Endothelial Progenitor Cell Vascular Endothelial Growth Factor Gene Transfer for Vascular Regeneration

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Background—Previous studies have established that bone marrow–derived endothelial progenitor cells (EPCs) are present in the systemic circulation. In the current study, we investigated the hypothesis that gene transfer can be used to achieve phenotypic modulation of EPCs.

Methods and Results—In vitro, ex vivo murine vascular endothelial growth factor (VEGF) 164 gene transfer augmented EPC proliferative activity and enhanced adhesion and incorporation of EPCs into quiescent as well as activated endothelial cell monolayers. To determine if such phenotypic modulation may facilitate therapeutic neovascularization, heterologous EPCs transduced with adenovirus encoding VEGF were administered to athymic nude mice with hindlimb ischemia; neovascularization and blood flow recovery were both improved, and limb necrosis/autoamputation were reduced by 63.7% in comparison with control animals. The dose of EPCs used for the in vivo experiments was 30 times less than that required in previous trials of EPC transplantation to improve ischemic limb salvage. Necropsy analysis of animals that received DiI-labeled VEGF-transduced EPCs confirmed that enhanced EPC incorporation demonstrated in vitro contributed to in vivo neovascularization as well.

Conclusions—In vitro, VEGF EPC gene transfer enhances EPC proliferation, adhesion, and incorporation into endothelial cell monolayers. In vivo, gene-modified EPCs facilitate the strategy of cell transplantation to augment naturally impaired neovascularization in an animal model of experimentally induced limb ischemia. (Circulation. 2002;105:732-738.)

Key Words: gene therapy ■ endothelium ■ angiogenesis ■ ischemia
After transduction, cells were applied, and the culture maintained through day 7. Nonadherent cells were removed by washing, new media was applied, and the culture was maintained through day 7.

Methods

EPC Culture

Ex vivo expansion of EPC was performed as recently described. In brief, peripheral blood mononuclear cells from human volunteers were plated on human fibronectin–coated (Sigma) culture dishes and maintained in EC basal medium-2 (EBM-2) (Clonetics) supplemented with 5% fetal bovine serum, human VEGF-A, human fibroblast growth factor-2, human epidermal growth factor, insulin, and 20 ng/mL human TNF-α.

EPC Gene Transfer

After 7 days in culture, cells were transduced with an adenovirus encoding the murine VEGF 164 gene (Ad/VEGF) or lacZ gene (Ad/lacZ) (generously provided by Kevin Peters). To establish optimum conditions for EPC adenovirus gene transfer serum concentration, virus incubation time and virus concentration were evaluated (Figure 1). After preliminary experiments were performed, human EPCs were transduced with 1000 MOI Ad/VEGF or Ad/lacZ for 3 hours in 1% serum media.

Figure 1. Profile of transfection efficiencies for Ad/β-gal in ex vivo–expanded human EPCs. Four different multiplicities of infection (MOI, 250, 500, 1000, and 2000) were tested in 2 different media conditions (1% or 5% serum EBM-2) for 1 or 3 hours of incubation. Error bars represent SEM of triplicate experiments. After these preliminary experiments, human EPCs were transduced with 1000 MOI Ad/VEGF or Ad/β-gal for 3 hours in 1% serum media.

Proliferative Activity Assay

At 24 hours after gene transfer, EPCs transduced with Ad/VEGF (Td/VEGFCs), Ad/β-gal (Td/β-galCs), or nontransduced EPCs (non-Td/ECs) were reseeded on 96-well plates coated with human fibronectin for assay of proliferative activity with the use of the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] Assay (Promega). After 48 hours in culture, MTS/PMS (phenazine methosulfate) solution was added to each well for 3 hours, and light absorbance at 490 nm was detected by ELISA plate reader (Biotecics Laboratory).

In Vitro Incorporation of Td-EPCs Into Human Umbilical Vein Endothelial Cell Monolayer

At 24 hours after gene transfer, Td/V-EPCs and Td/β-EPCs were stained with fluorescent carbocyanine Dil (Biomedical Technologies). Dil-labeled EPCs were incubated on a monolayer of human umbilical vein endothelial cells (HUVECs) in 4-well culture slides with or without pretreatment of tumor necrosis factor (TNF)-α (1ng/mL) for 12 hours. Three hours after incubation, nonadherent cells were removed by washing with PBS, new media was applied, and the culture was maintained for an additional 24 hours. The total number of adhesive EPCs in each well was counted in a blinded manner under a ×200 magnification field of a fluorescent microscope.

Td-EPCs Transplantation Animal Model

All procedures were performed in accordance with the St Elizabeth’s Institutional Animal Care and Use Committee. Female athymic nude mice (Jackson Laboratory, Bar Harbor, Maine), 8 to 9 weeks old and 17 to 20 g weight, were anesthetized with 160 mg/kg IP pentobarbital for operative resection of one femoral artery and subsequently for laser Doppler perfusion imaging.

As a preliminary experiment, we performed dose-dependent EPC transplantation to determine the minimum number of VEGF-transduced EPCs that was required to achieve a magnitude of therapeutic neovascularization similar to that which could be achieved with nontransduced EPCs. According to this result, 1.5×10^5 VEGF-transduced EPCs, 30 times less than the number required for cell therapy alone, were used in the current series of in vivo experiments. One day after unilateral femoral artery excision, 1.5×10^5 Td/V-EPCs (n=11), Td/β-EPCs (n=11), or non-Td/EPCs (n=5) in 100 µL EBM-2 media without growth factors were administered through the tail vein.

To track the fate of transplanted EPCs, 4 mice in each EPC cohort received EPCs that were marked with the fluorescent carbocyanine Dil dye (Molecular Probes). In brief, before cellular transplantation, EPCs in suspension were washed with PBS and incubated with Dil at a concentration of 2.5 µg/mL PBS for 5 minutes at 37°C and 15 minutes at 4°C. After two washing steps in PBS, the cells were resuspended in EBM-2 medium. At 30 minutes before the animals were killed, a subgroup (n=4 each group) of mice received an intravenous injection of 50 µg of Bandeiraea simplicifolia lectin 1 (BS-1) conjugated with FITC (Vector Laboratories).

Plasma VEGF Levels

To confirm that the Ad/VEGF could mediate successful gene transfer at the protein level, an enzyme-linked immunoassay (ELISA, R&D System) was used to quantify VEGF levels in plasma from animals 1, 4, 7, and 28 days after intravenous injections of Td/V-EPCs or Td/β-EPCs. The results were compared with a standard curve constructed with murine VEGF (each assay carried out in duplicate for each animal). Absorbance was measured at 450 nm by means of a microplate reader.

Physiological Assessment of Animals Given Transplantation

Laser Doppler perfusion imaging (LDPI) (Moor Instruments) was used to record blood flow measurements on day 0 and day 28 after surgery, as previously described. In these digital color-coded images, red hue indicates regions with maximum perfusion, medium perfusion values are shown in yellow, and lowest perfusion values are represented as blue. The resulting images display absolute values in readable units. For quantification, the ratio of readable units in ischemic to nonischemic hindlimb is determined.

Histological Assessment of Animals Given Transplantation

Vascular density was evaluated at the microvascular level through the use of light microscopic sections harvested from the ischemic hindlimbs at necropsy. Tissue sections from the lower calf muscles of ischemic limbs were harvested on days 7 and 28. Muscle samples were embedded in OCT compound (Miles), snap-frozen in liquid nitrogen, and cut into 5-µm-thick sections. Tissue sections were stained for alkaline phosphatase with an indoxyltetrazolium method to detect capillary endothelial cells as previously described and then were counterstained with eosin. A total of 20 different fields were randomly selected, and the number of capillaries and myofibers were counted (×40 magnification for 20 fields).
Statistical Analysis

All results are expressed as mean±SEM. Statistical significance was evaluated by means of a paired Scheffé test or ANOVA. A value of \( P < 0.05 \) was considered to denote statistical significance.

Results

Proliferative Activity Assay

MTS assay was used to determine proliferative activity of transduced EPCs. With the use of 5% serum-conditioned media, proliferative activity of Ad/VEGF-transduced EPCs exceeded proliferative activity of Ad/β-gal (Td/β-EPCs) and nontransduced EPCs (non-Td) (0.48±0.03 vs 0.37±0.01 corrected absorbance at 490 nm, \( P < 0.01 \)) and nontransduced EPCs (non-Td) (0.32±0.02, \( P < 0.05 \)) in vitro (Figure 2).

In Vitro Incorporation of Td-EPCs Into HUVEC Monolayer

At 24 hours after transduction, EPCs were labeled with the fluoroscein marker DIL for cell tracking. DiI-labeled, VEGF-transduced EPCs were incubated on a HUVEC monolayer with or without TNF-α (1 ng/mL) pretreatment for 12 hours (Figure 3A). After 3 hours of incubation, nonadherent cells were removed by washing with PBS, and DiI-marked cells adherent to the HUVEC monolayer were manually counted. In the quiescent HUVEC monolayer, adhesion of DiI-labeled EPCs was not significantly different between Td/V-EPCs and Td/β-EPCs (2.7±0.2 versus 2.2±0.3, \( P = \text{NS} \)) (Figure 3B). In activated HUVECs, however, adhesion of DiI-labeled Td/V-EPCs exceeded Td/β-EPCs (4.3±0.4 versus 2.9±0.3, \( P < 0.01 \)).

Alternatively, the same cells were incubated in new media and maintained for 24 hours on the HUVEC monolayer to confirm incorporation in vitro. In the quiescent HUVEC monolayer, incorporation of DiI-labeled Td/V-EPCs exceeded Td/β-EPCs (7.0±0.5 versus 3.5±0.5, \( P < 0.01 \)) (Figure 3B). In activated HUVECs, incorporation of DiI-labeled Td/V-EPCs also exceeded Td/β-EPCs (13.8±0.8 versus 5.3±0.6, \( P < 0.001 \)).

Transgene Expression After Td/V-EPC Transplantation

Ad/VEGF-mediated gene transfer and expression were confirmed by ELISA assay of plasma samples obtained from mice after Td/V-EPC transplantation. Mice transplanted with Td/V-EPCs disclosed significantly higher VEGF protein levels (day 1, 828.18±8.84 versus 4.56±2.21 pg/mL; day 4, 421.27±12.60 versus 5.16±2.79 pg/mL; day 7, 65.65±3.20 versus 3.78±2.26 pg/mL; day 28, 17.40±1.99 versus 4.42±1.88 pg/mL; \( P < 0.01 \)) at each time point than did mice transplanted with Td/β-EPCs (Figure 4).
EPC Incorporation Into Ischemic Hindlimb

Transplanted human EPC derived cells marked with DiI were identified in tissue sections by red fluorescence. In contrast, the mouse vasculature, stained by premortem administration of BS-1 lectin, was identified by green fluorescence in the same tissue sections. Transplanted animals disclosed increased numbers of EPC-derived vasculature as well as mouse vasculature compared with control animals (Td/β-EPC and non-Td groups). Td/V-EPC transplanted animals had increased numbers of EPC-derived vasculature in comparison to the other two groups (Figure 5A).

Both transplanted EPC-derived vasculature and mouse vasculature were analyzed quantitatively in the same microscopic field. The density of DiI-labeled EPCs in tissue sections of skeletal muscle removed from the ischemic limb was greater in the Td/V-EPCs group than either of the other two groups (non-Td and Td/β-EPCs; \(P<0.001\) vs non-Td and Td/β-EPCs, \(P<0.01\) vs non-Td and Td/β-EPCs).

Physiological Assessment of Animals Given Transplantation

The impact of human gene–modified EPC administration on neovascularization was investigated in a murine model of hindlimb ischemia. One day after operative excision of the femoral artery, athymic nude mice (n=27), in which angiogenesis is characteristically impaired,14,21 received an intravenous injection of 1.5\(\times\)10⁴ transduced EPCs (Td/V-EPCs, n=11) or Td/β-EPCs (n=11). As additional control animals, 5 mice with hindlimb ischemia were identically injected with non-Td/EPCs. Enhanced neovascularization in mice transplanted with Td/V-EPCs led to important biological consequences, compared with control animals.

After administration of Td/β-EPCs to 11 mice, 3 (27.2%) had extensive toe necrosis, and the remaining 8 (72.7%) underwent autoamputation of the ischemic limb (Figure 6A). In contrast, Td/V-EPC transplantation was associated with successful limb salvage in 7 (63.6%) of 11 animals; toe necrosis was limited to 3 (27.2%) mice, and only 1 (9%) had spontaneous limb amputation (Figure 6B).

Serial examination of hindlimb perfusion by LDPI was performed at days 0 and 28 (Figure 7A). The ratio of ischemic/normal blood flow in mice transplanted with Td/V-EPCs indicated significantly greater hindlimb perfusion compared with those mice transplanted with Td/β-EPCs and nontransduced EPCs at day 28 (0.71±0.15 versus 0.40±0.03 versus 0.34±0.04, \(P<0.05\)) (Figure 7B).

Histological Assessment of Animals Given Transplantation

To further evaluate the impact of EPC gene transfer on revascularization of the ischemic hindlimb, histological examination of skeletal muscle sections retrieved from the ischemic hindlimbs of mice killed at day 28 was performed as described above. Capillary density observed in the mice transplanted with Td/V-EPCs was significantly higher than in...
mice receiving Td/β-EPCs (421 ± 15 versus 230 ± 14/mm², P < 0.05) or nontransduced mice EPCs (183 ± 16/mm², P < 0.05) (Figure 8). Similarly, the capillary/muscle fiber ratios in the Ad/VEGF transplanted mice were significantly higher than in Td/β-EPCs or non-Td/EPCs transplanted mice (0.62 ± 0.02 versus 0.39 ± 0.03 versus 0.35 ± 0.05, P < 0.01).

Figure 6. Administration of hEPCs leads to reduced limb loss and increased limb salvage. A. Representative macroscopic photographs of mice show two different outcomes observed in the study. Left panel, Td/β-EPC–treated animals; right panel, Td/V-EPC–treated animals at day 28. B. Percent distribution of above outcomes among mice receiving Td/β-EPCs and Td/V-EPCs. *P < 0.01 vs Td/β-EPCs.

Figure 7. LDPI performed at day 28. A. LDPI was used to record serial blood flow measurements immediately before and 28 days after administration of transduced EPCs. In these digital, color-coded images, red hue indicates regions with maximum perfusion; medium perfusion values are shown in yellow; lowest perfusion values are represented as blue. Upper panel, Animal that received Td/β-EPCs; lower panel, animal that received Td/V-EPCs. Panel on right displays absolute values in readable units (RU). B. Color-coded recordings were analyzed by calculating the average perfusion for each foot (ischemic and nonischemic). To account for variations, including ambient light and temperature, perfusion is expressed as ratio of left (ischemic) and right (normal) hindlimbs. *P < 0.05 vs Td/β-EPCs and non-Td/EPCs.

Figure 8. Histological evidence of neovascularization in ischemic hindlimb. Extent of neovascularization was assessed by measuring capillary density in light microscopic sections prepared from muscles of ischemic hindlimbs 28 days after administration of Td/β-EPCs, Td/V-EPCs and non-Td/EPCs. *P < 0.05 vs Td/β-EPCs and non-Td/EPCs. Similar findings were documented for capillary/myocyte ratio (see text).

Discussion

The finding that circulating EPCs may home to sites of neovascularization and differentiate into ECs in situ is consistent with “vasculogenesis,” a critical paradigm for establishment of the primordial vascular network in the embryo. Although the proportional contributions of angiogenesis and vasculogenesis to postnatal neovascularization remain to be clarified, our findings together with the recent reports from other investigators suggest that growth and development of new blood vessels in the adult are not restricted to angiogenesis but encompass both embryonic mechanisms. As a corollary, augmented or retarded neovascularization—whether endogenous or iatrogenic—probably includes enhancement or impairment of vasculogenesis.

Therapeutic Vasculogenesis

We therefore considered a novel strategy of EPC transplantation to provide a source of robust ECs that might supplement fully differentiated ECs thought to migrate and proliferate from preexisting blood vessels according to the classic paradigm of angiogenesis developed by Folkman and colleagues. Our studies indicated that ex vivo cell therapy, consisting of culture-expanded EPC transplantation, successfully promotes neovascularization of ischemic tissues, even when administered as “sole therapy,” for example, in the absence of angiogenic growth factors.

Such a “supply side” version of therapeutic neovascularization in which the substrate rather than ligand comprises the therapeutic agent was first demonstrated in a immune deficient murine model of hindlimb ischemia, with the use of donor cells from human volunteers. These findings provided novel evidence that exogenously administered EPCs augment naturally impaired neovascularization in an animal model of experimentally induced critical limb ischemia. Not only did heterologous cell transplantation improve neovascularization and blood flow recovery, but important biological consequences—notably limb necrosis and autoamputation—were reduced by 50% in comparison with mice receiving differentiated ECs or control mice receiving media in which harvested cells were expanded ex vivo before transplantation.

More recently, this same strategy has been used successfully to enhance myocardial function after myocardial infarc-
tion in experimental animal models as well.\(^\text{15}\) Peripheral blood mononuclear cells obtained from healthy human adults were cultured in EPC medium, harvested 7 days later, and administered intravenously to Hsd:RH-mu (athymic nude) rats with myocardial ischemia induced by ligation of the left anterior descending coronary artery. Fluorescent microscopy of Dil-labeled EPCs revealed that transplanted EPCs accumulated to the ischemic area and incorporated into foci of myocardial neovascularization. Echocardiography disclosed ventricular dimensions that were significantly smaller and fractional shortening that was significantly greater in the EPC versus control animals. Necropsy examination disclosed that capillary density was significantly greater and the extent of left ventricular scarring was significantly less in rats receiving EPCs versus control animals.

### Logistics of Primary EPC Transplantation

Despite promising potential for regenerative applications, the fundamental scarcity of EPC populations in the hematopoietic system, combined with possible functional impairment of EPCs associated with a variety of phenotypes such as aging, diabetes, hypercholesterolemia, and homocysteinemia,\(^\text{17–20}\) constitute important limitations of primary EPC transplantation. Ex vivo expansion of EPCs cultured from the peripheral blood of healthy human volunteers typically yields \(5.0 \times 10^6\) cells per 100 mL of blood. Our animal studies\(^\text{14}\) suggest that heterologous transplantation requires \(0.5 \times 2.0 \times 10^6\) human EPCs/grams of weight (of the recipient mouse) to achieve satisfactory reperfusion of the ischemic hindlimb.

Rough extrapolation of this experience to humans suggests that a volume of as much as 12 L of peripheral blood may be necessary to harvest the EPCs required to treat critical limb ischemia. Even with the integration of certain technical improvements, the adjustment of species compatibility by autologous transplantation, and adjunctive strategies (eg, cytokine supplements) to promote EPC mobilization,\(^\text{3,4}\) the primary scarcity of a viable and functional EPC population constitutes a potential liability of therapeutic vasculogenesis based on the use of ex vivo expansion alone.

### Advantage of VEGF EPC Gene Transfer

Our current findings provide the first evidence that exogenously administered, gene-modified EPCs augment naturally impaired neovascularization in an animal model of experimentally induced limb ischemia. Previous studies from our laboratory\(^\text{14,21}\) established that neovascularization is impaired in nude rodents; failure of a satisfactory endogenous response to limb ischemia leads to extensive necrosis, including limb autoamputation, in nearly all animals. Transplantation of heterologous EPCs transduced with adenovirus encoding VEGF improved not only neovascularization and blood flow recovery but also had meaningful biological consequences: Limb necrosis and autoamputation were reduced by 63.7% in comparison with control animals.

The dose of EPCs used in the current in vivo experiments was subtherapeutic; that is, this dose of EPCs was 30 times less than that required in previous experiments to improve the rate of limb salvage above that seen in untreated control animals. Adenoviral VEGF EPC gene transfer, however, accomplished a therapeutic effect, as evidenced by a functional outcome, despite a subtherapeutic dose of EPCs. Thus, VEGF EPC gene transfer constitutes one option to address the limited number of EPCs that can be isolated from peripheral blood before ex vivo expansion and subsequent autologous readministration.

The results of our in vitro studies provide potential insights into the mechanisms responsible for the in vivo outcomes. First, VEGF gene transfer augmented EPC proliferative activity. Second, VEGF gene transfer enhanced adhesion and incorporation of EPCs into a quiescent endothelial cell monolayer as well as ECs activated by pretreatment with TNF-\(\alpha\). These findings suggest that modulation of adhesion molecule expression after VEGF gene transfer may promote homing of EPCs to foci of ischemia and are consistent with previous studies demonstrating VEGF-induced upregulation of certain EC integrins and matrix proteins.\(^\text{32}\) Given that EPCs by definition express VEGF receptors, the potential for autocrine effects—demonstrated previously by our laboratory for ECs—on proliferation, migration, and possibly survival of EPCs probably contributed to the reduced requirement of harvested EPCs.

Given the ELISA results demonstrating increased levels of VEGF protein in animals that received VEGF-transduced EPCs, it is also likely that indirect effects of VEGF transduction contributed to improved limb neovascularity. Because ex vivo–expanded EPCs are preferentially recruited to ischemic foci for neovascularization,\(^\text{14,15}\) EPCs may operate as a Trojan horse, promoting local overexpression of secreted VEGF that may in turn promote migration, proliferation, and remodeling of differentiated EC resident in the target ischemic tissue. The extent to which augmented neovascularity derives from phenotypically modified EPCs versus enhanced proliferation and migration of native ECs in response to VEGF secreted from transduced EPCs is difficult to discern because of the lack of valid methods available to quantify local VEGF overexpression.

Furthermore, it must be acknowledged that the possibility of similar outcomes achieved with non-EPC circulating cells that overexpress VEGF (or a combination of VEGF-expressing non-EPC cells administered together with nontransduced EPCs) has not been excluded by the current studies. Testing such alternative approaches, however, is currently precluded by the lack of non-EPC cells that can (a) be ex vivo–transduced with equal efficiency; (b) circulate in vivo for some reasonable time period; and (c) be recruited to as well as incorporate into foci of neovascularization.

### Vector-Specific Issues

Transient gene expression is characteristic of adenoviral vectors.\(^\text{27}\) For purposes of therapeutic neovascularization, this feature is unlikely to constitute a liability, given the plethora of previous preclinical and clinical studies suggesting that VEGF overexpression for 4 weeks or less, whether achieved by transfer of naked plasmid DNA\(^\text{28–31}\) or use of an adenoviral vector,\(^\text{32,33}\) is sufficient to augment angiogenesis. Indeed, previous work by others has demonstrated that protracted VEGF overexpression may result in hemangioma formation in normal skeletal muscle\(^\text{34}\) as well as myocardial...
um. This potential complication was not observed in the experiments described herein.

The potential for immunologic complications remains a concern attached to the use of adenoviral vectors, despite certain reports to the contrary. In the current application, however, the ex vivo transduction strategy used may preclude exposure of the adenovirus to the immune system of the transplant recipients. Thus, administration of the transgene by ex vivo viral gene transfer may not detract from the safety of this application for clinical gene therapy.

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