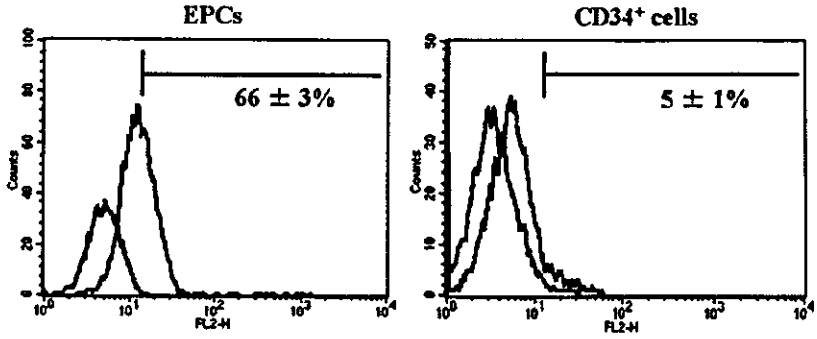

Figure 1. Analysis of CXCR-4 expression by flow cytometry. Results are shown as fluorescence histograms (blue, CXCR-4 expression; red, respective IgG control). Ex vivo expanded EPCs were positive by 66.0±3.1% for CXCR-4, and freshly isolated peripheral blood CD34⁺ cells by 5.2±1.1%. FL2-H indicates fluorescent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

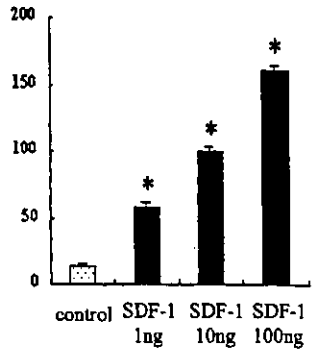

Figure 2. SDF-1 induced EPC migration. Migratory response of EPCs toward different dosages of SDF-1 stimulation was measured by modified Boyden chamber migration assay. Ex vivo expanded EPCs demonstrated a potent dose-dependent activity toward SDF-1. Control vs 10 ng/mL SDF-1 vs 100 ng/mL SDF-1, 24±2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

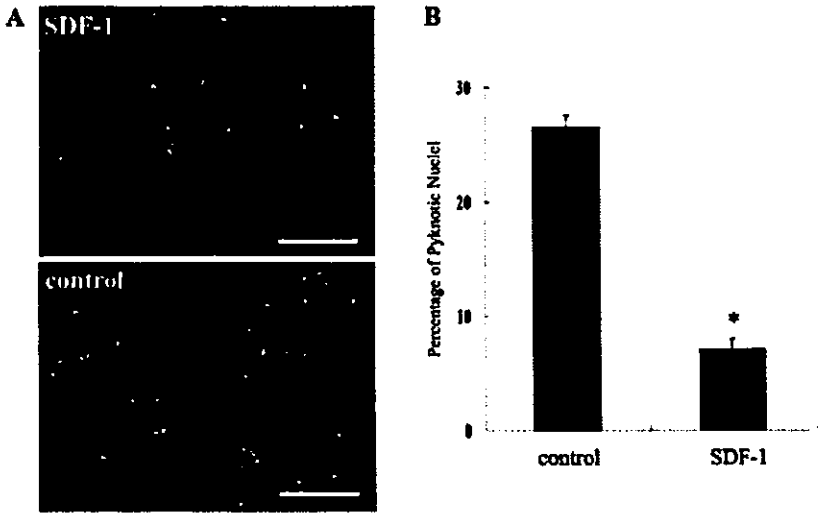

Figure 3. SDF-1 attenuated EPCs apoptosis. Serum starvation was used to induce apoptosis in ex vivo expanded EPCs. A, DAPI staining was performed to determine the proportion of apoptotic cells by manually counting pyknotic nuclei (white condensed nuclei in figures). Scale bars=100 μm. B, Quantification 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-

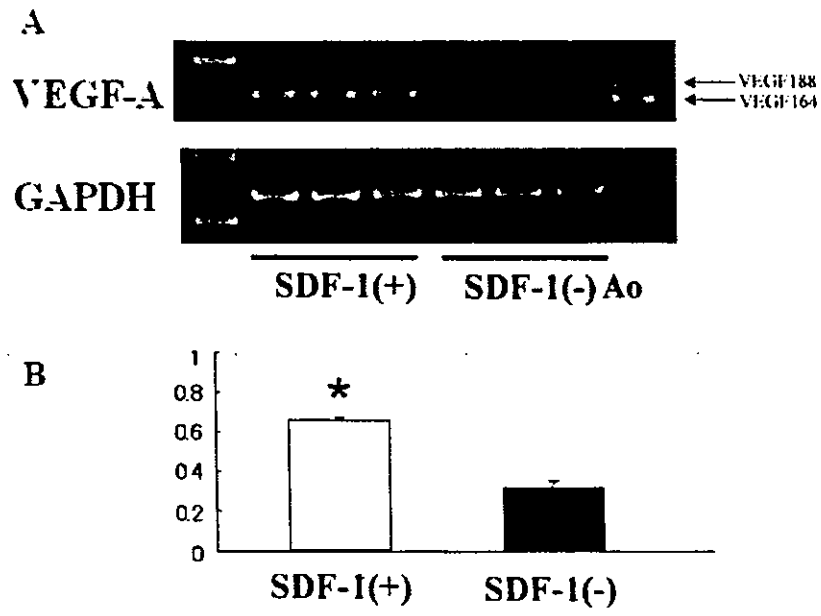

Figure 4. SDF-1 upregulated expression of VEGF-A mRNA in ischemic hindlimb. A, Expression of VEGF-A mRNA in SDF-1–treated and untreated muscle. Each panel shows RT-PCR products for VEGF-A and GAPDH. Ao indicates mouse aortic tissue as positive control. B, Densitometric analysis was performed; ratio of 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.

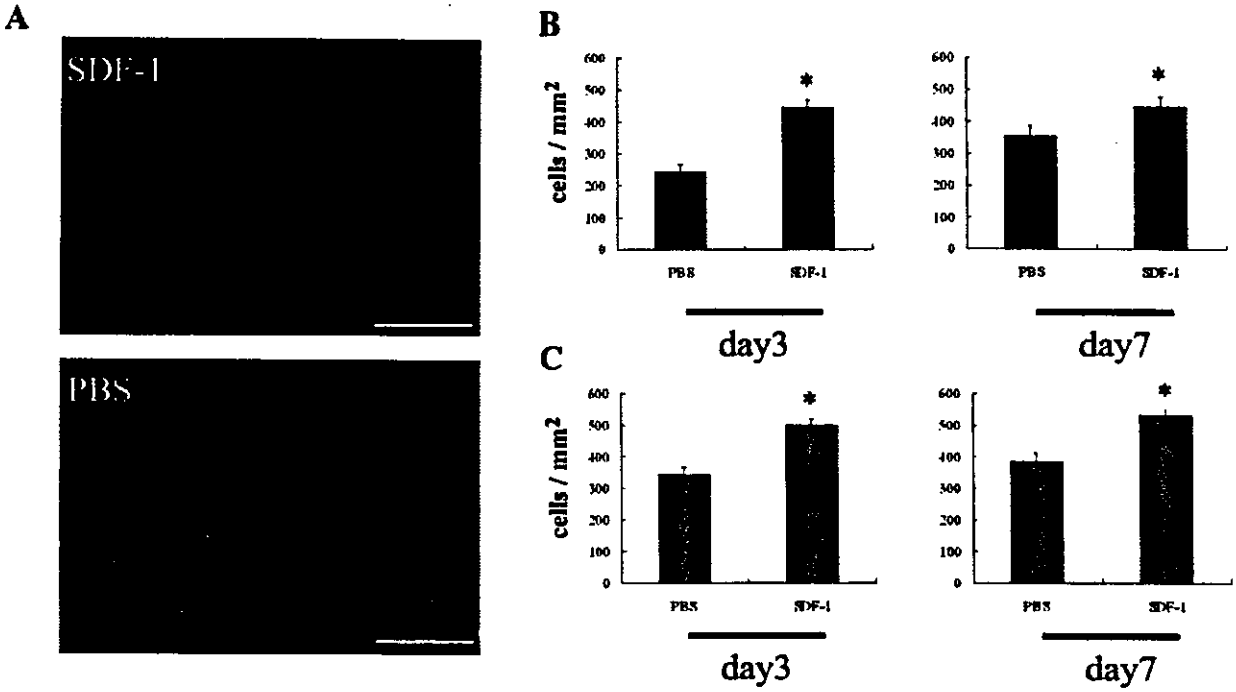

Figure 5. SDF-1 augmented EPC incorporation at an early time point. Fluorescence microscopic examination disclosed increased local accumulation of EPCs in SDF-1 treatment group compared with PBS group. A, Representative microscopic photographs of double fluorescence in ischemic muscles at day 3. Transplanted 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/mm², *P<0.0001; day 7, control vs SDF-1, 355±30 vs 446±31 cells/mm², *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/mm², *P<0.0001; day 7, control vs SDF-1, 386±25 vs 531±19 cells/mm², *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

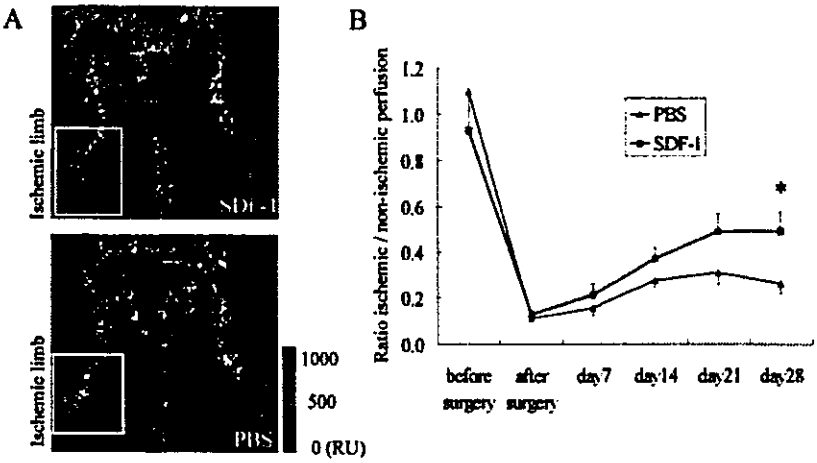

Figure 6. SDF-1 improved tissue perfusion. Hindlimb perfusion was measured by LDPI. A, Representative LDPI 28 days after induction of limb ischemia. Boxes indicate areas of interest. B, Quantitative analysis of perfusion recovery measured by LDPI. Ratios of ischemic/nonischemic limbs at day 28 were as 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.

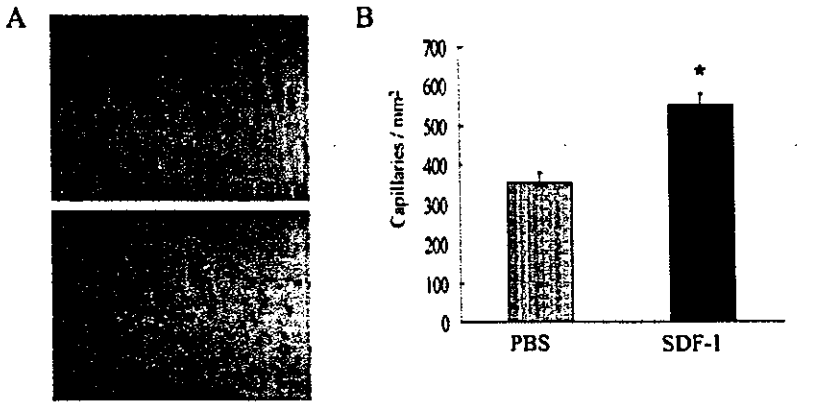

Figure 7. SDF-1 increased capillary density in ischemic tissue at day 28. Histological skeletal muscle section retrieved from ischemic hindlimbs at day 28 was examined for capillary density, an index of neovascularization, using endothelial-specific chemical staining of isolectin B4. A, Representative 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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Intramyocardial Transplantation of Autologous Endothelial Progenitor Cells for Therapeutic Neovascularization of Myocardial Ischemia

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Background—We investigated whether catheter-based, intramyocardial transplantation of autologous endothelial progenitor cells can enhance neovascularization in myocardial ischemia.

Methods and Results—Myocardial ischemia was induced by placement of an ameroid constrictor around swine left circumflex artery. Four weeks after constrictor placement, CD31+ mononuclear cells (MNCs) were freshly isolated from the peripheral blood of each animal. After overnight incubation of CD31+ MNCs in noncoated plates, nonadhesive cells (NA/CD31+ MNCs) were harvested as the endothelial progenitor cell–enriched fraction. Nonadhesive CD31− cells (NA/CD31− MNCs) were also prepared. Autologous transplantation of 10^7 NA/CD31+ MNCs, 10^7 NA/CD31− MNCs, or PBS was performed with a NOGA mapping injection catheter to target ischemic myocardium. In a parallel study, 10^5 human CD34+ MNCs, 10^5 human CD34− MNCs, or PBS was transplanted into ischemic myocardium of nude rats 10 minutes after ligation of the left anterior descending coronary artery. In the swine study, ischemic area by NOGA mapping, Rentrop grade angiographic collateral development, and echocardiographic left ventricular ejection fraction improved significantly 4 weeks after transplantation of NA/CD31+ MNCs but not after injection of NA/CD31− MNCs or PBS. Capillary density in ischemic myocardium 4 weeks after transplantation was significantly greater in the NA/CD31+ MNC group than the control groups. In the rat study, echocardiographic left ventricular systolic function and capillary density were significantly better preserved in the CD34+ MNC group than in the control groups 4 weeks after myocardial ischemia.

Conclusions—These favorable outcomes encourage future clinical trials of catheter-based, intramyocardial transplantation of autologous CD34+ MNCs in the setting of chronic myocardial ischemia. (***Circulation.* 2003;107:461-468.**)

Key Words: transplantation ■ cells ■ catheters ■ ischemia ■ vasculogenesis

Endothelial progenitor cells (EPCs) were first isolated as CD34+ mononuclear cells (MNCs) from adult peripheral blood.[1,2] Tissue ischemia mobilizes EPCs from bone marrow to peripheral blood, and mobilized EPCs home specifically to sites of nascent neovascularization and differentiate into mature endothelial cells (ECs).[3] The demonstrated role of EPCs in the physiological response to ischemia has led to the development of strategies of cell therapy for neovascularization in ischemic diseases. Intravenous transplantation of cultured human EPCs enhances neovascularization and improves limb salvage in nude mice with hindlimb ischemia.[4] A similar strategy applied in a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs incorporated into rat myocardial neovascularization, differentiated into mature ECs in ischemic myocardium, enhanced neovascularization, preserved left ventricular (LV) function, and inhibited myocardial fibrosis.[5] Recently, Kocher et al[6] attempted intravenous infusion of freshly isolated (not cultured) human CD34+ MNCs (EPC-enriched fraction) into nude rats with myocardial ischemia. This strategy resulted in preservation of LV function associated with inhibition of cardiomyocyte apoptosis. These experimental findings in immunodeficient animals suggest that both cultured and freshly isolated human EPCs have therapeutic potential in peripheral and coronary artery diseases.

Although these previous reports indicate a potential therapeutic role for EPCs in ischemic diseases, 2 major obstacles exist that must be overcome before considering actual clinical

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applications: dosage and immunologic rejection. In the previous study by our laboratory,[5] 1×10^6 cultured EPCs were used for each ≈200-g rat. Kocher et al[6] transplanted 1×10^6 freshly isolated EPCs/100-g rat. On a weight-adjusted basis, this would translate into 3×10^8 to 6×10^8 cells for an average-size human, requiring 8.5 to 120 L of peripheral blood. Although it may be possible to obtain enough EPCs from bone marrow in the clinical situation, it is a far from realistic strategy to isolate EPCs from peripheral blood by the previous methods. Moreover, these previous studies used an immunodeficient rat model to circumvent issues of cell rejection.

Accordingly, we designed a series of in vivo investigations to address the limitations of these previous approaches. First, we tested the hypothesis that local transplantation of EPCs, rather than systemic infusion, would permit a significant reduction in the number of EPCs required. Second, we developed a strategy that relies on freshly isolated, autologous EPCs that would allow us to evaluate the therapeutic potential of autologous EPC transplantation. We therefore performed catheter-based transplantation of a freshly isolated, autologous EPC-enriched fraction in a swine chronic myocardial ischemia model. To verify the therapeutic usefulness of the freshly isolated, human EPC-enriched fraction, we also performed intramyocardial transplantation in immunodeficient rats with myocardial ischemia using freshly isolated human CD34+ MNCs.

Methods

Animal Models of Myocardial Ischemia

Acute myocardial ischemia was induced by ligating the left anterior descending coronary artery (LAD) of male athymic nude rats (Hsd: RH-rnu rats, Harlan Sprague Dawley, Indianapolis, Ind) 6 to 8 weeks old.[5]

Male Yorkshire swine (Pine Acre Rabbitry Farm, Norton, Mass) weighing 20 to 25 kg were used to induce chronic myocardial ischemia. After left thoracotomy, an ameroid constrictor (Research Instruments SW) was placed around the proximal portion of the left circumflex (LCx) coronary artery.[7]

Isolation and Autologous, Percutaneous, Intramyocardial Transplantation of Swine EPCs

Four weeks after constrictor placement, 150 mL of peripheral blood was obtained from the ear vein of each pig. Total peripheral blood MNCs were isolated by density-gradient centrifugation. The MACS bead selection method for CD31 (Miltenyi Biotec) was used to isolate the EPC-enriched fraction from total MNCs (anti-swine CD34 antibody is not available). CD31+ MNCs resuspended in EC basal medium-2 (EBM-2, Clonetics) were cultured overnight in noncoated plastic plates at a density of 5×10^6 cells/10-cm plate. To remove macrophages, only nonadhesive CD31+ (NA/CD31+) MNCs were collected as the EPC-enriched fraction. CD31− MNCs were treated similarly, and nonadhesive CD31− MNCs (NA/CD31− MNCs) were obtained as a negative control.

To elucidate in vivo differentiation to endothelial lineage, 10^7 NA/CD31+ or the same number of NA/CD31− autologous MNCs were labeled with fluorescent carbocyanine 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) dye (Molecular Probes) and were injected via a 27-gauge needle to the LV lateral wall 4 weeks after constrictor placement. Four weeks after cell transplantation, 5 mg of *Bandeiraea simplicifolia* lectin I (BS-1 lectin) (Vector Laboratories), which is a murine- and porcine-specific (not human) EC marker, was infused into the left coronary artery, and the pigs were killed by an overdose of pentobarbital. Fluorescence microscopy was performed to examine incorporation of transplanted cells into foci of myocardial neovascularization.

After these preliminary studies, we examined the therapeutic potential of autologous, percutaneous, intramyocardial transplantation of an EPC-enriched MNC fraction in the swine chronic myocardial ischemia model. Four weeks after constrictor placement, NOGA nonfluoroscopic LV electromechanical mapping was performed to guide injections to foci of myocardial ischemia. The NOGA system (Biosense-Webster) of catheter-based mapping and navigation has been described in detail previously.[8–10] Ischemic myocardium was defined as a zone with unipolar voltage greater than the automatically determined cutoff, signified by red color on the unipolar voltage map and linear local shortening <3% on the linear local shortening map. This definition was consistent in all examinations throughout this study. Immediately after the ischemic territory was identified by NOGA mapping, 10^7 NA/CD31+ MNCs in 1 mL of PBS (n=7), 10^7 NA/CD31− MNCs in 1 mL of PBS (n=8), or 1 mL of PBS without cells (n=9) were injected into 5 sites within the ischemic myocardium (200 μL to each site) with the NOGA injection catheter (Biosense-Webster).

Fresh Isolation and Intramyocardial Transplantation of Human EPCs

Human total peripheral blood MNCs were isolated from healthy volunteers by density-gradient centrifugation, and CD34+ MNCs were isolated from total MNCs by the MACS bead selection method (Miltenyi Biotec) as the EPC-enriched fraction.[1] After the isolation, CD34− MNCs were also collected. CD34+ MNCs and CD34− MNCs were labeled with DiI. Ten minutes after the LAD of nude rats (n=2) had been ligated, 10^5 DiI-labeled CD34+ MNCs in 100 μL of PBS or 10^5 DiI-labeled CD34− MNCs in 100 μL of PBS were injected into 2 sites in the ischemic LAD territory with a 27-gauge needle (50 μL to each site). The ischemic zone was macroscopically identified by the pale color of the anterior and lateral walls after LAD ligation. This subgroup of rats was killed 10 days after myocardial ischemia. Thirty minutes before euthanization by overdose of pentobarbital, 500 μg of BS-1 lectin was administered intravenously. The hearts were fixed with 4% paraformaldehyde. The fixed tissues were embedded in OCT compound (Miles Scientific) and snap-frozen in liquid nitrogen for fluorescence microscopy. After this preliminary study to evaluate the incorporation of the cells into myocardial neovascularization, the therapeutic potential of CD34+ MNCs in myocardial ischemia was examined. Ten minutes after the LAD had been ligated, 10^5 human CD34+ MNCs in 100 μL of PBS (n=6), 10^5 human CD34− MNCs in 100 μL of PBS (n=6), or 100 μL of PBS (n=7) were injected into the myocardium as described above.

Physiological Assessment of LV Function and Ischemia

In the rat study, transthoracic echocardiography (SONOS 5500, Agilent Technologies) was performed to evaluate LV function 2 days before (baseline) and 4 weeks after myocardial ischemia. LV dimensions in end diastole (LVDd) and end systole (LVDs), fractional shortening (FS), and LV regional wall motion score[11] were examined.

In the swine study, transthoracic echocardiography (SONOS 5500), selective coronary angiography, and NOGA LV electromechanical mapping were performed 4 weeks after constrictor placement (just before injection of cells or PBS) and 4 weeks after the injections. LV ejection fraction was quantified by a computerized analysis system using a proprietary software package in the echo unit[12,13] in the LV short-axis view at the mid–papillary muscle level. Collateral flow to the LCx territory was graded angiographically in a blinded manner by use of the Rentrop scoring system.[14] The area of ischemia was quantified by NOGA mapping as previously described.[15]

All data were evaluated by blinded observers (echocardiography by Y.-S.Y., coronary angiography by J.-I.Y., and postprocessing analysis of the NOGA mapping by C.M.).

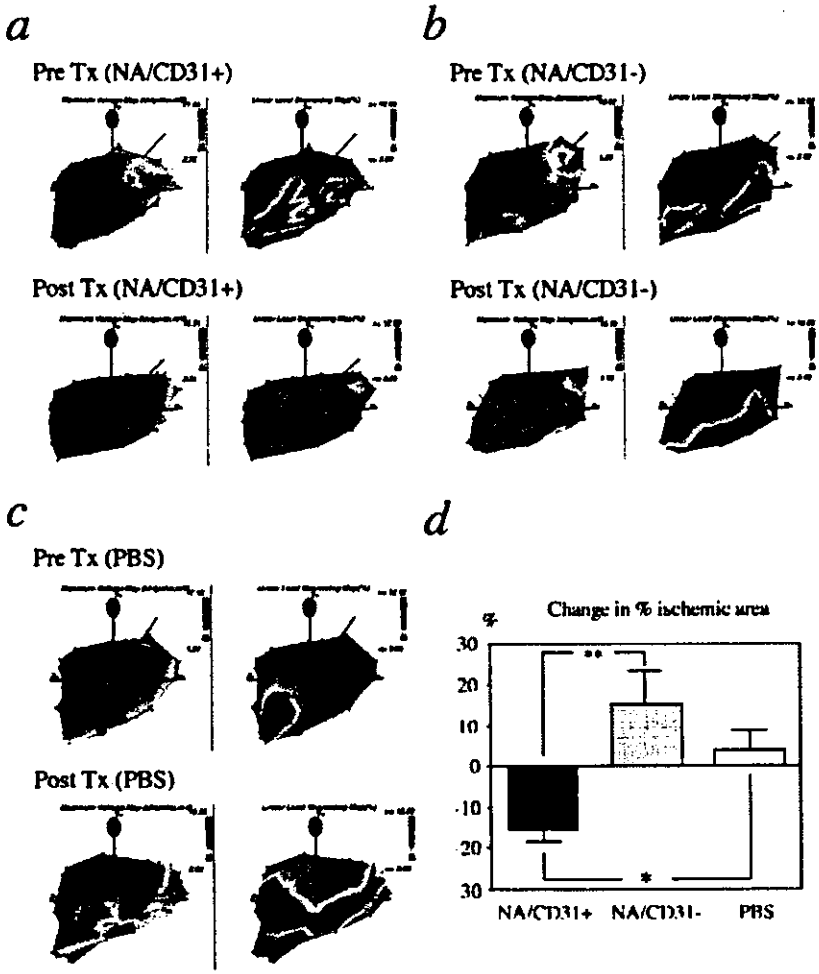

Figure 1. a, Representative findings of NOGA electromechanical mapping before (top) and 4 weeks after (bottom) NA/CD31+ MNC transplantation. Brown dots in pretreatment map show sites of cell transplantation. Red area on pretreatment linear local shortening map (top right) indicates area of decreased wall motion in lateral wall of left ventricle, consistent with ischemia in territory of LCx. Four weeks after local CD31+ cell transplantation, this area of ischemia is no longer evident (bottom right). b, Representative findings of NOGA electromechanical mapping before and 4 weeks after NA/CD31− MNCs transplantation. Area of ischemia on pretreatment map (top right) is unchanged or slightly increased 4 weeks after local transplantation of CD31− cells. c, Representative findings of NOGA electromechanical mapping before and 4 weeks after PBS injection reveal findings similar to those in CD31− transplant animals, with no improvement in ischemic area. d, Change in percentage ischemic area during 4 weeks after treatment. NA/CD31+, swine receiving NA/CD31+ MNCs; NA/CD31−, swine receiving NA/CD31− MNCs. *$P<0.05$; **$P<0.01$.

Histological Assessment of Animals Receiving Transplants

Both the rats and swine were killed 4 weeks after treatment. At necropsy, rat hearts were sliced in a bread-loaf manner into 8 transverse sections from apex to base and fixed with 100% methanol. To elucidate the severity of myocardial fibrosis, elastic tissue–trichrome staining was performed on paraffin-embedded sections from each tissue block, and the percentage area of fibrosis was calculated. Immunohistochemical staining with antibody prepared against the EC marker isolectin B4 (Vector Laboratories) was performed, and capillary density was evaluated by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD. Capillaries were recognized as tubular structures positive for isolectin B4. Immunohistochemical staining for the human-specific EC marker *Ulex europaeus* lectin type 1 (UEA-1 lectin) (Vector Laboratories) was also performed to identify transplanted human MNCs that had differentiated into mature ECs in the ischemic myocardium.

At necropsy, swine hearts were also sliced in a bread-loaf manner into 4 transverse sections from apex to base, and each section was separated into anterior, lateral, and posterior LV free wall; interventricular septum; and right ventricular free wall. All tissues obtained from each portion were fixed with 100% methanol. Immunohistochemistry for isolectin B4 was also performed to evaluate capillary density in the ischemic myocardium identified by NOGA mapping.

All morphometric studies were performed by 2 examiners (H.M. and A.H.) who were blinded to treatment.

Statistical Analysis

All values were expressed as mean±SEM. Student's paired t test was performed for comparison of data before and after treatment. ANOVA was performed to compare data among 3 groups. A probability value of $P<0.05$ was considered to denote statistical significance.

Results

Transplanted Autologous Swine EPCs Attenuate Chronic Myocardial Ischemia

Ischemic area determined by NOGA mapping before transplantation was not significantly different between the NA/CD31+, NA/CD31−, and PBS groups. A decrease in the size of the ischemic area was observed only after NA/CD31+ transplantation (before, 27.3±8.5%; after, 12.3±6.3%; $P=0.0034$), whereas the zone of ischemia increased in size after NA/CD31− or PBS injection. Similarly, the change in percentage ischemic area after transplantation was significantly improved only in the CD31+ group ($P=0.0017$ versus NA/CD31− group and $P=0.038$ versus PBS group) (Figure 1).

Transplanted Autologous Swine EPCs Enhance Neovascularization

Selective left coronary angiography was performed to evaluate collateral development before and after transplantation

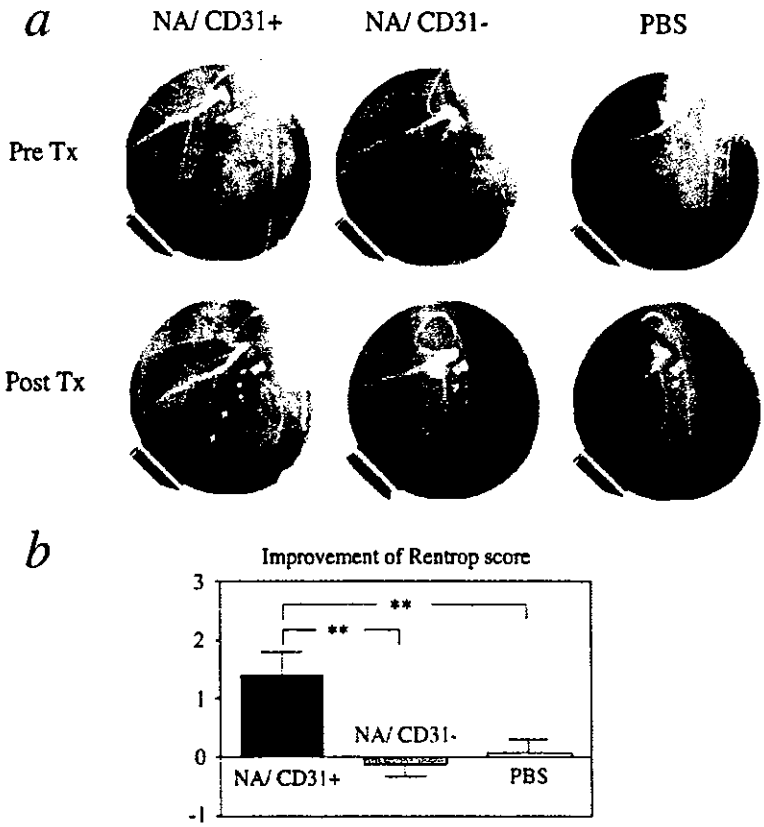

Figure 2. a, Representative left coronary angiographic findings in swine before and 4 weeks after cell transplantation. Well-developed collaterals (arrows) to LCx were observed in NA/CD31+ MNC group, resulting in complete opacification of LCx and its branches. b, Improvement of Rentrop angiographic score of collateral development after transplantation of NA/CD31+ MNCs, NA/CD31− MNCs, or PBS. $^{**}P<0.01$.

in the swine study. The mean value of the Rentrop score of collateral development to the LCx territory at baseline was 0.6±0.4 in the NA/CD31+ group, 1.1±0.4 in the NA/CD31− group, and 1.1±0.3 in the PBS group (P=NS). Rentrop scoring was improved significantly only after NA/CD31+ transplantation (0.6±0.4 versus 2.0±0.4, P=0.02) and not after NA/CD31− or PBS injection. Similarly, the change in the Rentrop score was significantly greater in the NA/CD31+ group than in either the NA/CD31− or PBS groups (P=0.002 versus NA/CD31− MNCs and P=0.006 versus PBS) (Figure 2).

Histochemical staining of isolectin B4 was performed to identify capillaries in ischemic myocardium 4 weeks after cell transplantation. Capillary density was significantly greater in the NA/CD31+ group than in the NA/CD31− and PBS groups (P=0.0033 versus NA/CD31− MNCs and P=0.0004 versus PBS). Capillary density in the NA/CD31− group was similar to that in the PBS group (Figure 3, a and b).

Transplanted Autologous Swine EPCs Improve LV Function

LV ejection fraction measured by echocardiography in the NA/CD31+ group was similar to that in the NA/CD31− and PBS groups 4 weeks after constrictor placement (Figure 3c). However, LV ejection fraction improved significantly only after NA/CD31+ transplantation (P=0.0037) and not after NA/CD31− or PBS injection. LV ejection fraction 4 weeks after transplantation was significantly greater in the NA/CD31+ group than in the NA/CD31− and PBS groups (P=0.0018 versus NA/CD31− and P=0.0017 versus PBS) (Figure 3c).

Swine EPCs Differentiate Into Endothelial Lineage After Catheter-Based Injection in Vivo

To examine in vivo differentiation of swine autologous EPCs after transplantation into ischemic myocardium, DiI-labeled NA/CD31+ or NA/CD31− MNCs were injected into the lateral LV wall 4 weeks after constrictor placement. Four weeks after transplantation, the majority of NA/CD31+ MNCs were positive for BS-1 lectin in the ischemic myocardium. In contrast, transplanted NA/CD31− MNCs positive for BS-1 lectin were rarely observed in the ischemic myocardium (Figure 3d).

Transplanted Human EPCs Enhance Neovascularization and Inhibit Myocardial Fibrosis

In the rat study, capillary density was significantly greater in the CD34+ group than in the CD34− and PBS groups (P=0.003 versus CD34− MNCs and P=0.003 versus PBS). Capillary density in the CD34− group was not significantly different from that in the PBS group (Figure 4, a and b). Elastic tissue–trichrome staining was performed to identify LV fibrosis after myocardial ischemia. The fibrotic area was significantly smaller in the CD34+ group than in either the CD34− or PBS group (P=0.001 versus CD34− and P=0.01 versus PBS) (Figure 5, a through d).

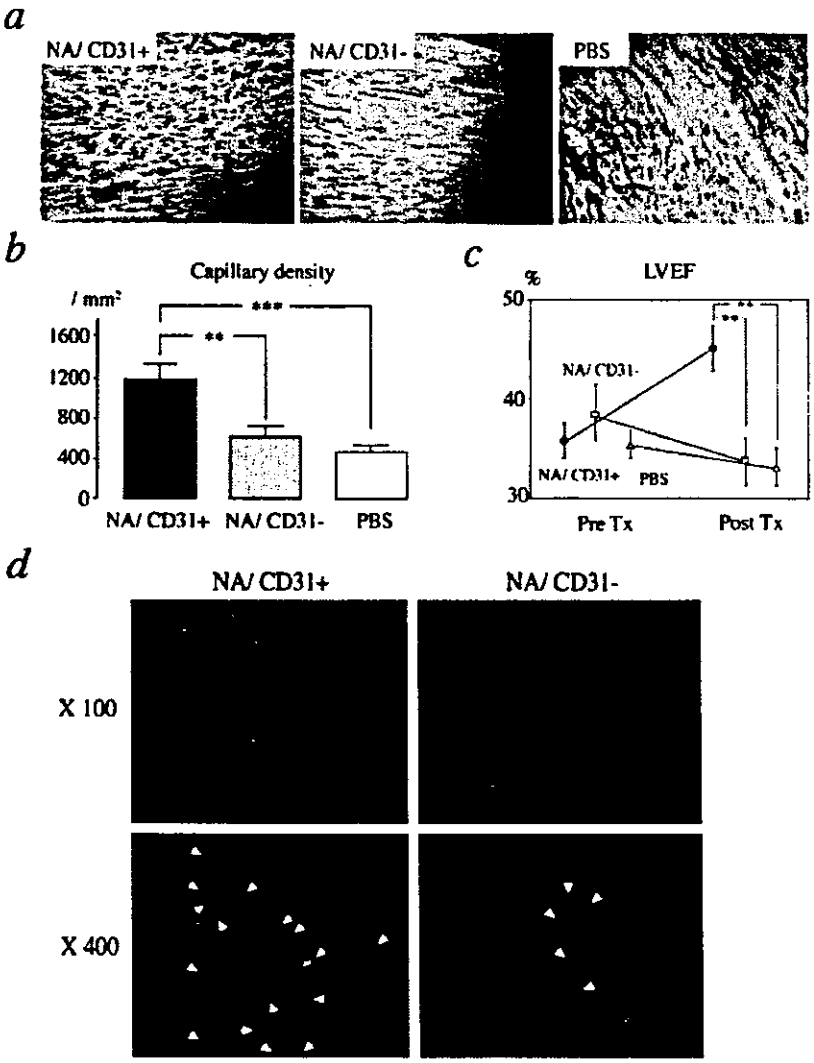

Figure 3. a, Representative immunohistochemical findings for isolectin B4 in swine ischemic myocardium 4 weeks after cell transplantation. b, Capillary density in swine ischemic myocardium 4 weeks after transplantation. $^{**}P<0.01$; $^{***}P<0.001$. c, Echocardiographic LV ejection fraction (EF) before and 4 weeks after intramyocardial cell transplantation in swine with chronic myocardial ischemia. Circle, pigs receiving NA/CD31+ MNCs; square, pigs receiving NA/CD31− MNCs; triangle, pigs receiving PBS. d, Representative fluorescence microscopic findings of swine ischemic myocardium 28 days after cell transplantation. Red (DiI) fluorescence marks all autologously transplanted cells; green fluorescence indicates BS-1 lectin binding, identifying ECs. Therefore, yellow fluorescence marks double-positive cells, ie, cells harvested from systemic circulation, that were autologously transplanted into myocardium and now express a marker of endothelial phenotype. A majority of transplanted NA/CD31+ MNCs differentiated into EC lineage in vivo, indicated by high percentage of double-positive (yellow) cells (arrows) in 2 left panels. In contrast, most of transplanted NA/CD31− MNCs are positive only for ex vivo DiI label and negative for endothelial phenotype (arrowheads). Double-positive cells were rarely observed in CD31− transplanted animals.

Transplanted Human EPCs Preserve LV Function

In the rat study, baseline LVDd, LVDs, FS, and regional wall motion score were similar between rats receiving human CD34+ MNCs, rats receiving CD34− MNCs, and rats receiving PBS. In all groups, all echocardiographic parameters worsened significantly 4 weeks after induction of myocardial ischemia ($P<0.01$ in all groups). Echocardiography performed 4 weeks after treatment revealed that LVDd was similar among the 3 treatment groups (Figure 5e). However, LVDs 4 weeks after ischemia was significantly smaller

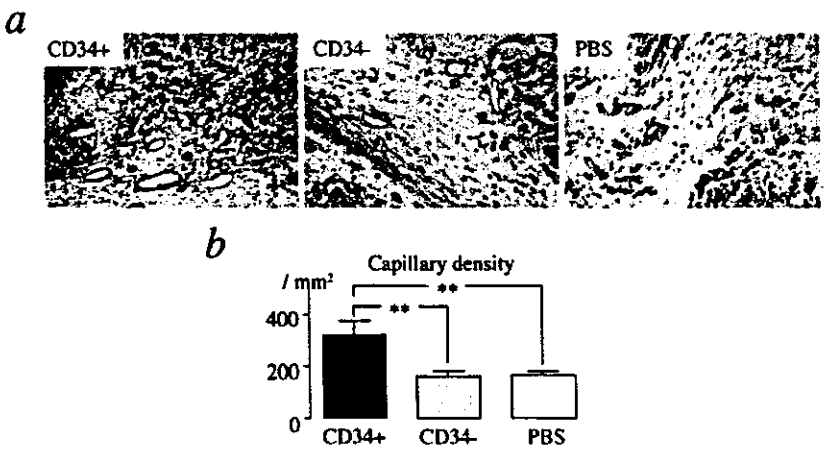

Figure 4. a, Representative immunohistochemical findings for isolectin B4 in ischemic myocardium of nude rats 4 weeks after cell transplantation. b, Capillary density in rat ischemic myocardium 4 weeks after cell transplantation. $^{**}P<0.01$.

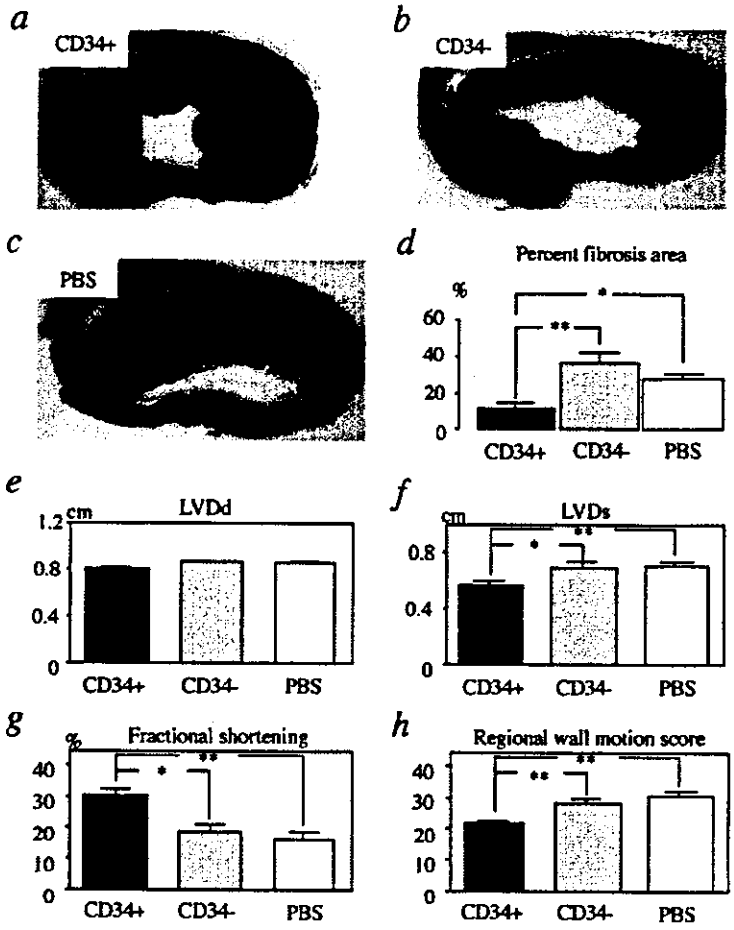

Figure 5. a through c, Representative elastic tissue–trichrome–stained sections from nude rats 4 weeks after receiving CD34+ MNCs (a), CD34− MNCs (b), and PBS (c). d, LV fibrosis was significantly reduced in CD34+ MNC–treated group compared with CD34− MNCs (*$P<0.05$) or PBS group (**$P<0.01$). e through h, Echocardiographic parameters 4 weeks after cell transplantation in nude rats with myocardial ischemia. LV dilatation was reduced (f), and fractional shortening (g) and regional wall motion scores (h) are significantly improved in CD34+ group compared with CD34− and PBS-treated animals. *$P<0.05$; **$P<0.01$.

($P=0.013$ versus CD34− MNCs and $P=0.005$ versus PBS) (Figure 5f), FS was significantly greater ($P=0.007$ versus CD34− MNCs and $P=0.001$ versus PBS) (Figure 5g), and regional wall motion score was significantly better ($P=0.005$ versus CD34− MNCs and $P=0.0002$ versus PBS) (Figure 5h) in rats receiving CD34+ MNCs compared with those treated with CD34− MNCs or PBS. LVDs, FS, and regional wall motion score 4 weeks after transplantation in the CD34− MNCs group were not significantly different from those in the PBS group (Figure 5, f through h).

Transplanted Human EPCs Incorporate Into Foci of Myocardial Neovascularization and Differentiate Into Mature ECs

Both Di-I labeled human CD34+ MNCs (EPC-enriched fraction) and CD34− MNCs (EPC-poor fraction) were distributed principally in the ischemic area of the rat myocardium. However, the number of cells incorporated into tubular structures consistent with neovasculature was much greater in rats receiving CD34+ MNCs than in those in which CD34− MNCs were transplanted (Figure 6a).

Differentiated human ECs derived from transplanted MNCs were frequently identified by UEA-1 lectin staining in the vasculature of the ischemic myocardium in rats receiving CD34+ MNCs. In contrast, mature human ECs were rarely identified in the ischemic myocardium of rats receiving CD34− MNCs (Figure 6b). Thus, locally transplanted human EPCs were incorporated into foci of neovascularization and differentiated into mature ECs in ischemic myocardium.

Discussion

In the present study, we demonstrate the therapeutic potential and technical feasibility of percutaneous, intramyocardial transplantation of autologous EPCs in the setting of chronic myocardial ischemia. This strategy was designed to overcome the 2 inherent limitations of previous approaches that would prevent application in humans. First, the requirement for a large number of EPCs was avoided by delivering the cells directly to the ischemic myocardium with the use of a novel, real-time ischemia mapping system. Second, the issue of immunologic compatibility was resolved by the use of autologous cells. Although transplantation of autologous cells, such as bone marrow MNCs[16] or skeletal myoblasts,[17] has been reported, the present study is the first to elucidate the therapeutic potential of autologous EPC transplantation.

Catheter-based, percutaneous intramyocardial transplantation of swine EPCs resulted in histological, angiographic, and functional evidence of enhanced neovascularization of ischemic myocardium. The incorporation of transplanted EPCs into the neovasculature was documented in pilot studies using labeled NA/CD31+ cells. Increased vascularity of the myocardium was observed only in animals in which EPCs were

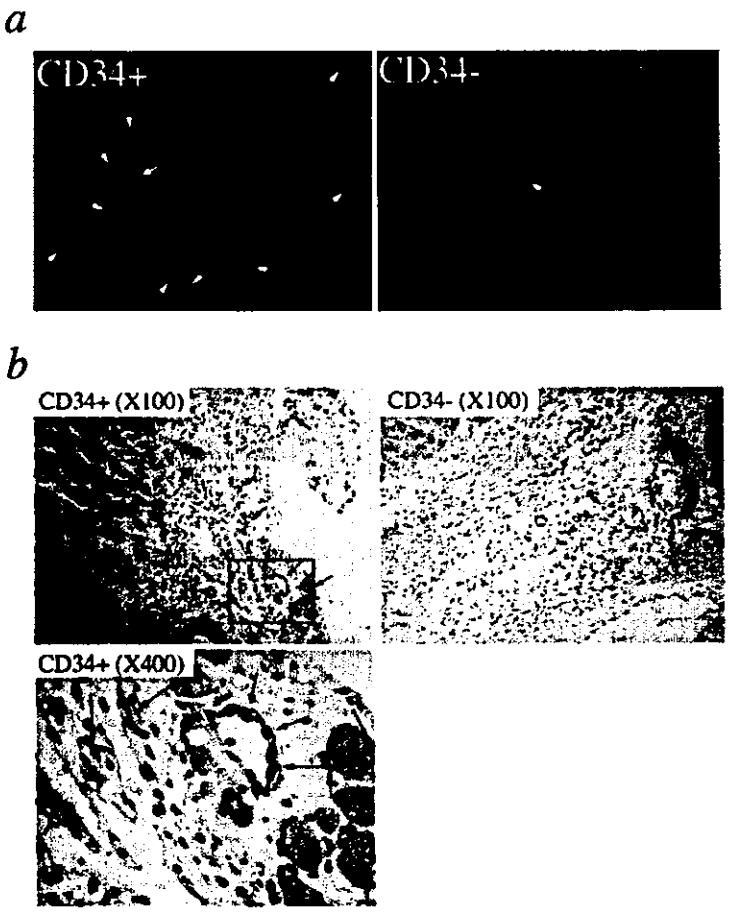

Figure 6. a, Representative fluorescence microscopic findings of ischemic myocardium of nude rats 10 days after cell transplantation. Red fluorescence indicates DiI labeling of transplanted human cells, and green fluorescence indicates BS-1 lectin, a marker for rat ECs. Transplanted cells with tube-like structures (arrows) were observed frequently in CD34+ MNCs group (left) and were rarely seen in CD34− MNCs group (right). b, Representative findings of immunohistochemical staining for UEA-1 lectin (human-specific EC marker) in ischemic myocardium of nude rats 28 days after cell transplantation. UEA-1 lectin-positive mature ECs (arrows) were observed more frequently in CD34+ group than in CD34− group.

delivered. The notion that inflammation is induced either by needle injury or trauma resulting from injection of cells is completely dispelled by these data.

The porcine model of chronic myocardial ischemia was chosen for these preclinical studies to evaluate the strategy of local delivery via the NOGA injection catheter. Although CD34+ MNCs would be used in future clinical situations, anti-swine CD34 antibody is not available. Therefore, we performed cell selection with anti-swine CD31 antibody instead. To complement these studies and verify that selected CD34 cells could also yield similar clinical benefit, we transplanted freshly isolated human CD34+ cells into the myocardium in a nude rat model of myocardial ischemia. The locally transplanted CD34+ cells incorporated into foci of myocardial neovascularization, differentiated into mature ECs, enhanced vascularity in the ischemic myocardium, preserved LV systolic function, and inhibited LV fibrosis. Once again, these benefits were absent after injection of negatively selected cells or PBS, providing further evidence against the "injury hypothesis" of neovascularization. These positive outcomes are similar to those in previous studies involving intravenous EPC transplantation.[5,6] However, the number of transplanted human CD34+ MNCs in this study was 20 times less than that in these previous studies of intravenous transplantation,[6] providing a practical solution to the requirement for large numbers of cells in these previous investigations.

These data suggest that percutaneous delivery of autologous, freshly isolated EPCs targeted to sites of ischemia may represent a practical strategy for revascularization of patients with chronic myocardial ischemia.

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Review

Post-natal endothelial progenitor cells for neovascularization in tissue regeneration

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Abstract

The isolation of endothelial progenitor cells (EPCs) derived from bone marrow (BM) was an outstanding event in the recognition of 'de novo vessel formation' in adults occurring as physiological and pathological responses. The finding that EPCs home to sites of neovascularization and differentiate into endothelial cells (ECs) in situ is consistent with 'vasculogenesis', a critical paradigm well described for embryonic neovascularization, but proposed recently in adults in which a reservoir of stem or progenitor cells contributes to vascular organogenesis. EPCs have also been considered as therapeutic agents to supply the potent origin of neovascularization under pathological conditions. This review provides an update of EPC biology as well as highlighting their potential use for therapeutic regeneration.

Keywords: Experimental; Vasculature; Cellular; Circulatory physiology

1. Introduction

Tissue regeneration by somatic stem/progenitor cells has been recognized as a maintenance or recovery system of many organs in adult. The isolation and investigation of these somatic stem/progenitor cells has described how these cells contribute to postnatal organogenesis. On the basis of the regenerative potency, these stem/progenitor cells are expected to develop as a key strategy of therapeutic applications for the damaged organs.

Recently endothelial progenitor cells (EPCs) have been isolated from adult peripheral blood (PB). EPCs are considered to share common stem/progenitor cells with hematopoietic stem cells and have been shown to derive from bone marrow (BM) and to incorporate into foci of physiological or pathological neovascularization. The finding that EPCs home to sites of neovascularization and differentiate into endothelial cells (ECs) in situ is consistent with 'vasculogenesis', a critical paradigm well described for embryonic neovascularization, but recently proposed in adults in which a reservoir of stem/progenitor cells contributes to post-natal vascular organogenesis. The discovery of EPCs has therefore drastically changed our understanding of adult blood vessel formation. The following review provides an update of EPC biology as well as highlighting their potential utility for therapeutic vascular regeneration.

2. Post-natal neovascularization

Through the discovery of EPCs in PB [1,2], our understanding of post-natal neovascularization has been expanded from angiogenesis to angio/vasculogenesis. As previously described [3], post-natal neovascularization was

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originally recognized to be constituted by the mechanism of 'angiogenesis', which is neovessel formation, operated by in situ proliferation and migration of preexisting endothelial cells. However, the isolation of EPCs resulted in the addition of the new mechanism, 'vasculogenesis', which is de novo vessel formation by in situ incorporation, differentiation, migration, and/or proliferation of BM-derived EPCs [4] (Fig. 1). More recently, tissue specific stem/progenitor cells with the potency of differentiation into myocytes or ECs were isolated in skeletal muscle tissue of murine hindlimb, although the origin remains to be clarified [5]. This finding suggests that the origin of EPCs may not be limited to BM, e.g. tissue specific stem/progenitor cells possibly provide 'in situ EPCs' as other sources of EPCs than BM.

In the event of minor scale neovessel formation, i.e. slight wounds or burns, 'in situ preexisting ECs' causing post-natal angiogenesis may replicate and replace the existing cell population sufficiently, as ECs exhibit the ability for self-repair that preserves their proliferative activity. Neovascularization through differentiated ECs, however, is limited in terms of cellular life span (Hayflick limit) and their inability to incorporate into remote target sites. In the case of large scale tissue repair, such as patients who experienced acute vascular insult secondary to burns, coronary artery bypass grafting (CABG), or acute myocardial infarction [6,7], or in physiological cyclic organogenesis of endometrium [4], BM-derived or in situ EPC kinetics are activated under the influence of appropriate cytokines, hormones and/or growth factors through the autocrine, paracrine, and/or endocrine systems. Thus the contemporary view of tissue regeneration is that neighboring differentiated ECs are relied upon for vascular regeneration during a minor insult, whereas tissue specific or BM-derived stem/progenitor cells bearing EPCs/ECs are important when an emergent vascular regenerative process is required (Fig. 1).

3. Profiles of EPCs in adults

3.1. The evidence of circulating EPCs in adults

In the embryo, evidence suggests that hematopoietic stem cells (HSCs) and EPCs [8,9] are derived from a common precursor (hemangioblast) [10,11]. During embryonic development, multiple blood islands initially fuse to form a yolk sac capillary network [12], which provides the foundation for an arteriovenous vascular system that eventually forms following the onset of blood circulation [8]. The integral relationship between the cells which circulate in the vascular system (the blood cells) and those principally responsible for the vessels themselves (ECs) is suggested by their spatial orientation within the blood islands; those cells destined to generate hematopoietic cells are situated in the center of the blood island (HSCs) while EPCs or angioblasts are located at the periphery of the blood islands. In addition to this arrangement, HSCs and EPCs share common antigens, including CD34, Vascular

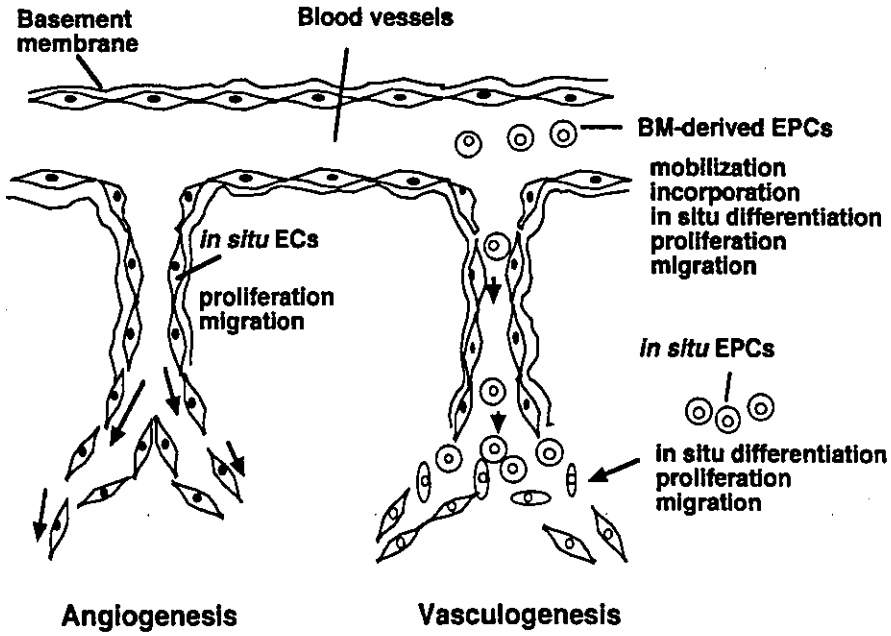

Fig. 1. Post-natal neovascularization in the physiological or pathological events is consistent with neovessel formation contributed by angiogenesis and vasculogenesis at the various rates between their two mechanisms. Angiogenesis and vasculogenesis are due to the activations of in situ ECs and BM-derived or in situ EPCs, respectively.

endothelial growth factor receptor-2 (VEGFR2), Tie-2, CD117, and stem cell antigen-1 (Sca-1) [13].

The existence of HSCs in the PB and BM, and the demonstration of sustained hematopoietic reconstitution with HSC transplantation led to the idea that a closely related cell-type, namely EPCs, may also exist in adult tissues. Recently, EPCs were successfully isolated from circulating mononuclear cells (MNCs) using VEGFR2, CD34, and CD133 antigens shared by both embryonic EPCs and HSCs [1,14,15]. In vitro, these cells differentiate into endothelial lineage cells, and in animal models of ischemia, heterologous, homologous, and autologous EPCs have been shown to incorporate into the foci of neovasculature, contributing to neovascularization. Recently, similar studies with EPCs isolated from human cord blood have demonstrated their analogous differentiation into ECs in vitro and in vivo [16–19].

These findings have raised important questions regarding fundamental concepts of blood vessel growth and development in adults. Does the differentiation of EPCs in situ (vasculogenesis) play an important role in adult neovascularization, and would impairments in this process lead to clinical diseases? There is now a strong body of evidence suggesting that vasculogenesis in fact significantly contributes to postnatal neovascularization. Recent studies with animal BM transplantation (BMT) models in which BM (donor)-derived EPCs could be distinguished have shown that the contribution of EPCs to neovessel formation may range from 5 to 25% in response to granulation tissue formation [20] or growth factor-induced neovascularization [21]. Also, in the tumor neovascularization, the range is approximately 35–45% higher than the former events [22]. The degree of EPC contribution to post-natal neovascularization is predicted to depend on each neovascularizing event or disease.

3.2. Isolation of EPCs in circulation

Under the current status, it is impossible to differentiate 'immature EPCs' from primitive HSCs, as those cells share common surface markers, i.e. AC133, CD34, or VEGFR2 as described above. In circulation, the cell population with the capacity of differentiation to EPCs is considered to be included in the cell population expressing AC133 and VEGFR2 markers in the subset of CD34 positive cells [15]. Circulating EPCs are constitutively expressing stem/progenitor markers, i.e. CD34 or VEGFR2 except AC133, and start expressing endothelial lineage specific markers, VE cadherin or E-selectin. On the other hand, following the commitment and differentiation to hematopoietic stem/progenitor cells, the surface markers of AC133 and VEGFR2 are extinguished. Such stem/progenitor cell markers do not express on the differentiated hematopoietic cells. Alternatively, kinds of surface markers are expressed to characterize individual hematopoietic cell populations. AC133 is a marker to differentiate immature EPCs or primitive HSCs from circulating EPCs. To differentiate EPCs from hematopoietic stem/progenitor cells, VEGFR2, VE cadherin, or E-selectin are useful. Also, circulating EPCs do not express monocyte or myeloid markers, such as CD14 or CD15. Accordingly, circulating EPCs may be isolated via selection by the antigenicity of CD34, VEGFR2, and/or VE cadherin and also circulating immature EPCs by AC133 (Fig. 2).

3.3. Diverse identification of human EPCs and their precursors

Since the initial report of EPCs [1,2], a number of groups have set out to define this cell population better. Because EPCs and HSCs share many surface markers, and no simple definition of EPCs exists, various methods of EPC isolation have been reported [1,2,15–18,23–31]. The term EPC may therefore encompass a group of cells that exist in a variety of stages ranging from hemangioblasts to fully differentiated ECs. Although the true differentiation lineage of EPCs and their putative precursors remains to be determined, there is overwhelming evidence in vivo that a population of EPCs exists in human.

Lin et al. cultivated peripheral MNCs from patients receiving gender-mismatched BMT and studied their growth in vitro. In this study, they identified a population of BM (donor)-derived ECs with high proliferative potential (late outgrowth); these BM cells likely represent EPCs [24]. Gunsilius et al. investigated a chronic myelogenous leukemia model and disclosed that BM-derived EPCs contribute to postnatal neovascularization in human [26]. Interestingly, in the report, BM-derived EPCs could be detected even in the wall of quiescent vessels without neovascularization events. This finding suggests that BM-derived EPCs may relate even to the turnover of ECs consisting of quiescent vessels.

Reyes et al. have recently isolated multipotent adult progenitor cells (MAPCs) from BM MNCs, differentiated them into EPCs and proposed MAPCs as an origin of EPCs [22]. These studies therefore provide evidence to support the presence of BM-derived EPCs that take part in neovascularization. Also, as described above, the existence of 'in situ EPCs' as derived from tissue specific stem/progenitor cells in murine skeletal muscle remains to be investigated also in the other tissues [5] (Fig. 2).

4. EPC kinetics in adults

4.1. EPC kinetics effected by endogenous agents

The incorporation of BM-derived EPCs into foci of physiological and pathological neovascularization has been demonstrated through various animal experiments. One well-established model that allows the detection of BM-

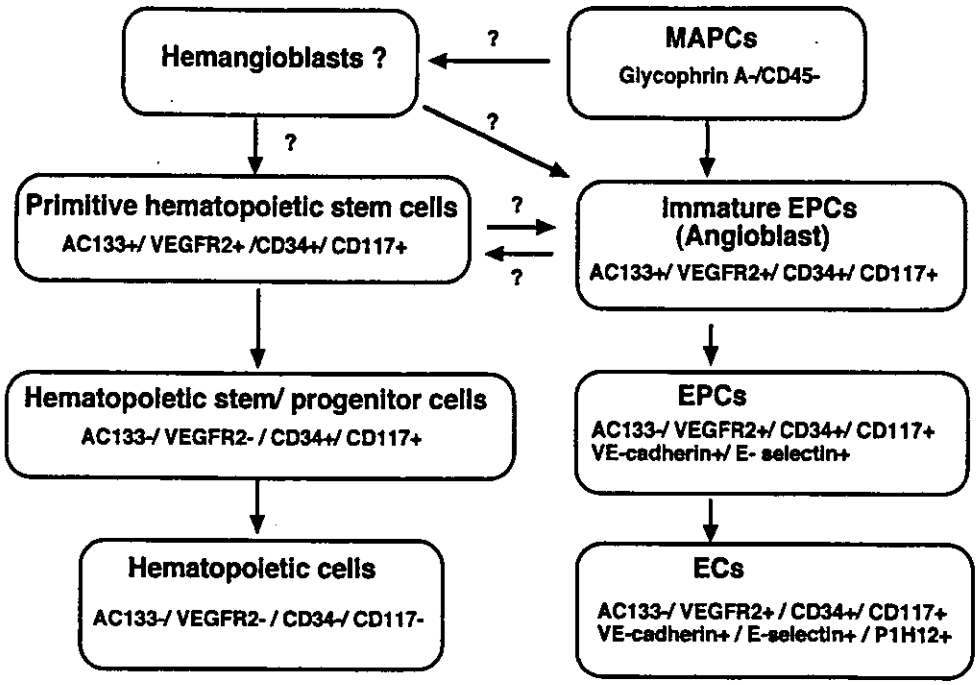

Fig. 2. Origin and differentiation of EPCs in adult BM. EPCs are thought to differentiate not only from putative hemangioblasts, common precursor cells with HSCs, as previously described, but also from MAPCs. Representative antigenicities to stem/progenitor cells are shown (+, positive; −, negative).

derived EPCs includes transplanting wild-type mice with BM cells harvested from transgenic mice in which LacZ expression is regulated by an EC lineage-specific promoter, flk-1 or Tie-2 (flk-1/lacZ/BMT, Tie-2/lacZ/BMT). Using such mice, flk-1- or Tie-2-expressing endothelial lineage cells derived from BM (EPCs) have been shown to localize to vessels during tumor growth, wound healing, skeletal and cardiac ischemia, corneal neovascularization, and endometrial remodeling following hormone-induced ovulation [4].

Tissue trauma causes mobilization of hematopoietic cells as well as pluripotent stem or progenitor cells from the hematopoietic system [32]. Consistent with the notion that EPCs and HSCs share a common ancestry, recent data from our laboratory have shown that mobilization of BM-derived EPCs constitutes a natural response to tissue ischemia. The aforementioned murine BMT model also provided direct evidence of enhanced BM-derived EPC incorporation into foci of corneal neovascularization following the development of hindlimb ischemia [33]. This finding indicates that circulating EPCs are mobilized endogenously in response to tissue ischemia and can incorporate into neovascular foci to promote tissue repair. These results in animals were recently confirmed by human studies illustrating EPC mobilization in patients following burns [6], CABG, or acute myocardial infarction [7].

As previous studies demonstrated, endogenous mobilization of BM-derived EPCs, we considered exogenous mobilization of EPCs as an effective means of augmenting the resident population of EPCs/ECs. Such a strategy is appealing for its potential to overcome the endothelial dysfunction or depletion that may be associated with older, diabetic, or hypercholesterolemic patients. Granulocyte macrophage colony-stimulating factor (GM-CSF) is well known to stimulate hematopoietic progenitor cells and myeloid lineage cells, but has recently been shown to exert a potent stimulatory effect on EPC kinetics. The delivery of this cytokine induced EPC mobilization and enhanced neovascularization of severely ischemic tissues and de novo corneal vascularization [33].

The exact mechanism by which EPCs are mobilized to the peripheral circulation remains unknown, but may mimic aspects of embryonic development. Vascular endothelial growth factor (VEGF), critical for angio/vasculogenesis in the embryo [34–36], has recently been shown to be an important stimulus of adult EPC kinetics. Our studies carried out first in mice [37] and subsequently in patients undergoing VEGF gene transfer for critical limb or myocardial ischemia [38] established that a previously unappreciated mechanism by which VEGF contributes to neovascularization is in part by mobilizing BM-derived EPCs. Similar modulation of EPC kinetics has been observed in response to other hematopoietic stimulators, such as granulocyte-colony stimulating factor (G-CSF) and stroma-derived factor-1 (SDF-1) [39].

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

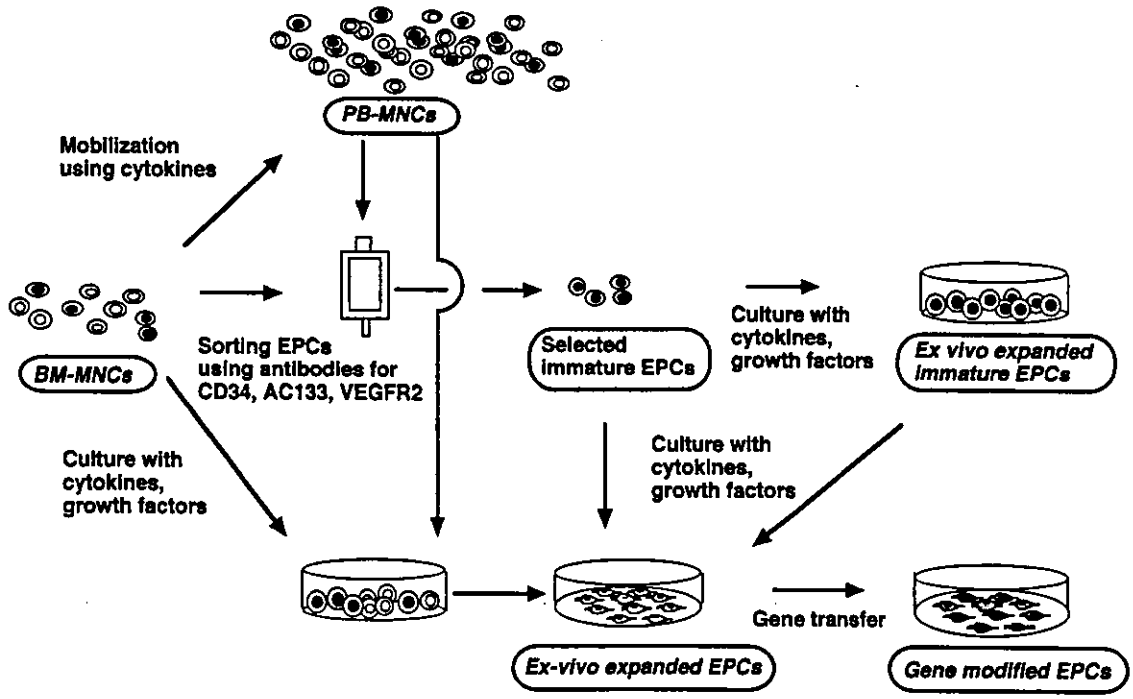

Fig. 3. EPC therapy using autologous EPCs derived from BM for vascular regeneration. Transplantation of BM- or mobilized PB-MNCs are considered 'crude EPC therapy', as EPCs are not selected. BM-MNCs have already been under clinical application. Following the manipulation to acquire the optimized quality 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-