Endothelial Progenitor Cells for Vasculogenesis

Postnatal vasculogenesis is considered to be involved in neovascularization of adult tissues, because bone marrow-derived endothelial progenitor cells (EPCs) were isolated from circulating mononuclear cells in peripheral blood and were shown to incorporate into sites of physiological and pathological neovascularization and to differentiate into mature endothelial cells. EPCs might have an attractive potential therapeutic application for cardiovascular ischemic diseases as a novel cell-based strategy mainly via a vasculogenesis mechanism.

The therapeutic implications of angiogenic growth factors were identified by the pioneering work of Folkman and colleagues over two decades ago (14). Their work documented the extent to which tumor development was dependent on neovascularization and suggested that this relationship might involve angiogenic growth factors that were specific for neoplasms. Subsequent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. This novel strategy for the treatment of vascular insufficiency was termed “therapeutic angiogenesis” (43).

More recent data suggest that the basis for native as well as therapeutic neovascularization is not restricted to angiogenesis but includes postnatal vasculogenesis as well. Our laboratory (2–4, 20, 22, 42) and others (8, 15–17, 30, 32, 39) have established that bone marrow (BM)-derived endothelial progenitor cells (EPCs) are present in the systemic circulation, are augmented in response to certain cytokines and/or tissue ischemia, and home to as well as incorporate into sites of neovascularization (FIGURE 1). Because of these features, EPCs have been investigated as therapeutic agents in studies of “supply-side” angiogenesis under pathological as well as physiological conditions. This review focuses on EPC isolation from adult peripheral blood, EPC kinetics in vivo, and the therapeutic potential of EPCs for ischemic diseases.

Isolation of EPCs

Available evidence suggests that hematopoietic stem cells (HSCs) and EPCs (33, 36) are derived from a common precursor (hemangioblast) (12, 18, 46). Growth and fusion of multiple blood islands in the yolk sac of the embryo ultimately give rise to the yolk sac capillary network (35); after the onset of blood circulation, this network differentiates into an arteriovenous vascular system (36). The integral relationship between the elements that circulate in the vascular system—the blood cells—and the cells that are principally responsible for the vessels themselves—endothelial cells (ECs)—is implied by the composition of the embryonic blood islands. The cells destined to generate hematopoietic cells are situated in the center of the blood island and are termed HSCs. EPCs, or angioblasts, are located at the periphery of the blood islands. In addition to this spatial association, HSCs and EPCs share certain antigenic determinants, including Flk-1, Tie-2, c-Kit, Sca-1, CD133, and CD34. These progenitor cells have consequently been considered to derive from a common precursor, putatively termed a hemangioblast (12, 18, 46).

The identification of putative HSCs in peripheral blood and BM and the demonstration of sustained hematopoietic reconstitution with these HSC transplants have constituted inferential evidence for HSCs in adult tissues (5, 24, 38, 41). Recently, the related descendents—EPCs—have been isolated along with HSCs in hematopoietic organs. Flk-1 and CD34, shared by embryonic EPCs and HSCs, were used to detect putative EPCs from the mononuclear cell fraction of peripheral blood (3). In vitro, these cells differentiated into endothelial lineage cells, and in animal models of ischemia, heterologous, homologous, and autologous EPCs were shown to incorporate into sites of active neovascularization. This finding was followed by diverse identifications of EPCs by several groups (15, 16, 27, 34, 39) using equivalent or different methodologies. It should be noted that no specific surface markers have been found between HSCs and EPCs in the immature stage. Although no specific markers are yet available, endothelial-specific surface markers, such as vascular endothelial cadherin, Tie-2, and Flk-1, disappear in HSCs; on the
other hand, those markers remain in EPCs following the differentiation step. It should be possible to divide EPCs and HSCs in the downstream by using these markers. The evidence that EPCs are descendents from HSCs is still unclear. Regarding a hierarchy of EPCs, EPCs could be descendents from HSCs or could be transdifferentiated from HSCs. Further precise investigation would be necessary to confirm the hierarchy of EPCs.

Therapeutic Mobilization of EPCs

Having demonstrated the potential for endogenous mobilization of BM-derived EPCs, we considered that iatrogenic expansion and mobilization of this putative EC precursor population might represent an effective means to augment the resident population of ECs that is competent to respond to administered angiogenic cytokines. Such a program might thereby address the issue of endothelial dysfunction or depletion that may compromise strategies of therapeutic neovascularization in older, diabetic, and/or hypercholesterolemic animals and patients. We should take into account that the plasma level of growth factors (e.g., VEGF) is attenuated in older patients with atherosclerosis and that the frequency of EPC mobilization is reduced, suggesting that frequency of EPC mobilization from BM depends on aging or diseases. Our preliminary data suggested that cornea neovascularization in nude mice was impaired by EPC transplants derived from older patients compared with those from healthy young volunteers. Tepper et al. (44) have shown the impairment of EPC incorporation into vascular structures in type 2 diabetic patients. Granulocyte macrophage colony stimulating factor, which stimulates hematopoietic progenitor cells and myeloid lineage cells as well as nonhematopoietic cells, including BM stromal cells and ECs, has been shown to exert a potent stimulatory effect on EPC kinetics (42) (FIGURE 1). Such cytokine-induced EPC mobilization could enhance neovascularization of severely ischemic tissues as well as de novo corneal vascularization (42). The mechanisms whereby these EPCs are mobilized to the peripheral circulation occur in the early stage of definition. Among all growth factors, VEGF is the most critical factor for vasculogenesis and angiogenesis (6, 11, 37). Recent data indicate that VEGF is an important factor for EPC kinetics

![FIGURE 1. Kinetics of endothelial progenitor cells for neovascularization](https://physiologyonline.physiology.org/)

Endothelial progenitor cells (EPCs) circulate in adult human peripheral blood and are mobilized from bone marrow by cytokines, growth factors, and ischemic conditions. Vascular injury is repaired by both angiogenesis and vasculogenesis mechanisms. Circulating EPCs contribute to repair of injured blood vessels mainly via a vasculogenesis mechanism.

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too. Our studies performed first in mice (4) and subsequently in patients undergoing VEGF gene transfer for critical limb ischemia (20) and myocardial ischemia (22) established that a previously unappreciated mechanism by which VEGF contributes to neovascularization is via mobilization of BM-derived EPCs. A similar timing of EPC mobilization has been observed in response to other hematopoietic stimuli, such as granulocyte colony stimulating factor (15) and stromal-derived factor-1 (34). In a pathological situation (e.g., ischemia, wound), the plasma level of cytokines and growth factors should be systemically augmented depending on the ischemic size or severity of the wound. The evidence that EPCs are mobilized from BM to peripheral blood confirms the elevation of the plasma level of cytokines or growth factors. Shintani et al. (40) have reported that plasma VEGF levels positively correlated with the number of CD34-positive cells derived from circulating mononuclear cells.

This therapeutic strategy of EPC mobilization has recently been implemented not only by using natural hematopoietic or angiogenic stimulants but also by using antihypercholesterolemia drugs. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, inhibit the activity of HMG-CoA reductase, which catalyzes the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis. The statins rapidly activate Akt signaling in ECs, and this stimulates EC bioactivity in vitro and enhances angiogenesis in vivo (26). Recently, we and Dimmeler et al. demonstrated a novel function for HMG-CoA reductase inhibitors that contributes to postnatal neovascularization by augmented mobilization of BM-derived EPCs through stimulation of the Akt signaling pathway (9, 29, 45). Because of their well-established pharmacological safety and their effectiveness against hypercholesterolemia, one of the risk factors for atherogenesis, the statins might thus become a potent medication against atherosclerotic vascular diseases both for patients with normal and with high cholesterolemia.

**Therapeutic Vasculogenesis of EPC Transplantation**

Recently the regenerative potential of stem cells has been under intense investigation. In vitro, stem and progenitor cells possess the capability for self-renewal and differentiation into organ-specific cell types. In vivo, transplantation of these cells may reconstitute organ systems, as shown in animal models of diseases (1, 3, 10, 13, 21, 28).

Direct repopulation capability of EPCs was investigated in murine and rodent models of hindlimb and myocardial ischemia, respectively. One day after the operative excision of one femoral artery, athymic nude mice (n = 17), in which angio genesis is characteristically impaired (7), received an intracardiac injection of 5 × 10⁵ culture-expanded human EPCs (hEPCs). Two control groups were similarly injected with either human microvascular ECs (HMVECs) (n = 12) or medium from the culture plates employed for hEPC ex vivo expansion (n = 14). Time-course studies demonstrated that peak hEPC incorporation into sites of neovascularization was achieved within 3–7 days after administration of hEPCs. Histological evaluation of skeletal muscle sections retrieved from the ischemic hindlimbs of mice killed 7, 14, and 28 days later showed that capillary density, an index of neovascularization, was markedly increased in hEPC-transplanted mice.

Enhanced neovascularization in mice transplanted with hEPCs led to important biological consequences. Among mice in which induction of hindlimb ischemia was followed by administration of HMVECs, limb salvage was limited to 1 (8.3%) of 12 animals, whereas the remainder developed extensive foot necrosis (n = 5, 41.7%), leading in 6 (50%) to spontaneous amputation. Likewise, a preserved limb was observed in only 1 (7.1%) of 14 mice treated with culture medium, whereas foot necrosis and/or autoamputation developed in 7 (50%) and 6 (42.9%) mice, respectively.

In contrast, hEPC transplantation was associated with successful limb salvage in 10 (58.8%) of 17 animals. Foot necrosis was limited to five (29.4%) mice, and only two (11.8%) experienced spontaneous limb amputation. The difference in outcomes between the hEPC-treated mice and both control groups was statistically significant (for hEPC vs. control medium, P = 0.003). The outcomes in mice receiving HMVECs or culture medium were similar (P = 0.9).

Similar outcomes have now been demonstrated in rats with myocardial ischemia (23). In this case, peripheral blood mononuclear cells obtained from healthy human adults were cultured in EPC medium and were harvested 7 days later. Myocardial ischemia was induced by ligation of the left anterior descending coronary artery in male C57BL/6J-athymic male) rats. In two rats, 10⁶ EPCs labeled with Dil were injected intravenously 3 h after induction of myocardial ischemia. Seven days later, fluorescence-conjugated BS-1 lectin, a murine-specific EC marker, was administered intravenously and the rats were immediately killed. Fluorescence microscopy revealed that transplant ed EPCs accumulated in the ischemic area and incorporated into foci of myocardial neovascularization.

To determine the impact on left ventricular (LV) function, five rats (EPC group) were injected intra-
venously with $10^6$ EPCs 3 h after induction of ischemia. Five other rats (control group) received culture medium. Echocardiography, performed just before and 28 days after induction of ischemia, disclosed ventricular dimensions that were significantly smaller and fractional shortening that was significantly greater in the EPC vs. the control group by day 28 (diastole = $0.87 \pm 0.03$ vs. $0.93 \pm 0.01$ cm, $P < 0.05$; systole = $0.68 \pm 0.03$ vs. $0.79 \pm 0.02$ cm, $P < 0.01$; fractional shortening = $21.3 \pm 0.6$ vs. $15.3 \pm 2.2\%$, $P < 0.001$). Regional wall motion was better preserved in EPC vs. control group (absolute value $25.3 \pm 0.8$ vs. $30.6 \pm 1.0$, $P < 0.01$). Following death on day 28, necropsy examination disclosed that capillary density was significantly greater in the EPC group than in controls ($290.1 \pm 21.5$ vs. $191.1 \pm 17.8/mm^2$, $P < 0.001$). Moreover, the extent of LV scarring was significantly lower in rats receiving EPCs than in controls ($8.9 \pm 0.9$ vs. $17.8 \pm 1.4\%$ of LV, $P < 0.01$). Immunohistochemistry revealed capillaries that were positive for human CD31 and UEA-1 lectin. Thus ex vivo-expanded EPCs administered intravenously to rats with myocardial ischemia incorporate into foci of myocardial neovascularization and have a favorable impact on the preservation of LV function.

Recently, Kocher et al. attempted intravenous infusion of freshly isolated (not cultured) human CD34+ mononuclear cells (EPC-enriched fraction) into nude rats with myocardial ischemia (25). This strategy resulted in preservation of LV function associated with inhibition of cardiomyocyte apoptosis. These experimental findings using immunodeficient animals suggest that both cultured and freshly isolated human EPCs have therapeutic potential in peripheral and coronary artery diseases.

It should be noted that ischemic or wound stimulus causes proliferation, migration, and mobilization of EPCs, and then mobilized EPCs are incorporated into the foci of neovascularization. The mechanism of angiogenesis is thought to be involved at any time, and it is not completely excluded when vasculogenesis is induced. Vasculogenesis is dominant in the case of severe ischemic or wound condition. In the foci of neovascularization, mobilized EPCs derived from a vasculogenesis mechanism act as both the provider for repairing the injured vessels and the producer of cytokines for stimulating other cells.

**Gene Therapy using EPCs**

Given these findings, together with the limited quantity of EPCs available even under healthy, physiological conditions, one must consider a

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**FIGURE 2. Strategies for EPC transplantation**

Patients with ischemic disorders often experience complications, e.g., hypertension, hyperlipidemia, and/or diabetes in addition to aging. To overcome the cell-functional impairment, it is necessary to increase the cell number or improve the cell quality. Cytokine administration or gene modification of ex vivo-expanded cells might be effective for clinical application.
strategy that addresses this shortfall and mitigates the possibility of dysfunctional EPCs for therapeutic vasculogenesis in ischemic disorders complicated by aging, diabetes, hypercholesterolemia, and/or hyperhomocysteinemia. Genetic modification of EPCs to overexpress angiogenic growth factors, enhance signaling activity of the angiogenic response, and rejuvenate the bioactivity and/or extend the life span of EPCs constitutes one potential strategy that might address these limitations of EPC transplantation and thereby optimize therapeutic neovascularization (FIGURE 2).

Our recent findings provide the first evidence that exogenously administered, gene-modified EPCs augment naturally impaired neovascularization in an animal model of experimentally induced limb ischemia (19, 31) (FIGURE 3). Most somatic cells of humans and other mammals undergo a finite number of cell divisions, ultimately entering a nondividing state termed senescence. Loss of telomerase activity has been suggested to constitute the molecular clock that triggers cellular senescence. In contrast to somatic cells, true stem cells and germline cells highly express the catalytic subunit of telomerase (human telomerase reverse transcriptase; hTERT), thus maintaining telomerase activity and full replication of telomeric DNA; these cells (by definition) are thereby able to divide indefinitely. Although they have demonstrated regenerative potentials for vascular development, EPCs are not pluripotent, self-renewing stem cells but rather are lineage-committed progenitors and thus are subject to a Hayflick life span via replicative senescence. Accordingly, we have deduced that constitutive expression of hTERT might induce a delay in senescence and recover/enhance regenerative properties of EPCs. Transplantation of heterologous EPCs transduced with adenovirus encoding VEGF or hTERT (Ad/VEGF, Ad/TERT) not only improved neovascularization and blood flow recovery but also had meaningful biological consequences: limb necrosis and autoamputation were reduced compared with controls. The dose of EPCs used in the current in vivo experiments was subtherapeutic, i.e., this dose of EPCs was 30 times less than that required in previous experiments done with nontransduced cells. Thus transplantation of EPCs transduced with Ad/VEGF or Ad/TERT successfully combines VEGF or hTERT gene therapy and stem cell therapy; it constitutes an attractive option to address the limited number of EPCs that can be isolated from peripheral blood before ex vivo expansion and subsequent autologous readministration. Although the potential risk is the evidence of malignant transformation or loss of functional and morphogenetic characteristics of the parental cells in the case of hTERT gene modification, no such evidence was observed in the experi-

FIGURE 3. Enhancement of neovascularization by gene-modified EPCs
Constitutive expression of telomerase reverse transcriptase (TERT) induces delay in senescence and recovers/enhances regenerative properties of EPCs. EPCs were isolated from healthy human volunteers and were cultivated for 7 days. An adenoviral TERT or green fluorescent protein (GFP) construct was introduced into EPCs the next day. Immunodeficient mice with hindlimb ischemia received TERT-EPCs or GFP-EPCs systemically. TERT-EPC transplantation resulted in superior rescue of ischemic legs and improvement of blood flow compared with GFP-EPC transplantation.
ment. To minimize these risks, we tested only temporary overexpression of hTERT.

Summary

EPCs were isolated from circulating mononuclear cells and shown to enhance neovascularization. EPC transplantation in ischemic diseases could be a future therapeutic strategy.

References


