

Endothelial Progenitor Cells in Diabetic Vasculopathy

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Abstract

BACKGROUND: The discovery of endothelial progenitor cell (EPC) a decade ago by Asahara et al has refuted the previous belief that vasculogenesis only occurs during embryogenesis. The reduced circulating concentration of EPCs is a surrogate marker of endothelial function and has been implicated in the pathogenesis of many vascular diseases.

CONTENT: Diabetes is linked to impaired vascular function, including alterations in both endothelial cells and EPCs. A number of studies have shown that individuals with diabetes have decreased level of circulating EPCs and that the severity of disease is inversely proportional to EPC levels. In vitro, hyperglycemia increases the rate of EPC senescence and the angiogenic function of EPCs from patients with either type 1 or type 2 diabetes is impaired such that they are poorly proliferative and fail to incorporate into forming vessel-like structures.

Given the comprehensive role of EPC alterations in diabetes complications, modulation of the levels and/or function of EPCs may be considered a potential therapeutic strategy.

SUMMARY: The available data demonstrating that decrease or dysfunction of EPCs may have a prominent role in the pathogenesis of all diabetes complications. Further approaches, such as EPC administration, may represent novel treatments for diabetic vasculopathy

in the future. To date, many barriers remain to such a therapeutic approach. Firstly, there is no specific marker for EPC at present. Secondly, techniques of EPC isolation are not standardized, preventing direct comparison between various studies. The long-term effects of transplanted EPCs are currently unknown.

Introduction

EPCs can promote postnatal vasculogenesis by homing to, differentiating, proliferating and incorporating into new vessels. EPCs may play a role in this process by physically incorporating or may influence neighbouring cells in a paracrine fashion. These normal roles of EPCs may explain why these cells have been implicated in the pathogenesis of many vascular disease states such as ischaemia, restenosis and pulmonary hypertension (1-6). EPC number and function can be affected by various cardiovascular risk factors such as cigarette smoking, hypertension and hyperlipidaemia (7-9). In fact, Hill et al. have demonstrated that EPC number may be a surrogate biologic marker for vascular function and cumulative cardiovascular risk in healthy men (10). Schmidt-Lucke et al. and Werner et al. later showed that the number of circulating EPCs can predict the occurrence of cardiovascular events and death from cardiovascular causes and may help to identify patients at increased cardiovascular risk (11,12). EPC dysfunction has also been observed in the high cardiovascular risk state of diabetes mellitus (DM).

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The impaired neovascularization found in the diabetic setting may be explained by the inability of diabetic EPCs to respond to stromal-derived factor-1 (SDF-1), which plays a crucial role in EPC homing (13). Dysfunctional EPCs in the diabetic setting are also associated with impaired re-endothelialization (14). Furthermore, EPC mobilization was also impaired after ischaemic reperfusion injury in a streptozotocin induced diabetic mouse model, a finding that was associated with altered SDF-1 and VEGF release as well as impaired hypoxia inducible factor-1a upregulation (15). These *in vivo* animal studies demonstrated that diabetes is associated with impaired EPC mobilization, differentiation and ability to form tubules.

The administration of both autologous and heterologous unfractionated BM cells, which contain EPCs, has produced dramatic improvement in neovascularization in various ischaemic states (16-26). However, the results of autologous EPC transplantation in the diabetic setting may not be as promising. As discussed above, in patients with DM, EPC numbers are lower and their function is impaired.

Diabetic Complications

Diabetes complications represent a huge burden for patients and health services. The fight against each single complication has led to significant improvements in diabetes care, especially for microvascular complications, yet macroangiopathy remains a major source of morbidity and mortality. A common approach for the prevention and treatment of diabetes complications relies on the understanding of their complex pathophysiology (27).

While it was originally thought that a single patient tends to develop the cluster of micro- or macrovascular complications, recent prospective studies show that typical markers of microvascular dysfunction, such as microalbuminuria or retinal vascular abnormalities, are associated with an increased risk of macrovascular events (28,29). These and other data suggest that there must be a unifying pathogenetic model underlying diabetes complications. To date, the most credited and supported model proposes that oxidative stress originating from mitochondria activates all subcellular damage pathways (30). However, subsequent events diverge for each complication, and there is not a supracellular unifying hypothesis. The discovery that a subset of circulating immature cells contributes to

vascular homeostasis has been a major achievement in many fields of basic science.

Endothelial progenitor cells (EPCs) are circulating immature cells that contribute to vascular homeostasis and compensatory angiogenesis. During the last decade, data have become available indicating that alterations in EPCs may have an important causative role in the development and progression of virtually all diabetes complications (27).

Endothelial Progenitor Cells (EPCs)

EPCs were discovered in 1997 as circulating cells with the ability to differentiate into mature endothelium and take part in neoangiogenesis (1). EPCs share markers of hematopoietic (CD34 and CD133) and endothelial (KDR, CD31, and vWf) lineages (31), are derived from bone marrow, and can be mobilized to the peripheral circulation in response to many stimuli (32). Tissue ischemia, through the release of growth factors and cytokines, mobilizes EPCs, which, once in the peripheral circulation, specifically home on the ischemic sites to stimulate compensatory angiogenesis (33). Moreover, EPCs constitute a circulating pool of cells able to form a cellular patch at sites of endothelial injury, thus contributing directly to the homeostasis and repair of the endothelial layer. Taken together, these observations suggest that EPCs have a major role in cardiovascular biology; in fact, the extent of the circulating EPC pool is now considered a mirror of cardiovascular health. Virtually all risk factors for atherosclerosis have been associated with decrease and/or dysfunction of circulating EPCs (34), while an expanded EPC pool is associated with a decreased cardiovascular mortality (11).

According to the initial discovery, EPCs or CEPCs (Circulating bone marrow – derived endothelial progenitor cells) were defined as cells positive for both hematopoietic stem cell markers such as CD34 and an endothelial marker protein as VEGFR2. Because CD34 is not exclusively expressed on hematopoietic stem cells but, albeit at a lower level, also on mature endothelial cells, further studies used the more immature hematopoietic stem cell marker CD133+ and demonstrated that purified CD133+ cells can differentiate to endothelial cells *in vitro* (35). CD133, also known as prominin or AC133, is a highly conserved antigen with unknown biological activity, which is expressed on hematopoietic stem cells but is

absent on mature endothelial cells and monocytic cells (36). Thus, CD133+VEGFR2+ cells more likely reflect immature progenitor cells, whereas CD34+VEGFR2+ may also represent shedded cells of the vessel wall. At present, it is unclear whether CD133 only represents a surface marker or has a functional activity involved in regulation of neovascularization. Overall, controversy exists with respect to the identification and the origin of endothelial progenitor cells, which are isolated from peripheral blood mononuclear cells by cultivation in medium favoring endothelial differentiation. In peripheral blood mononuclear cells, several possible sources for endothelial cells may exist: (1) the rare number of hematopoietic stem cells, (2) myeloid cells, which may differentiate to endothelial cells under the cultivation selection pressure, (3) other circulating progenitor cells (eg, "side population" cells), and (4) circulating mature endothelial cells, which are shed off the vessel wall (37) and adhere to the culture dishes (38).

A combination of CD34, kinase insert domain-containing receptor (KDR) and CD133 are commonly used to define EPCs at present. The use of fluorescence-activated cell sorter (FACS) analysis to define EPCs appears to be the most optimal approach but may be technically challenging and the best combination of the 'true' markers for EPCs is currently unknown (39).

The study of EPC biology consists of two related aspects: quantitative evaluation of the EPC pool and functional assessment. Circulating EPCs can be quantified directly *ex vivo* using flow cytometry, which is considered the gold standard for this purpose (40); typical surface antigens to identify EPCs are CD34, CD133, and KDR. Functional characteristics are explored *in vitro* using standardized protocols (38). Proliferation refers to the ability of EPCs to expand and form colonies in culture: EPCs should proliferate in response to growth factors released locally after vascular damage or tissue ischemia. Adhesion is a further step required for both reendothelization and angiogenesis; it is assessed as the ability of EPCs to adhere to a monolayer of mature endothelium in culture. Migration of EPCs through the extracellular matrix is crucial for the growth of new vessels and is generally assessed *in vitro* as the ability to invade the lower side of a Boyden-like chamber. Finally, after EPCs have adhered to the vessel wall, migrated into the interstitium, and expanded locally, they should spatially organize to form vascular structures; this property can be assessed *in vitro* as a tube formation assay in which EPCs are seeded with human umbilical

vein endothelial cells on a gel of extracellular matrix proteins.

Two types of EPC had different morphology, proliferation rates, and survival behaviors. They also had different gene expression profiles, leading to different function *in vitro*. Despite such differences in gene expression and *in vitro* function, they equally contributed to neovasculogenesis *in vivo* in that early EPC secreted angiogenic cytokines, whereas late EPC supplied a sufficient number of endothelial cells (41).

Late EPC was different from early EPC in the expression of VE-cadherin, Flt-1, KDR, and CD45. Late EPC produced more nitric oxide, incorporated more readily into human umbilical vein endothelial cells monolayer, and formed capillary tube better than early EPC. Early EPC secreted angiogenic cytokines (vascular endothelial growth factor, interleukin 8) more so than late EPC during culture *in vitro*. Both types of EPC showed comparable *in vivo* vasculogenic capacity (41).

Distinct origins of the different types of EPCs exist that have different functions in neovascularization. Mixed transplantation of these cells results in synergistic neovascularization through cytokines and MMPs (42).

EPCs in Neovascularization

Improvement of neovascularization is a therapeutic option to rescue tissue from critical ischemia (43). The finding that bone marrow-derived cells can home to sites of ischemia and express endothelial marker proteins has challenged the use of isolated hematopoietic stem cells or EPCs for therapeutic vasculogenesis.

However, the number of incorporated cells with an endothelial phenotype into ischemic tissues is generally quite low. How can such a small number of cells increase neovascularization? A possible explanation might be that the efficiency of neovascularization may not solely be attributable to the incorporation of EPCs in newly formed vessels, but may also be influenced by the release of proangiogenic factors in a paracrine manner (38).

Thus, EPCs may act similar to monocytes/macrophages, which can increase arteriogenesis by providing cytokines and growth factors. Indeed, EPCs cultivated from different sources showed a marked

expression of growth factors such as VEGF, HGF, and IGF-1. Moreover, adherent monocytic cells, which were cultivated under similar conditions, but do not express endothelial marker proteins, also release VEGF, HGF, and G-CSF (44). The release of growth factors in turn may influence the classical process of angiogenesis, namely the proliferation and migration as well as survival of mature endothelial cells (45). However, EPCs additionally incorporated into the newly formed vessel structures and showed endothelial marker protein expression *in vivo*.

EPCs in Endothelial Regeneration

In the past, the regeneration of injured endothelium has been attributed to the migration and proliferation of neighboring endothelial cells. More recent studies, however, indicate that additional repair mechanisms may exist to replace denuded or injured arteries.

Bone marrow transplantation experiments revealed that bone marrow-derived cells can contribute to reendothelialization of grafts and denuded arteries (46-48). However, in a model of transplant arteriosclerosis, bone marrow-derived cells appear to contribute only to a minor extent to endothelial regeneration by circulating cells (49). These data again indicate that there might be at least two distinct populations of circulating cells that principally are

capable to contribute to reendothelialization, namely mobilized cells from bone marrow and non-bone marrow-derived cells. The latter ones may arise from circulating progenitor cells released by non-bone marrow sources (eg, tissue resident stem cells) or represent vessel wall-derived endothelial cells (49, 46-48).

A rapid regeneration of the endothelial monolayer may prevent restenosis development by endothelial synthesis of antiproliferative mediators such as nitric oxide. Indeed, enhanced incorporation of β -galactosidase-positive, bone marrow-derived cells was associated with an accelerated reendothelialization and reduction of restenosis (46,47). Similar results were reported by Griese *et al*, who demonstrated that infused peripheral blood monocyte-derived EPC home to bioprosthetic grafts and to balloon-injured carotid arteries, the latter being associated with a significant reduction in neointima deposition (50). Likewise, infusion of bone marrow-derived CD34-/CD14+ mononuclear cells, which are not representing the population of the "classical hemangioblast," contributed to endothelial regeneration (51). The regenerated endothelium was functionally active as shown by the release of NO (51), which is supposed to be one of the major vasculoprotective mechanisms. Consistently, neointima development was significantly reduced after cell infusion (51). Whereas the regeneration of the endothelium by EPCs protects lesion formation, bone marrow-derived stem/progenitor cells may also contribute to plaque

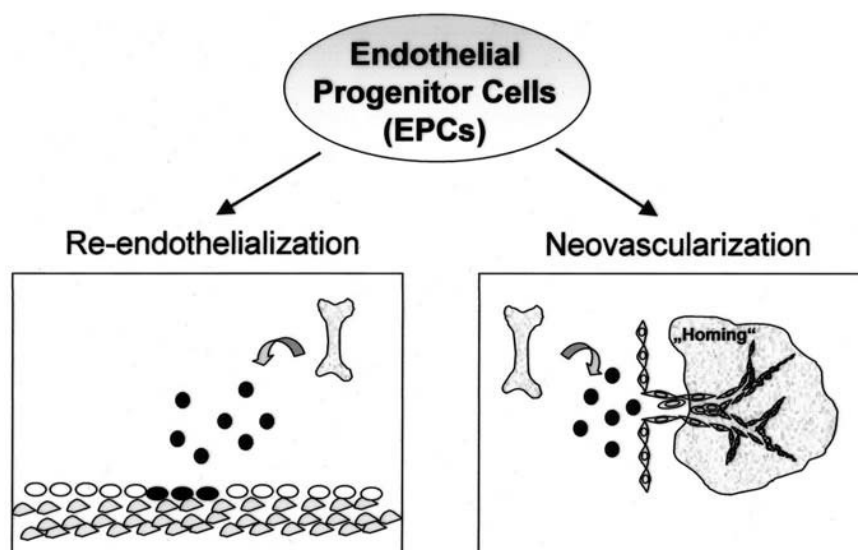


Figure 1. Role of EPCs in vascular biology. Reprinted by permission from Wolter Kluwer Health, *Circ.Res.*, Copyright (2004).

angiogenesis, thereby potentially facilitating plaque instability (52). However, in a recent study, no influence of bone marrow cell infusion on plaque composition was detected in nonischemic mice (53). An increase in plaque size was only detected in the presence of ischemia, suggesting that ischemia-induced release of growth factors predominantly accounts for this effect (53).

Overall, these studies implicate that regardless of the origin of circulating endothelial progenitor cells, this pool of circulating endothelial cells may exert an important function as an endogenous repair mechanism to maintain the integrity of the endothelial monolayer by replacing denuded parts of the artery (Figure 1). One can speculate that these cells may also regenerate the low grade endothelial damage

by ongoing induction of endothelial cell apoptosis induced by risk factors for coronary artery disease (54).

Moreover, various risk factors for coronary artery disease, such as diabetes, hypercholesterolemia, hypertension, and smoking, affect the number and functional activity of EPCs in healthy volunteers (10) and in patients with coronary artery disease (55).

In addition, factors that reduce cardiovascular risk such as statins (56,46,47,57) or exercise (58) elevate EPC levels, which contribute to enhanced endothelial repair. The balance of atheroprotective and proatherosclerotic factors, thus, may influence EPC levels and subsequently reendothelialization capacity.

EPCs Mobilization Differentiation and Homing

The release of EPCs from the bone marrow is regulated by a variety of growth factors, enzymes, ligands, and surface receptors. Activation of matrix metalloproteinase-9, which promotes the transformation of membrane-bound Kit ligand to a soluble Kit ligand and the subsequent move of cKit-positive stem and progenitor cells, including a common hematopoietic and angioblast precursor (hemangioblast), to the vascular zone of the bone marrow microenvironment, are initial steps in this complex process (59). The signals, which initiate the diversion of the hemangioblast to either hematopoietic precursor cells or EPCs, are largely unknown at present but may include angiogenic growth factors from the periphery.

To date, no clear definition exists as to when an endothelial progenitor cell turns into a mature, fully differentiated endothelial cell *in vivo*. One possibility could be the loss of CD133 and a parallel or subsequent expression of von Willebrand factor in conjunction with the appearance of other endothelial characteristics. The starting point of this differentiation process may be the migration of EPCs from the bone marrow to the systemic circulation. After homing, *ie*, after adhesion and insertion into the monolayer of surrounding mature vascular ECs, this process may be completed (60).

Recruitment and incorporation of EPCs requires a coordinated sequence of multistep adhesive and signaling events including chemoattraction, adhesion,

and transmigration, and finally the differentiation to endothelial cells (Figure 2) (38).

The local bone marrow microenvironment, the so-called stem cell niche consisting of fibroblasts, osteoblasts, and endothelial cells, governs the maintenance and mobilization of bone marrow stem cells (61,62,63). Mechanistically, cytokines inducing mobilization interfere with the interactions between stem cells and bone marrow stromal cells, which allow stem cells to disengage the bone marrow, and to pass through the sinusoidal endothelium to enter the blood stream. Stem cell mobilization is mediated by proteinases such as elastase, cathepsin G, and matrix metalloproteinases (MMPs) (64). A cytokine clinically used for the mobilization of CD34+ cells in patients is G-CSF, which releases the proteinases elastase and cathepsin G from neutrophils. These proteinases induce cleavage of adhesive bonds on stromal cells, which interact with integrins on hematopoietic stem cells (65). Moreover, these proteinases cleave the cytokine SDF-1, which is released by stromal cells and its receptor CXCR4 on stem and progenitor cells (66). Stem cell mobilization as a result of high levels of circulating SDF-1 appears to reverse the SDF-1 gradient across the bone marrow barrier, forcing CXCR-4+ cells to exit the bone marrow (67). However, VEGF, SDF-1, and placental growth factor (PIGF)-induced stem cell mobilization was shown to rely on MMP-9 (59,68). When PIGF is administered in the early phase of bone marrow recovery, it is chemoattractive

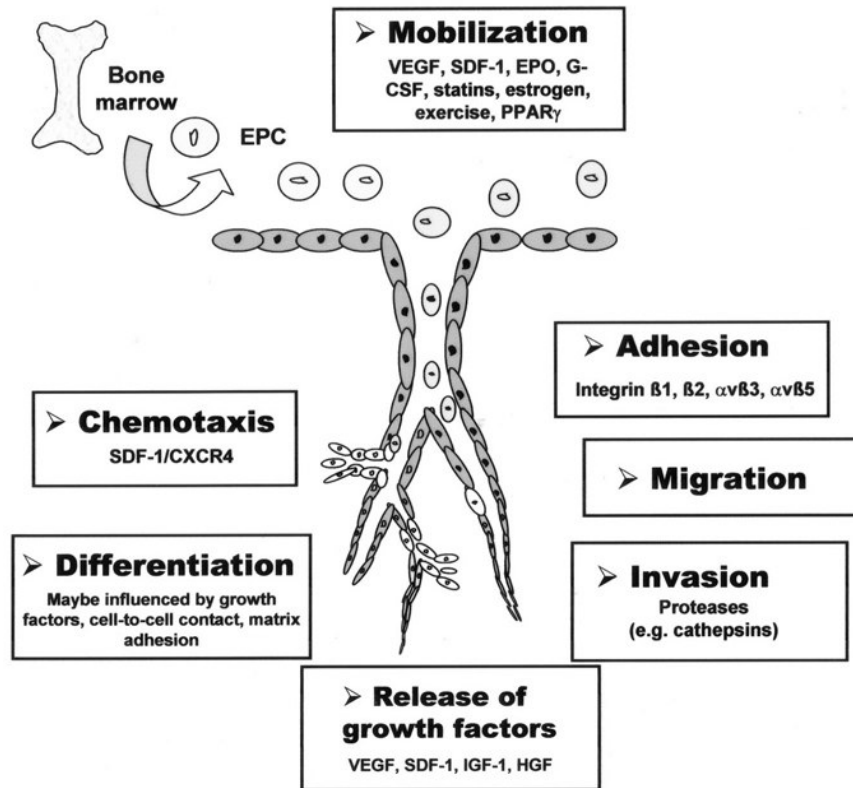


Figure 2. Mechanism of EPC homing and differentiation. Reprinted by permission from Wolters Kluwer Health, *Circ. Res.*, Copyright (2004).

for VEGF-receptor-1⁺ stem cells, whereas in later stages PIGF functions are mediated by MMP-9 (68). Thus, increasing the local concentration of MMP-9 in the bone marrow cleaves membrane bound Kit ligand (mKitL) and, finally, releases soluble Kit ligand (KitL; also known as stem cell factor) (59). After all, this process transfers endothelial and hematopoietic progenitor cells from the quiescent to the proliferative niche. However, the question of whether G-CSF-induced stem cell mobilization depends on MMP-9 is still a matter of debate (59,69,70). This controversy might be explained by the fact that MMP-9 plays a pivotal role in growth factor-induced hematopoietic progenitor mobilization in wild-type animals, whereas compensatory upregulation of enzymes with a similar activity profile to MMP-9 might mask the impact of MMP-9 deficiency in the knockout model. As discussed, eNOS is essential to maintain adequate progenitor cell mobilization in response to distinct stimuli, including VEGF, statins, exercise, and estrogen, in the regulation of stem and progenitor cell mobilization (58,62,71,72). The defective mobilization was caused by the lack of eNOS (Nos3) provided by the bone marrow stromal microenvironment.

Therefore, eNOS deficiency in the bone marrow microenvironment impaired the mobilization of stem and progenitor cells from the bone marrow. In contrast, intravenous injection of stem and progenitor cells circumvented the defective mobilization from the bone marrow and improved the neovascularization after induction of hind limb ischemia. Therefore, eNOS-derived NO is a physiological regulator of stem and progenitor cell mobilization in the bone marrow stromal microenvironment.

Given that patients with coronary artery disease showed a diminished NO bioavailability in peripheral endothelial cells, one may speculate that this also translates into the bone marrow. EPC numbers are lower in patients with coronary artery disease or diabetes (55,74) and are correlated with NO-dependent vasorelaxation measured in the forearm (10).

Hypoxia, caused by an imbalance between oxygen supply and consumption, stimulates vasculogenesis in embryonic and adult tissue (75-79). Similarly, hypoxia, and the subsequent alteration of HIF-1, can induce VEGF and SDF-1, which stimulates migration of endothelial precursors during vasculogenesis (78,80). Therefore, it can be suggested that hypoxia

in embryonic and adult target tissue leads to an expression of chemotactic factors inducing migration of angioblasts and EPCs toward the region to be vascularized by vasculogenesis.

SDF-1 gene expression is regulated by the transcription factor hypoxia-inducible factor-1 in endothelial cells, resulting in selective in vivo expression of SDF-1 in ischemic tissue in direct proportion to reduced oxygen tension. HIF-1-induced SDF-1 expression increases the adhesion, migration and homing of circulating CXCR4-positive progenitor cells to ischemic tissue.

These data show that the recruitment of CXCR4-positive progenitor cells to regenerating tissues is mediated by hypoxic gradients via HIF-1-induced expression of SDF-1 (80).

In brief, the directed migration of angioblasts is regulated by a combination of different soluble and interaction molecules that build overlapping attractive and repulsive gradients of soluble factors forming the migratory route for the endothelial precursor cells. It can be suggested that the correct positioning of the endothelial cells after they start to migrate toward the target tissue is dependent on a fine balance of signals. Soluble factor from regions that should be vascularized diffuse to the endothelial precursors, attract them and keep them in their migratory phenotype state until they reach their final position (81).

Vascular endothelial growth factor caused the phosphorylation of AMPK, acetyl-coenzyme A (CoA) carboxylase (ACC), and eNOS in human cord blood-derived EPCs. The expression of EC markers, including VE-cadherin and intercellular adhesion molecule1 (ICAM-1), was also increased but blocked by Compound C, an AMPK inhibitor. AICAR, an AMPK agonist, increased the phosphorylation of ACC and eNOS and the expression of EC markers in a time- and dose-dependent manner, which reinforces the positive effect of AMPK on EPC differentiation.

The activation of eNOS by AMPK during EPC differentiation provides a novel mechanism for the pleiotropic effects of statins in benefiting the cardiovascular system (82).

There is accumulating evidence that platelets mediate the effect of hematopoietic cytokines to recruit bone marrow-derived cells to the vasculogenic niche. Thrombopoietic cytokines thrombopoietin (TPO) and stem cell factor (SCF) are significantly elevated shortly after ischemic tissue injury, and transgenic mice deficient in TPO or the TPO receptor (c-MPL) demonstrate significantly impaired hind - limb

revascularization and inhibited angiogenic tumor growth (83).

Aggregation of activated platelets was necessary for the recruitment of bone marrow-derived c-Kit+ ScaI+ Lin- progenitor cells and CD34+ cells to sites of endothelial disruption (84). Hematopoietic cells were unable to adhere directly to the exposed extracellular matrix, but were

tethered to platelet surface P-selectin via P-selectin glycoprotein ligand (PSGL)-1 binding. Platelet release of SDF-1 provided an ongoing retention signal for bone marrow-derived cells at these sites (84). The mechanism by which platelets release SDF-1, VEGF and other growth factors in a site-specific fashion is likely to involve glycoprotein (GP) IIb- dependent platelet aggregation (84), inward receptor signaling, and MMP-9-mediated SDF-1 release (83). In addition to platelet-derived SDF-1, exposed smooth muscle cells also produce SDF-1 after arterial injury, and platelet activation via CXCR4 signaling leads to P-selection upregulation, further promoting arrest of bone marrow-derived cells to activated endothelium (85).

Taken together, these data suggest that aggregation and activation of platelets at sites of exposed subendothelium and vasculogenesis play a major role in the recruitment, differentiation, and incorporation of bone marrow-derived progenitor cells.

These insights have profound clinical implications; inhibition of platelet-deployed growth factors or their receptors may be an effective strategy to block tumor growth, whereas activation of these pathways may be used to accelerate revascularization and tissue regeneration (86).

Endothelial progenitor cells (EPCs) and hematopoietic progenitor cells are recruited to ischemic regions, improving neovascularization. $\beta 1$ and $\beta 2$ integrins play a crucial role for progenitor cell homing to ischemic tissues. Integrin activity is regulated by chemokines and their respective G protein- coupled receptors. The phosphatidylinositol-3-kinase catalytic subunit γ (PI3K γ) is the PI3K isoform that selectively transduces signals from G protein-coupled receptors. A pharmacological PI3K γ inhibitor significantly reduced chemokine - induced chemotaxis and stromal cell-derived factor (SDF)1 α -induced transmigration of human EPCs. Moreover, the PI3K γ inhibitor significantly reduced SDF1 α -induced adhesion of EPCs to intercellular adhesion molecule-1 and human umbilical vein endothelial cell monolayers (87,88).

Adverse effects of EPC mobilization have been described as contribution of EPCs to tumor neovascularization in some tumor models (89). Moreover, circulating progenitor cells have been implicated in the neovascularization of arteriosclerotic lesions of allografts and in further atherosclerotic plaque progression in an ischemic setting (52,53). However, transfusion of EPCs enhanced re-endothelialization

and reduced neointima formation after vascular injury (47). One may speculate that the endothelial repair capacity might override the potential harmful effects of plaque neovascularization. Thus, future studies have to determine the overall influence of EPC levels on atherosclerotic disease progression and prognosis (32).

Cardiovascular Risk Factors and EPCs

Small clinical studies have shown that the number of circulating EPCs inversely correlates with risk factors for atherosclerosis (10,55). Circulating CD34/KDR-positive progenitor cells are reduced to ~50% in patients with CAD compared with control groups. In addition, EPCs isolated from patients with CAD displayed an impaired migratory response, which was inversely correlated with the number of cardiovascular risk factors (55).

In patients with arterial hypertension, systolic blood pressure negatively correlates with the number of circulating CD133+ and CD34+/KDR+ EPCs, whereas the clonogenic potential (number of colony forming units-ECs) is not impaired by arterial hypertension (55). Experimental data demonstrate that angiotensin II, a potent mediator of detrimental effects in arterial hypertension, can accelerate the onset of EPC senescence by gp91 phox - mediated increase in oxidative stress, leading to an impaired proliferation activity of EPCs. Angiotensin II-induced EPC senescence was inhibited by treatment with the angiotensin II type 1 receptor blocker valsartan (90).

Recent studies have underlined the detrimental effects of types 1 and 2 diabetes on EPC function (74,91). Tepper et al demonstrated that in type 2 diabetes proliferation capacity of EPCs was reduced, adhesion capacity on activated human ECs was impaired.

Hypercholesterolemia was associated with reduced EPC numbers in 20 age-matched patients with hypercholesterolemia (8). Proliferative capacity, migratory activity, and in vitro vasculogenesis were negatively influenced by hypercholesterolemia. The underlying mechanisms are probably an increased rate of EPC senescence/apoptosis, as demonstrated after incubation of EPCs with oxidized LDL (92).

A role for HDL in promoting the repair of injured endothelium by stimulating the recruitment of

endothelial progenitor cells (EPCs) into the endothelial layer (93).

Smoking has been identified as an important risk factor for reduced EPC numbers in one of the first studies on cardiovascular risk factors by Vasa et al (55). However, Wang et al recently demonstrated that the role of nicotine is more complex than initially expected (94). In an experimental study, they demonstrated that low concentrations of nicotine (10-8-10-12 mol/L) increased EPC number and activity, whereas higher (toxic) concentrations (>10-6 mol/L) were associated with cytotoxicity. In humans, Kondo et al demonstrated that chronic smokers (n=15) exhibit reduced EPC levels that can be restored after smoking cessation within 4 weeks (9).

In humans, a significant increase in progenitor cell numbers was observed in patients who resumed a standardized physical activity during a rehabilitation program (58), in patients with CAD (95), and in healthy individuals exercising for ≥30 minutes (96).

CRP, at concentrations known to predict adverse vascular outcomes, directly inhibits EPC differentiation, survival, and function, key components of angiogenesis and the response to chronic ischemia. This occurs in part via an effect of CRP to reduce EPC eNOS expression. The PPAR γ agonist rosiglitazone inhibits the negative effects of CRP on EPC biology. The ability of CRP to inhibit EPC differentiation and survival may represent an important mechanism that further links inflammation to cardiovascular disease (97).

To contribute to tissue repair, EPCs, and stem cells in general, have to be equipped with antioxidative defense systems to survive in necrotic and ischemic tissues. Interestingly, a high resistance to oxidative stress has been considered a characteristic feature of stem cells (98,99).

He et al demonstrate that human EPCs possess a unique property to withstand oxidative injury and that elevated expression of manganese superoxide dismutase (MnSOD, a mitochondria-located SOD) is a critical intrinsic mechanism protecting EPCs against oxidative stress (100).

The finding that GPx-1 expression is essential for EPC functions may also have clinical implications, given that patients with chronic heart failure (101) and with type 2 diabetes (102) showed a downregulation of GPx-1. This in turn may contribute to the reduced EPC numbers and functions in patients with coronary artery disease and severe heart failure (55,103). However, other antioxidative enzymes such as superoxide dismutases and catalases are also downregulated in these patients (101,102). Thus, it seems mandatory to understand the specific role of the various antioxidative enzymes for EPC functions. In the future, a specific interference with the expression and activity of antioxidative enzymes in progenitor cells from patients might be a therapeutic strategy to improve their regenerative potential (104).

Schroeter et al (105) have now demonstrated that infusion of leptin-stimulated human EPCs reduces neointima formation and enhanced reendothelialization through upregulation of $\alpha v\beta 5$ - and $\alpha 4$ -integrin-dependent adhesion to platelets in ferric chloride-induced vascular injury. With this elegant approach, the effect of leptin on EPC-mediated endothelial recovery can be readily discerned from leptin-enhanced thrombosis. This not only extends the mechanisms by which platelet-derived chemokines, P-selectin, and $\beta 2$ integrins can support the arrest of EPCs and CD34+ progenitor cells at sites of arterial injury (85,106) but also adds a new dimension to the functional profile of leptin.

PPAR- δ activation with agonist (GW501516 or L-165041) increased the proliferation of human EPCs and protected them from hypoxia-induced apoptosis. In addition, PPAR- δ activation enhanced EPC functions, such as transendothelial migration, and tube formation. These actions by PPAR- δ activation in EPCs were dependent on the phosphatidylinositol 3-kinase/Akt pathway (107).

What are the mechanisms by which risk factors mediate their negative effect on progenitor cells? It appears that the signaling pathways mediating progenitor cell impairment are similar to the previously identified regulators of endothelial cell function and

atherosclerosis and include a dysregulation of nitric oxide (NO) and reactive oxygen species (ROS). A diabetic environment or high glucose exposure in vitro is associated with reduced nitric oxide (NO) bioavailability in cultured EPCs, and plasma levels of endogenous NO – synthase inhibitors (asymmetrical dimethyl-L-arginine) are associated with clinically reduced EPC numbers (108). In experimental studies, endothelial NO synthase (eNOS)-derived NO was shown to be essential for basal, VEGF-, and SDF-1-induced migration of EPCs or bone marrow-derived progenitor cells, and eNOS-/- progenitor cells showed a reduced homing capacity in ischemia models in vivo (109,110). The underlying mechanisms mediating the NO effects, eg, on chemokines or integrin expression or signaling, need to be further defined. Despite various studies supporting an important role of eNOS for progenitor cell mobilization and function (58,71,109), under certain conditions, eNOS uncoupling may lead to an increased ROS production. To what extent the redox balance in stem cells (as opposed to cultured EPCs or crude bone marrow homogenates used in the study(111) favors such uncoupling processes is unclear and deserves further studies.

In healthy individuals and patients with coronary artery disease, age is associated with a reduced number and function of cultured EPCs, circulating CD34+KDR+ cells, or CD133+ cells and of granulocyte macrophage colony forming units (GM-CFUs) in the bone marrow (11,55,112).

On a molecular level aging is linked to a reduction of telomere length. The proliferative history of a cell is written on telomeres: telomere erosion reflects the number of past divisions experienced by a cell and its proliferative potential (113). In addition, telomere erosion may contribute to telomere shortening. When long telomeres protect chromosomal ends, cells can undergo repeated cell divisions. Conversely, telomere shortening beyond a critical length leads to genomic instability, DNA damage, p53 activation, and ultimately cell cycle arrest (114).

In summary, experimental and clinical studies demonstrated that aging interferes with progenitor cell functions. To what extent the dysfunction is exclusively related to age-associated telomere shortening and intrinsic cell dysfunction (eg, senescence) or might also involve age-dependent changes in paracrine activities remains to be defined (115).

EPCs Alteration in Diabetes

Both cytometric and culture methods have extensively demonstrated that type 1 and type 2 diabetic patients have less circulating EPCs than matched healthy subjects. Moreover, diabetic EPCs display functional impairment, such as reduced proliferation, adhesion, migration, and incorporation, into tubular structures (74,91,116). The mechanisms underlying EPC reduction in diabetes include weak bone marrow mobilization, decreased proliferation, and shortened survival in peripheral blood (127).

Poor collateral formation in diabetes may be attributed to weaker bone marrow stimulation from the ischemic tissue. Fadini *et al* (27) have recently confirmed this hypothesis, showing that bone marrow mobilization of EPCs after ischemia-reperfusion injury is defective in diabetic rats. Inability to mobilize EPCs was associated with downregulation of HIF-1 α and weakened release of marrow – stimulating factors, such as VEGF and SDF-1, ultimately leading to insufficient compensatory angiogenesis (15). Another study has shown that progenitor cell mobilization restored blood flow in diabetic mice (117). It is conceivable that HIF-1 α deregulation in diabetes depends on an overproduction of reactive oxygen species (ROS).

Although molecular mechanisms that regulate EPC release in peripheral blood are complex and not fully understood, a role for the phosphatidylinositol (PI) 3-kinase/protein kinase-B and endothelial nitric oxide (NO) synthase pathways has been shown (72,118). As diabetes is characterized by altered activation of PI 3-kinase/Akt pathways and by reduced NO bioavailability (119), dysfunction of these subcellular pathways may be involved in the defective mobilization of EPCs from bone marrow. Hyperglycemia may be the common feature that affects survival and function of EPCs because similar alterations have been demonstrated in both type 1 and type 2 diabetes. *In vivo*, hyperglycemia induces oxidative stress by increasing the production of ROS and alters leukocyte and endothelial function (30).

Recently, Krankel *et al.* (120) have convincingly demonstrated that high glucose impairs proliferation, survival, and function of cultured EPCs, with concomitant-decreased NO production and matrix metalloproteinase-9 activity. Furthermore, activation of mitogen-activated protein kinases has been revealed as a potential mechanism of EPC dysfunction induced by high glucose (121). A definite demonstration is

that correction of hyperglycemia by insulin therapy (15,122) can indeed restore the normal EPC pool.

Fadini *et al* (27) shown that patients with the metabolic syndrome have decreased levels of CD34+KDR+ EPCs compared with patients without the syndrome (116). Circulating CD34+ cells are synergically decreased by clustering components of the metabolic syndrome, and their levels negatively correlate with the homeostasis model assessment value, a measure of insulin resistance (123). In fact, insulin resistance, the typical hallmark of metabolic syndrome, is characterized by a defective activation of the PI3-kinase/ Akt pathway and decreased endothelial NO synthase activity, which are considered essential for EPC mobilization and function. Oxidative stress plays a crucial role in the pathogenesis of diabetes complications (30), as well as in the entire atherogenic process. Therefore, stress-induced apoptosis may be one mechanism of EPC reduction in diabetes. The literature provides ample evidence that EPCs might decrease because of increased apoptosis and that EPCs are stress sensitive (124).

In summary, reduction in circulating EPCs in diabetic patients may recognize at least three pathophysiological explanations: impaired bone marrow mobilization, defective proliferation, and enhanced apoptosis. Remarkably, in accordance with Brownlee's unifying hypothesis (30), oxidative stress appears as a major determinant of all of these mechanisms. Interestingly, two very recent studies have demonstrated that the natural transketolase activator, benfotiamine, which is theoretically able to prevent the subcellular damage pathways triggered by oxidative stress, restored EPC-mediated healing of ischemic diabetic limbs in mice (125) and prevented hyperglycemia – mediated EPC dysfunction via modulation of the Akt pathway (126).

EPCs and the Diabetic Paradox

High blood glucose is an extremely detrimental factor for the retinal microvasculature. Hyperglycemic damage results in increased permeability, blood and serum leakage to the extravascular space, and progressive decline in retinal blood flow. Retinal ischemia and release of angiogenic factors stimulate the proliferation of microvessels, leading to proliferative retinopathy. Recently, intriguing novelties have been added to this pathogenetic model. In animals, bone

marrow-derived cells are mobilized and recruited at sites of retinal neovascularization in response to VEGF and SDF-1 (127,128). Therefore, not only local endothelial cells, but also EPCs may be involved in the development of proliferative retinopathy. This seems counterintuitive, as diabetes complications that may affect the same patient, such as diabetic retinopathy and PAD, can have opposing EPC alterations, the one being associated with increased and the other with decreased EPC levels. Parallely, a single diabetic patient can present at the same time with complications of excessive angiogenesis (proliferative diabetic retinopathy) and of poor angiogenesis (symptomatic PAD) (27).

An unexplained paradox puzzles diabetologists: diabetic patients must face both poor vessel growth in ischemic heart and limbs and increased angiogenesis in retinal complications (129,130). Endothelial progenitor cells (EPCs) are marrow-derived cells involved in adult neovascularization and endothelial homeostasis (1,131). It has been postulated that low EPCs in peripheral blood may have a role in cardiovascular disease, and we have demonstrated that EPCs are reduced in macrovascular diabetes complications (10,116). On the other hand, an excess of EPCs may be involved in pathologic neoangiogenesis of cancer and proliferative retinopathy (132,133). Therefore, diabetes complications may be associated with both decreased and increased EPCs. Recently, novel therapeutic approaches have been directed to enrich the EPC pool in ischemic diseases and to block EPC function in proliferative diseases (128,134). These approaches in diabetic subjects require cautious evaluation of the implications carried by the paradox and new studies to unravel its causes (135,136).

Fadini et al (141) show that CD34+ cells and CD34+KDR+ cells are differentially altered in the presence of DR and PAD. These two common diabetes complications exhibit a very different behavior in terms of angiogenic response to ischemia, and this contrast has been termed "diabetic paradox." Many growth factors have been proposed to have a role in this phenomenon (137-139), all of which are potent stimuli for progenitor cell mobilization and homing to ischemic tissues (32). Indeed, recent data indicate that marrow-derived cells are involved in retinal neovascularization and that DR+ patients have increased levels of circulating progenitors (133,140). Conversely, macrovascular complications are characterized by reduced angiogenesis and exhausted EPC levels (116). This findings suggest that enhanced endothelial differentiation of circulating progenitors characterize DR, as shown by the high CD34+KDR+ proportion and the enhanced efficiency of EPC culture in contrast with the poor endothelial differentiation of PAD patients (141).

Therefore, the differential regulation of circulating progenitors, possibly in association with different oxygen gradients and local accumulation of growth factors, may explain why peripheral ischemia cannot stimulate angiogenesis as retinal ischemia does. Going deeper into the systemic events accompanying retinal vascular proliferation may provide novel therapeutic targets against peripheral ischemic complications. The notion that EPCs may be involved in retinal vascular proliferation should induce caution when trying to expand the EPC pool to ameliorate the cardiovascular profile. For instance, erythropoietin itself is an angiogenic factor that may worsen proliferative diabetic retinopathy (142).

Therapeutic Potential of EPC in Neovascularization

In vivo models of limb ischemia and myocardial infarction supported a direct role for bone marrow-derived cells in neovascularization, concluding that these cells differentiate into smooth muscle and endothelial cells that incorporate into neovessels (143-146). However, more recently, other groups have contested these findings and suggest that bone marrow-derived progenitor cells act primarily via paracrine mechanisms, secreting chemokines such as angiopoietin-1 and vascular endothelial growth factor (VEGF) at sites of vascular injury to enhance

local angiogenic function (147-149). It remains to be determined whether these discrepancies are attributable to differences in study design, such as the choice of bone marrow cell populations selected and the ischemic models used. Based on the current evidence, however, it appears that EPCs may make both direct contributions to neovascularization as well as indirectly promote the angiogenic function of local endothelial cells via secretion of angiogenic factors(150).

A number of factors have been shown to enhance EPC function either at the site of mobilization from the bone marrow and/or at sites of homing to damaged blood vessels. Age-associated decreases in a wide range of factors, including VEGF and PDGF signaling and circulating estrogen levels have been suggested in animal models to be important factors in the decrease in EPC mobilization from the bone marrow, cardiac homing, and regeneration (151-153).

Clinical trials in patients with coronary artery disease or limb ischemia showed improvement after treatment with plasmid DNA encoding VEGF165 (154-157); thus VEGF may prove to be a feasible and successful therapy for vascular injury.

PDGF acts to promote the angiogenic activity of local vascular cells after myocardial infarction as well as to recruit bone marrow cells that differentiate into both endothelial cells and cardiomyocytes (158,159). Intramyocardial treatment with PDGF therefore appears to enhance the interactions between bone marrow and cardiac stem cell niches and provides functional benefit to the injured heart. Additionally, studies recently demonstrated that PDGF pathways are essential for maintaining the cardiomyogenic potential of Oct3/4+ bone marrow cells that is decreased with age (160).

Tenascin-C, which we have shown to be a downstream mediator of PDGF signaling in the cardiac vasculature, is associated with sites of EPC recruitment in the heart and is also important for bone marrow cell-mediated mechanisms of cardiac angiogenesis (161). This protein is downregulated in the aging bone marrow and may also be depleted in the aging heart (161). Thus mechanisms that restore tenascin-C may have multiple actions that promote cardiovascular repair mechanisms, including CSC-mediated cardiac regeneration.

Another factor acting both in the cardiac and bone marrow stem cell niches is stromal cell-derived factor (SDF)-1. In the bone marrow, SDF-1 is among a number of proteins, including VEGF and placental growth factor, that induce matrix metalloproteinase (MMP)-9, leading to the translocation of stem cells to the vascular zone of the bone marrow before mobilization (59). SDF-1 has also been shown to promote bone marrow cell proliferation and angiogenesis (162).

Other factors that regulate EPC function include the hematopoietic growth factor granulocyte macrophage-colony stimulating factor, which increases the number of circulating EPCs *in vivo*, while enhancing differentiation of EPCs *in vitro* (33).

Similarly G-CSF also has stimulated EPC mobilization in clinical trials, but these EPCs display functional impairment of migratory properties *in vitro*.

Pharmacologically, the class of factors known as the statins, or 3-hydroxy-3-methyl glutaryl coenzyme A reductase inhibitors, has also been shown to enhance EPC-mediated angiogenesis in models of ischemic tissue injury. *In vivo*, statin treatment increases the numbers of circulating EPCs and enhances both neovascularization in corneal assays and reendothelialization of injured vessels, promoting incorporation of labeled bone marrow-derived cells into these vessels (46,56,163). Mechanistically, statin treatment *in vitro* appears to inhibit EPC senescence, via induction of telomere repeat binding factor-2, which inhibits induction of the DNA damage checkpoint-kinase 2 (164). Simvastatin activates the serine-threonine kinase Akt in endothelial cells, promoting endothelial cell survival and migration (165). Akt also acts downstream of VEGF and may therefore represent a key regulator of VEGF-mediated neovascularogenesis (166). Thus, these data suggest that statin therapy may constitute an important approach in the development of strategies to improve EPC survival and function and to improve cardiac repair pathways in the aging population. Indeed, the TOPCARE-AMI clinical trial demonstrated that the treatment of *ex vivo* cultured blood-derived progenitor cells with atorvastatin was found to be safe and potentially effective for the enhancement of cardiac regeneration (167).

EPC Transplantation in Diabetes

Accelerated atherosclerosis is probably the most devastating among diabetes complications. The atherogenetic process in diabetic subjects is similar to that observed in their nondiabetic counterparts. However, diabetic vasculopathy is characterized by high prevalence, early development, bilaterality, rapid progression, and typical involvement of multiple distal sites. The severity of macrovascular complications in diabetes has been attributed to a profoundly impaired collateralization of vascular ischemic beds (168), which is insufficient to overcome the loss of blood flow, and leads to critical limb ischemia that often requires amputation. The mechanisms that hinder ischemia-induced neovascularization in diabetes had remained elusive until the discovery of EPCs. In animal models of diabetic vasculopathy, defective collateralization

was counteracted by administration of EPCs from control animals. Conversely, diabetic EPCs were not able to stimulate vascularization, even becoming antiangiogenic (169,170). Additionally, EPCs appeared important for vascularization and healing of diabetic wounds (171).

Patients with diabetes and PAD had a further significant decrease in circulating EPC levels, especially in the presence of ischemic foot lesions. Remarkably, EPC levels strongly correlated with the ankle-brachial index, the most objective diagnostic and prognostic test for lower extremity arterial disease (116).

Lower-limb atherosclerosis: higher degrees of carotid stenosis, as well as worse stages of leg claudication and ischemic lesions, were associated with lower levels of EPCs, suggesting that EPC count may be considered a valuable marker of atherosclerotic involvement. Indeed, cytometric techniques, which allow EPC count, are widely used for routine laboratory testing, and the determination of EPCs is sufficiently reproducible to be used in the clinical practice (11,40,123).

In the case that EPCs are defined as CD34+KDR+ cells. The clinical usefulness would stand in that EPCs not only mirror vascular function and atherosclerotic burden but also reflect the endogenous vasculoregenerative potential. Importantly, there are data suggesting that measuring EPCs would provide additional information over the classical risk factor

analysis; in one study (11), CD34+KDR+ EPC count predicted cardiovascular events independently of risk factors and hard indexes, such as left ventricular ejection fraction. Moreover, EPCs isolated from diabetic patients with PAD exhibited impaired proliferation and adhesion to mature endothelium (172). We suggest that impaired collateralization leading to the clinical manifestations and complications of atherosclerosis in diabetes may be attributable to decreased and dysfunctional EPCs.

Huang et al. (23) have transplanted bone marrow-mobilized cells, as an EPC enriched fraction, into critically ischemic limbs of diabetic patients. Compared with standard therapy, cell therapy improved angiographic scores and ankle-brachial index values and reduced relevant end points, such as ulcer size and need for limb amputation (27). Recently, Körbling et al. were able to isolate a large quantity of clinically graded EPC from healthy volunteers by leukapheresis after G-CSF stimulation (173). This method may offer promise as a means of improving the efficacy of EPC transplantation for therapeutic neovascularization in the diabetic setting. However, previous data, while suggesting that G-CSF or granulocyte-macrophage colony-stimulating factor (GM-CSF) administration did increase EPC mobilization, the therapeutic effect in patients with coronary artery disease was conflicting (174,175).

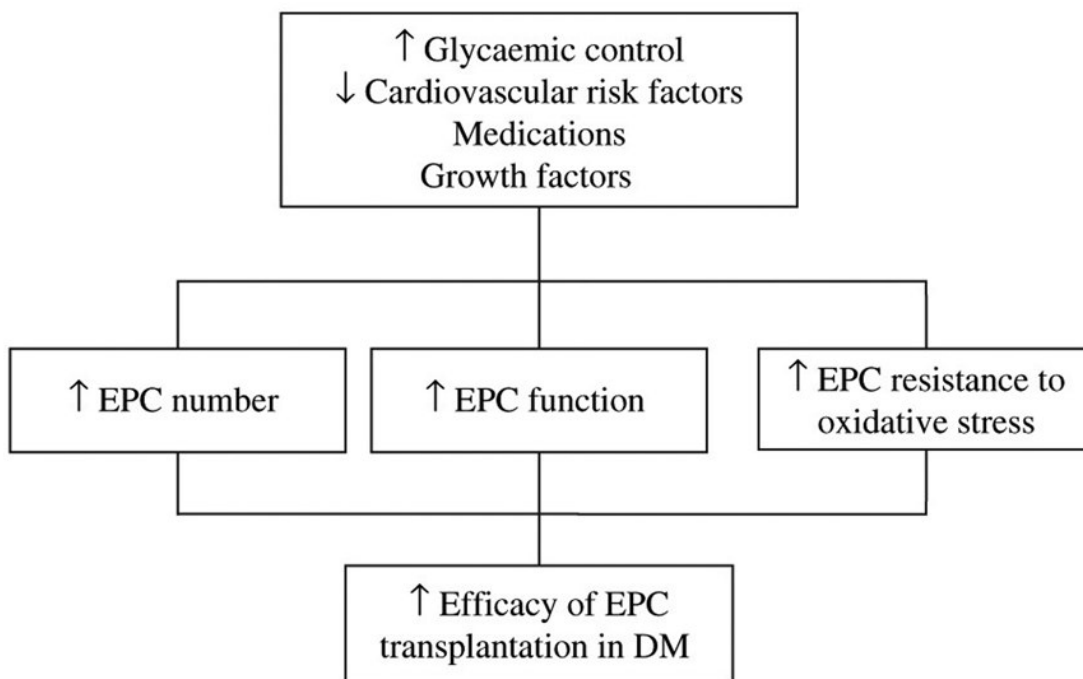


Figure 3. Approaches to improve efficacy of EPC transplantation in diabetic setting. (Reprinted by permission from Wiley-Blackwell Publisher, Diabetes Obesity and Metabolism, Copyright 2007).

More recent data have shown that transplantation of G-CSF-mobilized EPCs for the treatment of critical limb ischaemia in patients with DM is safe and effective (23). Nevertheless, further studies are required to ensure the safety of this approach. EPC number can also be increased by augmenting the differentiation ability of MNC by co-administration of growth factors with unfractionated BM-MNC. For instance, co-administration of placental growth factor and BM-MNC into a diabetic hindlimb mouse model augments EPC differentiation from diabetic BM-MNCs *in vitro* (176).

Improved neovascularization can also be achieved in a diabetic hindlimb ischaemic mouse model by inhibition of NADPH oxidase-derived reactive oxidative species overproduction (177). Silencing of apoptotic gene expressions such as p53 renders EPCs derived from patients with T2DM more resistant to oxidative stress. This method also reduces EPC senescence and improves their ability to differentiate and form tubules *in vitro* (178). Lastly, administration of benfotiamine has been recently shown to increase EPC number and reverse EPC dysfunction in patients with DM (125,126).

Other growth factors such as VEGF, transforming growth factor- β 1, angiopoietin-1, SDF-1, constitutive telomerase reverse transcriptase, hepatocyte growth factor, PDGF-B, basic fibroblast growth factor and

medications such as erythropoietin, oestrogen, phosphodiesterase-5 inhibitor, puerarin and Ginkgo biloba have been shown to augment EPC number and function in a non-diabetic settings (179). These factors, however, have not been studied in the setting of diabetes but may be beneficial for therapeutic neovascularization in DM. The approaches to improve efficacy of EPC transplantation in diabetic setting is summarized in figure 3 (39).

Conclusion

Diabetes complications represent a huge burden for patients and health services. A common approach for the prevention and treatment of diabetes complications relies on the understanding of their complex pathophysiology.

During the last decade, data have become available indicating that alteration in EPCs may have an important causative role in all diabetes complications. New approaches such as EPC administration may represent novel treatments for diabetic vasculopathy in the future. To date, many barriers remain to such a therapeutic approach, hopefully in the near future these problems can be resolved.

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