SPANDIDOS PUBLICATIONS INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 30: 1000-1006, 2012

Contribution of endothelial progenitor cells to neovascularization (Review)

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Received June 1, 2012; Accepted July 30, 2012

DOI: 10.3892/ijmm.2012.1108

Abstract. Endothelial progenitor cells (EPCs) are a cell population mobilized from bone marrow into the peripheral circulation and recruited into sites of vessel injury to participate in blood vessel formation in both physiological and pathological conditions. Due to the lack of unique surface markers and different isolation methods, EPCs represent heterogeneous cell populations including cells of myeloid or endothelial origin. Evidence suggests that EPCs play a critical role in postnatal blood vessel formation and vascular homeostasis and provide a promising therapy for vascular disease. However, the mechanisms by which EPCs participate in new vessel formation are still incompletely understood. We review the process of EPCs in neovascularization including EPC mobilization, migration, adhesion and effect on new vessel formation, in an attempt to better understand the underlying mechanisms and to provide potential effective management for the treatment of patients with vascular disease.

Contents

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Abbreviations: EPCs, endothelial progenitor cells; ECs, endothelial cells; CFU-Hill, colony-forming unit-Hill; CACs, circulating angiogenic cells; ECFCs, endothelial colony-forming cells; MNCs, mononuclear cells; PB, peripheral blood; CB, cord blood; UEA-1, lectin *Ulex europeus* agglutinin-1; UCB, umbilical cord blood; VEGF, vascular endothelial growth factor; SDF-1, stromal cell-derived factor-1; PNT, pleiotrophin; HMGB1, high-mobility group box 1; SMCs, smooth muscle cells; eNOS, endothelial nitric oxide synthase

Key words: endothelial progenitor cells, neovascularization, cytokines

1. Introduction

Vascular disorders, possessing high morbidity and mortality, are suffered by a multitude of patients due to the limitations of current therapies. Efficient repair of damaged endothelium and enhancement of the ability to form new blood vessels are crucial for treatment of these disease. Disruption of endothelial integrity initiates proliferation and migration of endothelial cells (ECs) from adjacent preexisting blood vessels, which promotes reendothelialization and neointimal formation of vascular lesions. The postnatal formation of new vessels occurs exclusively through proliferation and migration of existing neighboring ECs, referred to as angiogenesis. This traditional concept of blood vessel formation has been challenged by Asahara *et al* (1), who first described the cell population derived from bone marrow contributing to new vessel formation and termed these cells endothelial progenitor cells (EPCs). In contrast to angiogenesis, the formation of new blood vessels via mature ECs from proliferation and differentiation of bone marrow-derived progenitor cells is defined as vasculogenesis. Therefore, EPCs proposed as a promising treatment for vascular disease have been extensively studied by many investigators. However, due to the absence of specific surface markers, these progenitor cells represent heterogeneous cell populations including cells of myeloid or endothelial origin (2,3). Recently, three types of cells have been most studied as EPCs including colony-forming unit-Hill (CFU-Hill) cells, circulating angiogenic cells (CACs) and endothelial colony-forming cells (ECFCs). CFU-Hill cells and CACs are usually referred to as early outgrowth EPCs according to their obtention from short term blood sample culture. In contrast, ECFCs are termed as late outgrowth EPCs. Although the contribution of endothelial progenitor cells including early outgrowth EPCs and late outgrowth EPCs to new vessel formation has been established, the underlying mechanisms of bone marrow EPC-induced vascularization remain unclear, and need to be further studied.

2. Putative endothelial progenitor cells

Putative EPCs encompass different cell populations mainly containing hematopoietic and endothelial progenitor cells. These different populations result in a mixed ability to enhance the formation of new blood vessels (2,4). Recently, there are mainly three culture methods used to isolate EPCs.

The first method originally described by Asahara *et al* (1) has been modified (5,6), and can be performed using a commercially available kit. Mononuclear cells (MNCs) isolated from adult peripheral blood (PB) or cord blood (CB) are plated on fibronectin-coated tissue culture surfaces. After 48 h, the adherent macrophages and mature ECs in the sample are depleted, and the nonadherent colony cells are replated on fresh fibronectin-coated dishes. Over the next 5-9 days, adherent colonies emerge centrally, comprised of round cells with spindle-shaped cells sprouting at the periphery. These colonies are often referred to as colony-forming unit-Hill (CFU-Hill) or CFU-ECs. CFU-Hill cells have been shown to express the cell surface antigens CD31, CD105, CD144, CD146, vWF and KDR (VEGFR2). These cells also display the ability to ingest acetylated low-density lipoprotein and binding of the lectin Ulex europeus agglutinin-1 (UEA-1). These phonotypes and functions are often ascribed to ECs. CFU-Hill cells also express several monocyte/macrophage markers including CD14 and CD115 and hematopoietic-specific cell surface antigen CD45, and display non-specific esterase activity (1-3,7). Recently, evidence indicates that these progenitor cells originate from hematopoietic origins (3,8), and enhance new blood vessel formation by paracrine action (4,9).

Another widely used method to isolate putative EPCs involves the culture of PB MNCs in supplemented endothelial growth media for 4 days, and the nonadherent cells are washed away, thus an adherent cell target population remains (7,10). These adherent cells, referred to as CACs, have been shown to express the endothelial cell surface antigens, CD31, CD144, vWF and KDR, bind *Ulex Europaeus* selectin (7,10,11), and have been shown to uptake acetylated low-density lipoprotein (ac-LDL) (10). This particular method of CAC isolation and culture is contaminated by platelets, and these platelet membrane proteins may be transferred to the target cells by adhering to MNCs in the culture. CFU-Hill cells and CACs are also referred to as early outgrowth EPCs.

The third method of culture yields isolation and identification of ECFCs. In this method, adult PB MNCs or umbilical cord blood (UCB)-derived MNCs are collected and plated onto collagen I-coated plates in endothelial-specific growth media (3,12). Non-adherent cells are discarded during gentle washing steps. ECFC colonies emerge from the adherent cell population 10-21 days for PB and 5-7 for UCB after plating and display a cobblestone EC appearance (3,12-14). Some studies have demonstrated that ECFCs express the cell surface antigens, CD31, CD105, CD144, CD146, vWF and KDR and uptake ac-LDL (3,12). In contrast to CFU-Hill cells, ECFCs do not express the hematopoietic or monocyte/macrophage cell surface antigens, CD14, CD45 or CD115 (3), and exhibit robust capacity in proliferation and forming secondary endothelial cell colonies. Further studies showed that ECFCs possess the ability of forming capillary-like *in vitro* and perfused blood vessels *in vivo* (3,15).

The process of EPCs in the formation of new blood vessels include multiple steps in which many cytokines and modulators are involved. Although there has been a great deal of progress in this field, a comprehensive understanding of mechanisms by which EPCs participate in neovascularization will contribute to the development of effective therapies for vascular disorders.

3. Mobilization of endothelial progenitor cells

It has been indicated that levels EPCs in peripheral circulation are low under physiological conditions, and that these cells may reside in the bone marrow niche. The majority of EPCs are quiescent and tethered by integrins to stromal cells in a microenvironment within the bone marrow (16,17). The mobilization of EPCs from the bone marrow into the peripheral circulation is the crucial step for these cells to participate in postnatal vasculogenesis. The precise mechanism of EPC mobilization is not entirely elucidated and is still under investigation. It has been demonstrated that these cells can be converted into functional cells and released from the stem cell niche in response to various special cytokines and factors.

Vascular endothelial growth factor (VEGF), a pleiotropic cytokine, functions in neovascularization as an endothelial cell mitogen (18), chemotactic agent (19) and inducer of vascular permeability (20-22). Further studies have demonstrated that VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived EPCs (23), and VEGF gene transfer has been shown to augment circulating EPCs in human subjects (24). In burn injures, VEGF levels are elevated in the plasma and are responsible for the enhancement of mobilization of $VEGFR2^+$ EPCs in circulation (25). In addition, VEGF has the ability to upregulate the levels of granulocyte colony-stimulating factor (G-CSF) (26), which can induce the release of progenitor cells from bone marrow (27). The interaction between VEGF and VEGFR leads to the activation of bone marrow NOS, then produces nitric oxide (NO), which is responsible for MMP-9 activation. The activated MMP-9 contributes to the release of soluble kit ligand (sKitL) which enhances the mobility of $VEGFR2^+$ EPCs and stimulates the mobilization of these cells from bone marrow to the peripheral circulation (28).

The CXC chemokines, playing a role as significant regulators, have been implicated in the mobilization of bone marrow-derived stem or progenitor cells. Stromal cell-derived factor-1 (SDF-1) is the most characterized player in EPC mobilization and a potent chemokine in EPC adhesion and migration, therefore it exerts a beneficial effect on neovascularization. A range of stimuli such as inflammation and hypoxia account for upregulated levels of SDF-1 expressed in the extracellular matrix (29). Ischemic environments appear to promote the release of SDF-1, in proportion to the degree of hypoxia, with elevated intracellular SDF-1 mRNA expression in ischemic ECs (29). This occurs within the first hour of ischemia (30). The elevation of SDF-1 in plasma stimulates mobilization of $CXCR4^+$ bone marrow cells, including hematopoietic stem cells (HSCs) and EPCs (28,31). CXCR4 is a receptor of SDF-1 highly expressed by hematopoietic progenitor and endothelial progenitor cells (32,33). The interaction of SDF-1 and CXCR4 not only initiates the mobilization of EPCs from bone marrow, but stimulates the recruitment and retention of stem cells to

ischemic areas (17,34,35). SDF-1 can be secreted by platelets and induce chemotaxis of EPCs (36). EPCs themselves can also release SDF-1 in a paracrine fashion (37). The first response to vascular injury is the adhesion of platelets to the exposed subendothelium (38-41), which provides the signals of target for the mobilization and homing of stem cells to the damaged area (42). SDF-1α gene transfer promotes the mobilization of EPCs into the peripheral blood and enhances neovascularization in ischemic animal models. However, the effect of SDF-1 on the mobilization of EPCs is ablated in the absence of injury. Furthermore, blockade of VEGF or NOS signaling prevents all SDF-1-induced effects, indicating that VEGF/eNOS signaling is involved in the benefit from SDF-1 upregulation in neovascularization (43). Studies have shown that the mobilization of bone marrow-derived stem or progenitor cells requires the activation of bone marrow NOS, which are responsible for releasing progenitor cells from bone marrow via the NO-MMP-9-soluble kit ligand cascade (28,44). SDF-1 also enhances the mobilization of EPCs by upregulating the levels of VEGF which contribute to the release of EPCs from the bone marrow to the peripheral circulation. Interleukin-8 (IL-8) is an inflammatory chemokine originally described as a chemotactic factor for leukocytes. Recent data have implicated IL-8 as a regulater in mobilizing EPCs into the peripheral circulation by binding both CXCR1 and CXCR2. This function is synergized by G-CSF in animal models (45,46).

Nitric oxide (NO) initially discovered as an endothelium-derived relaxing factor plays a key role in regulating the physiological properties of blood vessels, including vasodilation, vascular permeability, and antithrombotic properties (47). NO also participates in maintaining vascular integrity and blood flow by modulating platelet-endothelial interactions (48). In addition to vasoprotective effects, NO is now recognized as a key determinant in EPC mobilization from the bone marrow into circulation resulting in enhancement of ischemic limb perfusion and wound healing (49,50). Production of NO relies on the conversion of L-arginine to L-citrulline catalyzed by nitric oxide synthases (51,52). The enzyme has four isoforms, NOS1 or neuronal nitric oxide synthase (nNOS), NOS2 or inducible nitric oxide synthase (iNOS), NOS3 or endothelial nitric oxide synthase (eNOS) and NOS4 or mitochondrial nitric oxide synthase (mtNOS). mtNOS recently identified appears to be a constitutively active eNOS-like isoform (53). Among these, eNOS selectively expressed in vascular endothelial cells and surrounding stromal cells plays a central role in vascular biology. NO has also been shown to be expressed by various EPC subtypes. eNOS plays a crucial role in regulation of mobilization and function of EPCs (44). Many patients with diabetes suffer from delayed or nonhealing wounds of the lower extremities and diabetic foot ulcers. Likely, EPCs which play a crucial role in postnatal neovascularization are impaired by hyperglycemia in diabetes. Hyperglycemia and diabetes are associated with impaired eNOS function which results in depressed EPC mobilization into circulation. The decreased levels of phosphorylated eNOS, but not the level of eNOS protein, was found to be responsible for impaired mobilization of EPCs (54). It is known postnatally that stem cells including EPCs reside in the bone marrow via adherence to stromal cells in the stem cell niche by integrins and can be released into the peripheral circulation in response to cytokines and other angiogenic factors (16,17). The mechanisms of EPC mobilization from bone marrow are still incompletely understood. The NO-MMP-9-sKitL-ckit cascade may play a central role in this process. eNOS can be stimulated in bone marrow by many cytokines involved in the event, then NO is produced. NO can stimulate MMP-9, which result in the release of sKitL from the stromal cell membrane-bound kit ligand (mKitL). c-Kit expressed by EPCs contributes to the retention of EPCs within the bone marrow niche. c-Kit is also the receptor for sKitL and can be released from bone marrow in response to binding to sKitL, resulting in mobilization of c-Kit$^+$ EPCs from the cell niche into circulation (Fig. 1). Certainly, the mechanisms associated with the mobilization of EPCs induced by these factors in the process of new blood vessel formation required further study.

4. Migration of endothelial progenitor cells

EPCs are released from bone marrow and recruited to sites of injury to enhance new blood vessel formation and wound healing. However, the signals that target EPCs to the sites of vascular injury are still poorly understood. Chemokines are important factors controlling cellular migration. The CXC chemokines have been involved in the field of vascularization because of their ability to modulate the functions of vascular endothelial cells and EPCs by increasing the migratory capacity of these cells to the sites of injured vessels. SDF-1 is the most potent chemoattractant of EPCs. The formation of SDF-1 concentration gradients from the periphery to ischemia plays a pivotal role in the migration of EPCs (Fig. 2). CXC receptor (CXCR4) is the predominant receptor for SDF-1 and is selectively expressed in vascular endothelial cells and EPCs (29,55). Studies have demonstrated that SDF-1 is able to track various types of CXCR4$^+$ cells (29,56,57). It is upregulated in ischemic tissue and acts as a homing signal for EPCs (58). The migration of EPCs is significantly reduced by anti-CXCR4 neutralizing antibodies (29). Further evidence suggest that SDF-1-induced EPC migration is mediated through the PI3K/Akt/eNOS signal transduction pathway (59). IL-6 is a multifunctional cytokine which possesses the ability to modulate cell proliferation and differentiation in physiological conditions (60-62). Studies indicate that IL-6 contributes to cerebral EC and smooth muscle cell (SMC) proliferation and migration *in vitro* (63-65). Furthermore, IL-6 *in vitro* was found to enhance EPC proliferation and migration in a dose-dependent manner. EPCs express IL-6 receptor (gp80 and gp130), and the molecular mechanism of EPC proliferation and migration is mediated by IL-6 via gp80/gp130 signaling pathways including downstream ERK1/2 and STAT-3 phosphorylation (66). The signal pathways widely exist in many cell types including EPCs. Activation of the ERK1/2 and STAT-3 pathways plays a crucial role in EC proliferation, migration and microvascular tube formation (65,67,68). This process may include the modulation of cytoskeletal reorganization in EPCs, which is associated with cell migration. Studies also demonstrate that activation of the CXCR1 is transient and characterized by stress fiber formation, whereas activation of CXCR2 is prolonged and associated with cell contraction (69). Additionally, pleiotrophin (PNT), produced under ischemic conditions, also chemotactically attracts early EPCs to sites of vessel injury (70).

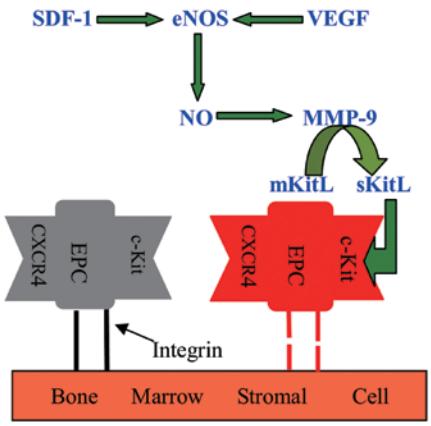

Figure 1. Mobilization of EPCs from bone marrow. Injured tissues release cytokines including VEGF and SDF-1. These cytokines interact with their respective receptors, which initiates the production of NO through activated eNOS. NO stimulates MMP-9, then soluble kit-ligand is released, leading to the release of EPCs from the bone marrow into circulation. SDF-1, stromal cell-derived factor-1; eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; VEGF, vascular endothelial growth factor; MMP-9, matrix metalloproteinase-9.

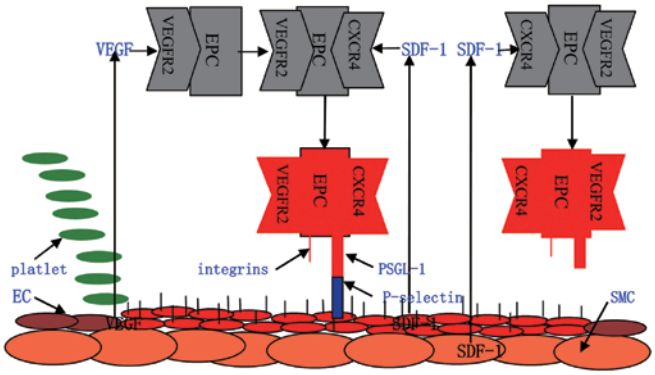

Figure 3. Adhesion of EPCs to the injured vessel wall. Within minutes after vessel injury, platelets aggregate on the exposed subendothelium. Adherent platelets express P-selectin on the surface and secrete high levels of SDF-1. In this process, circulating EPCs also upregulate PGSL-1 via the stimulation of SDF-1, which interact with their ligand P-selectin, thereby leading to EPC adhesion. Within the next hours and days after endothelial disruption, apoptotic SMCs mainly contribute to SDF-1 release, which is required to sustain the process of vascular remodeling and repair. EPCs, endothelial progenitor cells; SDF-1, stromal cell-derived factor-1; VEGFR2, vascular endothelial growth factor receptor 2; PSGL-1, P selectin glycoprotein ligand-1; EC, endothelial cell.

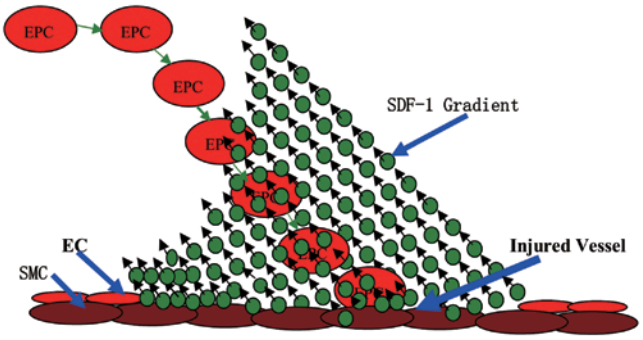

Figure 2. The migration of EPCs to the injury vessel. Disruption of endothelial integrity initiates the release of high levels of SDF-1, a critical chemoattractant for EPCs. After the release, a gradient between peripheral blood and impaired endothelium is established, which directs EPC migration along a low to high SDF-1 gradient. EPCs, endothelial progenitor cells; SDF-1, stromal cell-derived factor-1.

It is well known that cell mobility is closely associated with the cytoskeleton. Various cell activities, including migration, morphological change, and polarity formation are regulated by actin filament dynamics including actin filament disassembly and severing and reorganization. During cell migration, actin filaments are assembled to form the lamellipodial protrusion at the leading edge of the cell. Addition of actin monomers to the barbed ends of actin flaments at the tip of the lamellipodium provides the force with which to propel the membrane forward. These events are modulated by a variety of actin-binding proteins (71,72). As an important actin-binding protein, cofilin modulates actin filament assembly and disassembly, especially in stimulus-induced lamellipodium formation. Our group studied *in vitro* the mechanism of the impaired migration of EPCs induced by H_2O_2 using proteomic analysis, and indicated that the oxidative levels of actin and/or cofilin, not its number, are responsible for the attenuated ability of EPC migration (73). We hypothesized that modulators involved in the migration of EPCs activate actin-binding proteins which regulate the actin filament and result in the migration of EPCs into areas of peripheral ischemic or wound tissue. Further studies are needed to more specifically identify the underlying mechanism of EPC migration.

5. Endothelial progenitor cell adhesion

Accumulating data indicate that $CD34^+$ bone marrow-derived progenitor cells recruit and differentiate into ECs in the vascular intima in response to vessel injury (74-77). The mechanisms involved in the recruitment of EPCs from the circulation to adhesion to the sites of neovascularization, although incompletely understood, appear to mainly rely on the interaction between P selectin glycoprotein ligand-1 (PSGL-1) expressed on EPCs and P-selectin expressed on platelets (Fig. 3). Vascular injury initiates the adhesion of platelets to the exposed subendothelium (38-41). Upon adhesion, platelets become activated and express P-selectin and release SDF-1, which are both implicated in the adhesion of EPCs to the sites of vascular injury (42). SDF-1 released into the microenvironment of sites of vascular injury may play a crucial role in the modulation of this process via the SDF-1/CXCR4 pathway (58).The interaction of SDF-1 and CXCR4 upregulates the PSGL-1 expression on the surface of EPCs, which is the major ligand of P-selectin. The bonding contributes to the adhesion of EPCs to the sites of vessel injury and enhances their pro-angiogenic capacity. The ability of EPCs homing to the ischemic myocardium is impared by the inhibition of the SDF-1/CXCR4 pathway (78), and the adhesion of EPCs to the sites of injury is also significantly inhibited by CXCR4 blockade as noted in a model of hindlimb ischemia (32). Interestingly, SDF-1 30 min after vascular injury is highly expressed in the aggregated platelets at the sites of vessel foci, whereas 4 h after endothelial disruption, SDF-1 is abundantly expressed in both SMCs and the aggregated platelets (42). It seems that platelets constitute the initial and

presumably more short-lived source of SDF-1 that first directs EPCs to the site of vessel damage. In contrast, SMCs appear to account for the long-term SDF-1 release over the ensuing days and weeks after vessel injury required to sustain the process of vascular remodeling and repair (79,80). Integrins, a type of cell adhesion molecule, regulate cell adhesion and migration by interaction with the extracellular matrix. EPCs selectively express β_1- and β_2-integrins which contribute to the homing of these cells by strengthening adhesion to the damaged endothelial monolayer and by enhancing migration into the endothelial monolayer (81). High-mobility group box 1 (HMGB1) released into the extracellular space by necrotic cells also participates in EPC adhesion to ischemic areas via activation of β_1- and β_2-integrins (82). ECFCs, however, are not of hematopoietic origin and do not express β_2-integrins in contrast to early EPCs; the homing of these cells to the ischemic zone may be related to the expression of E-selectin under specific conditions (83). GPIIb-dependent platelet aggregation plays a critical role in the recruitment of EPCs at sites of vascular injury. GPIIb may promote the homing of EPCs into vascular foci by forming a link between platelets and EPCs using a GPIIb-dependent bridging mechanism previously reported to occur during platelet-EC interactions (84). Furthermore, platelets aggregated by GPIIb can form a cross-linking structure in which PCs get trapped (42). In addition, α_4-integrin seems to participate in circulating progenitor cell homing to the sites of neovascularization and improves blood perfusion in impaired tissue (85,86).

6. Endothelial progenitor cells in new blood vessel formation

The contribution of EPCs to vascularization has been demonstrated in animal models and in humans. Infusion of EPCs, capillary density and new vessel formation are augmented in impaired tissues. In ischemic animal models, injection of EPCs was found to significantly enhance neovascularization in the sites of foci thus improving blood flow and injured tissue recovery (87-90). In human trials, transplantation of EPCs generated a trend towards functional improvement in patients with acute myocardial infarction (91,92). These studies indicate that EPCs play a pivotal role in new blood vessel formation. However, putative EPCs and their role in neovascularization remain controversial. These controversies may be due to discrepancies in their identification and the complex mechanisms involved in EPC-induced neovascularization. Putative EPCs encompass different cell populations including hematopoietic or endothelial origin. Accumulating data suggest that hematopoietic cells facilitate new blood vessel formation without direct incorporation into the endothelial intima in the process of postnatal vasculogenesis, and mainly depend on humoral and cell-mediated support functions (93-96). In contrast, the contribution of late outgrowth endothelial cells (ECFCs) to neovascularization may combine the incorporation into newly formed vessels and the release of pro-angiogenic factors in a paracrine manner, although there is less direct evidence showing that vascular neointimal formation occurs via mature ECs from proliferation and differentiation of EPCs. Studies have shown that EPCs are essential in wound healing by facilitating new vessel formation (54,97). Delayed wound healing in diabetes results from impaired eNOS activition and hence reduces mobilization of EPCs from the bone marrow into circulation, which can be reversed by the treatment of hyperoxia. However, increased levels of circulating EPCs induced by hyperoxia fail to enhance wound healing. One possible explanation is that the downregulated production of SDF-1 in local wound lesions in diabetes is responsible for the ablated effects. Administration of SDF-1 into wounds improves efficiency of EPC migration consequently enhancing nevascularization and wound healing (54), indicating that thorough understanding of the mechanisms of EPC-mediated new vessel formation are required. These unanswered questions need to be further elucidated.

In conclusion, the contribution of EPCs to neovascularization provides a promising therapeutic target for treating patients with vascular disorders. However, the putative EPCs are heterogeneous cell populations and demonstrate a mixed capacity of contribution to vessel formation. Furthermore, vasculogenesis is a complex process including EPC mobilization from the bone marrow, circulation in blood vessels, recruitment to the impaired sites, adhesion to the injured vessel intimal and exertion effect on new blood formation, which are controlled by multiple cytokines and modulators via different mechanisms, a better understanding of which will elucidate the effect of EPCs in neovascularization and eventually lead to the development of an efficient approach for treating patients with vascular disease.

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