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REVIEW

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Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis?

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Abstract The integrity and functional activity of the endothelial monolayer play a crucial role in the prevention of atherosclerosis. Increasing evidence suggests that risk factors for coronary artery disease increase endothelial cell apoptosis and lead to a disturbance in the endothelial monolayer. Recent insights suggest that the injured endothelial monolayer is regenerated by circulating bone marrow derived endothelial progenitor cells, which accelerates reendothelialization and limits atherosclerotic lesion formation. However, risk factors for coronary artery disease such as age and diabetes reduce the number and functional activity of these circulating endothelial progenitor cells, thus limiting the regenerative capacity. The impairment of stem/progenitor cells by risk factors may contribute to atherogenesis and atherosclerotic disease progression. We discuss this novel concept of endothelial regeneration and highlight possible novel strategies to interfere with the balance of injury and repair mechanisms.

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Abbreviations *EC:* Endothelial cell · *eNOS:* Endothelial nitric oxide synthase · *EPC:* Endothelial progenitor cells · *LDL:* Low-density lipoprotein · *VEGF:* Vascular endothelial growth factor

Introduction

The integrity and functional activity of the endothelial monolayer play a critical role in artherogenesis. Injury of endothelial monolayer by mechanical removal of the endothelium (e.g., by PTCA or stenting) or inflammatory activation of the endothelial cells induces a cascade of proinflammatory events resulting in infiltration of monocytic cells and smooth muscle cell proliferation [1]. These processes can lead to the formation of atherosclerotic lesions, plaque rupture, and finally myocardial infarction, which is still the leading cause of death in the Western world. The maintenance of the endothelial integrity is therefore of crucial importance in preventing the trig-

gering of processes. In the past the turnover of endothelial cells was believed to be very low [2]. However, increasing evidence suggests that risk factors for coronary artery disease increase endothelial cell (EC) apoptosis and there lead to a disturbance of the endothelial monolayer [3, 4]. Recent insights additionally suggest that the injured endothelial monolayer is regenerated by circulating endothelial cells. This contribution discusses this novel concept of endothelial regeneration and highlights possible novel strategies for interfering with the balance of injury and repair mechanisms.

Endothelial cell apoptosis and endothelial regeneration

Risk factors for atherosclerotic lesion formation can induce a proinflammatory response in endothelial cells and directly induce endothelial injury by promoting the apoptotic suicide pathways. Various in vitro studies show such proapoptotic events induced by a variety of risk factors, including proinflammatory cytokines, reactive oxidant species, oxidized low-density lipoprotein (LDL), and angiotensin II (reviewed in [3, 4]). An elegant animal study found elevated EC apoptosis in aortas and femoral arteries of aged monkeys to be associated with vascular endothelial dysfunction [5], the latter being a well recognized premanifestation feature during the early initiation phases of atherosclerosis. As the most direct in vivo evidence for the occurrence of EC apoptosis as yet available, histopathological analysis of human carotid atherosclerotic plaques demonstrated the occurrence of EC apoptosis in the downstream part of the plaque [6]. As early as in 1978 a significant increase in endothelemia was reported in patients with myocardial infarction and severe but not mild angina, which was sustained for several days [7]. Remnants of apoptotic cells, the apoptotic microparticles, have been detected in the peripheral blood particularly of patients with acute coronary syndromes. About 30% of these microparticles are of endothelial origin [8]. These data have been confirmed by multicolor flow cytometry, which detects annexin V (as a marker for apoptosis) and endothelial marker proteins (KDR and von Willebrandt factor) (S.D., personal communication).

What happens after endothelial injury? In the past the regeneration of injured endothelium was attributed to the migration and proliferation of neighboring endothelial cells. As early as 1972 Kaplan and Schwartz investigated the rate of endothelial cell proliferation; they detected only a rather low number of proliferating cells. These cells were found predominantly in the turbulent flow areas of the vascular tree, the lesion prone regions where atherosclerotic lesions preferentially develop. These observations are in accordance with data obtained more than 20 years later that laminar flow prevents endothelial cell apoptosis in vitro and in vivo [6, 9]. More recent studies, however, suggest that additional repair mechanisms exist to replace denuded or injured arteries (Fig. 1). Implanted Dacron grafts were shown to be rapidly recovered by

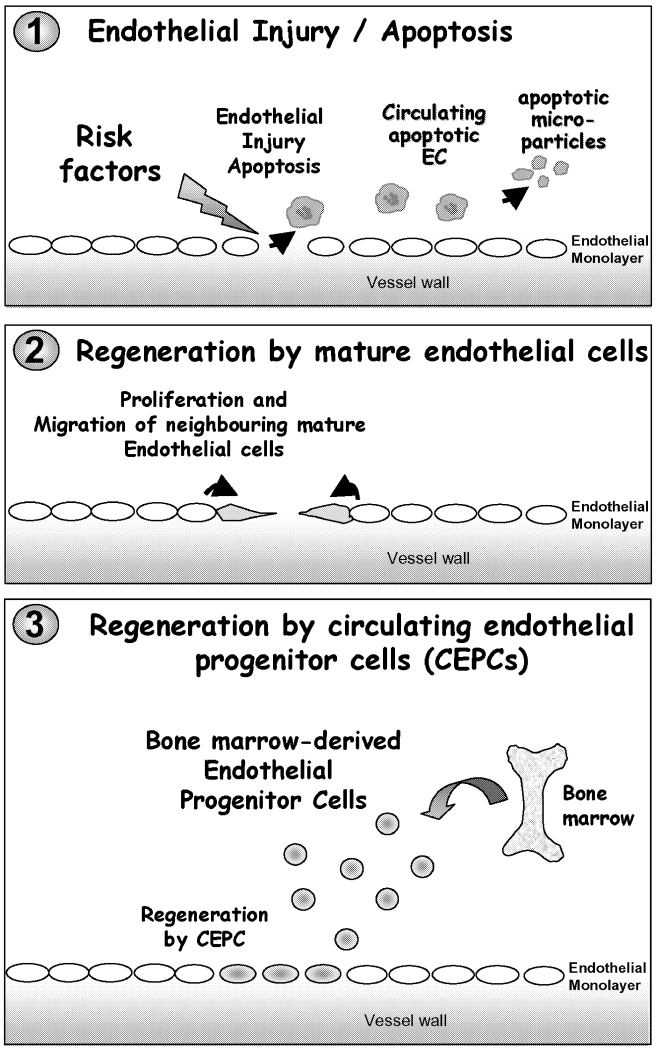

Fig. 1 Regeneration of the endothelial monolayer after injury. After induction of endothelial injury (*1*) two possibilities exist to regenerate the injured endothelial monolayer (*2*, *3*). *2* Regeneration by mature endothelial cells which migrate and proliferate to regenerate the endothelial layer. *3* Regeneration by bone marrow derived endothelial progenitor cells

bone marrow-derived cells deriving from $CD34^+$ hematopoietic stem cells in a dog model [10]. In humans the surface of ventricular assist devices was found to be covered by even more immature $CD133^+$ hematopoietic stem cells, which concomitantly express the vascular endothelial growth factor (VEGF) receptor 2 [11]. Additionally, Walter and coworkers [12] demonstrated that circulating endothelial precursor cells can home to denuded parts of the artery after balloon injury. The incorporated cells were shown to derive from the bone marrow, using mice which had had bone marrow transplantion with β-galactosidase expressing cells. Enhanced incorporation of β-galactosidase positive bone marrow derived cells is associated with accelerated reendothelialization and reduced restenosis [12, 13]. Similar results were reported by Griese et al. [14] who demonstrated that peripheral blood derived monocyte-derived endothelial pro-

genitor cells (EPC) home into bioprosthetic grafts and to balloon-injured carotid arteries, the latter being associated with a significant reduction in neointima deposition. Likewise, infusion of bone marrow-derived $CD34^{-}/CD14^{+}$ mononuclear cells contributes to endothelial regeneration [15]. Overall these findings suggest that circulating endothelial cells make an important contribution to reendothelialization.

The origin of the circulating pool of endothelial cells is not entirely clear. Various studies provide compelling evidence that EPC can derive from hematopoietic stem cells, which express the marker proteins CD133 or CD34 [11, 16, 17]. However, this does not exclude the presence of other sources in the bone marrow (e.g., mesenchymal stem cells, stem/progenitor cells) or even tissue resident stem cells. Moreover, $CD14^{+}$ monocytic cells (even in the absence of the hematopoietic marker CD34 [15]) can differentiate to endothelial cells [18, 19]. Regardless of the origin of the circulating EPC this pool of circulating endothelial cells may play an important function in endogenous repair mechanism to maintain the integrity of the endothelial monolayer, thereby preventing thrombotic complications and atherosclerotic lesion development. Although this concept has not yet been confirmed, several hints from recently presented data are supportive. For example, transplantation of apolipoprotein $E^{-/-}$ mice with wild-type bone marrow reduced atherosclerotic lesion formation [20]. Moreover, various risk factors affect the number and functional activity of EPC both in vitro and in patients with coronary artery disease, whereas the reduction in risk factor load elevates EPC levels (see next section).

By improving neovascularization bone marrow derived stem/progenitor cells may also contribute to plaque angiogenesis thereby potentially facilitating plaque instability [21]. However, a recent study in nonischemic mice detected no effect of bone marrow cells infusion on plaque composition [22]. Moreover, an increase in plaque size was detected only in the presence of ischemia, suggesting that ischemia induced release of growth factors predominantly accounts for this effect [22].

Risk factors and endothelial progenitor cells

The number of circulating EPC, which might have a repair capacity is significantly downregulated in patients with coronary artery disease [23]. Classical risk factors for atherosclerosis such as age and smoking are associated with reduced numbers of circulating $CD34/KDR^{+}$ and CD133/KDR cells [23]. Likewise, the number of cultured EPC from peripheral blood mononuclear cells of patients with risk factors for coronary artery disease was found to be significantly reduced. Two other studies reported lower numbers of EPC as assessed by outgrowth assays in patients with type II [24] or type I diabetes [25]. Most convincingly, classical risk factors for atherogenesis are associated in healthy men with a reduction in peripheral blood-derived endothelial cells [26]. Interestingly, the number of outgrowing endothelial colonies is correlated with endothelial function as assessed by measurement of flow-dependent dilation [26]. Since the measurement of endothelial-mediated vasodilatory capacity gives direct insight into the functional activity of the endothelium and is closely linked to the prognosis of patients with coronary artery disease [27], these data suggest that EPC do indeed have a regenerative capacity. The reduction in EPC by risk factors may contribute to a vicious cycle resulting in endothelial dysfunction (Fig. 2)

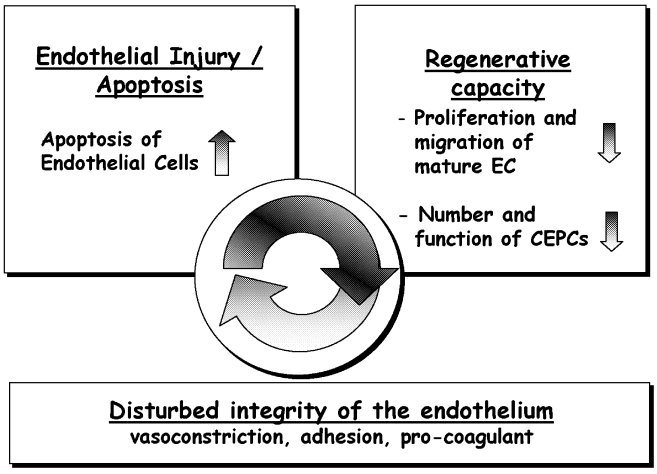

Fig. 2 Effect of risk factors on endothelial regeneration and function. Risk factors induce apoptosis of endothelial cells. In the presence of ongoing apoptosis the endothelial monolayer needs to be regenerated. However, risk factors also reduce the migratory capacity of mature endothelial cells and lower the number and function of circulating endothelial progenitor cells. The enhanced apoptosis and reduced repair may lead to a vicious cycle promoting endothelial dysfunction. Indeed, the number of endothelial progenitor cells is inversely correlated with endothelial function [26]

Mechanisms for impaired regenerative capacity due to reduced EPC levels

The reduction in circulating EPC may have different causes: (a) exhaustion of the pool of stem/progenitor cells in the bone marrow, (b) reduced mobilization, or (c) reduction of survival and/or differentiation (Fig. 3). The maintenance and mobilization of hematopoietic stem cells (the precursors of EPCs) in the bone marrow is determined by the local microenvironment, the "stem cell niche," which consists of stromal cells [28]. The direct influence of the overall risk factors for coronary artery disease on the bone marrow microenvironment is not clear. However, the effect of aging has been extensively studied. Whereas basal hematopoiesis is maintained in aging, the capacity to react to stress-induced mobilization gradually declines with increased age (for review see [29]). Thus one may speculate that elderly patients would show a limited response towards EPC mobilizing/differentiation stimuli and therefore a reduced number of circulating EPCs [30]. Indeed, a lower increase in circulating EPC has been found in elderly patients after surgery [31].

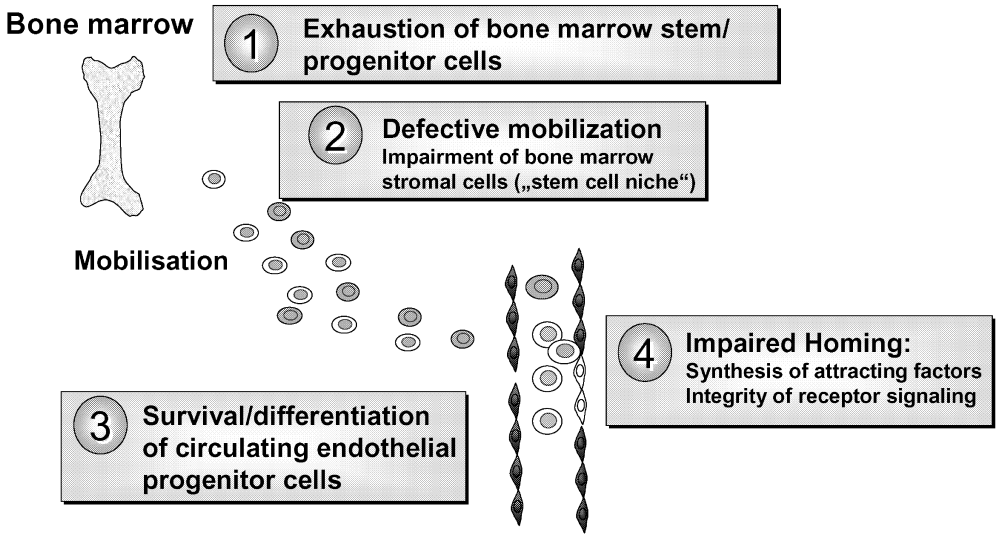

Fig. 3 Possible targets of risk factors for coronary artery disease to reduce circulating endothelial progenitor cells (*1–4*). Risk factors for coronary artery disease may interfere with hematopoietic stem cells in the bone marrow, reducing mobilization or affecting survival and differentiation of circulating progenitor cells. Additionally, risk factors such as age may also reduce homing by reducing stimulatory factors such as VEGF and the block receptor dependent signaling of EPCs

In apolipoprotein $E^{-/-}$ mice age significantly reduced intermediate vascular progenitor cells in the bone marrow (which were defined as $CD31^+/CD45^-$ cells) but did not affect the overall number of hematopoietic stem cells or mature endothelial cells [20]. Interestingly, similar findings have been observed in patients with coronary artery disease, where the number of circulating EPC was significantly reduced while the overall hematopoietic precursor cell levels ($CD34^+$ or $CD133^+$ cells) were not changed [23]. Age itself may also interfere with the functional activity of stem/progenitor cells. Edelberg and coworkers [32] previously demonstrated that only the transplantation of young bone marrow derived cells restores age-associated impaired neovascularization, while bone marrow of aged mice was not effective. Furthermore, young bone marrow derived cells provide protection against atherosclerotic lesion formation [20]. The antiatherosclerotic effect was detected only when young bone marrow derived cells were used [20].

What is the effect of other risk factors on the bone marrow environment and the stem cell niche? The bone marrow stromal cells partially consist of endothelial cells. Given that risk factors systemically impair endothelial cell functions, one may speculate that stromal cells also have impaired functional activity. Indeed, deficiency for the endothelial nitric oxide synthase (eNOS) leads to an impairment of VEGF-induced mobilization of hematopoietic stem cells and EPC and blunted hematopoietic recovery after myelosuppression [33].

$eNOS^{-/-}$ mice additionally showed a reduction in exercise-induced EPC mobilization [34]. Moreover, bone marrow derived $eNOS^{-/-}$ stem cells exhibit a lower engraftment and reduced capacity to augment neovascularization after intravenous injection in a hind limb ischemia model [33]. Interestingly, bone marrow derived cells from patients with chronic heart failure showed a reduced migratory response ex vivo and significant impairment to home to sites of ischemia and to improve neovascularization after hind limb ischemia [35]. These experimental data correspond to initial clinical observations showing a correlation between the migratory capacity of the bone marrow derived cells and improvement in cardiac function in patients after cell therapy [36]. Since nitric oxide bioavailability is reduced in patients with coronary artery disease and heart failure, one may speculate that the reduction in nitric oxide may lead to reduced mobilization and impair maintenance of functional active stem cells.

A second possibility, which may underlie the reduced EPC levels, is enhanced apoptosis and/or deregulation of EPC differentiation. An increased turnover rate in the progenitor cell population with increased susceptibility to apoptosis may be due to an imbalance in pro- and antiapoptotic factors or be caused by a decline in antioxidant defense, as has been suggested for the coronary artery disease process in general. Moreover, angiostatin, an antiangiogenic molecule, was shown to block proliferation of EPCs [37]. In contrast, increased EPC levels by statins or estrogens (see next section) are associated with inhibition of EPC apoptosis [38, 39]. In addition to apoptosis, dysregulation of EPC differentiation by environmental factors may change the balance between cells determined to the endothelial lineage or to inflammatory cells. Monocytic cells or $CD34^+$ cells can be differentiated to endothelial-like cells, when grown under appropriate medium conditions with endothelial growth factors (e.g., VEGF). However, changing the medium conditions and including macrophage-colony stimulating factors or a combination of granulocyte-macrophage-colony-stimulating factor and interleukin 4 results in generation of macrophage or dendritic cells from the same starting cell population [18]. Moreover, bone marrow derived cells can even give rise to smooth-muscle cells and, thereby, contribute to atherosclerotic lesion development [40]. In vitro, oxidized LDL inhibits VEGF-induced EPC differentiation [41]. These data suggest a critical role of the local environment to determine the cell fate promoting either EPC development, with a presumed vasculoprotective effect, or preventing EPC differentiation, which may limit endo-

thelial regeneration. Particularly under conditions of blocked endothelial differentiation, one may speculate that this forces differentiation to proinflammatory cell types or smooth-muscle progenitors, which are considered to promote atherosclerotic lesion development.

Finally, mechanisms which facilitate homing of EPC to the ischemic tissue, could be affected by risk factors. Indeed, aging has been shown to significantly impair hypoxia-induced expression of VEGF [42] and VEGF-expression after balloon injury [43]. In patients with coronary artery disease an impairment of hypoxia-induced VEGF release was found to be associated with a reduced collateral circulation development [44]. Moreover, a reduced increase in VEGF plasma levels is associated with lower mobilization of EPC in elderly patients after coronary artery bypass graft [31].

Other factors which may act as chemoattractants, such as hematopoietic growth factor and stromal cell derived factor 1, may also be affected. Alternatively, the expression of the receptors or the integrity of the receptor-dependent signaling pathways could be targets for risk factor induced inhibitory effects. Receptors could be cleaved by inflammation-induced activation of proteases, as shown for granulocyte colony-stimulating factor triggered proteolysis of the stromal cell derived factor 1 receptor CXCR4 [45]. Initial evidence that intracellular signaling pathways are targeted by risk factors was observed in mature endothelial cells; VEGF-dependent activation of intracellular signaling pathways (such as Akt) was disturbed by incubation with oxidized LDL [46]. Likewise, isolated peripheral blood derived EPC from patients with coronary artery disease show a blunted migratory response towards VEGF despite unchanged VEGF receptor expression [23], suggesting that the response towards VEGF is blocked intracellularly.

Although it may be premature to rush any conclusions based on these preliminary findings, risk factors might interfere with EPC-mediated vascular protection via various possible mechanisms, thereby modulating the endothelial regeneration process.

Regulation of EPC levels

If the hypothesis is correct that the impaired circulating EPC numbers and/or function contributes to atherosclerotic disease progression in patients with risk factors for coronary artery disease, the augmentation of EPC may offer an attractive therapeutic approach. Initial evidence for potential pharmacological intervention came from studies using 3-hydroxy-3-methylgluaryl coenzyme A reductase inhibitors (statins). Statins were shown to increase the number and the functional activity of EPC in vitro [39, 47] in mice [39, 47] and in patients with stable coronary artery disease [48]. The increase in EPC numbers was associated with increased bone marrow derived cells after balloon injury and accelerated endothelial regeneration [12, 13]. The mechanism for enhancing the EPC numbers and function include an increase in proliferation, mobilization, and prevention of EPC senescence and apoptosis [39, 47, 49]. The molecular signaling pathways have not been identified thus far. However, several studies indicate that the activation of the phosphatidylinositol 3-kinase/Akt pathway, which has first been shown to be activated in mature endothelial cells by statins [50], may also play an important role in EPC [47, 49]. Thus, statins may share similar down-stream signaling pathways described for growth factors such as VEGF. VEGF is one of the first factors identified to mobilize EPCs in mice [51]. Clinical studies using gene therapy with plasmids encoding for VEGF demonstrated an augmentation of EPC levels in humans [52]. Recently estrogen was shown to increase bone marrow derived EPC levels and reduced neointimal thickening [38]. Furthermore, erythropoietin, which is routinely used for stimulation of erythropoiesis, also potently augmented EPC levels in mice [53] and man [54]. Importantly, plasma levels of erythropoietin were significantly associated with EPC levels in patients with coronary artery disease [53], suggesting an important role of erythropoietin levels for basal EPC levels. Finally, a recent study suggests that physical exercise also increased EPC levels in mice and man [34]. Physical exercise and the other factors, which increased EPC levels, such as estrogen and erythropoietin are well established activators of the phosphatidylinositol 3-kinase/Akt pathway similar to statins, suggesting that this may be a common pathway to support EPC survival. Although this obviously does not rule out the possible importance of other signaling pathways, for example, that of Janus kinase/signal transducer and activator of transcription, activation of Akt may be useful in improving stem/progenitor cell therapy. Interestingly, overexpression of Akt recently has been shown to increase the efficiency of mesenchymal stem cell therapy after myocardial ischemia in mice [55].

Other mobilizing factors which are known to augment circulating stem cells and EPCs include granulocyte-macrophage colony-stimulating factor, stromal cell derived factor 1, and placenta-derived growth factor [56, 57, 58]. For clinical purposes the long-term use of these mobilizing factors for endothelial regeneration may be hampered by a proinflammatory response and therefore potentially proatherosclerotic side effects.

Conclusion

Intensive investigation is underway to determine the functional activity of EPC for endothelial repair and vasculoprotection. Preliminary data suggest that EPC have the capacity to regenerate the injured endothelial monolayer and thereby reduce atherosclerotic lesion formation. Improvement in EPC levels and/or function by pharmacological interventions could therefore be an attractive novel therapeutic option for antiatherosclerotic therapy. However, in light of the many unanswered questions it is premature to rush any conclusion. Thus, in addition to replacing injured endothelial cells in the

conductance vessels, EPC also promote neovessel formation. It is still controversial whether plaque angiogenesis accelerates atherosclerotic lesion development. Further studies are necessary to elucidate the definitive contribution of circulating progenitor cells for prevention of atherosclerosis.

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