4.2. EPC kinetics effected by exogenous agents

EPC mobilization has recently been implicated not only by natural hematopoietic or angiogenic stimulants but also by pharmacological agents. For instance, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are known to rapidly activate Akt signaling in ECs, thereby stimulating EC bioactivity in vitro and enhancing angiogenesis in vivo [40]. Recent studies by Dimmeler et al. and our laboratory have demonstrated a novel function of statins by mobilizing BM-derived EPCs through the stimulation of the Akt signaling pathway [41–44]. Therefore this newly appreciated role of statins, along with their already well-established safety and efficacy on hypercholesterolemia, suggests that they can offer benefit in treating various forms of vascular diseases. On the other hand, some antiangiogenic agents, i.e. angiostatin or soluble flk-1, have been shown to inhibit BM-derived EPC kinetics, leading to tumor regression [45], as BM-derived EPC kinetics is a critical factor for tumor growth, in terms of tumor neovascularization [46].

4.3. Clinical profile of EPC kinetics

There is a strong body of evidence to suggest that impaired neovascularization results in part from diminished cytokine production. However, endogenous expression of cytokines is not the only factor leading to impaired neovascularization. Diabetic or hypercholesterolemic animals—like clinical patients—exhibit evidence of dysfunction in mature endothelial cells. While the cellular dysfunction does not necessarily preclude a favorable response to cytokine replacement therapy, the extent of recovery in limb perfusion in these animals fails to reach that of control animals; this suggests another limitation imposed by a diminished responsiveness of EPCs/ECs [47–49].

The aging characterized by impaired neovascularization [50,51] might be associated with dysfunctional EPCs and defective vasculogenesis. Indeed, preliminary results from our laboratory indicate that transplantation of BM (including EPCs) from old mice into young mice led to minimal neovascularization in a corneal micropocket assay, relative to transplantation of young BM. We also demonstrated that EPCs from older patients with clinical ischemia had significantly less therapeutic effect in rescuing ischemic hindlimb of mice compared with those from younger ischemic patients [52]. These studies provide evidence to support an age-dependent impairment in vasculogenesis (as well as angiogenesis) that is heavily influenced by the EPC phenotype. Moreover, analysis of clinical data from older patients at our institution disclosed a significant reduction in the number of EPCs at baseline, as well as that in response to VEGF165 gene transfer [38]. Thus impaired EPC mobilization and/or activity in response to VEGF may contribute to the age-dependent defect in postnatal neovascularization. Recently Vasa et al. have further investigated EPC kinetics and their relationship to clinical disorders, showing that the number and migratory activity of circulating EPCs inversely correlates with risk factors for coronary artery disease, such as smoking, family history and hypertension [53]. On the basis of these findings, monitoring of BM-derived EPC kinetics in the patients with vascular diseases is expected to be valuable in the evaluation of lesion activity and/or therapeutic efficacy.

5. Therapeutic vasculogenesis

5.1. The potential of EPC transplantation

The regenerative potential of stem/progenitor cells is currently under intense investigation. In vitro, stem/progenitor cells possess the capability of self-renewal and differentiation into organ-specific cell types. When placed in vivo, these cells are then provided with the proper milieu that allows them to reconstitute organ systems. The novel strategy of EPC transplantation (cell therapy) may therefore supplement the classic paradigm of angiogenesis developed by Folkman and colleagues. Our studies indicated that cell therapy with culture-expanded EPCs can successfully promote neovascularization of ischemic tissues, even when administered as 'sole therapy,' i.e. in the absence of angiogenic growth factors. Such a 'supply-side' version of therapeutic neovascularization in which the substrate (EPCs/ECs) rather than ligand (growth factor) comprises the therapeutic agent, was first demonstrated by intravenously transplanting human EPCs to immunodeficient mice with hindlimb ischemia [25]. These findings provided novel evidence that exogenously administered EPCs rescue impaired neovascularization in an animal model of critical limb ischemia. Not only did the heterologous cell transplantation improve neovascularization and blood flow recovery, but also led to important biological outcomes—notably, the reduction of limb necrosis and auto-amputation by 50% in comparison with controls. A similar strategy applied to a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs localize to areas of myocardial neovascularization, differentiate into mature ECs and enhance neovascularization. These findings were associated with preserved left ventricular (LV) function and diminished myocardial fibrosis [54]. Murohara et al. reported similar findings in which human cord blood-derived EPCs also augmented neovascularization in a hindlimb ischemic model of nude rats, followed by in situ transplantation [17].

More recently, other researchers have explored the therapeutic potential of freshly isolated human CD34+ MNCs (EPC-enriched fraction). Shatteman et al. conducted local injection of freshly isolated human CD34+ MNCs into diabetic nude mice with hindlimb ischemia, and showed an increase in the restoration of limb flow [29].

Similarly Kocher et al. attempted intravenous infusion of freshly isolated human CD34+ MNCs into nude rats with myocardial ischemia, and found preservation of LV function associated with inhibition of cardiomyocyte apoptosis [55]. Thus two approaches of EPC preparation (i.e. both cultured and freshly-isolated human EPCs) may provide therapeutic benefit in vascular diseases, but as described below, will likely require further optimization of techniques to acquire the ideal quality and quantity of EPCs for EPC therapy (Fig. 3).

5.2. Future strategy of EPC cell therapy

Ex vivo expansion of EPCs cultured from PB-MNCs of healthy human volunteers typically yields 5.0×10^6 cells per 100 ml of blood on day 7. Our animal studies [25] suggest that heterologous transplantation requires systemic injection of $0.5–2.0\times10^4$ human EPCs/g body weight of the recipient animal to achieve satisfactory reperfusion of an ischemic hindlimb. Rough extrapolation of these data to human suggests that a blood volume of as much as 12 l may be necessary to obtain adequate numbers of EPCs to treat critical limb ischemia in patients. Therefore, the fundamental scarcity of EPCs in the circulation, combined with their possible functional impairment associated with a variety of phenotypes in clinical patients, such as aging, diabetes, hypercholesterolemia, and homocyst(e)inemia (vide infra), constitute major limitations of primary EPC transplantation. Considering autologous EPC therapy, certain technical improvements that may help to overcome the primary scarcity of a viable and functional EPC population should include: (1) local delivery of EPCs, (2) adjunctive strategies (e.g. growth factor supplements) to promote BM-derived EPC mobilization [33,37], (3) enrichment procedures, i.e. leukapheresis or BM aspiration, or (4) enhancement of EPC function by gene transduction (gene modified EPC therapy, vide infra), (5) culture-expansion of EPCs from self-renewable primitive stem cells in BM or other tissues. Alternatively, unless the quality and quantity of autologous EPCs to satisfy the effectiveness of EPC therapy may be acquired by the technical improvements described above, allogenic EPCs derived from umbilical cord blood or culture-expanded from human embryonic stem cells [17,56], may be available as the sources supplying EPCs.

5.3. Gene modified EPC therapy

A strategy that may alleviate potential EPC dysfunction in ischemic disorders is considered reasonable, given the findings that EPC function and mobilization may be impaired in certain disease states. Genetic modification of EPCs to overexpress angiogenic growth factors, to enhance signaling activity of the angiogenic response, and to

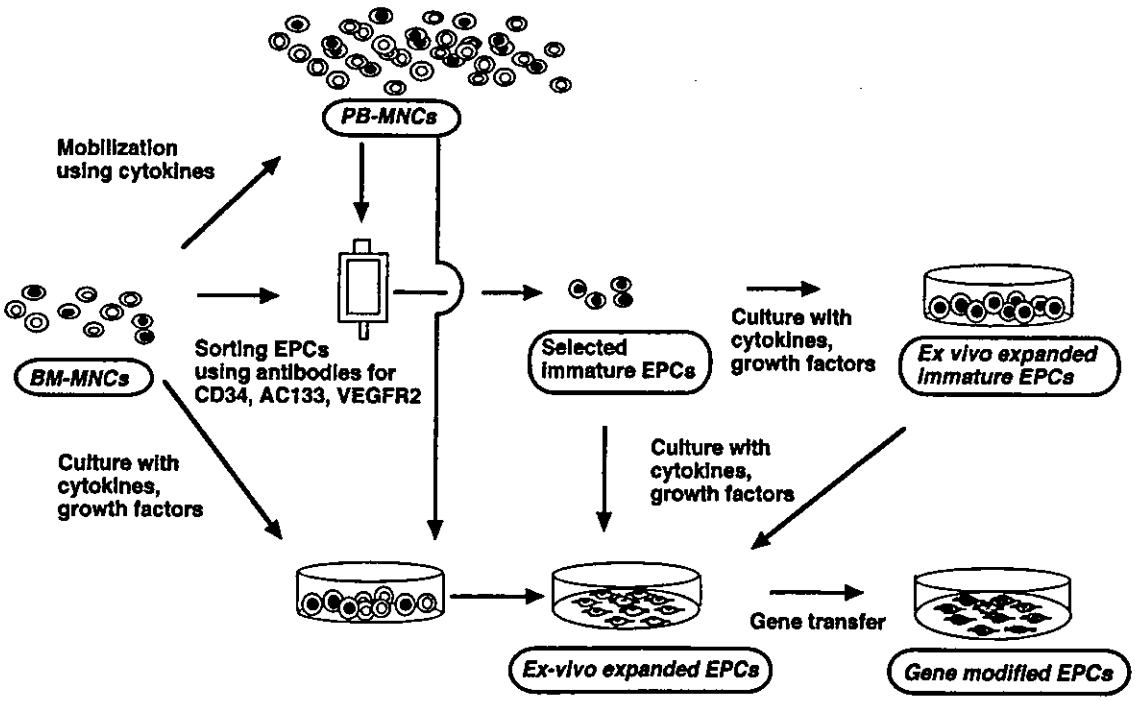

Fig. 3. EPC therapy using autologous EPCs derived from BM for vascular regeneration. Transplantation of BM- or mobilized PB-MNCs are considered 'crude EPC therapy', as EPCs are not selected. BM-MNCs have already been under clinical application. Following the manipulation to acquire the optimized quality and/or quantity, e.g. sorting by surface markers, ex vivo culture-expansion and/or gene transfection, EPC therapy is expected to be the useful strategy for vascular regeneration.

rejuvenate the bioactivity and/or extend the life span of EPCs, can constitute such potential strategies.

We have recently shown for the first time that gene-modified EPCs rescue impaired neovascularization in an animal model of limb ischemia [57]. Transplantation of heterologous EPCs transduced with adenovirus encoding human VEGF165 not only improved neovascularization and blood flow recovery, but also had meaningful biological consequences, i.e. limb necrosis and auto-amputation were reduced by 63.7% in comparison with controls. Notably, the dose of EPCs needed to achieve limb salvage in these in vivo experiments was 30 times less than that required in the previous experiments involving unmodified EPCs [25]. Thus, combining EPC cell therapy with gene (i.e. VEGF) therapy may be one option to address the limited number and function of EPCs that can be isolated from peripheral blood in patients.

5.4. BM-MNC transplantation

Nonselected total BM cells or BM-MNCs including immature EPC population have also been investigated for their potential to induce neovascularization. Several experiments have reported that autologous BM administration into rabbit [58] or rat [59] hindlimb ischemic model, and porcine myocardial ischemic model [60,61] could augment neovascularization in ischemic tissue mainly through the production of angiogenic growth factors and less through the differentiation of a portion of the cells into EPCs/ECs in situ. Although there are no long-term safety and efficacy data for local delivery of such cell population mostly composed of inflammatory leukocytes, these strategies have already been applied to clinical patients in some institutions and preliminary results are expected soon.

6. Other devices of EPCs for clinical application

EPCs have recently been applied to the field of tissue engineering as a means of improving biocompatibility of vascular grafts. Artificial grafts first seeded with autologous CD34+ cells from canine BM and then implanted into the aortae were found to have increased surface endothelialization and vascularization compared with controls [62]. Similarly, when cultured autologous ovine EPCs were seeded onto carotid interposition grafts, the EPC-seeded grafts achieved physiological motility and remained patent for 130 days versus 15 days in non-seeded grafts [63]. Alternatively, as previously reported, the cell sheets of cultured cardiomyocytes may be effective for the improvement of cardiac function in the damaged hearts, i.e. ischemic heart disease or cardiomyopathy [64,65]. The cell sheets consisting of cardiomyocytes with EPCs expected to induce neovessels may be attractive, as blood supply is essential to maintain the homeostasis of implanted cardiomyocytes in such cell sheets.

EPCs have also been investigated in the cerebrovascular field. Embolization of the middle cerebral artery in Tie2/lacZ/BMT mice disclosed that the formation of new blood vessels in the adult brain after stroke involves vasculogenesis/EPCs [66]. Similar data were reported using gender-mismatched wild-type mice transplanted with BM from Green Fluorescein Protein transgenic mice [67]. However, whether autologous EPC transplantation would augment cerebral revascularization has yet to be examined.

To date, the role of EPCs in tumor angiogenesis has been demonstrated by several groups. Davidoff et al. showed that BM-derived EPCs contribute to tumor neovasculature and that BM cells transduced with an anti-angiogenic gene can restrict tumor growth in mice [45]. Lyden et al. recently used angiogenic defective, tumor resistant Id-mutant mice and showed the restoration of tumor angiogenesis with BM (donor)-derived EPCs throughout the neovessels following the transplantation of wild-type BM into these mice [46]. These data demonstrate that EPCs are not only important, but also critical, to tumor neovascularization. Given the findings, 'anti-tumor EPC mediated gene therapy' by transplantation of EPCs transferred genes to inhibit tumor growth may be developed in the near future.

Orlic et al. recently demonstrated that lineage marker negative (non-committed) and CD117 positive BM cells can regenerate de novo myocardium and ECs and improve cardiac function when they were locally delivered into murine myocardial infarction model [68]. They also reported that mobilization of BM cells by G-CSF and stem cell factor leads to a reduction in infarct size, improves cardiac function and decreases the mortality in this animal model [69]. Jackson et al. showed that BM-derived stem cells (side population cells defined by dye exclusion) can differentiate into cardiomyocytes and ECs at a very low rate in murine cardiac reperfusion injury model following BMT [70]. These studies suggest a clinical use of BM for cardiovascular diseases other than EPCs/therapeutic vasculogenesis. Given the extensive plasticity of BM cells differentiating into neural, hepatic and mesenchymal lineages, BM-derived EPCs may also exhibit such a potential, as seen in the report suggesting the transdifferentiation of endothelial lineage cells into cardiomyocytes [71].

7. Conclusion

As the concepts of BM-derived EPCs in adults and postnatal vasculogenesis are further established, clinical applications of EPCs to regenerative medicine are likely to follow. To acquire the more optimized quality and quantity of EPCs, several issues remain to be addressed, such as the development of a more efficient method of EPC purification and expansion, the methods of administration and senescence in EPCs. Alternatively, in the case of im-

possible utility of autologous BM-derived EPCs in the patients with impaired BM function, an appreciable number of EPCs isolated from umbilical cord blood or differentiated from tissue specific stem/progenitor or embryonic stem cells need to be optimized for EPC therapy. However, the unlimited potential of EPCs along with the emerging concepts of autologous cell therapy with gene modification suggests that they may soon reach clinical fruition.

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Stromal Cell–Derived Factor-1 Effects on Ex Vivo Expanded Endothelial Progenitor Cell Recruitment for Ischemic Neovascularization

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Background—Stromal cell–derived factor-1 (SDF-1) is a chemokine considered to play an important role in the trafficking of hematopoietic stem cells. Given the close relationship between hematopoietic stem cells and endothelial progenitor cells (EPCs), we investigated the effect of SDF-1 on EPC-mediated vasculogenesis.

Methods and Results—Flow cytometric analysis demonstrated expression of CXCR4, the receptor of SDF-1, by 66±3% of EPCs after 7 days in culture. In vitro modified Boyden chamber assay showed a dose-dependent EPC migration toward SDF-1 (control versus 10 ng/mL SDF-1 versus 100 ng/mL SDF-1, 24±2 versus 71±3 versus 140±6 cells/mm^2; $P<0.0001$). SDF-1 attenuated EPC apoptosis (control versus SDF-1, 27±1 versus 7±1%; $P<0.0001$). To investigate the effect of SDF-1 in vivo, we locally injected SDF-1 into athymic ischemic hindlimb muscle of nude mice combined with human EPC transplantation to determine whether SDF-1 augmented EPC-induced vasculogenesis. Fluorescence microscopic examination disclosed increased local accumulation of fluorescence-labeled EPCs in ischemic muscle in the SDF-1 treatment group (control versus SDF-1=241±25 versus 445±24 cells/mm^2, $P<0.0001$). At day 28 after treatment, ischemic tissue perfusion was improved in the SDF-1 group and capillary density was also increased. (control versus SDF-1, 355±26 versus 551±30 cells/mm^2; $P<0.0001$).

Conclusion—These findings indicate that locally delivered SDF-1 augments vasculogenesis and subsequently contributes to ischemic neovascularization in vivo by augmenting EPC recruitment in ischemic tissues. (***Circulation.*** **2003;107: 1322-1328.)**

Key Words: chemokines ■ angiogenesis ■ ischemia ■ endothelium

Stromal cell–derived factor-1 (SDF-1) is a member of the chemokine CXC subfamily originally isolated from murine bone marrow stromal cells.[1] It has a single substantial open reading frame of 267 nucleotides encoding an 89-amino acid polypeptide and expressed on stromal cells of various tissues. On the other hand, CXCR4, a 7-transmembrane-spanning G protein–coupled receptor, is the only known receptor for SDF-1 and is also a coreceptor for HIV type 1 infection.[2] SDF-1/CXCR4 interaction is reported to play an important physiological role during embryogenesis in hematopoiesis,[3] vascular development, cardiogenesis,[4] and cerebellar development.[5]

Recently, several investigators reported that CD34$^+$ cells, classically considered to be hematopoietic stem cells, expressed CXCR4, and that SDF-1 could induce CD34$^+$ cell migration in vitro.[6] Accordingly, SDF-1 is considered as one of the key regulators of hematopoictic stem cell trafficking between the peripheral circulation and bone marrow. SDF-1 has also been shown to effect CD34$^+$ cell proliferation[7] and mobilization[8] and to induce angiogenesis in vivo.[9]

Bone marrow–derived endothelial progenitor cells (EPCs) have been isolated from the peripheral blood of adult species.[10,11] These cells participate in not only physiological but also pathological neovascularization in response to certain cytokines and/or tissue ischemia.[12–14] More recently, ex vivo expanded EPCs from peripheral blood, transplanted into animal models of ischemic hindlimbs and acute myocardial infarction, successfully augmented neovascularization resulting in physiological recovery documented as limb salvage and improvement in myocardial function.[15,16]

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At present, however, enthusiasm for the therapeutic potential of strategies of EPC transplantation is limited by certain practical considerations. For example, adjusting the number of EPCs for injection according to body weight, ≈6 L of blood would be required for harvesting of EPCs in an average-size patient to administer a dose equivalent to that which yielded therapeutic effects in limb and myocardial ischemia in small animal models. Accordingly, we investigated the hypothesis that locally administered SDF-1 could augment the local accumulation of transplanted EPCs, thereby resulting in enhanced neovascularization. Here we report that EPCs express CXCR4 and that the combination of SDF-1 local administration and EPC transplantation has potential as a strategy for therapeutic neovascularization.

Methods

Cell Isolation and Culture

Ex vivo expansion of EPCs was performed as described.[10] In brief, total human peripheral blood mononuclear cells were isolated from healthy human volunteers by density-gradient centrifugation with Histopaque-1077 (Sigma) and plated on culture dishes coated with human fibronectin (Sigma). The cells were cultured in endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with 5% FBS, human vascular endothelial growth factor (VEGF)-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, ascorbic acid, and antibiotics. After 4 days in culture, nonadherent cells were removed by washing with PBS, new medium was applied, and the culture was maintained through day 7.

$CD34^+$ cells from isolated human peripheral blood mononuclear cells were positively selected using the MiniMACS immunomagnetic separation system (Milteney Biotec) according to the manufacturer's instructions as recently described.[7]

Fluorescence-Activated Cell Sorting

Fluorescence-activated cell sorting (FACS) detection of EPCs was performed after 7 days in culture. The procedure of FACS staining was described previously.[16] In brief, a total of 2 to 3×10^5 cells were resuspended with 200 μL of Dulbecco's PBS (BioWhittaker) containing 10% FBS and 0.01% NaN_3 and incubated for 20 minutes at 4°C with phycoerythrin-conjugated monoclonal antibodies against CXCR4 (PharMingen). After staining, the cells were fixed in 2% paraformaldehyde. Quantitative FACS was performed on a FACStar flow cytometer (Becton Dickinson). All groups were studied at least in triplicate.

Migration Assay

To investigate EPC migration activity, a modified Boyden chamber assay was performed using a 48-well microchemotaxis chamber (NeuroProbe) as described.[17] In brief, SDF-1 (PharMingen) is diluted to appropriate concentrations in EBM-2 supplemented with 0.1% BSA, and 30 μL of the final dilution was placed in the lower compartment of a Boyden chamber. Human EPCs cultured for 7 days were harvested, 3×10^4 cells were suspended in 50 μL of EBM-2 supplemented with 0.1% BSA, and antibiotics were reseeded in the upper compartment. After incubation for 5 hours at 37°C, the filter was removed, and the cells on the filter were counted manually in random high-power fields (×100) in each well. All groups were studied at least in triplicate.

Apoptosis Assay

EPC apoptosis, induced by serum starvation, was quantified to determine whether SDF-1 exerts a survival effect on EPCs. The proportion of apoptotic EPCs after serum starvation was determined by manually counting pyknotic nuclei after DAPI (Roche) staining. In brief, day 7 EPCs were reseeded onto 4-chamber slides (1×10^5 cells per well with 500 μL of EPC culture medium). After 24 hours of incubation, culture medium was removed and replaced with 500 μL of EBM-2 without any supplement. After 48 hours of serum deprivation, the medium was supplemented with 100 ng/mL of SDF-1 (versus medium alone) and incubated for 3 hours. DAPI-stained pyknotic nuclei were counted as percentage of 100 cells in each well. Each group was studied at least in triplicate.

Animal Model of Ischemic Hindlimb

All procedures were performed in accordance with the Institutional Animal Care and Use Committee of St Elizabeth's Medical Center. Male athymic nude mice (CBy-Cg-Foxnl^{nu}, The Jackson Laboratory), age 8 to 10 weeks and weighing 18 to 22 g, were anesthetized with sodium pentobarbital (160 mg/kg IP) for operative resection of one femoral artery as described.[16] For euthanization, mice were injected with an overdose of pentobarbital.

RNA Extraction and Reverse Transcriptase–Polymerase Chain Reaction Analysis

Tissue RNA was extracted from frozen muscle samples (day 7 after hindlimb ischemia) using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcriptase–polymerase chain reaction (RT-PCR) of the VEGF and GAPDH genes was performed using 1 μg of total RNA. PCR was performed for 35 cycles for VEGF-A and 25 cycles for GAPDH, with each cycle consisting of 94°C for 30 seconds and 64°C for 3 minutes. Amplification was carried out in 20-μL reaction mixtures containing 0.4 U *Taq* polymerase.

Transplantation of Ex Vivo Expanded EPCs

The impact of local administration of SDF-1 after EPC transplantation on therapeutic neovascularization was investigated in a murine model of hindlimb ischemia.[16] Just after operative excision of one femoral artery, athymic nude mice, described above, in which angiogenesis is characteristically impaired, received a local intramuscular injection of 1 μg SDF-1 versus PBS in the center of the lower calf muscle followed immediately by an intravenous injection of 1.5×10^5 culture-expanded EPCs. To evaluate EPC incorporation into the vasculature in ischemic muscles, some mice were transplanted with EPCs labeled with the fluorescent carbocyanine 1,1'-dioctadecyl-1 to 3,3,3'3'-tetramethylidocarboyanine perchlorate (DiI) dye (Molecular Probes). Before transplantation, EPCs in suspension were washed with PBS and incubated with DiI at a concentration of 2.5 μg/mL PBS for 5 minutes at 37°C and 15 minutes at 4°C. After 2 washing steps in PBS, the cells were resuspended in EBM-2. Five mice in the placebo and SDF-1 groups each received 1.5×10^5 DiI-labeled EPCs intravenously as described above. Thirty minutes before euthanization at day 3 and day 7, 5 mice in each group received an intravenous injection of 50 μg of *Bandeiraea simplicifolia* lectin 1 (BS-1 lectin, Vector Laboratories) to identify the mouse vasculature.

Physiological Assessment of Transplanted Animals

Laser Doppler perfusion imaging (LDPI, Moor Instruments) was used to record serial blood flow measurements over the course of 4 weeks postoperatively, as previously described.[16] There were 8 mice in the SDF-1 group and 9 in the PBS group. In these digital color-coded images, a red hue indicates the region of maximum perfusion, medium perfusion values are shown in yellow, and the lowest perfusion values are represented by blue. Figure 5B displays absolute values in readable units.

Histological Assessment of Transplanted Animals

Tissue sections from the lower calf muscles of ischemic and healthy limbs were harvested on days 3, 7, and 28. To examine EPC incorporation at early time points after transplantation (at days 3 and 7) and SDF-1 effect on host endothelial cells, tissues from the mice injected with DiI-labeled EPCs and BS-1 lectin were embedded for frozen section samples. A total of 20 different fields (4 cross sections from each animal) were randomly selected, and the DiI-labeled EPCs were counted (×40 magnification).

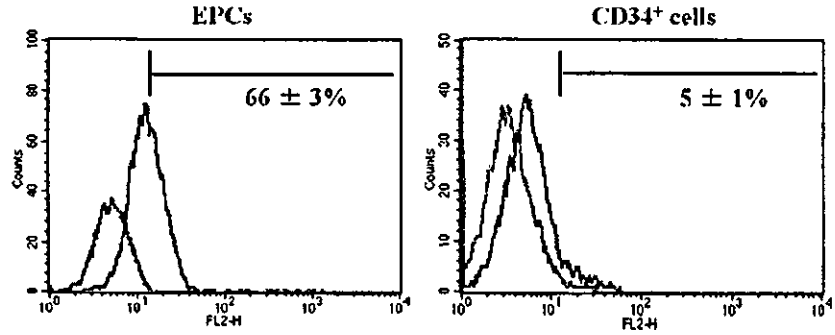

Figure 1. Analysis of CXCR-4 expression by flow cytometry. Results are shown as fluorescence histograms (blue, CXCR-4 expression; red, respective IgG control). Ex vivo expanded EPCs were positive by 66.0±3.1% for CXCR-4, and freshly isolated peripheral blood CD34+ cells by 5.2±1.1%. FL2-H indicates fluorescent intensity.

The extent of neovascularization at day 28 was assessed by measuring capillary density in light microscopic sections.[16] Paraffin-embedded sections of 5-μm thickness were stained for the mouse endothelial cell marker isolectin B4 (Vector Laboratories) and counterstained with eosin to detect capillary endothelial cells as previously described.[15] A total of 20 different fields were randomly selected (2 or 3 cross sections from each animal), and the capillaries were counted (×40 magnification).

Statistical Analysis

All results are expressed as mean±SEM. Statistical significance was evaluated using the unpaired Student *t* test for comparisons between 2 means. Multiple comparisons between >3 groups were done by ANOVA. Probability value of $P<0.05$ denoted statistical significance.

Results

Fluorescence-Activated Cell Sorting

After 7 days of culture, ex vivo expanded EPCs derived from peripheral blood of healthy human volunteers exhibited spindle-shaped morphology. These progenitor cells have qualitative properties of endothelial lineage cells.[16] FACS analysis elucidated that 66.0±3.1% of day 7 cultured EPCs express CXCR4, whereas only 5.2±1.1% of freshly isolated human peripheral blood CD34+ cells showed CXCR4 expression (Figure 1). In addition, 50.6±4.7% of CD34+ cells cultured 24 hours with EPC culture medium expressed CXCR4, which is consistent with previous reports.[7]

Migration Assay

To investigate the migratory response of ex vivo expanded EPCs toward an SDF-1, we performed a modified Boyden chamber assay in vitro. SDF-1 induced EPC migration in a dose-dependent manner (Figure 2). The magnitude of migration was similar to that induced by VEGF (data not shown). SDF-1 induced a small, statistically insignificant increase in EPC proliferative activity (data not shown).

Apoptosis Assay

To examine the effect of SDF-1 on ex vivo expanded EPC survival, we quantified apoptosis induced by serum starvation. After 48 hours of serum starvation, ex vivo expanded EPCs were treated with 100 ng/mL of SDF-1 for 3 hours. DAPI staining was performed to determine the proportion of apoptotic cells by manually counting pyknotic nuclei (Figure 3A). SDF-1 reduced apoptosis of EPCs from 26.6±1.0% to 7.1±0.9% ($P<0.0001$) (Figure 3B).

SDF-1 Upregulates Endogenous VEGF Expression in Hindlimb Ischemic Muscle

To investigate whether SDF-1 upregulates endogenous VEGF expression, we examined the expression of VEGF-A in the hindlimb ischemic muscle. Figure 4A shows temporal expression of VEGF-A mRNA in hindlimb muscle from mice treated with SDF-1 or PBS. Seven days after the treatment, VEGF-A mRNA expression was increased in SDF-1–treated muscle. Quantitative analysis of expression is shown in Figure 4B.

EPC Incorporation Into Ischemic Hindlimb Neovasculature

To elucidate the SDF-1 effect on local recruitment of transplanted EPCs from the systemic circulation and of host endothelial cells, we quantified incorporation of transplanted EPCs into the microvasculature of ischemic limbs and the number of host endothelial cells after local SDF-1 administration in nude mice hindlimbs. Transplanted human EPCs labeled with DiI were identified in tissue sections by red fluorescence, whereas the native mouse vasculature stained by premortem BS-1 lectin administration was identified by green fluorescence in the same tissue sections (Figure 5A). Histological examination disclosed increased local accumulation of DiI-labeled EPCs in the SDF-1 group compared with PBS controls (day 3, 445±24 versus 241±25 cells/mm², $P<0.0001$; day 7, 446±31 versus 355±30 cells/mm²,

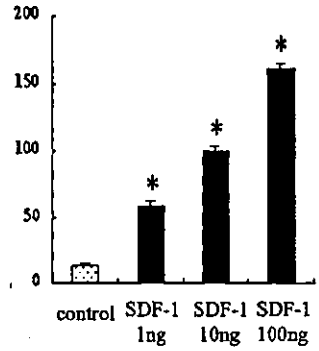

Figure 2. SDF-1 induced EPC migration. Migratory response of EPCs toward different dosages of SDF-1 stimulation was measured by modified Boyden chamber migration assay. Ex vivo expanded EPCs demonstrated a potent dose-dependent activity toward SDF-1. Control vs 10 ng/mL SDF-1 vs 100 ng/mL SDF-1, 24±2 vs 71±3 vs 140±6 cells/mm²; *$P<0.0001$.

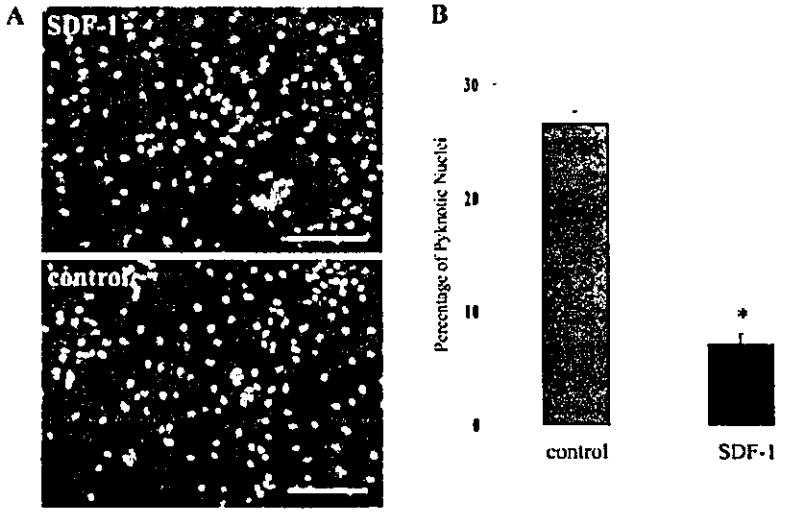

Figure 3. SDF-1 attenuated EPCs apoptosis. Serum starvation was used to induce apoptosis in ex vivo expanded EPCs. A, DAPI staining was performed to determine the proportion of apoptotic cells by manually counting pyknotic nuclei (white condensed nuclei in figures). Scale bars=100 μm. B, Quantification of percentage of pyknotic nuclei. Control vs SDF-1, 27±1% vs 7±1%; *$P<0.0001$.

$P<0.05$) (Figure 5B). Moreover, increased numbers of host endothelial cells were observed in the SDF-1 group compared with the PBS group (day 3, 500±19 versus 343±23 cells/mm^2, $P<0.0001$; day 7, 531±19 versus 386±25 cells/mm^2, $P<0.05$) (Figure 5C).

Physiological Assessment of Transplanted Animals

After systemic human EPC transplantation with local intramuscular administration of SDF-1 or PBS, serial measurements of hindlimb perfusion by LDPI were performed at days 7, 14, 21, and 28. LDPI disclosed profound differences in the limb perfusion 28 days after induction of limb ischemia (Figure 6A). By day 28, the ratio of ischemic/nonischemic blood flow in the SDF-1 treatment group improved to 0.50±0.08 versus 0.26±0.04 in the PBS group ($P<0.05$, Figure 6B). Thus, the homing effect of local SDF-1 injection documented above was accompanied by physiological evidence for enhanced neovascularization, suggesting that the EPCs that were attracted to the ischemic limb by SDF-1 were subsequently incorporated into the developing vasculature. To provide anatomic evidence of EPC-increased vasculature in the SDF-1–treated limbs, histological examination for capillary density was performed.[16]

Histological Assessment of Transplanted Animals

Staining with the endothelial cell marker isolectin B4 was performed on skeletal muscle sections retrieved from the ischemic hindlimbs of mice at day 28 to quantify capillary density (Figure 7A). Capillary density, an index of neovascularization, was significantly higher in the SDF-1 treatment group (551±30 cells/mm^2) than in the PBS treatment group (241±25 cells/mm^2, $P<0.0001$) (Figure 7B).

Discussion

Our previous studies indicated that ex vivo cell therapy, consisting of systemic implantation of culture-expanded hu-

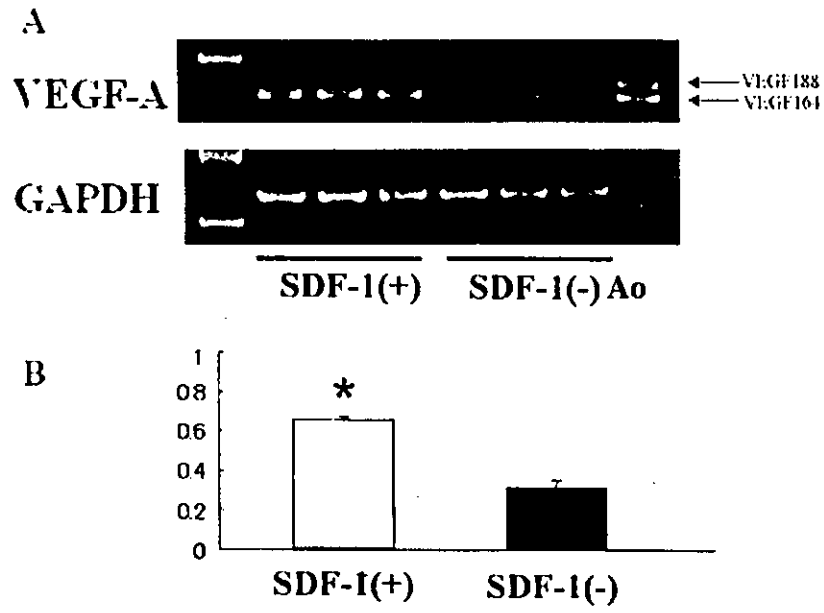

Figure 4. SDF-1 upregulated expression of VEGF-A mRNA in ischemic hindlimb. A, Expression of VEGF-A mRNA in SDF-1–treated and untreated muscle. Each panel shows RT-PCR products for VEGF-A and GAPDH. Ao indicates mouse aortic tissue as positive control. B, Densitometric analysis was performed; ratio of RT-PCR product of VEGF-A ($VEGF_{164}$) to that of GAPDH is shown. Data were obtained from 3 separate experiments and are presented as arbitrary units over controls. *$P<0.01$ (unpaired t test) vs SDF-1 (−) group.

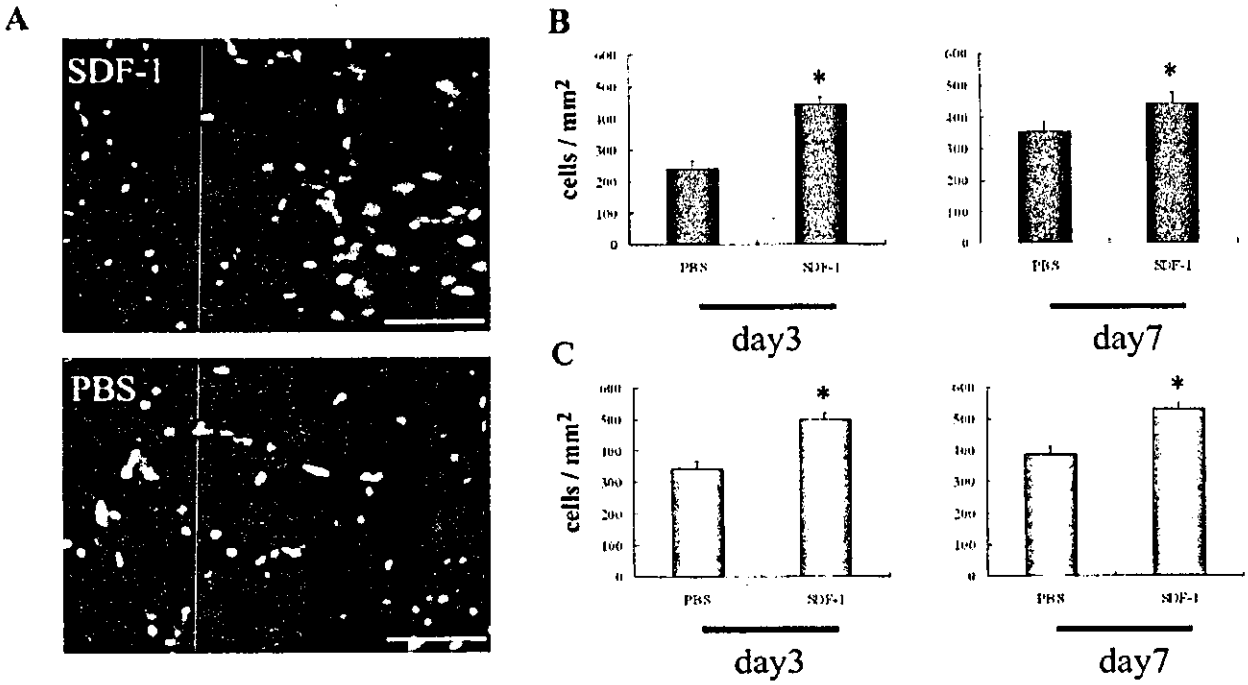

Figure 5. SDF-1 augmented EPC incorporation at an early time point. Fluorescence microscopic examination disclosed increased local accumulation of EPCs in SDF-1 treatment group compared with PBS group. A, Representative microscopic photographs of double fluorescence in ischemic muscles at day 3. Transplanted human DiI-labeled EPC-derived cells were identified by red fluorescence in histological sections retrieved from ischemic muscles. Host mouse vasculature was identified by green fluorescence in the same tissue sections. Scale bars=100 μm. B, Quantitative analysis of incorporated EPCs. Density of DiI-labeled EPCs (red fluorescence) in tissue sections retrieved from ischemic muscles was greater in SDF-1 treatment group than in PBS group at both days 3 and 7 (day 3, control vs SDF-1, 241±25 vs 445±24 cells/mm^2, *P<0.0001; day 7, control vs SDF-1, 355±30 vs 446±31 cells/mm^2, *P<0.05). C, Quantitative analysis of host endothelial cells. Density of host endothelial cells (green fluorescence) in tissue sections retrieved from ischemic muscles was greater in SDF-1 treatment group than in PBS group at both days 3 and 7 (day 3, control vs SDF-1, 343±23 vs 500±19 cells/mm^2, *P<0.0001; day 7, control vs SDF-1, 386±25 vs 531±19 cells/mm^2, *P<0.05).

man EPCs, successfully promotes neovascularization of ischemic hindlimbs[16] and acute myocardial infarction[15] in immune-deficient animal models. In these studies, heterogenous cell transplantation not only improved neovascularization but also reduced adverse biological consequences such as limb necrosis and autoamputation in the mouse ischemic hindlimb model. These studies also disclosed that systemic EPC transplantation improved myocardial neovascularization and cardiac function corresponding to reduced left ventricular scarring.

SDF-1 Effect on Vasculogenesis

Recent reports[6,7] indicated that SDF-1 was a strong chemoattractant for CD34$^+$ cells, which express CXCR4, the receptor for SDF-1, and played an important role in hematopoietic stem cell trafficking between the peripheral circulation and bone marrow. In addition, certain evidence suggests that SDF-1 may have direct effects on vasculogenesis. Tachibana et al[4] reported that mice lacking SDF-1 had defective formation of large vessels supplying the gastrointestinal tract. More recently, Hattori et al[8] reported that plasma elevation of SDF-1 induced mobilization of mature and immature hematopoietic progenitors and stem cells, including EPCs.

SDF-1 Contributes to Neovascularization by Augmenting Local Accumulation of Transplanted EPCs in Ischemic Tissues

Given the close relationship between hematopoietic stem cells and EPCs, we focused on the chemoattractant properties of SDF-1. We investigated the hypothesis that locally administered SDF-1 might augment the accumulation of EPCs to the site of ischemia, resulting in enhancing the efficacy of neovascularization after systemic EPC transplantation. The factors mediating the recruitment of circulating progenitors to ischemic tissue are not well characterized. Western analysis detected no SDF-1 protein in ischemic muscles (data not shown). We hypothesized that exogenous SDF-1, administered into ischemic tissue, could exert a strong chemoattractant effect for circulating EPCs, augmenting the effect of endogenous angiogenic/chemoattractant factors.

Our in vitro data verified the feasibility of this approach. CXCR4, the receptor for SDF-1, is expressed by EPCs, and the percentage of EPCs expressing CXCR4 was 13-fold higher compared with that of freshly isolated peripheral blood–derived CD34$^+$ cells. SDF-1 induced EPC migration and also exerted a survival effect on cultured EPCs.

In vivo, local SDF-1 administration augmented EPC accumulation 3 days after the treatment, which is consistent with

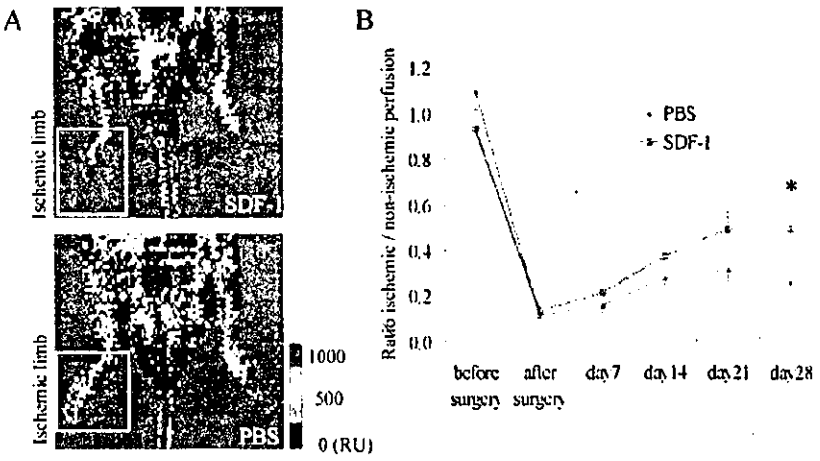

Figure 6. SDF-1 improved tissue perfusion. Hindlimb perfusion was measured by LDPI. A, Representative LDPI 28 days after induction of limb ischemia. Boxes indicate areas of interest. B, Quantitative analysis of perfusion recovery measured by LDPI. Ratios of ischemic/nonischemic limbs at day 28 were as follows: for PBS, 0.26±0.04; for SDF-1, 0.50±0.08; *$P<0.05$.

a chemoattractant effect in excess of the native locally expressed factors. The magnitude of EPC incorporation in the SDF-1 treatment group at day 3 was 1.8-fold higher than in the control group. The magnitude of EPC incorporation was similar between days 3 and 7, suggesting that the homing of exogenously administered EPCs occurs early after transplantation. Subsequent physiological and histological evaluations were performed to determine whether this increase in EPC local accumulation culminated in an increase in neovascularization. Serial LDPI measurements indicated significant differences in limb perfusion 28 days after induction of ischemia, whereas histological analysis revealed that capillary density, a direct anatomic reflection of neovascularization, was significantly greater in the SDF-1 treatment group than in the control group. These data provide evidence that the ultimate degree of physiological improvement is critically dependent on sufficient EPC recruitment at an early time point.[18,19]

It seems likely that in addition to transplanted EPCs, SDF-1 might stimulate host endothelial cells from preexisting blood vessels and host EPCs derived from bone marrow. Indeed, Salcedo et al[9] reported that subcutaneous serial SDF-1 injections into mouse skin induced formation of local small blood vessels and that SDF-1 treatment enhanced VEGF release from human umbilical vein endothelial cells in vitro. We have also observed enhanced VEGF release from EPCs treated with SDF-1 in vitro (data not shown).[20] Taken together with these observations, SDF-1 appears to have effects on endogenous angiogenesis (direct or via certain secondary cytokines) as well as vasculogenesis.

However, SDF-1 administered locally as the sole therapy for hindlimb ischemia in the same animal model resulted in autoamputation within 7 days in all animals (n=5, data not shown). Accordingly, at least under the experimental conditions used in this study, the effect of SDF-1 on neovascularization appears to result primarily from its ability to enhance the recruitment and incorporation of transplanted EPCs.

To the best of our knowledge, this study represents the first experimental proof of principle for the feasibility and therapeutic effectiveness of augmenting local accumulation of EPCs. EPCs widely express CXCR4, and local administration of SDF-1 enhanced vasculogenesis and subsequently contributed to neovascularization in vivo inducing in situ recruitment of transplanted EPCs in ischemic tissues. To apply SDF-1 treatment in clinical ischemic patients, certain issues will need to be considered, such as the effect of SDF-1 on atherosclerosis. Additional experiments using atherosclerotic animal models may shed light on this concern. Nevertheless, we believe that the concept of augmenting local accumulation of transplanted EPCs opens perspectives for the clinical strategy of EPC therapies.

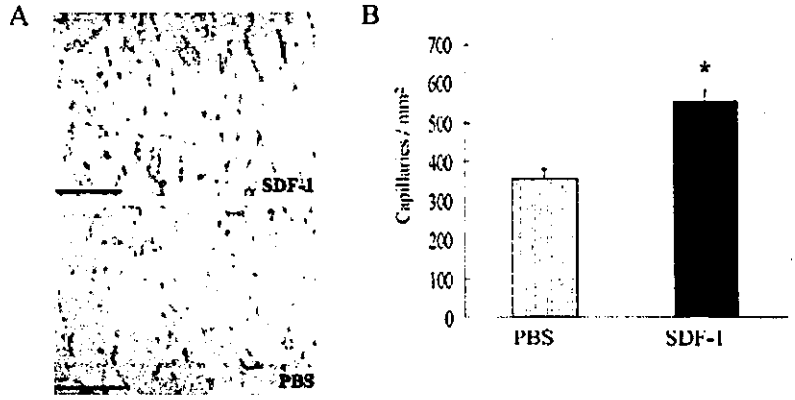

Figure 7. SDF-1 increased capillary density in ischemic tissue at day 28. Histological skeletal muscle section retrieved from ischemic hindlimbs at day 28 was examined for capillary density, an index of neovascularization, using endothelial-specific chemical staining of isolectin B4. A, Representative microscopic photographs of isolectin B4 histochemical staining in ischemic muscles at day 28. Brown indicates isolectin B4–positive vasculatures. Scale bars=100 μm. B, Quantitative analysis of capillary density. PBS vs SDF-1, 355±26 vs 551±30 cells/mm² (*$P<0.0001$).

Acknowledgments

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This article is dedicated to Dr Jeffrey M. Isner, who died on October 31, 2001. We gratefully acknowledge him for his inspirational leadership, friendship, and encouraging support.

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Endothelial Progenitor Cells for Vasculogenesis

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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

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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

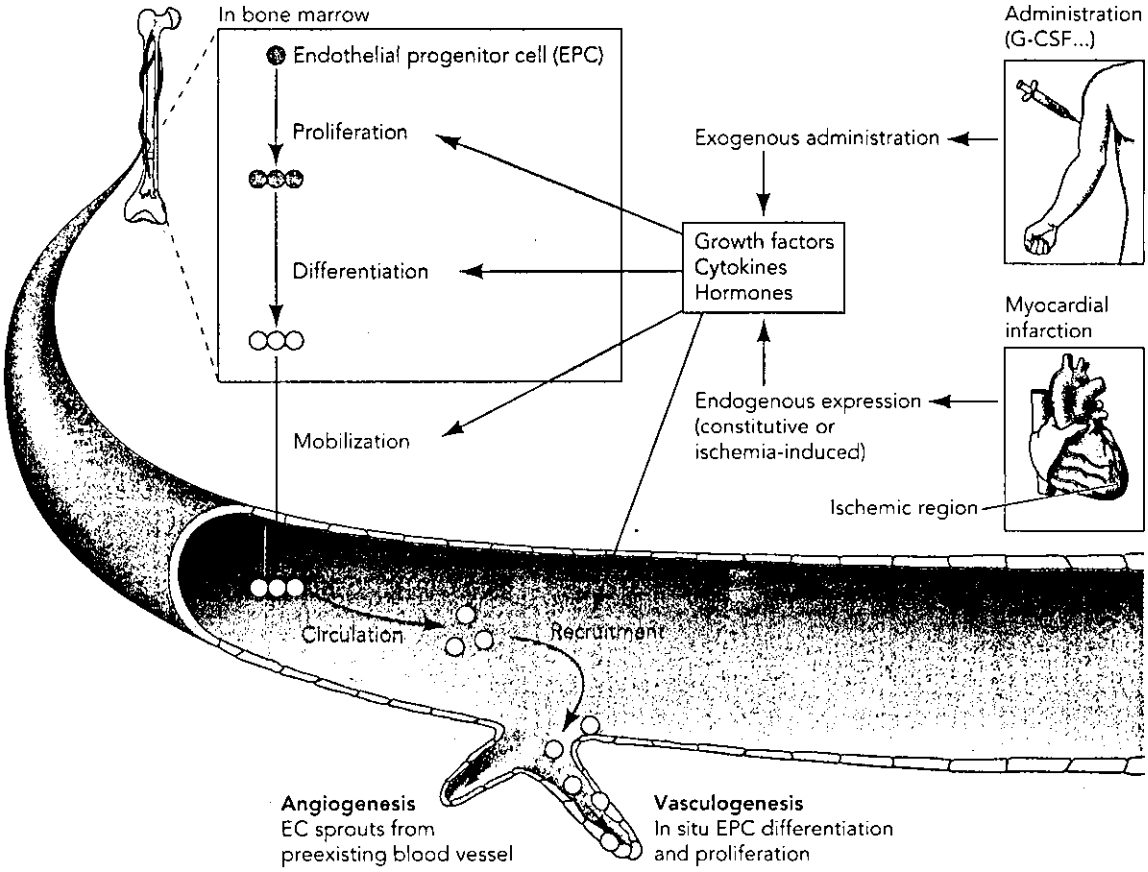

FIGURE 1. **Kinetics of endothelial progenitor cells for neovascularization**
Endothelial progenitor cells (EPCs) circulate in adult human peripheral blood and are mobilized from bone marrow by cytokines, growth factors, and ischemic conditions. Vascular injury is repaired by both angiogenesis and vasculogenesis mechanisms. Circulating EPCs contribute to repair of injured blood vessels mainly via a vasculogenesis mechanism.

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 mobilization of EPCs has been observed in response to other hematopoietic stimulators, 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 angiogenesis is characteristically impaired (7), received an intracardiac injection of 5×10^5 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 forefoot 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. HMVEC, P = 0.006; for hEPC vs. control medium, P = 0.003). The outcomes in mice receiving HMVECs vs. 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 Hsd:RH-rnu (athymic nude) rats. In two rats, 10^6 EPCs labeled with DiI 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 transplanted 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 ± 0.03 vs. 0.93 ± 0.01 cm, $P < 0.05$; systole = 0.68 ± 0.03 vs. 0.79 ± 0.02 cm, $P < 0.01$; fractional shortening = 21.3 ± 0.6 vs. 15.3 ± 2.2%, $P < 0.001$). Regional wall motion was better preserved in EPC vs. control group (absolute value 25.3 ± 0.8 vs. 30.6 ± 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 ± 21.5 vs. 191.1 ± 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 ± 0.9 vs. 17.8 ± 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

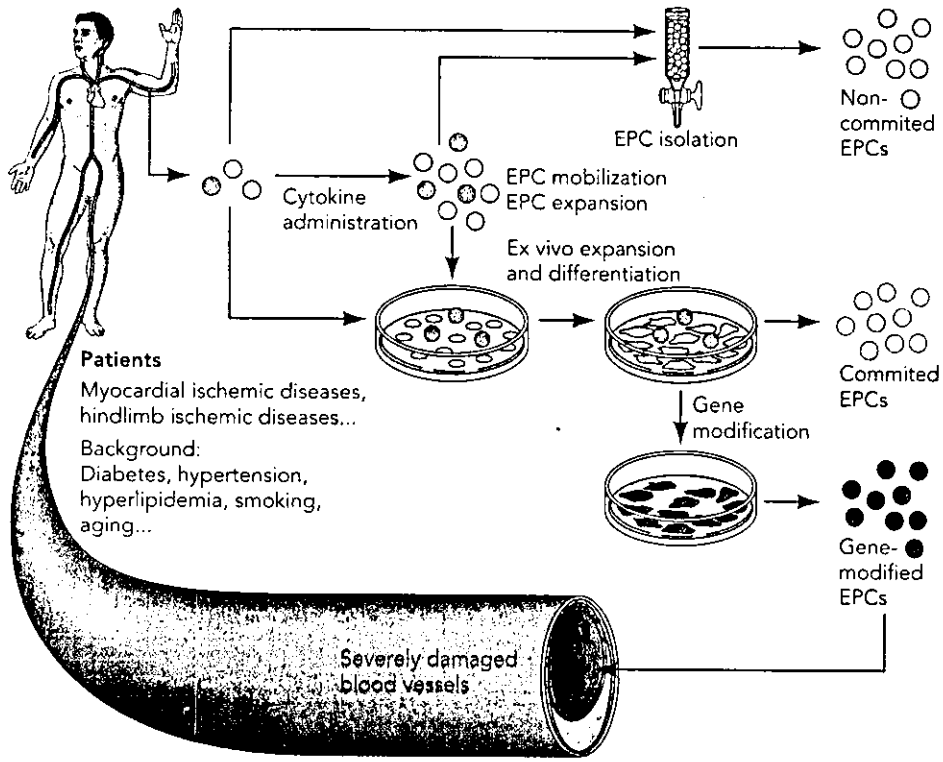

FIGURE 2. **Strategies for EPC transplantation**
Patients with ischemic disorders often experience complications, e.g., hypertension, hyperlipidemia, and/or diabetes in addition to aging. To overcome the cell-functional impairment, it is necessary to increase the cell number or improve the cell quality. Cytokine administration or gene modification of ex vivo-expanded cells might be effective for clinical application.

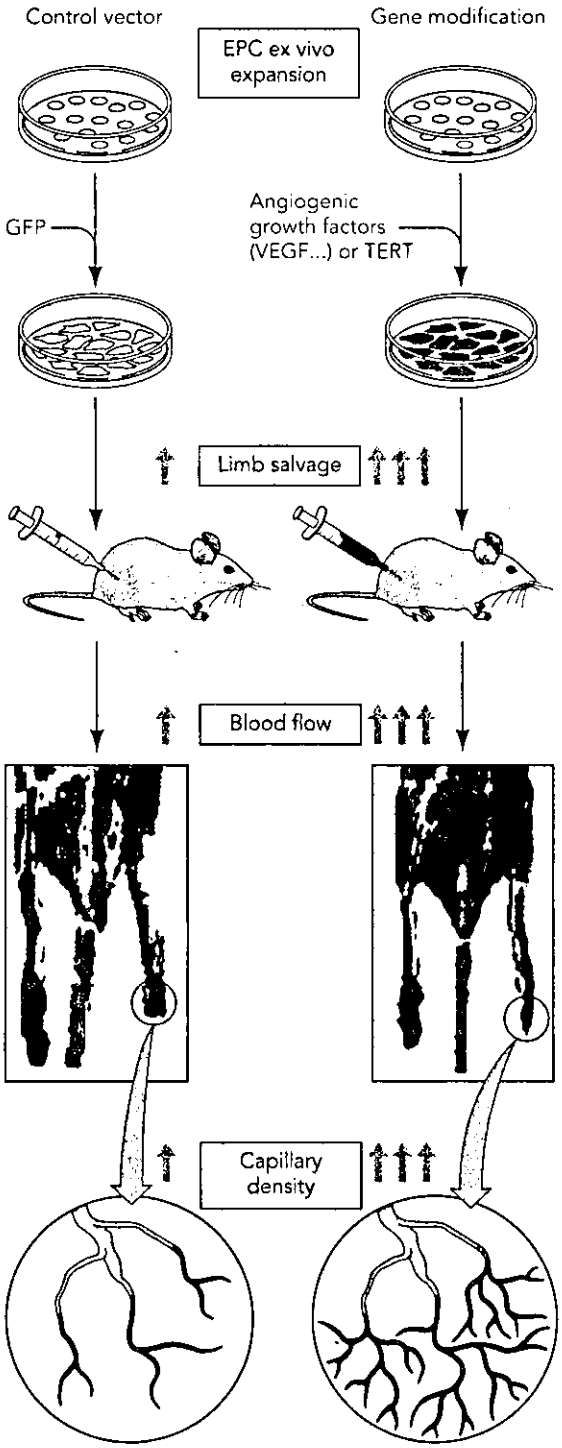

FIGURE 3. **Enhancement of neovascularization by gene-modified EPCs**
Constitutive expression of telomerase reverse transcriptase (TERT) induces delay in senescence and recovers/enhances regenerative properties of EPCs. EPCs were isolated from healthy human volunteers and were cultivated for 7 days. 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.

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-

mental procedure. 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. ■

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ANNUAL REPORT

2003年における遺伝子・再生医学研究

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はじめに

再生医学とは，細胞移植や，サイトカインや成長因子などの投与により，失われた器官や組織(あるいはその一部)を再生するための，基礎・臨床研究，および臨床応用に関わる学問である。近年，心血管領域での再生医学に関する研究が進んでいるが，これには，血管再生や心筋再生の概念が含まれる。世界に先駆けて，ボストン・タフツ大学のIsner教授らがVEGF(Vascular Endothelial Growth Factor)プラスミドの遺伝子治療を末梢動脈閉塞症患者に試みて血管再生による効果を報告して以来，再生医療と遺伝子治療が密接な関連をもつことが示唆されるようになった。本稿では心血管領域における最近の遺伝子・再生医学研究の動向について紹介をする。

遺伝子治療の動向

1994年にIsner教授らによってVEGF$_{165}$プラスミド遺伝子導入による閉塞性動脈硬化症(ASO)への臨床研究が開始されて以来，細胞増殖因子を用いた血管新生療法の概念が臨床応用に適用されるようになった。1998年にはBaumgartner[1]らが重症ASO患者に対して，またLosordo[2]らが，重症狭心症患者に対してそれぞれ，VEGF$_{165}$プラスミド遺伝子治療を行い，自覚症状の改善とともに，客観的評価による側副血行路の増加，および末梢血流の改善をもたらすことを報告した。

虚血性心疾患に対する遺伝子導入経路はその後，胸部小切開法による心筋内注入から，血管内を経由したカテーテルによる選択的注入へと変わり，より低侵襲の治療が可能になった。その後，Losordo[3]らは，血管内を経由するカテーテルを用いて，VEGF-2プラスミドを注入する第Ⅰ/Ⅱ相試験を開始した。カテーテルを用いた方法による安全性が確認され，第Ⅲ相試験への試みがなされている。一方，開胸によるVEGF-2プラスミドの心筋への直接注入による第Ⅰ相試験が重症狭心症患者に対して行われ，1年のフォローアップの報告がFortuin[4]らによって最近なされたが，この中では，遺伝子治療による臨床所見の改善は認めたものの，客観的評価による血管新生の所見は得られず，プラセボ効果の関与が示唆されている。これらの効果を明らかにするため，現在第Ⅲ相試験が行われているものと思われる。一方，国内ではVEGFと同様に血管内皮増殖作用が報告されているHGF(Hepatocyte Growth Factor)を用いた重症下肢虚血患者に対する臨床応用が大阪大学の森下らによって進められている。

細胞増殖因子を用いた血管新生療法には，VEGFの遺伝子治療のほかに，FGF(Fibroblast Growth factor)蛋白による治療が行われている。1998年のSchumacherら[5]，1999年にLaham[6]ら，また2000年にHendel[7]らがいずれも重症狭心症患者に対して臨床研究を行い，有効性を報告している。しかし一方で，動物実験レベルで確認される血管新生治療効果が，重症冠動脈疾患患者で十分に発揮されないことが，これまでのデータから示唆されるようになった。Ruelらは[8]，局所のNO(nitric oxide)が血管新生に関与するという基礎研究の結果をもとにして，高コレステロール食による内皮障害ミニブタと通常食によるミニブタに対して，それぞれ慢性心筋虚血を作製し，FGF-2蛋白を投与して心筋の血流改善を比較したところ，内皮障害モデルにおいて明らかにFGF-2に対する血管再生の反応が障害されているという結