Endothelial Progenitor Cells: More Than an Inflammatory Response?

Ton J. Rabelink, Hetty C. de Boer, Eelco J.P. de Koning, Anton-Jan van Zonneveld

Abstract—The formation of new capillaries (angiogenesis) may be of clinical importance in facilitating reperfusion and regeneration of hibernating cardiac tissue after myocardial infarction and in microvascular ischemia. Evidence is accumulating that as part of the response to hypoxia, bone marrow-derived circulating endothelial progenitor cells (CEPs) are mobilized and subsequently differentiate into proper endothelial cells. There are also indications that such CEPs can facilitate endothelial repair and angiogenesis in vivo. It is not clear yet, however, whether these CEPs are essential for these adaptive processes or what the relative contribution of CEP is compared with that of other mononuclear inflammatory cells that are mobilized to areas of ischemia. Moreover, there are still many uncertainties about how cardiovascular risk factors alter CEP function. Particularly when therapeutically mobilizing CEPs, a further understanding of this issue is essential to assess the risk of potentially harmful side effects of altered CEP function.

Key Words: angiogenesis ■ atherosclerosis ■ endothelial progenitor

Over the past 7 years, the discovery of the phenomenon that mononuclear cells in peripheral blood have the potential to differentiate into endothelial cells ex vivo as well as in vivo has opened up a new field of cardiovascular research. It thus appears that such endothelial progenitor cells (EPCs) can be used therapeutically to restore damaged endothelium. They can also incorporate into the endothelial monolayer and stimulate proliferation of neighboring endothelial cells, thus inducing the formation of new blood vessels. Although the biology is not really understood, several pilot studies have suggested beneficial effects of infusion of mononuclear cells after myocardial infarction in animal models and in humans. The aim of the current review is to discuss some of the potential regulatory mechanisms involved in this phenomenon. In particular, we address the question of whether these progenitor cells are a specific subpopulation with stem cell properties or whether these cells merely reflect plasticity of the normal inflammatory response that occurs in occlusive vascular disease. Ultimately, understanding this biology will be a critical success factor for bringing progenitor cell therapy into the clinical arena.

Vascular Occlusion Leads to Inflammation

In the adult, vessels grow either via capillary sprouting (angiogenesis) or via remodeling of pre-existing arteriolar connections into collateral vessels (arteriogenesis). Both processes occur on occlusion of a vessel, thus improving blood delivery and local perfusion of ischemic tissue. The regulation of the cellular processes involved in arteriogenesis has recently been reviewed in this journal. In this review, we focus on the contribution of bone marrow-derived progenitor cells in the (microcirculatory) angiogenic response to ischemia, a process referred to as (postnatal) vasculogenesis. The endothelial cell is key in initiating vasculogenesis. First, on vascular occlusion, endothelial cells will sense altered shear stress. Recent studies have shown that at low shear stress or oscillatory shear stress, endothelial cells typically will increase the expression of pro-oxidant enzymes, such as NADH oxidase, and reduce the expression of anti-oxidant enzymes such as manganese superoxide dismutase, thioredoxin reductase, and glutathione reductase. As a result, altered shear stress during vascular occlusion will result locally in increased redox signaling. One of the activated transcription factors is NF-κB, which plays a central role in the inflammatory response. NF-κB activation in the endothelium results in the expression of adhesion molecules and the release of chemotactic factors for inflammatory cells. In agreement, in situ nuclear translocation of NF-κB has been found in the vessel wall near regions of disturbed blood flow, like bifurcations, curvatures, and branching points. The second parameter that endothelial cells sense during vascular occlusion is hypoxia. Basically, the endothelial cell is equipped with 2 systems to sense such hypoxia. One is the transcription factor HIF-1α. Under normoxic conditions, this transcription factor will be hydroxylated on a conserved prolyl residue in a reaction with molecular oxygen. The hydroxylated prolyl group allows the Von Hippel-Lindau protein to bind and polyubiquinate the molecule, thereby...

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Figure 1. In atherosclerotic vascular disease, the endothelial cells will be exposed to hypoxia and altered shear stress. Both mechanisms induce redox signaling in the endothelial cell, with subsequent release of chemokines and surface expression of leukocyte adhesion molecules. The CD34+ hematopoietic stem cell is equipped with several receptors, which allow for chemotaxis, and with ligands for adhesion. Local cues such as the concentration of VEGF and basic FGF probably determine differentiation into endothelial-like cells.

Earmarking HIF-1α for destruction by the proteasome. However, in hypoxia, this hydroxyproline is reduced, allowing HIF-1α to accumulate and translocate to the nucleus to activate transcription of genes such as VEGF, EPO, and inducible NOS. The other oxygen-sensing mechanism in the endothelial cell is located in the mitochondria where, under normoxic conditions, oxidative phosphorylation occurs. This reaction is constitutively inhibited by nitric oxide that is also produced in the mitochondria. When hypoxia occurs, nitric oxide starts to dominate and reduces electron transport toward its final acceptor, complex IV. As a result, intermediate products such as hydrogen peroxide and reactive nitrogen species are produced by the mitochondria, again leading to redox signaling and subsequent activation of the endothelial cell. Taken together, altered shear stress and hypoxia will result in redox-mediated endothelial cell activation and release of chemokines, cytokines, and growth factors. Thus, occlusive vascular disease inevitably results in a local inflammatory response of the endothelium (Figure 1).

Endothelial Progenitor Cells: Part of the Inflammatory Response?

For some time, it has been suggested that as a consequence of such an inflammatory response, the ensuing recruitment of monocytes are instrumental not only in inducing collateral formation but also in promoting angiogenesis. It is believed that paracrine release of cytokines and growth factors with known angiogenic properties, such as bFGF and TNFα, mediate these effects of monocytes on capillary sprouting.

More recently, attention has also been drawn to other vasculogenic cell populations present in the mononuclear fraction of peripheral blood that may also be recruited to the activated endothelium in response to an ischemic insult. These are referred to as pluripotent stem cell or progenitor cell populations and include the CD34+ hematopoietic stem cells and subpopulations of CD34+ mononuclear cells, and even subpopulations of the peripheral blood monocytes. Isolation of each of these subpopulations and subsequent culture in vitro could give rise not only to classical circulating blood cells such as monocytes/macrophages but also to unexpected phenotypes such as endothelial cells and myocytes. Hence, the concept has evolved that vascular progenitors are recruited from the bone marrow to sites of tissue revascularization, where they participate in a paracrine way and also directly by differentiating into mature endothelial cells. In particular, the CD34+ hematopoietic stem cell has raised a lot of attention in this respect because of the similarities with the embryonic hemangioblast, which gives rise not only to circulating blood cell lineages but also to vascular cells. Hematopoietic stem cells appear to be essential for angiogenesis in the mouse embryo and, when durably engrafted in adult mice, were shown to have functional hemangioblast activity and develop into endothelial cells that participate in the neovasculature that evolved after retinal ischemia.

Although bone marrow transplantation experiments have shown unequivocally that bone marrow-derived cells can also differentiate into vascular cells in situ, the frequency of this phenomenon and the identification of the cell type involved are still matter of debate. Only recently have some specific surface markers for “true” EPCs emerged from detailed studies characterizing mammalian embryogenesis and angiogenesis. Flk-1/KDR is a receptor for vascular endothelial cell growth factor (VEGFR-2), which appears to be critical for embryonic endothelial cell differentiation and vasculogenesis. Also, it was reported that Flk-1-positive cells, derived from differentiated embryonic stem cells, can give rise to endothelial cells and vascular smooth muscle cells in vitro and in vivo.

Together with the essential role of Flk-1 in hematopoiesis, these observations are consistent with the existence of a Flk-1-positive hemangioblast that serves as a common origin of endothelial cells and blood cells. AC133 is a second early hematopoietic stem cell marker that is downregulated on differentiation and is therefore a marker for early EPCs. Indeed, AC133-positive cells from human peripheral blood were shown to differentiate into endothelial cells in vitro. Using these stem cell markers, it has become clear that only a very small subset of circulating mononuclear cells in peripheral human blood stains (0.002%) positively for CD34, AC133, and Flk-1 simultaneously. The most detailed phenotypic description of the circulating EPC (CEP) proposes the co-expression of several common endothelial and hematopoietic antigens: CD34+, c-kit+, CD31+, PECAM1, and VE-cadherin. In addition, it represents even a subfraction of these.

Are these CEP part of the inflammatory response on vascular occlusion and, if so, does contribution of these cells matter in view of the reported effects of the abundantly present monocytes on these processes? To play such a role, CEP should have the capacity to home exclusively on sites of angiogenesis. They should be able to attach to activated endothelium or extracellular matrix, to (trans)differentiate into an endothelial phenotype, and be able to proliferate. We recently demonstrated that CD34+ hematopoietic stem cells specifically home and migrate to angiogenic endothelium (unpublished observation). Although CD34+ cells probably...
do not adhere to normal endothelium, they can attach to activated endothelium. Platelets may play an important modulating role in this attachment. Platelets can adhere to inflamed endothelial cells or to exposed extracellular matrix, where they express P-selectin and thus can provide an adhesive surface for CEPs,

however, CD34+ cells express the binding determinant for P-selectin (PSGL-1).

In particular, the issue whether endothelial progenitor cells can proliferate after homing and (trans)differentiation may be important in appreciating the in vivo relevance of these CEPs as sources of paracrine factors and as sources for endothelial cells versus the effects that other inflammatory cells have on resident endothelium. Bone marrow transplantation experiments show low to very low percentages of in situ differentiation of bone marrow-derived cells into endothelial cells, making the role for CEPs as a major source of endothelial cells in the short-term perspective of these experiments less likely. However, in acute ischemic events such as myocardial infarction, the number of circulating CD34+ cells was increased and a direct correlation with plasma levels of VEGF was shown.

Furthermore, in patients experiencing a acute vascular insult secondary to burns or coronary bypass grafting, a rapid increase (50×) in the number of CEP was noted within 6 to 12 hours after injury, which coincided with an elevation in VEGF level. Clearly, increased numbers of circulating CD34+ cells, in combination with efficient homing and ultimately (trans)differentiation and proliferation at the site of vascular injury, may increase the contribution of these progenitor cells relative to other inflammatory cells. This phenomenon can be used therapeutically by artificially increasing the number of circulating progenitor cells in conditions such as ischemia. VEGF, basic fibroblast growth factor, angiopoietin-1, placental growth factor, and stromal cell-derived growth factor-1 have all been shown to induce mobilization and recruitment.

What most of these factors have in common is that they stimulate the Akt/PKB pathway; evidence is accumulating that the Akt/PKB pathway plays a central role in stem cell recruitment and survival.

Activation of this pathway may also explain some of the effects of statins on in vivo (re)endothelialization, an effect that appears, at least partly, dependent on incorporation of bone marrow-derived cells into the endothelial cell monolayer.

Because CEPs are thought to be derived from the CD34+ hematopoietic stem cell, an additional method to increase circulating CEPs is to use stem cell mobilizing factors, such as stem cell factor and granulocyte-macrophage colony-stimulating factor. In experimental myocardial infarction and the ischemic hindlimb, it was found that during therapy with such cytokines, circulating CEPs were mobilized into the ischemic regions and augmented neovascularization of ischemic tissue. Recently, a stimulatory effect of erythropoietin (Epo) has also been described on EPC recruitment and angiogenesis in the mouse model of inflammation and ischemia-induced neovascularization.

Also, in renal anemia patients, recombinant Epo markedly increased the number of CD34+ cells in the circulation. Although these observations are exciting, it should be noticed that the beneficial effects of mobilizing CEPs on ischemia were observed in otherwise healthy animals. In disease states or in the presence of cardiovascular risk factors, mobilization of such cells may also promote the formation of potentially harmful CD34+ derived phenotypes such as macrophages or fibrocytes. It thus appears that cardiovascular risk factors may shift the balance between cells that can induce repair and neovascularization toward cells that contribute to a harmful inflammatory reaction. In addition, nitric oxide availability appears to be essential for mobilization of circulating CEPs from the bone marrow stroma.

Strategies that allow the beneficial side of inflammation such as the endogenous capacity to form endothelial-like cells while at the same time reducing differentiation of harmful cellular phenotypes by drugs that enhance nitric oxide bioavailability or activate the Akt/PKB signaling pathway (eg, statin therapy) may therefore prove to be even more useful than mobilizing or infusing progenitor cells.

Attaching Cells: The Other EPC

The low numbers of CD34+ CEP (100 to 500 per mL blood) are in sharp contrast with the relatively large numbers of attached cells that are obtained (~100 000 from 1 mL blood) after culturing the blood mononuclear cell fraction on fibronectin or gelatin for 4 days in the presence of endothelial growth factors and that, unfortunately, often also are referred to as “EPC” (Figure 2). These spindle-shaped attaching cells (hereafter referred to as AT cells) exhibit endothelial characteristics such as the potential to take-up acetylated LDL and expression of endothelial markers such as ULEX and von Willebrand factor.

This remarkable plasticity of cells present in the AT cell cultures cannot be explained by the presence of a few co-isolated CEPs and more likely originate from a more abundant circulating mononuclear cell type, such as monocytes. The concept that has developed over the years is that the number of these AT cells quantitatively reflect subpopulations within the blood mononuclear cells that have the potential to differentiate into an endothelial phenotype in vivo. Interestingly, the number of AT cells is reduced in patients with cardiovascular risk factors. Recently, this reduction has been related to intermedi-
ate endpoints of cardiovascular disease, such as impaired flow-mediated dilatation.\textsuperscript{61} However, one has to realize that the AT cell cultures are an in vitro phenomenon and thus subject to methodological influences. For example, increased expression of matrix adhesion molecules such as the vitronectin receptor \(\alpha_v\beta_3\) in mononuclear cells (eg, by statin therapy) may yield higher numbers of AT cells in culture conditions in which vitronectin is used as an adhesive surface.\textsuperscript{4,62} Thus, when certain adhesion receptors are altered in mononuclear cells, either disease- or therapy-related, then this will be noticed only when the mononuclear cells are cultured on the relevant adhesive protein(s). To date, \(\approx \)6 different adhesive surfaces have been used in the EPC culture assay: FN, FN plus gelatin, gelatin, VN plus gelatin, FN plus collagen, and collagen type I. To what extent these different surfaces have led to conflicting interpretations is not clear. However, we feel that the concept that the number of AT cells quantitatively reflects the number of circulating EPCs has to be carefully interpreted. Nevertheless, these AT cells appear to offer spectacular therapeutic opportunities. Intravenous infusion of autologous expanded AT cells in patients with myocardial infarction potentially of ex vivo expanded endothelial progenitor cells for myocardial ischemia. \textit{Circulation}. 2001;104:1046-1052.


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