cells is the special area where stem cells are both in undifferentiated condition and in differentiated form [12, 64]. The subsidiary cells in the niche counteract the stem cells and regulate their self-maintenance and differentiation. These cells leave the niche and are put into circulation occur under the influence of different stimuli, including hypoxia. In response to vessel injury or physiolog-

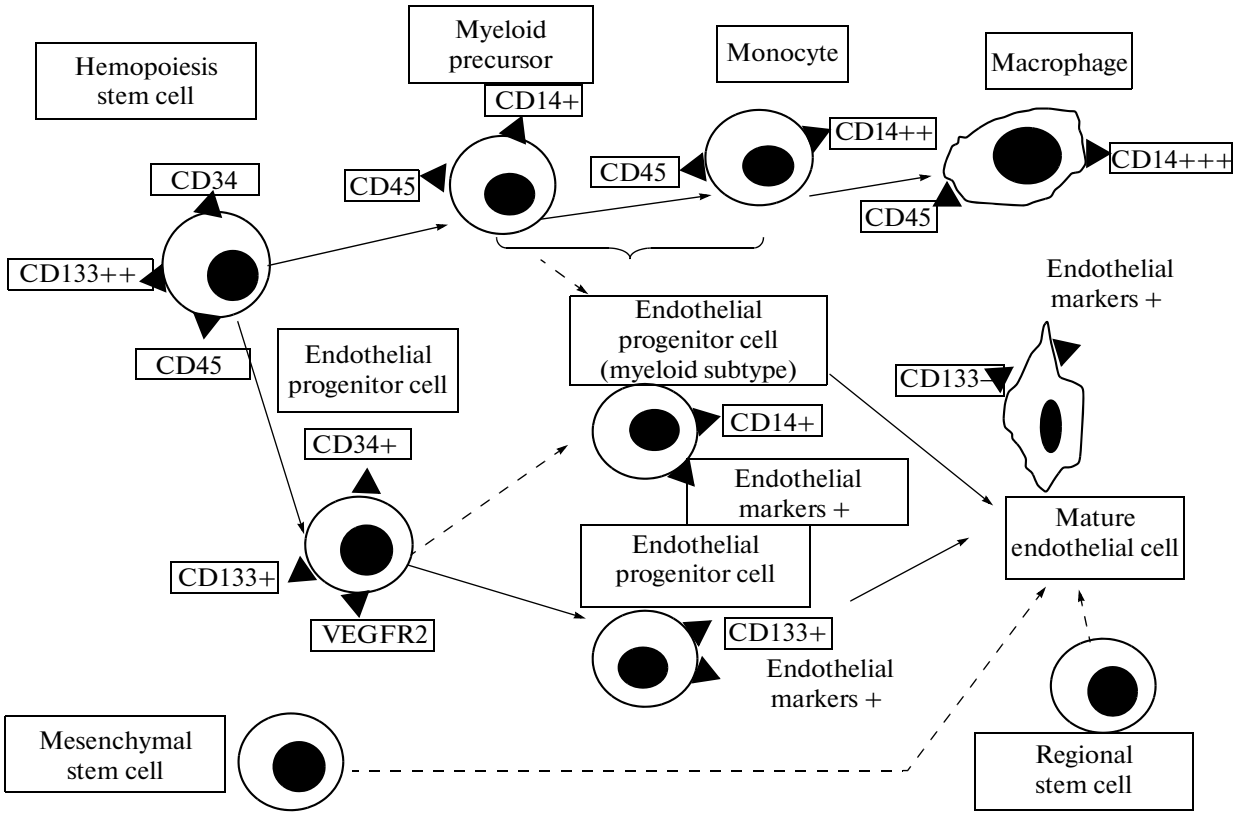

Fig. 1. Differentiation of endothelial progenitor cells.

ical stress, the stem cell is immediately mobilized and recruited to the area of change. VEGF is an effective mobilizator of EPC and potential inductor of angiogenesis. In tissue alteration processes, when the formation of new vessels is necessary, VEGF mediates the proliferation, differentiation, and chemotaxis of endothelial cells. The rapid increase in the circulating VEGF level leads to the activation of matrix metalproteinase 9 (MMP-9) and an increase in the NO level, which leads to the stimulation of several ligand-receptor pairs. The chemokine SDF-1 and its CXCR4 receptor is the most studied factor of stem-cell homing [40]. Endothelial progenitors move from the osteoblast to the vascular region of the niche, are mobilized into the peripheral circulation, and persist in the ischemic area, where they they stimulate neovascularization [24, 33]. SDF-1 is expressed constitutively, but its level increases in response to stimuli such as inflammation mediators, hypoxia, and changes in extracellular matrix architectonics.

The migration of stem cells from the bone marrow to peripheral blood can be enhanced by applying several inducing agents, i.e., G-CSF, GM-CSF, zymosan, statins, chemokines (IL-8), growth factors (VEGF), and CXCR4 antagonist. To increase the pool of circulating EPC in clinics, G-CSF is used via the series of successive injections. G-CSF induces expansion of neutrophils and their precursors in bone marrow, which excrete proteases (elastase, catepsine G, MMP-9), which leads to the degradation and inactivation of adhesion and chemotaxis between hemopoiesis stem cells and bone marrow [27, 42]. Erythropoietin (Epo), which, in addition to its basic function of erythropoiesis stimulation, also stimulates the proliferation of endothelial cells, enhances EPC mobilization in experimental models, and increases the pool of circulating $CD34^+CD45^+$EPC in human peripheral blood, is alternative to G-CSF [23, 45, 51].

EPC HOMING

Maintaining the integrity of the endothelial monolayer is extremely significant, not only because the endothelium is a barrier between blood and proteins of the subendothelial matrix, but also because it prevents infiltration by inflammation cells and thrombogenesis, modulates vascular tonus, and controls the proliferation of smooth muscles [18].

Recruiting stem cells to the ischemic area is similar to the inflammation response. Progenitor cells interact with the damaged monolayer of the endothelium similarly to how leukocytes interact with activated endothelium cells. Adhesion molecules involved into the movement and adhesion of leukocytes were identified as integrative regulators of EPC homing. Homing is a process of moving circulating cells to the target

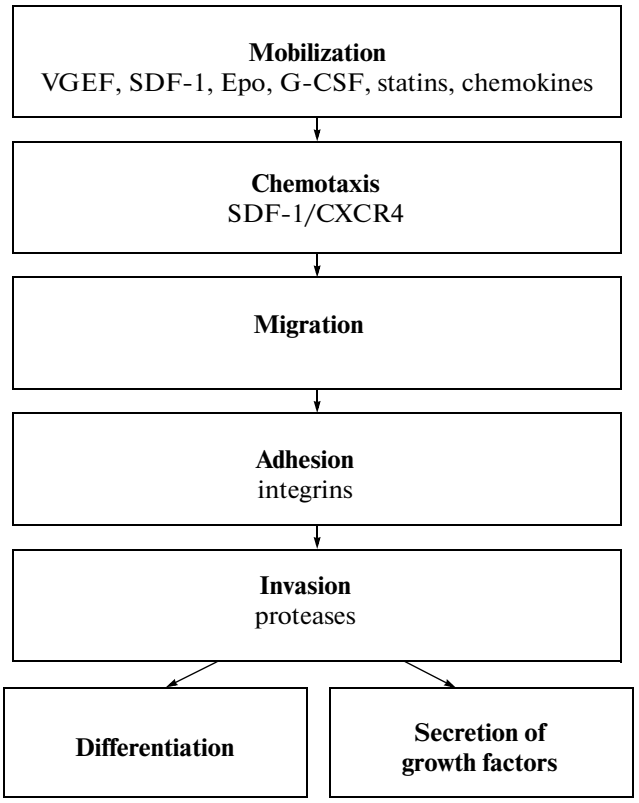

Fig. 2. Homing of EPCs to ischemic tissues.

tissues, e.g., to the heart after myocardial infarction, or to bone marrow. The initial stage of progenitor-cell homing to ischemic tissue includes the adhesion of progenitor cells to endothelial cells and the transmigration of progenitor cells through the endothelial monolayer conditioned by integrins (Fig. 2) [57]. Selectins (P-selectin, E-selectin) condition the initial process; integrins (β2-integrins) and adhesion molecules, including the intercellular adhesion molecule-1 (ICAM-1) and vascular endothelial cell adhesion molecule-1 (VCAM-1) promote the adhesion and transmigration of EPCs to the damaged endothelial monolayer; and cathepsins (cathepsin L) and MMP-2 promote the degradation of the matrix and EPC invasion. Progenitor cells begin to differentiate into endothelial cells, even during movement to damaged tissues. Cytokines (VEGF and SDF-1) and the mechanic pressure of blood flow initiate this cascade [11].

ROLE OF EPCs IN NEOVASCULARIZATION

The regeneration of the damaged endothelium is conducted either through the migration and proliferation of adjacent endothelial cells or through circulating endothelial progenitors. Thus, both populations of circulating cells, i.e., those mobilized form bone marrow and tissue-resident stem cells, are able to contribute to reendothelialization [61, 71].

The ability of stem cells to recruit to ischemic areas and express markers of endothelial cells allows hematopoiesis and endothelial progenitors to be used for therapeutic vasculogenesis. In multiple experimental studies, it has been shown that the infusion of different types of cells extracted from bone marrow or peripheral blood or cultivated ex vivo leads to an increase in the capillary density and the neovascularization of ischemic tissues. Thus, in models of myocardial infarction in animals, it has been shown that the injection of EPCs after expansion in vitro improves the functional state of the cardiac muscle and decreases the cicatrix area and apoptosis of cardiomyocytes [14]. In the models of lower-limb ischemia, EPCs also increase the capillary density; muscle perfusion; and, in certain investigations, promote the formation of a new vascular network [37, 44, 56, 67].

It has been shown in clinical studies that bone-marrow cells or progenitor cells that circulate in blood flow enhance the blood supply of ischemic tissues in different diseases. Thus, the transplantation of autologous stem cells from bone marrow has a positive effect both during acute myocardium infarction [46] and chronic myocardial ischemia in humans [35]. The expansion of EPCs isolated from the peripheral blood ex vivo assists in myocardium neovascularization [31]. Progenitor cells assist in the increase in blood flow and enhance the functional state of the left ventricle in patients with myocardium infarction [52] and significantly influence the post-infarction remodeling of cardiac muscle [47]. The autologous transplantation of mononuclear cells of bone marrow to patients with critical lower-limb ischemia allows one to prevent the development of gangrene and avoid amputations [59].

The angiogenic capacity of EPCs has also been studied on models of tumor growth and angiogenesis. It was shown that inhibiting VEGF, the growth factor of endothelial cells, blocks tumor growth and the formation of vessels [36].

Despite different experimental models, different cellular populations ($CD34^+$, $CD133^+$, EPC), and the introduction of different amounts of cells, the transplantation of EPCs leads to similar functional improvements [43, 56, 65, 67]. Like early progenitor cells, late progenitor cells also have similar vasculogenesis ability [29, 53]; however, terminally differentiated mature endothelial cells, which do not have certain functional characteristics of EPCs (the presence of chemokine and integrin receptors, conditioning homing) do not promote neovascularization. EPCs cultivated in vitro, as well as both $CD14^+$ and $CD14^-$ cells similarly stimulate neovascularization, whereas isolated mononuclear cells do not have this ability [56], though they may play an integral role in bypass growth (arteriogenesis), which is conditioned by their excretion of hemoattractants [58]. The therapeutic effect is achieved by local introduction, whereas system infusion leads to an increasing number of circulating monocytes.

Despite that the the role of EPCs in the improvement of the blood supply is determined, how EPCs promote neovascularization remains an open question and is the subject of active investigations.

The transplantation of genetically modified cells of bone marrow allows one to control the integration of EPCs into tissues. It was established that the incorporation of progenitor cells into the intact tissue is very low. According to the data of different authors, in ischemic tissue, the percentage of integrated genetically labeled cells of bone marrow is in the range of 0–90% [13, 38, 41]. In addition, the difference of the integration of bone-marrow cells in cerebral artery after stroke has been shown [25, 39]. At the same time, in models of tumor angiogenesis, a significant amount (50%) of EPCs was found in tumor tissues [17]. The effectiveness of transplant during engraftment also differs in separate subpopulations of progenitor cells (hematopoiesis cells or integrate cells of bone marrow). At the same time, in several investigations, it has been shown that, despite that bone-marrow cells are identified in vessels after implantation, they have a positive effect on revascularization and do not always express endothelial markers [13]. The method of introduction also influences the engraftment of introduced cells. Intravenous introduction increases the incorporation of EPCs into 20% compared to endogen-mobilized cells (2%) [1, 48]. Nevertheless, in general, the number of introduced cells with the endothelial phenotype is rather low. A possible explanation for the effectiveness of vascularization is not only the introduction of EPCs to the tissue with the subsequent formation of new vessels, but also the paracrine effect, i.e., the excretion of pro-angiogenic factors by cells. EPCs act similarly to monocytes and promote arteriogenesis by the secretion of cytokines and growth factors, such as VEGF, hepatocyte growth factor (HGF), and fibroblast growth factor (FGF). Growth factors are also able to affect the angiogenesis process (proliferation and migration) and the survival of mature endothelial cells. However, due to the paracrine effect, EPCs integrate with tissues and form new vascular structures in vivo. Unlike EPCs, macrophages, which also excrete growth factors, do not promote the formation of vascular network [32].

Nevertheless, the maturation of EPC to the endothelial cells is important for the functional integration of vessels. Signal cascades that regulate differentiation are not fully known; however, it has been shown that the differentiation of hemangioblast to hemopoiesis, as well as endothelial precursors is controlled by the *Hex* gene [21].

CONCLUSIONS

Endothelial progenitor cells are a cell fraction that, similar to embryonic angioblasts, proliferate and migrate in response to angiogenic growth factors and differentiate into the mature endothelial cells in situ. These cells are able to form vessels in the adult organism and an increase in the number of EPCs leads to neovascularization and the recovery of ischemic tissues. The identification of the sources of progenitor cells and understanding how molecules mobilize, migrate, and differentiate these cells are the basis of an effective therapeutic strategy. Vasculogenesis by endothelial progenitor cells can be a determining factor in the start of tissue cascade and organ regeneration.

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