Published in final edited form as:

Microvasc Res. 2010 May ; 79(3): 200–206. doi:10.1016/j.mvr.2010.01.012.

Role of Endothelial Progenitor Cells During Ischemia-induced Vasculogenesis and Collateral Formation

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Abstract

Cell-based therapy has emerged as a promising therapeutic tool for treatment of ischemic cardiovascular disease. Both unselected bone marrow-derived mononuclear cells (BMNCs), which include stem/progenitor cells and several other cell types, and endothelial progenitor cells (EPCs), a subpopulation of BMNCs, display regenerative potential in ischemic tissue. Abundant evidence supports the involvement of EPCs in capillary growth, and EPCs also appear to participate in the formation of collateral vessels. Collectively, these effects have led to improved perfusion and functional recovery in animal models of myocardial and peripheral ischemia, and in early clinical trials, the therapeutic administration of EPCs to patients with myocardial infarction or chronic angina have been associated with positive trends in perfusion. EPCs also contribute to endothelial repair and may, consequently, impede the development or progression of arteriosclerosis. This review provides a brief summary of the preclinical and clinical evidence for the role of EPCs in blood-vessel formation and repair during ischemic cardiovascular disease.

Keywords

Endothelial progenitor cell; Vasculogenesis; Collateral formation

Introduction

In the western world, modern medical researchers and physicians continue to be challenged by the ever-increasing socio-economic burden of cardiovascular disease. Arteriosclerotic diseases, such as coronary artery disease (CAD) and peripheral arterial disease (PAD) (Rosamond et al., 2007), are particularly problematic. Gold-standard conventional therapies (e.g., medical treatment, percutaneous coronary intervention, revascularization surgery) are designed to limit ischemic damage and progressive organ dysfunction; however, research performed in the last decade suggests that the damaged tissues can be restored and,

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consequently, refutes the commonly held belief that terminally differentiated organs cannot regenerate. Early regenerative strategies, such as cardiovascular gene therapy, were followed by the discovery of endothelial progenitor cells (EPCs), which led to the development of stem- and progenitor-cell-based strategies for treatment of ischemic cardiovascular disease (Asahara et al., 1997).

Numerous stem/progenitor cell populations from a variety of sources have been proposed for cell-based strategies. Unselected bone-marrow mononuclear cells (BMNCs), which include several stem/progenitor cell populations as well as many other cell types, have been used successfully in preclinical disease models and in the clinical setting of ischemic disease; however, the regenerative potency of cell therapy appears to increase when certain subpopulations, such as EPCs, are selected and used for treatment (Kawamoto et al., 2006). Regenerative potency likely also depends on the type of tissue damaged and the biological role of the cell type. Because EPCs, by definition, display an endothelial-like phenotype, these cells appear to be particularly well-suited for therapeutically modifying the microcirculation in ischemic tissue.

Endothelial Progenitor Cells – Characterization and Regenerative Mechanisms

The key biological characteristics of stem cells include their capacity to self-renew, to transform into dedicated progenitor cells, and to produce large numbers of differentiated progeny cells. The differentiation potential of progenitor cells is more restricted, generally to a particular lineage, and they can proliferate for only a finite number of cell divisions. EPCs are difficult to define precisely because of a lack of consensus regarding the best EPC source, the optimal isolation and culture techniques, and (especially) the phenotypes and characteristics that are crucial for EPC identity.

EPCs were first isolated from the mononuclear-cell fraction of human peripheral blood (Asahara et al., 1997), but other sources, such as the bone marrow, have also been used. Because progenitor cells are believed to be more proliferative than mature cells and to have a greater capacity for expansion, the primary isolation strategy relies on culturing mononuclear cells via procedures designed to enrich the EPC population. The isolated cells have sometimes been identified descriptively, such as early outgrowth cells (also called circulating angiogenic cells) or late outgrowth cells (endothelial colony forming cells) (Hirschi et al., 2008), and EPCs have also been characterized by the uptake of diacetylated low-density lipoprotein (LDL), by the binding of fluorescently labeled *Ulex europaeus* agglutinin 1 lectin, or by in-vitro and in-vivo functional assays, such as colony formation, tube formation, and vascular integration; however, these methods of identification lack specificity and can be inconclusive. Another conventional method of EPC identification is based on the co-expression of hematopoietic stem-cell markers (e.g., CD133, CD34) and endothelial-cell markers (e.g., vascular endothelial growth factor [VEGF] receptor-2 or kinase-insert domain receptor [KDR], von Willebrandfactor [vWF], endothelial nitric oxide synthase [eNOS]) (Urbich and Dimmeler, 2004). Marker expression can also be used to trace the pathway of EPC differentiation; CD133 and CD34 are expressed by primitive, largely undifferentiated (i.e., more stem-cell-like) EPCs, but CD133 expression declines as differentiation progresses, whereas CD34 expression is maintained, and the expression of endothelial-lineage markers increases. Consequently, marker expression has been used to distinguish between early EPCs (e.g., CD133⁺CD34⁺ cells), early and late circulating EPCs (e.g., CD133⁻CD34⁺ cells), and EPCs that are nearing maturity (e.g., vWF⁺ cells).

EPC levels in the peripheral blood are low under normal conditions but increase as EPCs residing in the bone marrow are mobilized in response to physiological and pathological

stimuli, including myocardial and peripheral ischemia (Masuda et al., 2007; Takahashi et al., 1999) (Figure 1). The mobilized EPCs then travel (via a process called EPC recruitment or homing) to the sites of new vessel growth in the ischemic tissue (Asahara et al., 1999). EPC recruitment requires a coordinated sequence of multi-step adhesive and signaling events, including chemoattraction, adhesion, and migration. Early observations suggested that EPCs contributed the "hardware" of neovascularization by differentiating into cells that form structural components of the growing vasculature (Orlic et al., 2001b). However, as more recent insights into the mechanism of EPC-mediated neovascularization have accumulated, the paracrine hypothesis has become more plausible. This hypothesis states that EPCs (whether mobilized endogenously from the bone marrow or administered as cell therapy) contribute to vascular growth primarily by secreting proteins (e.g., growth factors, chemokines, cytokines) that inhibit cell death, enhance cell proliferation, activate stem/progenitor cells already present in the ischemic tissue (i.e., resident stem/progenitor cells), and recruit additional stem/progenitor cells to the injury site (Kamihata et al., 2001; Kinnaid et al., 2004a; Kinnaid et al., 2004b; Rehman et al., 2003; Wollert and Drexler, 2005). Thus, cell therapy appears to improve tissue perfusion by increasing the number of "cytokine factories," rather than the structural resources, available for vessel growth (Heil et al., 2004).

Because of the diversity of factors secreted by both unselected mononuclear cells and EPCs (Korf-Klingebiel et al., 2008) and the potential feedback between these cells and the microenvironment, cell therapy may induce mechanisms similar to those that support endogenous repair and, consequently, could be more effective than therapies that are limited to a fixed dose of a single gene (Losordo and Dimmeler, 2004). Furthermore, the cardiomyocyte population in adult human hearts may not be static, as previously believed. Endogenous cardiomyocyte turnover is common in adult organisms of more primitive species (Curado and Stainier, 2006; Lepilina et al., 2006; Poss et al., 2002), and recent evidence suggests that a small proportion (1% or less) of cardiomyocytes in human hearts are replaced annually (Bergmann et al., 2009). This endogenous mechanism of cardiomyocyte turnover is not clinically relevant during ischemia but could (in theory) be boosted by the therapeutic administration of stem and progenitor cells.

EPCs in Vessel Growth and Repair

In adults, microvascular blood-vessel formation (e.g., capillary growth) occurs through both angiogenesis – the sprouting and proliferation of endothelial cells located in pre-existing blood vessels – and vasculogenesis – de novo vessel growth (Figure 2). Macrovascular vessels develop through arteriogenesis – the transformation of small arterioles into larger conductance arteries. Collectively, arteriogenesis, angiogenesis, and vasculogenesis are referred to as neovascularization (Simons, 2005).

Historically, capillary growth was thought to occur exclusively through angiogenesis; however, more recent findings demonstrate that capillaries can also develop de novo in previously avascular tissue. This process (i.e., vasculogenesis) is initiated by the recruitment of progenitor cells (including EPCs) to ischemic tissue (Asahara et al., 1997; Asahara et al., 1999), which was first reported in the seminal study by Asahara et al. (Asahara et al., 1997). EPCs contribute to new vessel formation after the surgical induction of both myocardial infarction and hind-limb ischemia in mice (Asahara et al., 1999; Kalka et al., 2000; Kawamoto et al., 2001; Shintani et al., 2001b; Urbich et al., 2003), and the recruited EPCs have been shown to express marker proteins associated with endothelial cells and/or cardiomyocytes (Goodell et al., 2001; Kocher et al., 2001; Orlic et al., 2001a; Orlic et al., 2001b). These observations suggested that EPCs promote vascularization by differentiating into tissue-specific vascular cells or by transdifferentiating into cells of other lineages;

however, Ziegelhoeffer et al. found GFP⁺ cells in the perivascular space, but not in the developing vessels, of mice transplanted with GFP⁻ expressing bone marrow (Ziegelhoeffer et al., 2004), and similar observations have been reported by other groups (Hillebrands et al., 2002; Wagers et al., 2002; Ziegelhoeffer et al., 2004). Overall, the proportion of injected cells that are incorporated into the endothelium of growing vessels can vary from more than 50% to nearly 0%, and subsequent research into the mechanisms of EPC-mediated cardiovascular repair suggested that the apparent transdifferentiation occurred through fusion between transplanted and resident cells (Balsam et al., 2004; Murry et al., 2004). These findings, coupled with clear evidence in support of the paracrine mechanism (Korf-Klingebiel et al., 2008), argues against the structural importance of EPCs in the growing vasculature; however, whether paracrine mechanisms alone are responsible for the benefits of cell therapy, or whether other known or unknown mechanisms are also involved, has yet to be determined. Furthermore, the regenerative mechanism(s) may differ depending on characteristics of the patient population, the condition and/or tissue treated, the type and source of cells administered, the delivery route, and the techniques used for cell isolation and characterization. Studies comparing the mechanisms and regenerative potency of a variety of cell types are pending.

Arteriogenesis (Carmeliet, 2000; Heil and Schaper, 2004) is not organ-specific, occurs independently of hypoxia or ischemia, and is crucial for maintaining bulk blood flow and for limiting tissue damage in response to an abrupt occlusion or to progressive narrowing of the vasculature (Hansen, 1989; Sasayama and Fujita, 1992; Schaper and Ito, 1996). Vascular occlusions produce a pressure gradient that forces more blood to pass through arterioles connecting the vessels proximal and distal to the occlusion. This pressure gradient induces shear stress (Troidl et al., 2009), which has been shown to promote proliferation, differentiation, and tube formation in endothelial cells (Yamamoto et al., 2003), and the mitotic activity of both endothelial cells and smooth-muscle cells is elevated in remodeling arterioles (Schaper et al., 1990). On a molecular level, shear stress induces the expression of actin-binding Rho-activating protein through a cascade triggered by nitrous oxide production, and endothelial-cell activation leads to the infiltration and adhesion of monocytes, which subsequently secrete growth factors, chemokines, and proteases (Schaper and Scholz, 2003; Schierling et al., 2009). Collectively, these processes promote arteriole enlargement, and the enlarging arterioles eventually form the collateral vasculature that diverts blood flow around the occlusion. Thus, collateral vessels develop via the enlargement of pre-existing, microscopic, vascular structures, rather than through a dedicated, autonomous mechanism.

EPCs are sensitive to the shear stress that induces collateral formation. EPCs cultured from human peripheral blood elongated in response to laminar shear stress, and their long axes re-oriented to the direction of flow. Shear stress also markedly increased the expression of VEGF receptors and activated Akt signaling, which induced EPC differentiation into endothelial cells (Ye et al., 2008). In a rabbit hind-limb ischemia model, cells derived from BMNCs contributed to collateral formation that could be detected angiographically (Shintani et al., 2001a), and in mice transplanted with GFP⁻ expressing bone marrow, GFP⁺ fibroblasts, pericytes, and leukocytes were found adjacent to collateral vessels that developed in response to myocardial infarction; the GFP expression co-localized with the expression of endothelial or smooth-muscle-cell markers (Kawamoto et al., 2004). Furthermore, BMNC mobilization via the injection of granulocyte colony stimulating factor (G-CSF) was associated with greater arteriole density in the ischemic border zone and with improvements in functional recovery and survival after myocardial infarction in mice (Deindl et al., 2006), and pericyte recruitment, arteriole density, collateral formation, and functional recovery were enhanced in mice administered BMNCs isolated from diabetic patients after G-CSF mobilization; interestingly, the benefits associated with BMNCs from

diabetic patients were attenuated in mice predisposed to diabetes (Zhou et al., 2006). These findings are consistent with those reported in swine models of myocardial infarction; angiographic assessments found evidence of enhanced collateral development after the injection of BMNCs or peripheral-blood mononuclear cells into the border zone of ischemia (Kamihata et al., 2002; Kamihata et al., 2001), and Rentrop classification (Rentrop et al., 1985) of the ischemic tissue indicated that the collateral arteries were well developed (Kawamoto et al., 2003). These enhancements were accompanied by significant improvements in echocardiographic assessments of left-ventricular ejection fraction 4 weeks after EPC injection but not after the injection of CD31⁺ mononuclear cells or saline. Collectively, these reports suggest that cell therapy may enhance collateral formation.

If EPCs participate in collateral formation, they are unlikely to play a structural role, because collateral vessels develop from pre-existing arterioles and do not expand into an intervening capillary bed. Monocytes and macrophages are known to facilitate collateral formation by secreting matrix metalloproteinases (Carmeliet, 2000; Heil and Schaper, 2004), and locally administered EPCs could enhance collateral formation through a similar paracrine mechanism. EPC recruitment to sites of arteriogenesis (Carmeliet and Luttun, 2001) is regulated, in part, by interactions between stromal-cell– derived factor 1 and CXCR4 chemokine receptor 4, which also are involved in collateral formation (Carr et al., 2006), and several of the factors secreted by EPCs are known to induce collateral development (Schaper and Scholz, 2003). Furthermore, local injection of labeled, marrow-derived stromal cells (MSCs) after femoral artery ligation in mice led to greater collateral development and to higher protein levels of basic fibroblast growth factor (bFGF) and VEGF with no evidence of MSC incorporation (Kinnaird et al., 2004b).

EPC function is not limited to vessel formation in ischemic tissue; EPCs also participate in the repair and restoration of the endothelium, which is a functionally active tissue that extends from conduit vessels to the microcirculation. Arteriosclerosis develops in response to endothelial damage and inflammation; consequently, endothelial dysfunction can exacerbate arteriosclerosis and lead to cardiovascular diseases (Landmesser et al., 2004; Lerman and Zeiher, 2005). Before the discovery of EPCs, endothelial repair was thought to occur exclusively through the proliferation and migration of mature endothelial cells surrounding the lesion. However, CD34⁺ EPCs contribute to the endothelialization of implanted grafts (Shi et al., 1998), and cells descended from the bone marrow have been identified on the luminal surface of injured carotid arteries in mice transplanted with traceable bone-marrow cells (Walter et al., 2002). Furthermore, both EPCs and BMNCs interact with the endothelium (Rafii and Lyden, 2003) and promote re-endothelialization (Walter et al., 2002; Werner et al., 2003). Thus, EPCs likely have a beneficial role during endothelial repair, thereby maintaining vascular homeostasis and impeding the manifestation or progression of arteriosclerosis.

Clinical Perspective

The vasculogenic efficiency of cell therapy cannot be directly evaluated in patients, because capillaries are too small to be detected by established imaging techniques, and because clinical specimens are not available for histological analyses. Indirect assessments can be obtained with conventional measurements of tissue perfusion, such as single-photon emission computed tomography (SPECT) or magnetic-resonance imaging (MRI), but the sensitivity of these techniques is limited. Nevertheless, in a substudy of the REPAIR-AMI (i.e., Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction) trial, intracoronary Doppler measurements indicated that coronary flow reserve in the infarct-related artery was restored 4 months after the infusion of bone-marrow progenitor cells (Erbs et al., 2007), and in our phase I/IIa pilot study, positive trends in

SPECT perfusion imaging were observed after CD34⁺ progenitor cells were injected into the hibernating myocardium of patients with chronic angina (Losordo et al., 2007). These trends are further substantiated by findings in our phase IIb ACT34-CMI trial (Losordo et al., 2009).

Efficient collateral formation is a critical determinant of tissue vulnerability and preservation during ischemia. The formation of collaterals is a time-dependent process (Werner et al., 2001) that occurs only after vessel occlusion or obstruction (Cohen et al., 1989) and varies substantially, even among individuals with similar vascular anatomies (Koerselman et al., 2003; Pohl et al., 2001; Wustmann et al., 2003). The source of this variation is unknown; chronic ischemia is believed to stimulate collateral growth, but an individual's anatomic, genetic, and epigenetic predisposition to collateral formation is influenced by humoral and cellular mechanisms. Furthermore, age- and cardiovascular-risk– associated impairments of the peripheral circulation are accompanied by declines in the expression of many angiogenic factors that are typically upregulated in ischemic tissue (Conway et al., 2001; Koerselman et al., 2005; Waltenberger, 2001).

Although little is known about the involvement of stem/progenitor cells during collateral formation in humans, the available evidence suggests that EPCs could play a supportive role. Collateral formation was positively associated with higher levels of circulating EPCs that co-expressed VEGF receptor-2 and CD133 in patients with non-ST elevated myocardial infarction (Lev et al., 2005), and Lambiase et al. identified a strong, positive correlation between coronary collateral flow index and the number of circulating CD34⁺/CD133⁺ cells in patients with stable CAD. Furthermore, endothelial-cell–marker expression was more common in EPCs isolated from patients with adequate collateral flow than in EPCs from patients with poor collateral support, and when human umbilical-vein endothelial cells were cultured with plasma from patients with varying degrees of collateral flow, the chemotactic response was greater in cells exposed to plasma from patients with well-developed collateral networks (Lambiase et al., 2004). EPC functionality and the extent of collateral formation have also been investigated in patients who had chronic total occlusion of one major coronary artery before undergoing a bypass procedure. EPCs were isolated from the peripheral blood of patients with more (Rentrop class 2 and 3) or less (Rentrop class 0 and 1) collateral development, characterized by acetylated-LDL uptake and the binding of fluorescently labeled Ulex europaeus agglutinin 1 lectin, and cultured. EPCs from patients with more extensive collateral development produced more colony-forming units, fewer senescent cells, and a greater concentration of bFGF in the supernatant of the culture medium (Matsuo et al., 2006). Notably, the extent of collateral formation and the number of circulating EPCs correlate inversely with insulin resistance, adiponectin level, PAI-1 concentration (He et al., 2006; Ouchi et al., 2004; Xiang et al., 2004) and with many of the characteristics associated with metabolic syndrome (i.e., a combination of medical disorders that increase the risk of developing cardiovascular disease and diabetes) (Mouquet et al., 2009).

Because the ability to assess collateral formation in patients is limited, the potential impact of cell therapy on this process is difficult to determine. Angiographic assessments of collateral coronary vessels are influenced by vascular tone, the volume of contrast agent injected, the force of the injection, and the spatial resolution of the diagnostic technique, as well as the presence of pre-existing collateral networks (Gibson et al., 1999; Simons, 2005); consequently, only vessels with diameters >100 μ m can be detected, and they are notoriously difficult to quantify. Measurements of tissue perfusion provide only an indirect measure of collateral development, and the currently available methods (e.g., SPECT) lack sufficient sensitivity to reveal changes in smaller vessels. Synchrotron radiation microangiography has been used to assess the effect of local CD34⁺ cell application on

vessels as small as 20 μm diameter in an *ex-vivo* setting (Iwasaki et al., 2007) but has yet to be validated for clinical use. This and other advanced diagnostic tools and high-resolution imaging techniques must be developed before the potential benefits of cell therapy on collateral-vessel development can be accurately measured. Nevertheless, evidence from pilot studies suggests that cell therapy increases collateral formation: new collateral vessels could be detected angiographically in 3 out of 5 patients with severe PAD (Boda et al., 2009) and in 2 patients with critical limb ischemia (Kudo et al., 2003) who benefited from intramuscular injection of autologous CD34⁺ cells.

Summary

In summary, the involvement of EPCs in capillary development (vasculogenesis) and endothelial repair is supported by abundant preclinical evidence, and EPCs also appear to participate in collateral vessel formation (arteriogenesis) in ischemic tissue. Although the mechanisms of EPC-mediated vessel growth and repair are not fully understood, the vasculogenic effects of EPCs are most often attributed to the variety of angiogenic factors produced by EPCs. Both unselected BMNCs and EPCs have been shown to promote functional recovery in preclinical models of myocardial and peripheral ischemia, and positive trends in perfusion have been observed in early clinical trials. The regenerative potency of these and other cell populations will continue to be evaluated in ongoing, randomized, controlled clinical trials; however, clinical assessments of capillary growth, collateral formation, and (most importantly) perfusion are limited, because the currently available imaging techniques cannot detect capillaries or small collateral vessels, and human specimens are not available for histological analyses. More advanced diagnostic tools and high-resolution imaging techniques must be developed before the role of EPCs in vessel growth and, by extension, the potential benefits of cell therapy can be thoroughly characterized.

Acknowledgments

We thank W. Kevin Meisner, PhD, ELS, for editorial assistance and Ashley Peterson for administrative support. This work was supported in part by NIH grants R01 HL53354, R01 HL77428, R01 HL80137, and R01 HL95874 awarded to Douglas W. Losordo. Jörn Tongers was supported by a Midwest Affiliate Postdoctoral Fellowship from the American Heart Association, the German Heart Foundation, and Solvay Pharmaceuticals. Jerome Roncalli was supported by the French Society of Cardiology.

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Figure 1. EPCs in Ischemic Tissue Repair

The regenerative effects of EPCs in ischemic tissue are currently believed to occur primarily through the release of multiple factors that alter the microenvironment in a paracrine fashion. The secreted factors, in turn, recruit additional stem/progenitor cells, activate resident stem cells, suppress cell death, and may enhance the proliferation of resident cells. Collectively, these effects promote the growth of new blood vessels and improve tissue perfusion.

Figure 2. Mechanisms of Neovascularization

(A) Exceptionally detailed images depict the mechanisms of neovascularization in ischemic cardiovascular disease. (B) In response to the obstruction of bulk blood flow, collateral vessels form via arteriogenesis – the transformation of pre-existing arterioles into larger conduction vessels. (C) In ischemic tissues, capillary formation can occur via both angiogenesis – the sprouting of capillaries from pre-existing vessels – and vasculogenesis – de novo formation in previously avascular tissue, which is stimulated by the recruitment of stem and progenitor cells; the incorporation of EPCs into the microvasculature has been detected via the use of genetically modified cells.