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% 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/mm²; 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/mm²; 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/mm²; 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 chemokine CXC subfamily originally isolated from murine bone marrow stromal cells. 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. SDF-1/CXCR4 interaction is reported to play an important physiological role during embryogenesis in hematopoiesis, vascular development, cardiogenesis, and cerebellar development. 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. Accordingly, SDF-1 is considered as one of the key regulators of hematopoietic stem cell trafficking between the peripheral circulation and bone marrow. SDF-1 has also been shown to effect CD34+ cell proliferation and mobilization and to induce angiogenesis in vivo. Bone marrow–derived endothelial progenitor cells (EPCs) have been isolated from the peripheral blood of adult species. These cells participate in not only physiological but also pathological neovascularization in response to certain cytokines and/or tissue ischemia. 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.
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, e.g. 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. 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 (Miltenyi Biotec) according to the manufacturer’s instructions as recently described.

**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. In brief, a total of 2 to 3×10^6 cells were resuspended with 200 μL of Dulbecco’s PBS (BioWhittaker) containing 10% FBS and 0.01% NaN3, 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. 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, 3×10^5 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 EBW-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-Foxn1, 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. 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. 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 1.5×10^6 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-3,3,3',3'-tetramethylindocarbocyanine 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 1.5×10^6 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. 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).
The extent of neovascularization at day 28 was assessed by measuring capillary density in light microscopic sections. 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. 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. 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.

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, P<0.0001).

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.
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/mm², \(P<0.0001\); day 7, 531±19 versus 386±25 cells/mm², \(P<0.05\)) (Figure 5C).

**Physiological Assessment of Transplanted Animals**

After systemic human EPC transplantation with local intra-muscular 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.

**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/mm²) than in the PBS treatment group (241±25 cells/mm², \(P<0.0001\)) (Figure 7B).

**Discussion**

Our previous studies indicated that ex vivo cell therapy, consisting of systemic implantation of culture-expanded hu-
man EPCs, successfully promotes neovascularization of ischemic hindlimbs and acute myocardial infarction in immune-deficient animal models. In these studies, heterogeneous 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 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 reported that mice lacking SDF-1 had defective formation of large vessels supplying the gastrointestinal tract. More recently, Hattori et al reported that plasma elevation of SDF-1 protein in ischemic tissues was greater in SDF-1 treatment group than in PBS group at both days 3 and 7 (day 3, control vs SDF-1, 343±25 vs 500±19 cells/mm², *P<0.0001; day 7, control vs SDF-1, 386±25 vs 531±19 cells/mm², *P<0.05).

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...
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.

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. 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). 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 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.

**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 isoelectin B4. A, Representative microscopic photographs of isoelectin B4 histochemical staining in ischemic muscles at day 28. Brown indicates isoelectin 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).
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References
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