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Neoangiogenesis With Endothelial Precursors for the Treatment of Ischemia

P. Sepúlveda, J. Martinez-León, and J.M. García-Verdugo

ABSTRACT

In the embryo, blood vessels and hematopoietic cells arise from the hemangioblast, a common precursor cell. Compelling evidence suggests that bone marrow from adult individuals contains endothelial cell precursors (EPCs), similar to embryonic hemangioblast. They are able to increase neovascularization of tissue after ischemia. Herein we have discussed the ontogeny of these cells, their phenotypes, and their isolation from various sources. We also have presented experimental studies indicating that EPCs are able to induce neovascularization and angiogenesis when transplanted into ischemic tissues. Furthermore, endogenous EPCs can be mobilized using factors that promote their homing to sites of tissue injury. We also have discussed the ongoing clinical trials using these cells to treat ischemic diseases.

TWO MECHANISMS drive the development of the vascular system, vasculogenesis and angiogenesis. Vasculogenesis is defined as de novo vessel formation from mesodermal undifferentiated precursors. These cells assemble, forming cords that develop an internal cavity yielding primitive capillaries. Angiogenesis is the cellular process by which already formed vessels produce ramifications that invade tissues in response to normal or pathological stimuli. The main physiological factors implicated in angiogenesis are ischemia and mechanical factors.[1] Although the mechanisms and concrete stimuli that orchestrate the complete angiogenic program have not been elucidated, it is known to involve vascular endothelial growth factor (VEGF), its receptor (VEGFR2), and basic fibroblast growth factor (FGFb).[2] Angiogenesis consists of various stages. First, preexisting vessels expand and increase their permeability, allowing the diffusion of plasma proteins and the degradation of extracellular matrix by metalloproteinases (MMPs).

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Second, the newly created space favors the migration of endothelial cells (ECs) that secrete more FGFb, VEGF, and IGF-1, producing positive feedback that induces the proliferation and migration of new ECs. These cells then organize into solid cords that progressively increase in diameter. Last, the periendothelial cells synthesize and store growth factors, cytokines, and extracellular matrix, which act as scaffold through which the neoformed ramifications migrate.

It was believed that vasculogenesis and angiogenesis coexist in embryos, while angiogenesis is the only mechanism of vascular development in the mature organism. However, recent findings suggest that endothelial cell precursors (EPCs) promote vasculogenesis processes in adults. EPCs may play an important role in endothelial homeostasis, being implicated in both regeneration and neovascularization. These cells may serve as the substrate for new vessel formation and simultaneously exert a paracrine effect to promote angiogenesis.[3,4] Evidence for the contribution of EPCs to adult vasculogenesis comes from ex vivo and in vivo studies. Either freshly isolated or ex vivo expanded EPCs are able to form capillary-like structures and contribute to new vessel formation.[5,6] Moreover, when bone marrow (BM) is used to reconstitute lethally irradiated mice, EPCs from the donor BM are able to migrate and engraft into sites of injury, promoting neovascularization of ischemic tissues.[7,8]

CHARACTERIZATION OF THE EPC

During embryogenesis, a common precursor, the hemangioblast, precedes the EPC and hematopoietic stem cell (HSC). This early precursor has been identified in the hematopoietic compartment of adult BM.[9–11] In an attempt to purify EPCs, several groups have enriched BM cell populations targeting antigens associated with HSCs: CD34, CD117(c-kit), CD133, sca-1 (mouse), and VEGFR-2(KDR) or their combinations.[10,12–14] Initially EPCs were identified by their expression of $CD34^+$ and $VEGFR2^+$ among populations isolated from either BM or umbilical cord blood (UCB).[15] Unfortunately, these antigens are modulated in response to the environment (antigenic plasticity), making it difficult to define a unique EPC phenotype.[16] Another problem is that mature endothelium and subsets of myelomonocytic hematopoietic cells express the CD34 antigen, leading to confusion in the identification and isolation of these cells (Table 1).[17,18] Expression of CD133 antigen has also been used to isolate EPC, since it is expressed on early hematopoietic precursor cells (HPCs) but not in mature endothelium.[19]

In addition to hematopoietic EPCs, other sources and types of EPCs also capable to differentiate to the endothelial lineage exist not only in the BM, like the $CD34^-$/$CD133^+$/$VEGFR2^+$ mesenchymal stem cells,[11,20] multipotent adult progenitor cells (MAPCs),[21] and side population cells,[8] but also in the peripheral blood (PB), like circulating endothelial precursors (CEPs).[18] As a result of these complex scenarios, the identification and purification of the genuine EPCs has progressively lost its initial interest.[16] Lately, efforts have been focused on designing strategies to improve the repair of damaged tissue either by recruitment of endogenous EPCs or by direct transplantation of these precursors.

Table 1. Phenotype of Human EPCs, CEPs, CECs, HSCs, and HPCs

Cell type	Origin	Specific Markers
EPC	BM, vascular parenchyma	CD133, VEGFR2, CD34, VE-Cad
CEP	PB, UCB	CD133, VEGFR2, CD34, VE-Cad
CEC	Vessel mature endothelium	CD133, VEGFR2, CD34, VE-Cad
HSC	BM	$CD133^+$/$CD34^+$, $CD38^-$/$CD34^+$
HPC	BM	$CD45^+$, lineage-specific markers

EPC RECRUITMENT IN ISCHEMIC TISSUE

Evidence suggests that tissue injury involves the recruitment of circulating or resident stem cell populations that complement neoangiogenesis supported by preexisting endothelium, leading to organ revascularization.[18,22,23]

Hypoxia-inducible factors (HIFs) are transcription factors that activate pathways with the ability to increase the oxygen supply and promote adaptative responses to stress. Among the multiple targets of HIF genes are VEGF, erythropoietin, angiopoietin, placental growth factor, and platelet-derived growth factor, indicating that HIF-1 functions as a master regulator of angiogenesis in ischemic tissues.[24] These angiogenic factors recruit subsets of proangiogenic hematopoietic cells (HSCs and HPCs) along with EPCs and CEPs. These, in turn, may also release new angiogenic factors contributing to the repair by paracrine effects. Indeed, hypoxic preconditioning of EPCs, can increase their ability to repair ischemic limbs through the activation of the angiogenic program.[25]

VEGF is one of the main factors involved in the recruitment of EPCs in response to ischemia. Increased expression of VEGF after either vascular injury or exogenous delivery recruits EPC.[11,12,26] Additionally, cross talk between SDF-1 and VEGF has been recently described. Grunewald et al provided evidence that VEGF induced perivascular expression of SDF-1 that functions, in turn, to position recruited BM-derived circulating cells in this strategic location, where they act in a paracrine fashion to enhance in situ proliferation of resident, activated endothelial cells.[4] SDF-1 gene expression is also regulated by HIF-1 in endothelial cells, resulting in selective in vivo expression of SDF-1 in ischemic tissue. SDF-1 binds to the chemokine receptor CXCR4, which is highly expressed in EPC,[27,28] thus increasing the adhesion, migration, and homing of CXCR4-positive progenitor cells to ischemic tissue.[22] In addition, in humans, cells expressing the CXCR4 are EPCs of both hematopoietic ($CXCR4^+$/$CD34^+$/$CD45^+$) and nonhematopoietic origin ($CXCR4^+$/$CD34^+$/$CD133^+$/$CD45^-$). The latter population

has been proven to express tissue-specific markers with the ability to migrate to sites of injury.[29–31]

EPC MOBILIZATION FROM BM

Since Orlic et al described the ability of mobilized EPCs from BM to repair infarcted myocardium,[32] interest has turned to several proangiogenic growth factors, such as SDF-1, angiopoietin-1, placental growth factor, and erythropoietin, which are able to augment EPC levels and improve neovascularization.[33–37] Increased levels of SDF-1 following intramyocardial injection of syngeneic cardiac fibroblasts that were stably transfected to express SDF-1 induced homing of CD177-positive cells to injured myocardium.[38] Estrogens mobilize BM-derived EPCs contributing to reendothelization after arterial injury.[39] Other types of EPC mobilization, like physical training[40] or treatment with HMG CoA reductase inhibitors,[37] have also been described. However, such functional improvements are not entirely due to EPC mobilization, but also to direct prongiogenic or antipoptotic effects.[41]

THERAPEUTIC ANGIOGENESIS WITH EPCs

The ability of EPCs to repair ischemic tissues has been studied in animal models of ischemia like ischemic limb, retina, or infracted myocardium.

Revascularization of Ischemic Limbs

In 2000 ex vivo expanded EPCs were used for the first time to treat ischemic diseases.[42] Human PB mononuclear cells (hPBMCs) cultured in fibronectin-coated dishes with EC basal medium allowed expansion of a spindle-shaped population after 7 to 10 days. These cells were positive for VEGFR-2, VE-cadherin, and CD31, with ability to endocytose acLDL and bind UEA-1, consistent with an endothelial lineage. Transplantation of this population to athymic nude rats with hind limb ischemia resulted in blood flow recovery and increased capillary density in the ischemic hind limb.[26,43,44] Another study showed that autologous BM mononuclear cell transplantation in a rabbit ischemic limb improved collateral vessel formation and blood perfusion in the injured area.[45] Moreover, EPC-mediated neovascularization can still occur under disease conditions, since in the ischemic limbs of diabetic rodents neovascularization improved after EPC transplantation.[46,47]

Cellular Cardiomyoplasty

The therapeutic benefits of EPCs have also been shown in a model of myocardial infarction. EPC-enriched fraction of hPBMC were labeled and injected intravenously 3 hours after myocardial ischemia in nude rats, resulting in increased vasculogenesis, improved cardiac regional wall motion, and reduced infarct size.[48] It is noteworthy that the repair induced by these cells was clearly associated with neovascularization, contributing to improved myocardial blood flow. Other authors have demonstrated the benefits to treat ischemia of a more primitive EPC population, the $CD34^+$ fraction. Intravenous injection of human $CD34^+$ cells from PB showed that they were able to induce neoangiogenesis in the hearts of nude rats with myocardial infarctions.[49] In addition, the authors demonstrated that injection of $CD34^+$ cells with phenotypic features of mature endothelium was not sufficient for the induction of neoangiogenesis. They proposed that injection of a fraction containing angioblastic activity was required. In additional work by this group, the authors also proposed that improved cardiac function was also mediated by proangiogenic factors that protect cardiomyocytes against apoptosis and induce cardiomyogenesis.[50] More recently, it has been demonstrated that the ability of $CD34^+$ cells to repair infarcted myocardium is mediated by concurrent mechanisms of vasculogenesis and cardiomyogenesis.[51] This work is consistent with in vitro studies showing the ability of $CD34^+$ cells to transdifferentiate into cardiomyocytes, endothelial cells, and smooth muscle cells.[52,53] It differs from postulates of a lack of transdifferentiation of EPCs to other nonhematopoietic phenotypes.[54,55]

FACTORS GOVERNING THE SUCCESS OF THE CELL THERAPY IN ISCHEMIC PROCESSES

Routes of Application

EPCs for the treatment of ischemic diseases have been delivered in two ways—intravenously or intramuscularly. The advantage of intravenous infusion is that cells can travel directly into viable tissues, thus ensuring their survival. However, it requires the homing of the transplanted cells to the injured tissue, which may result in a less efficient delivery. Besides, it has been demonstrated that an important fraction of cells delivered by this route are arrested in other organs like the liver or the spleen.[56] In contrast, direct delivery of EPCs overcomes these problems. For these reason, the most successful preclinical studies were performed by intramyocardial injection via a small needle of stem cells into the healthy myocardium, the infarcted area, the border zone, or a combination of these. Unfortunately, often only a relatively small proportion of transplanted cells survives in the host myocardium.[57] An alternative approach would be the combination of cells with a biodegradable matrix or with survival factors that preserve the viability of the implant.[58–60]

The Survival of EPCs in the Hypoxic Environment

In the acute phase of the ischemic injury, there is an overexpression of cytokines and growth factors that not only govern the inflammatory process, but also dictate the behavior of the recruited or transplanted stem cells in the ischemic niche.[61] The effect of these cytokines on the transplanted cells must be taken into account, since it is possible that even using an appropriate dose and route of injection for tissue regeneration, the transplanted cells may

die or be removed from the site of injection shortly after transplantation due to a paucity of adhesion or proliferation signals or to excessive apoptotic stimuli.[62] For instance, 7 days after the acute myocardial infarction, the levels of the cytokines transforming growth factor β-3, interleukin (IL)1, IL6, and the chemokines IL8 and CXCl12 (SDF-1) are still increased.[28] As a result of this activation, other proteins like collagen I (12-fold increase), MMP9 (sixfold), and ANP (60-fold) are also overexpressed in the peri-infarct region.[63–66] It is believed that this cocktail constitutes the response to ischemia-induced stress.[61] We recently demonstrated that IL6 plays an important role in the survival and self-renewal capacity of $CD34^+$ cells ex vivo through the expression of Bcl-2 as mediated by the JAK/STAT3 pathway.[67] Thus, it is possible that the overexpression of this and other cytokines plays a role in the survival of EPCs after intramyocardial transplantation.

CLINICAL TRIALS WITH EPCs

Transplantation of ECPs either derived from BM or PB in rodent animal models of ischemia (hind limb, myocardial infarction, etc) augmented the number of vessels and angiogenesis, leading to improved blood flow and tissue performance. These cells have also been transplanted in large animal models using catheter-based intramyocardial transplantation, with favorable outcomes.[68] Encouraged by these results, several clinical trials have been initiated and are presently underway. The initial results point to the feasibility and safety of autologous EPC transplantation. Among those completed transplantation of ex vivo expanded EPCs improved coronary flow reserve and left ventricular function among patients with acute myocardial infarction.[69,70] The transplanted group also showed a significant improvement in stroke volume index, left ventricular end systolic volume, contractility, and perfusion of the infarct region. Other studies performed with an autologous, total BM mononuclear fraction (BM-MNC), showed improved myocardial perfusion and modulated heart rate variability in patients with myocardial infarction.[71,72]

Transplantation of autologous BM-MNC was also performed to treat ischemic limbs associated with peripheral arterial disease. Augmented ankle-brachial indices and reduced rest pain were observed among treated patients with ischemic limbs.[73] These results were attributed to the ability of BM cells to initiate therapeutic angiogenesis by supplying both EPCs and angiogenic factors.

Enriched-EPC populations like BM-CD133 were also assessed by intracoronary injection, showing improved left ventricular performance, but the treatment was associated with increased coronary events.[19] Taken together, these studies suggested that intracoronary infusion of EPCs can improve outcomes after myocardial infarction. However, larger-scale randomized trials are required to evaluate the long-term clinical outcomes. Concerning the clinical investigation of the use of autologous adult stem cells for the repair of the heart, the consensus of the task force of the European Society of Cardiology[74] was that treatment with autologous stem cells cannot be recommend in habitual clinical practice, but it was recommended to continue preclinical research in this area. In this context, it is important to know the cell type used for a given therapy for two reasons. First, the transplantation of complex cellular mixtures, like BM-MNCs, involves implantation of a low fraction of stem cells. Second, we cannot obviate the fact that transplanted cells also influence the host, although purified populations are less toxic than complex mixtures.[75]

In conclusion, these studies supported the idea that both cultured and freshly isolated human EPCs have therapeutic potential to treat ischemic damage. Important advances have been made in our understanding of the basic mechanisms controlling the recruitment, differentiation, and function of these cells. However, it is necessary to better understand the process of ischemia, the mechanism of physiological repair, and the mechanisms that each therapy unchain, in order to improve these therapies and diminish adverse effects. In this context, we must consider whether a given process in ischemic tissue inhibits or promotes the survival, differentiation, and integration of a replacement cell type.[75,76] Thus, in vitro analysis of the main physiological routes of transplanted cells that are activated or inhibited by factors overexpressed in ischemic tissues would help to elucidate the outcomes of a given cell therapy.

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