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

5.2. Future strategy of EPC cell therapy

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

5.3. Gene modified EPC therapy

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

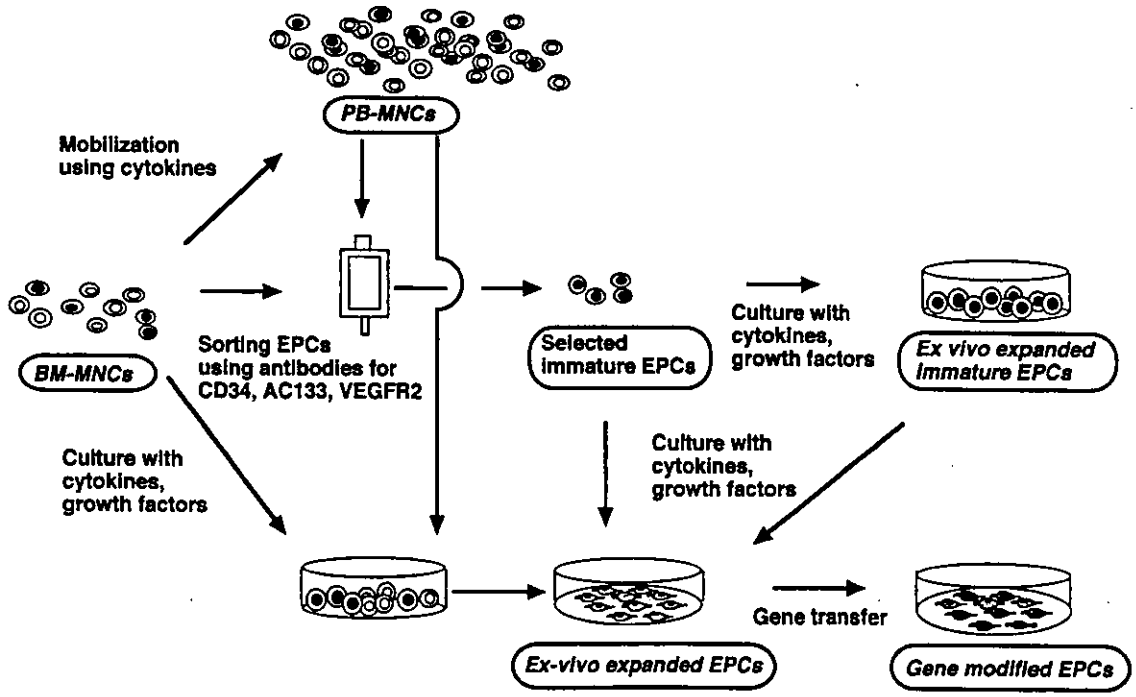

Fig. 3. EPC therapy using autologous EPCs derived from BM for vascular regeneration. Transplantation of BM- or mobilized PB-MNCs are considered 'crude EPC therapy', as EPCs are not selected. BM-MNCs have already been under clinical application. Following the manipulation to acquire the optimized quality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

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

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

5.4. BM-MNC transplantation

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

6. Other devices of EPCs for clinical application

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

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

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

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

7. Conclusion

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

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

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

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

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

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

Key Words: chemokines ■ angiogenesis ■ ischemia ■ endothelium

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

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

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

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

Methods

Cell Isolation and Culture

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

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

Fluorescence-Activated Cell Sorting

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

Migration Assay

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

Apoptosis Assay

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

Animal Model of Ischemic Hindlimb

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

RNA Extraction and Reverse Transcriptase–Polymerase Chain Reaction Analysis

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

Transplantation of Ex Vivo Expanded EPCs

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

Physiological Assessment of Transplanted Animals

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

Histological Assessment of Transplanted Animals

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

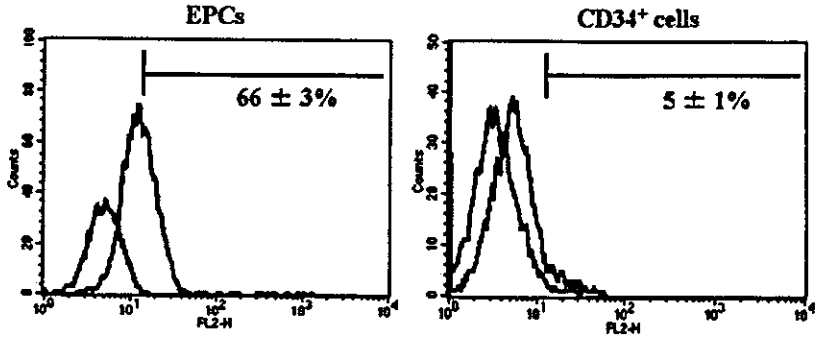

Figure 1. Analysis of CXCR-4 expression by flow cytometry. Results are shown as fluorescence histograms (blue, CXCR-4 expression; red, respective IgG control). Ex vivo expanded EPCs were positive by 66.0±3.1% for CXCR-4, and freshly isolated peripheral blood CD34+ cells by 5.2±1.1%. FL2-H indicates fluorescent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

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

Statistical Analysis

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

Results

Fluorescence-Activated Cell Sorting

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

Migration Assay

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

Apoptosis Assay

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

SDF-1 Upregulates Endogenous VEGF Expression in Hindlimb Ischemic Muscle

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

EPC Incorporation Into Ischemic Hindlimb Neovasculature

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

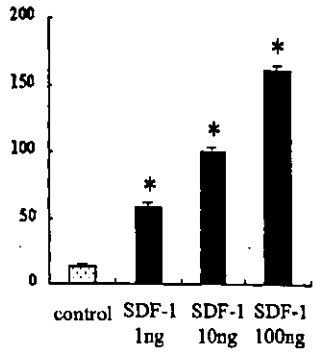

Figure 2. SDF-1 induced EPC migration. Migratory response of EPCs toward different dosages of SDF-1 stimulation was measured by modified Boyden chamber migration assay. Ex vivo expanded EPCs demonstrated a potent dose-dependent activity toward SDF-1. Control vs 10 ng/mL SDF-1 vs 100 ng/mL SDF-1, 24±2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

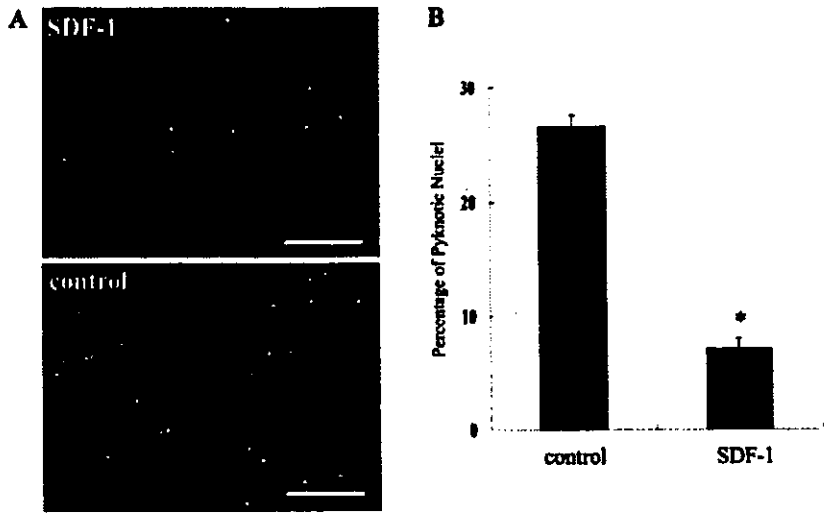

Figure 3. SDF-1 attenuated EPCs apoptosis. Serum starvation was used to induce apoptosis in ex vivo expanded EPCs. A, DAPI staining was performed to determine the proportion of apoptotic cells by manually counting pyknotic nuclei (white condensed nuclei in figures). Scale bars=100 μm. B, Quantification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

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

Physiological Assessment of Transplanted Animals

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

Histological Assessment of Transplanted Animals

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

Discussion

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

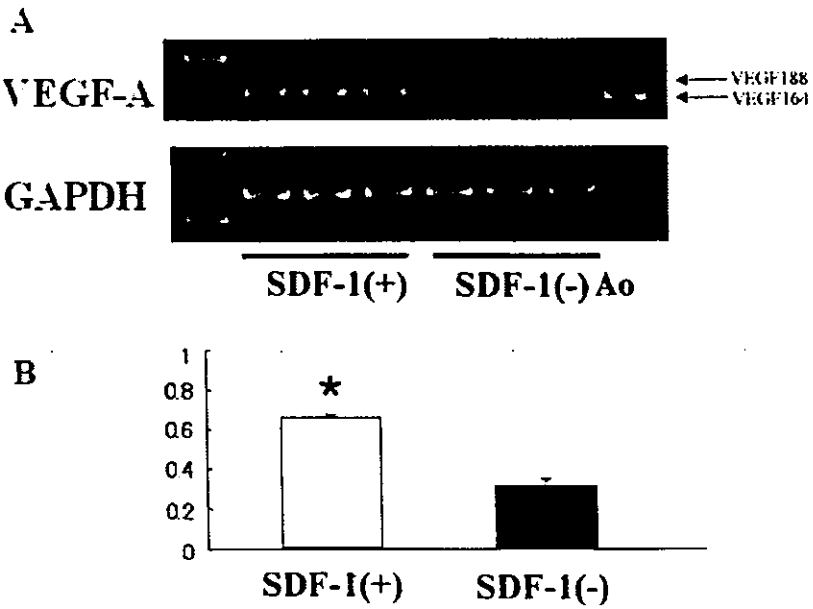

Figure 4. SDF-1 upregulated expression of VEGF-A mRNA in ischemic hindlimb. A, Expression of VEGF-A mRNA in SDF-1–treated and untreated muscle. Each panel shows RT-PCR products for VEGF-A and GAPDH. Ao indicates mouse aortic tissue as positive control. B, Densitometric analysis was performed; ratio of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

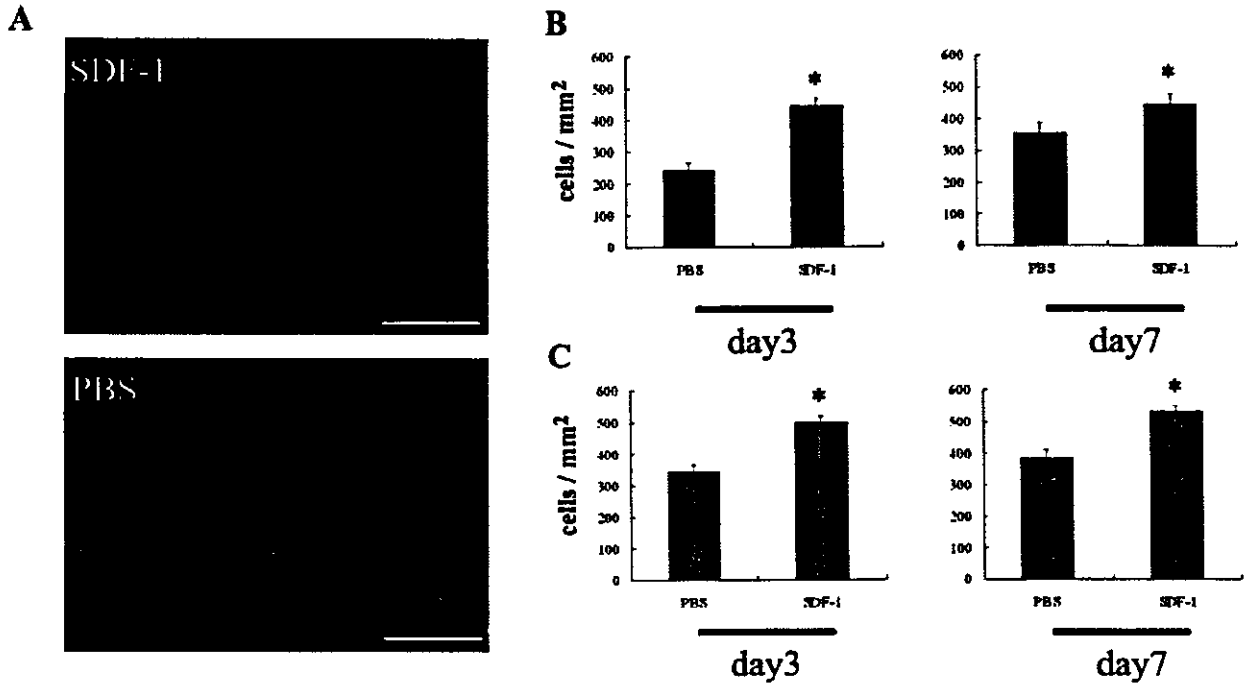

Figure 5. SDF-1 augmented EPC incorporation at an early time point. Fluorescence microscopic examination disclosed increased local accumulation of EPCs in SDF-1 treatment group compared with PBS group. A, Representative microscopic photographs of double fluorescence in ischemic muscles at day 3. Transplanted human DiI-labeled EPC-derived cells were identified by red fluorescence in histological sections retrieved from ischemic muscles. Host mouse vasculature was identified by green fluorescence in the same tissue sections. Scale bars=100 μm. B, Quantitative analysis of incorporated EPCs. Density of DiI-labeled EPCs (red fluorescence) in tissue sections retrieved from ischemic muscles was greater in SDF-1 treatment group than in PBS group at both days 3 and 7 (day 3, control vs SDF-1, 241±25 vs 445±24 cells/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

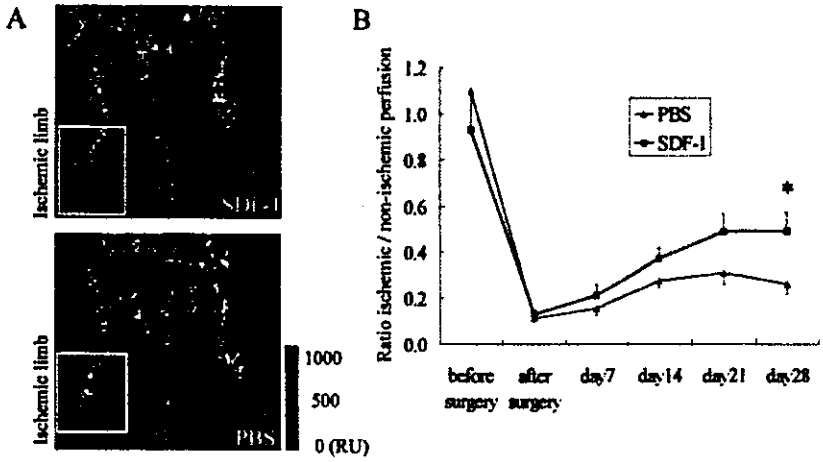

Figure 6. SDF-1 improved tissue perfusion. Hindlimb perfusion was measured by LDPI. A, Representative LDPI 28 days after induction of limb ischemia. Boxes indicate areas of interest. B, Quantitative analysis of perfusion recovery measured by LDPI. Ratios of ischemic/nonischemic limbs at day 28 were as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

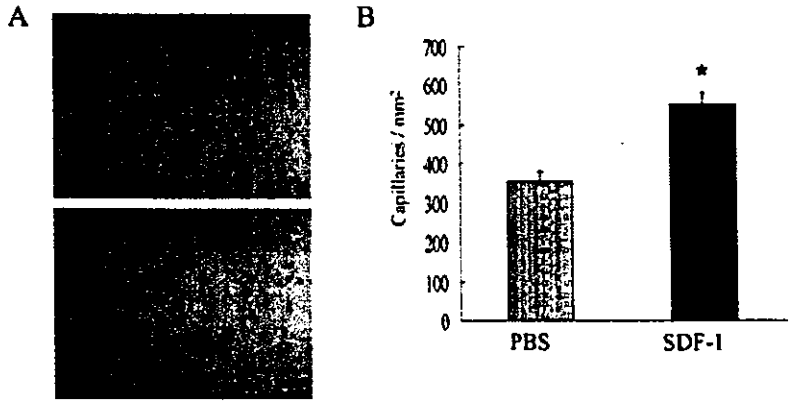

Figure 7. SDF-1 increased capillary density in ischemic tissue at day 28. Histological skeletal muscle section retrieved from ischemic hindlimbs at day 28 was examined for capillary density, an index of neovascularization, using endothelial-specific chemical staining of isolectin B4. A, Representative 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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Cell sheet engineering for myocardial tissue reconstruction

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Abstract

Myocardial tissue engineering has now emerged as one of the most promising treatments for the patients suffering from severe heart failure. Tissue engineering has currently been based on the technology using three-dimensional (3-D) biodegradable scaffolds as alternatives for extracellular matrix. According to this most popular technique, several types of 3-D myocardial tissues have been successfully engineered by seeding cardiomyocytes into poly(glycolic acid), gelatin, alginate or collagen scaffolds. However, insufficient cell migration into the scaffolds and inflammatory reaction due to scaffold biodegradation remain problems to be solved. In contrast to these technologies, we now propose novel tissue engineering methodology layering cell sheets to construct 3-D functional tissues without any artificial scaffolds. Confluent cells on temperature-responsive culture surfaces can be harvested as a viable contiguous cell sheet only by lowering temperature without any enzymatic digestions. Electrical communications are established between layered cardiomyocyte sheets, resulting in simultaneous beating 3-D myocardial tissues. Layered cardiomyocyte sheets in vivo present long survival, macroscopic pulsation and characteristic structures of native heart tissue. Cell sheet engineering should have enormous potential for fabricating clinically applicable myocardial tissues and should promote tissue engineering research fields.

Keywords: Myocardial tissue engineering; Cell sheet; Cardiac myocyte; Transplantation; Temperature-responsive culture surface

1. Introduction

Recently, alternative treatments for cardiac transplantation have been strongly requested to repair damaged heart tissue, because the utility of heart transplantation is limited by donor shortage. Cell therapy is now considered to be one of the most effective treatments for impaired heart tissue [1,2]. Direct transplantation of cell suspension has been researched since the early 1990s [3]. In these studies, survival of transplanted cells, integration of native and grafted cells, and improvement of host cardiac function have been reported. It is a critical point how to isolate and expand clinically transplantable myocardial cell source. Autologous myoblast transplantation has been performed clinically and the contraction and viability of grafted myoblasts have been confirmed [4]. Multipotent bone marrow cells or embryonic stem cells have been now aggressively investigated as possible candidates for human implantable myocardial cell source [5–8].

In direct injection of dissociated cells, it is difficult to control shape, size and location of the grafted cells. Additionally, isolated cell transplantation is not enough for replacing congenital defects. To overcome these problems, research on fabricating three-dimensional (3-D) cardiac grafts by tissue engineering technology has also now begun [9]. Tissue engineering has currently been based on the concepts that 3-D biodegradable scaffolds are useful as alternatives for extracellular matrix (ECM) and that seeded cells reform their native structure in according to scaffold biodegradation [10]. This context has been used for every type of tissue. In myocardial tissue engineering, poly(glycolic acid) (PGA), gelatin and alginate have been used as prefabricated biodegradable scaffolds. Papadaki et al. engineered 3-D cardiac constructs by using PGA scaffolds processed into porous meshes and rotating bioreactors [11]. Li et al. have demonstrated that transplantation of tissue-engineered cardiac grafts using biodegradable gelatin sponges replaced myocardial scar and right ventricular outflow track defect [12,13].

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Furthermore, Leor et al. reported that bioengineered heart grafts using porous alginate scaffolds attenuated left ventricular dilatation and heart function deterioration in myocardial infarction model [14]. As the technique premixing cells and ECM alternatives instead of seeding cells into preformed scaffolds, Zimmermann et al. engineered 3-D heart tissue by gelling the mixture of cardiomyocytes and collagen solution [15,16]. The construct has allowed direct measurement of isometric contractile force as heart tissue model.

In spite of these desirable results, insufficient cell migration into scaffolds and inflammatory reaction due to scaffold biodegradation remain problems to be solved [13,14]. In native myocardial tissue, cells are considerably dense (Fig. 1A) in comparison with other tissues including cartilage, vascular, and heart valve, which are cell-sparse tissues and have been successfully engineered by using biodegradable scaffolds (Fig. 1B). Cardiomyocytes are also tightly interconnected with gap junctions, which mediated the reciprocal exchange of small molecules and ions resulting in electrically synchronous beating [17]. In myocardial tissue engineering, biodegradable scaffolds themselves attenuate cell-to-cell connections and scaffold biodegradation leads to fibrous tissues containing excessive amount of ECM, which is shown in pathological states including ischemic heart disease or dilated cardiomyopathy. Investigators are now trying to fabricate more porous structure of biodegradable scaffolds and to develop new techniques seeding more cells into the scaffolds. In particular, structural balance between cells and ECM should be controlled to fabricate native heart-like tissues.

By contrast, we now propose novel tissue engineering methodology that is to construct 3-D functional tissues by layering 2-D cell sheets without any biodegradable alternatives for ECM. To obtain viable cell sheets, we have exploited intelligent culture surfaces, from which cultured cells detach as a cell sheet simply by reducing temperature. In this paper, we present the new technology "cell sheet engineering" and its application to myocardial tissue reconstruction.

2. Temperature-responsive culture surfaces

Temperature-responsive culture surfaces were developed among the research to control cell adhesion to biomaterials. Cells adhere to culture surfaces via membrane receptors and cell adhesive proteins, including fibronectin, that reside in serum or are secreted from the cells in culture (Fig. 2A). The interaction between adhesive proteins and culture surfaces depends on the wettability of the surface. Normal tissue culture polystyrene (TCPS) dishes are hydrophobic and absorb ECM proteins resulting in cell attachment and proliferation. To harvest cells from the surfaces, enzymatic digestion including trypsin and dispase are usually utilized. In that case, both adhesive proteins and membrane receptors are disrupted, then cells detach with considerable damages (Fig. 2B). On the other hand, we graft temperature-responsive polymer, poly(*N*-isopropylacrylamide)(PIPAAm) to TCPS dishes covalently by electron beam. The surfaces are hydrophobic and cells adhere and proliferate under culture condition at 37°C. By lowering temperature below 32°C, the surfaces change reversibly to hydrophilic and not cell adhesive due to rapid hydration and swelling of the grafted PIPAAm. This unique surface change allows cultured cells to detach spontaneously from these grafted surfaces simply by lowering temperature [18]. As against using enzymatic digestion, only the interaction between adhesive proteins and material surfaces is released and cells detach together with intact membrane proteins and adhesive proteins (Fig. 2C) [19]. As a result, cells recovered by using PIPAAm-grafted surfaces maintain their differentiated functions more strongly than the cells recovered by protease digestion [20]. For example, trypsin-treated hepatocytes decrease albumin production, on the other hand, those cells harvested from PIPAAm-grafted surface preserve albumin secretion [21].

In addition to the passive mechanism of the surface change from hydrophobic to hydrophilic, cell-mediated active processes have been ascertained as cell detachment mechanisms [22]. Sodium azide, an ATP synthesis inhibitor, considerably retarded cell release from PIPAAm-grafted surfaces, indicating that energy-dependent metabolic process is one of major mechanisms. The active processes are also mediated by intracellular signal transduction, including tyrosine phosphorylation and cytoskeltal reorganization and lead to the cell morphological change from spread to round after surface property change [23].

3. Cell sheet engineering

When cells are cultured confluently, they connect to each other via cell-to-cell junction proteins and ECM (Fig. 3A). With enzymatic digestions, these proteins are disrupted and each cell is released separately (Fig. 3B). In the case using PIPAAm-grafted surfaces, cell-to-cell connections are not disrupted and cells are harvested as a contiguous cell sheet by decreasing temperature (Fig. 3C). Furthermore, adhesive proteins underneath cell sheets are also maintained and they play a desirable role as an adhesive agent in transferring cell sheets onto other culture materials or other cell sheets [24]. These viable cell sheets are composed of cells and biological ECM without any artificial scaffolds. Various types of cell sheets have been successfully lifted up and transferred on other surfaces [25–32].

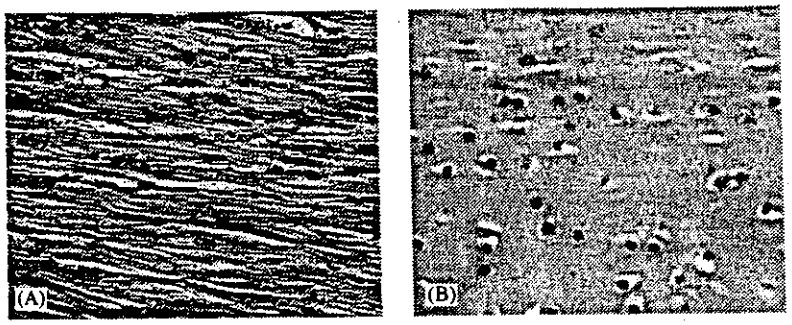

Fig. 1. Histological comparison between cell-dense and cell-sparse tissues. Hematoxilin and eosin staining shows that cells are dense and tightly connected in myocardial tissue (A) On the other hand, cartilage tissue includes sparse cells and large amount of ECM (B).

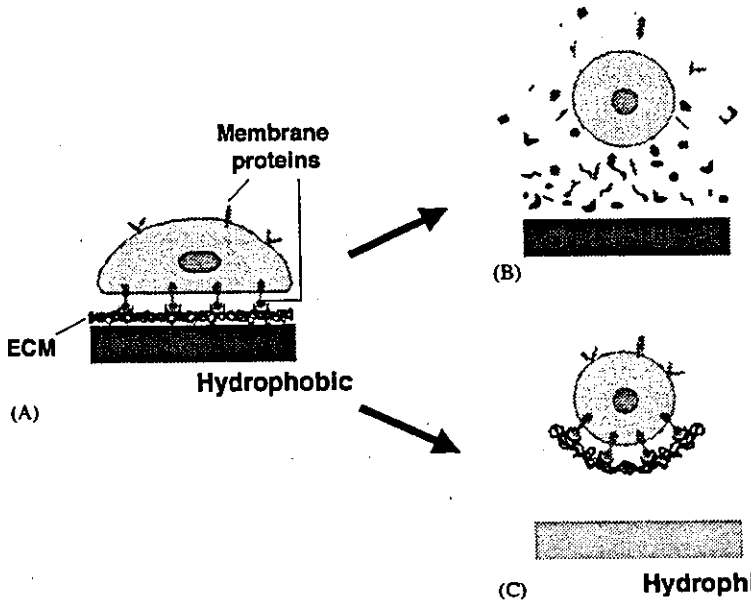

Fig. 2. Cell harvest mechanism by using temperature-responsive culture surfaces. (A) Cells attach to hydrophobic culture surfaces via cell membrane proteins and ECM, which reside in serum or are secreted from the cells. (B) When enzymatic digestion is used, both membrane and ECM proteins are disrupted, resulting in cell detachment. (C) When cells are cultured on temperature-responsive culture surfaces, the interconnection between ECM and hydrophilic culture surfaces is released only by lowering temperature. Then the cells detach together with intact proteins.

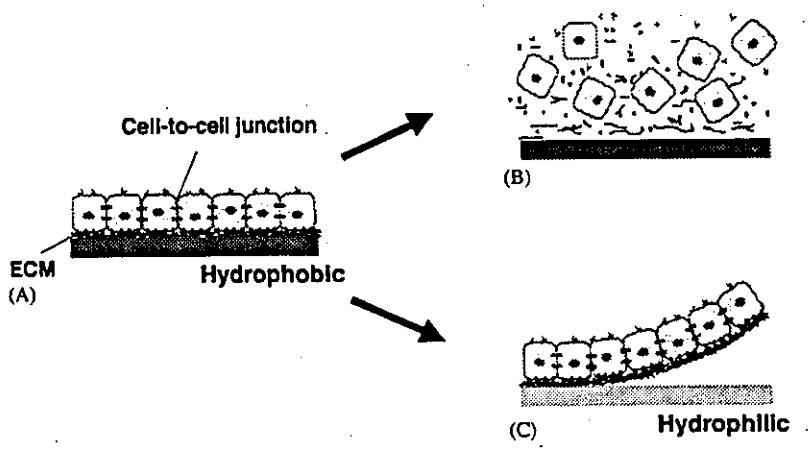

Fig. 3. Cell sheet release from temperature-responsive culture surfaces. (A) When cells are cultured confluently, the cells connect to each other via cell-to-cell junction proteins. (B) When harvested by protease treatments, cell-to-cell connections are disrupted and cells are released separately. (C) When PIPAAm-grafted surfaces are used, cell-to-cell connections are completely preserved and the cells are released as a contiguous cell sheet. ECM retained underneath the cell sheets play a role as a adhesive agent.

As cell sheet manipulation, two techniques have been performed according to cell types and objects. One is to manipulate cell sheets directly with forceps or pipetting after the sheets are completely harvested resulting in proportionally shrunk and thicker constructs due to active cytoskeletal reorganization. As indicated by synchronized beating of shrunk cardiomyocyte sheets, cell-to-cell connections are preserved after this procedure [31]. The other is to use support membranes including a hydrophilically modified poly(vinylidene difluoride)(PVDF) membrane for preserving cell sheet morphology without any shrinkage. Before cell sheets release, support membranes are placed over the confluent cells. Then the cell sheets physically attached to the support membranes are harvested from PIPAAm-grafted surfaces below 32°C and transferred onto other surfaces. Incubation at 37°C causes reattachment of the cell sheets to new surfaces via remaining adhesive proteins. Finally, only the support membranes are removed. The latter technique has realized the cell sheet manipulation preserving their structure and function [26–30].

These cell sheet manipulation techniques without using any biodegradable scaffolds have been applied to tissue engineering in three types of contexts (Fig. 4). First is transplanting single cell sheet for skin and cornea reconstruction. Advantages of skin epithelial cell sheets harvested by using PIPAAm-grafted surfaces have been confirmed in comparison with those harvested by dispase treatments. E-cadherin, which is an essential protein for skin cell-to-cell junctions, and laminin 5, which is a major component of epithelial basement membranes, were retained in skin cell sheets released from PIPAAm-grafted surfaces [27]. It should attenuate the risk of infection after artificial skin transplantation. Second is to layer same cell sheets for reconstructing homogeneous tissues including myocardium. Third is to layer several types of cell sheets for fabricating laminar structures including liver, kidney and vascular. Layered co-culture comprising a hepatocyte sheet and an endothelial cell sheet has revealed the differentiated cell shape and extensive albumin expression of hepatocytes, which have never been seen in hepatocyte mono-culture [32]. We have been now applied these technologies "cell sheet engineering" to reconstructing various types of tissues. Among them, myocardial tissue engineering based on the second context is described below.

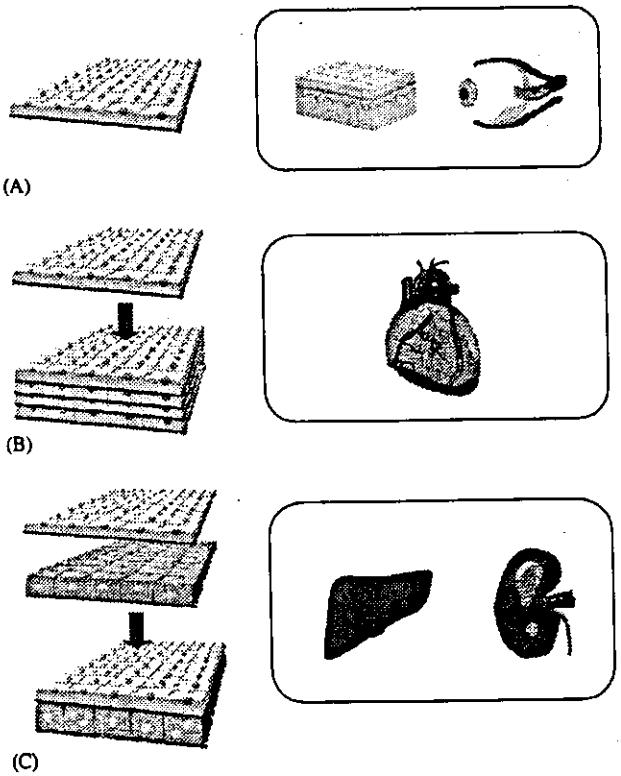

Fig. 4. Three contexts in cell sheet engineering. (A) Single cell sheet is useful for skin or cornea transplantation. (B) Same cell sheets are layered to reconstruct homogeneous 3-D tissues including myocardium. (C) Several types of cell sheets are co-layered to fabricate laminar structures including liver and kidney.

4. Myocardial tissue reconstruction by layering cardiomyocyte sheets [28,30,31]

Cardiomyocytes are tightly interconnected with gap junctions and pulsate simultaneously in native heart tissue. It is also well-known that confluent cultured cardiomyocytes on culture surfaces connect via gap junctions and beat simultaneously [33]. Therefore, in myocardial tissue engineering by layering cell sheets, it is a crucial point whether electrical and morphological communications are established between bilayer cell sheets. Chick embryo or neonatal rat cardiomyocyte sheets released from PIPAAm-grafted surfaces presented synchronized pulsation. To examine the electrical communication, two cardiomyocyte sheets were overlaid partially as schematically illustrated in Fig. 5. Two

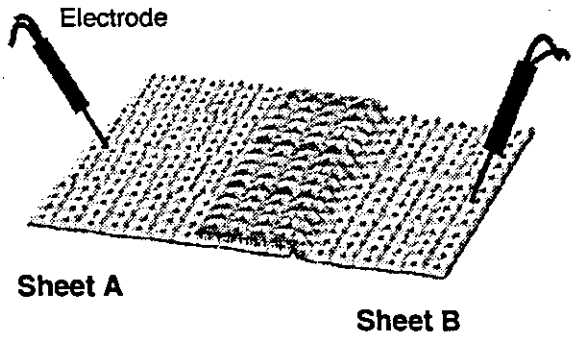

Fig. 5. Schematic illustration of electrical analysis of layered cardiomyocyte sheets. To examine the electrical synchronization, two cardiomyocyte sheets (A, B) are overlaid partially. Two electrodes are set over monolayer parts of both cell sheets to detect the electrical potentials separately.

electrodes were set over monolayer parts of both cell sheets. Detected electrical potentials of the two sheets completely synchronized (Fig. 6). Furthermore, electrical stimulation to the single-layer region of one sheet was transmitted to the other cell sheet and the two cell sheets pulsated simultaneously. Histological analysis showed that bilayer cardiomyocyte sheets contacted intimately resulting in homogeneous tissue. Cell-to-cell connections including desmosomes and intercalated disks were confirmed by transmission electron microscopic images. These data indicate that electrical and morphological communications are established between layered cardiomyocyte sheets.

Under conventional culture conditions, cardiac myocytes are fixed to rigid material surfaces and their motion is highly limited. To minimize the interaction between cell sheets and culture materials, the sheets were overlaid on several types of materials including polyethylene meshes, elastic polyurethane meshes or frame-like collagen membranes. In any cases, the constructs pulsated simultaneously with higher amplitude than the cells fixed on rigid culture surfaces. When cardiomyocyte sheets were layered on frame-like collagen membranes, the center part of them is free from any culture materials. In result, 4-layer cardiac constructs on the frame-like collagen membranes pulsated spontaneously in macroscopic view.

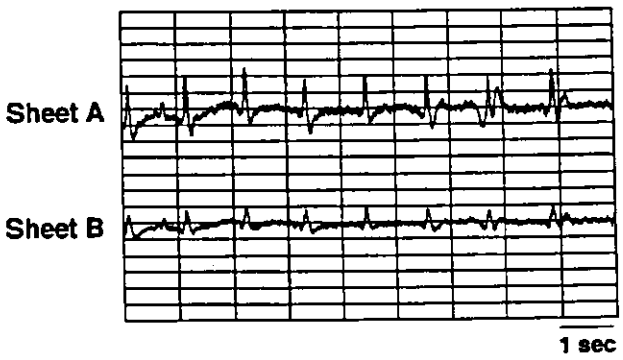

Fig. 6. Synchronization of layered cardiomyocyte sheets. Representative tracings of electrical potentials of sheet A and sheet B show complete synchronization.

To examine in vivo survival and function of layered cardiomyocyte sheets, the constructs were transplanted into dorsal subcutaneous tissues of nude rats. Surface electrograms originating from transplanted constructs were detected independently from host electrocardiograms, in the earliest case, at 2 weeks after the operation (Fig. 7). When transplantation sites were opened, macroscopic simultaneous graft beatings were observed at the earliest period, 3 days after the transplantation. Furthermore, graft survival was confirmed at least up to 1 year. Morphological analysis demonstrated that neovascularizations occurred in a few days and that vascular network was organized within a week (Fig. 8A). Cross-sectional views revealed stratified cell-dense myocardial tissues (Fig. 8B), well-differentiated sarcomeres and diffuse formation of gap junctions. In comparison between 2-layer and 4-layer cardiac tissue grafts, fractional shortening increased depending on the number of layered cell sheets.

Thus, the basic technology has been established to fabricate electrically communicative, pulsatile myocardial tissues by using cell sheets both in vitro and in vivo.

5. Future perspectives

Recently, research on myocardial tissue engineering has been accelerated to develop further advanced therapy for severe heart failure. Transplantation of layered cardiomyocyte sheets on the myocardial scar may be more beneficial than that of bioengineered heart tissue including biodegradable scaffolds in the point of scaffold-mediated disadvantages. However, there are several common problems in myocardial tissue

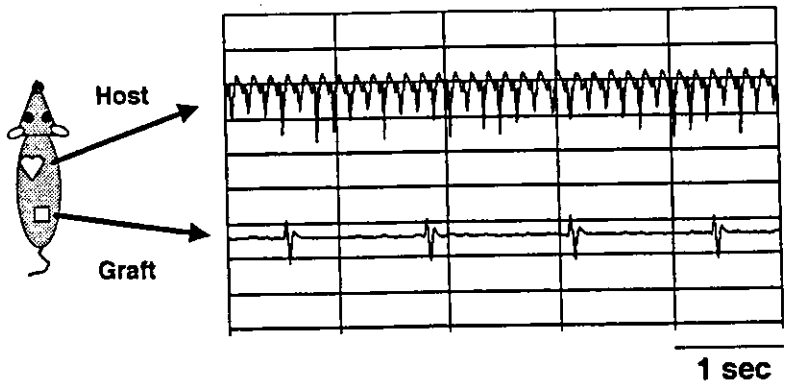

Fig. 7. Skin surface electrogram of transplanted cardiomyocyte sheets. Representative tracings of the host electrocardiogram (upper) and the electrical potential detected via the electrode set at the skin just above the transplanted heart graft (lower) are shown. Skin surface electrogram originating from the graft is detected independently from host electrocardiogram.

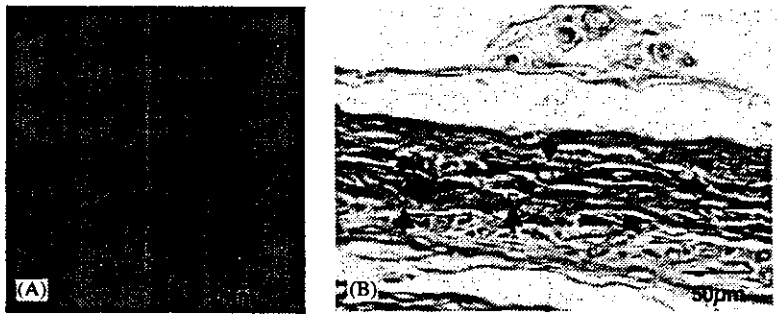

Fig. 8. (A) Macroscopic view of the transplanted cardiac graft. Multiple neovascularization is shown in the square-designed cardiac graft transplanted into dorsal subcutaneous tissue. (B) Azan staining shows a stratified cardiac tissue graft including elongated cardiomyocytes and microvasculars (arrows).

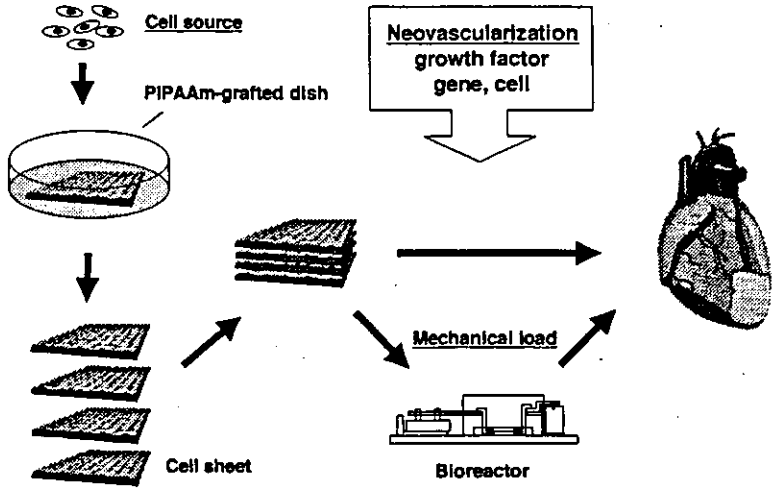

Fig. 9. Schematic illustration of myocardial reconstruction based on cell sheet engineering. We now propose the application of "cell sheet engineering" to myocardial tissue reconstruction. Cell sourcing remains a crucial problem. Neovascularization for oxygen and nutrition supply is also critical to fabricate human applicable myocardial tissue. Growth factors, gene delivery and the utility of gene-modified cells or endothelial cells may be helpful. Mechanical load by using bioreactors should strengthen the engineered myocardial tissues. Transplantation of engineered tissue into myocardial infarction model is now in progress.

engineering. As described in Section 1, myocardial cell sourcing remains a crucial problem. Further advance in stem cell biology for cardiomyocytes will be needed to realize clinical application of bioengineered myocardial tissues.

Vascular reconstruction is also one of the most critical issues in myocardial tissue engineering. Sufficient supply of oxygen and nutrition is required for functionally beating heart tissue. It has been reported that cells are dense in the graft periphery, but sparse in the interior part due to insufficient oxygen perfusion in scaffold-based heart tissue grafts [34]. Although, in our studies, multiple neovascularization arose in transplanted cardiac grafts in a few days, primary insufficient oxygen and nutrition permeation also limit the number of transplanted cardiomyocyte sheets. Hence, new methods to accelerate blood vessel formation are now requested to engineer larger or thicker constructs for heart tissue repair. As examined in isolated cell injection, gene-modified cells may be also applicable for engineering more vascularized heart tissues [35]. Using cell sheet technology, it has been reported that a single layer of endothelial cell sheet enhances the capillary formation in vivo [36]. Therefore, heterogenous layering of endothelial cell sheets between cardiomyocyte sheets may promote neovascularization. Further research and development will be needed to engineer vascular networks sufficient for fabricating clinically applicable heart tissues.

In native heart, cardiomyocytes are gradually elongated and hypertrophied by mechanical load increase in accordance with the growth of the body. Therefore, some investigators have attempted to strengthen bioengineered heart tissues by using mechanical devices. Carrier et al. used a rotating bioreactor for culturing cardiomyocytes on PGA scaffolds [37]. Fink et al.

clearly demonstrated that application of stretch devices to engineering heart tissues strengthened the contraction power and oriented the cells unidirectionally [38]. We are now trying to stretch layered cardiomyocyte sheets to fabricate more powerful cardiac constructs in vitro.

Finally, our concept of myocardial tissue engineering is schematically illustrated in Fig. 9. Although further interdisciplinary research will be needed to clear the existing several problems, cell sheet engineering should have enormous potential for constructing clinically applicable heart grafts and should promote tissue engineering research fields.

Acknowledgements

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虚血性心疾患に対するtissue engineering

Tissue engineering for ischemic heart disease

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Key words：組織工学，生体吸収性高分子，細胞シート

はじめに

近年，不全・欠損組織の補完を目的に，細胞単独あるいは組織工学(tissue engineering)により再構築した組織の移植を行う臨床および研究が盛んに行われている．循環器領域においても筋芽細胞や再生血管の移植が既に臨床応用されており，今後の更なる発展が期待されている．冠動脈疾患に対する細胞治療としては，冠動脈バイパス術に用いる小口径の血管組織の再構築，細胞移植による血管新生あるいは心筋組織再生療法，移植用心筋グラフトの作製が追究されている．単離細胞の移植療法に関しては前項で既出であり，本稿ではtissue engineeringによる血管および心筋組織の再構築に関する研究の動向について概説する．

1. Tissue engineering

tissue engineeringは1993年LangerとVacantiが提唱した概念であり，医学と工学の融合により生まれた新たな学問である[1]．彼らは組織再生には細胞，細胞の足場となる細胞外マトリックス(extracellular matrix: ECM)，細胞の分化・増殖のためのサイトカインが必要であるとし，その足場のECMの代替としてポリグリコール酸(polyglycolic acid: PGA)とポリL乳酸(polylactic acid: PLLA)およびその共重合体からなる生体吸収性の3次元支持体を用いた．この支持体に細胞を培養し生体内に移植することにより支持体が徐々に分解，生体が作り出すECMと置換され生体類似の組織が再構築されるという手法である(図1)．実際，この手法で軟骨組織が再生され臨床応用されている．現在，あらゆる組織に関して細胞の足場として生体吸収性の3次元生体材料を用いた組織再生の研究が行われている．

2. 血管組織の再構築

現在，狭窄あるいは閉塞血管に対する外科的治療法としては人工血管を用いた置換術が行われ，ポリエチレンテレフタレート(PET，ダクロン)やポリテトラフルオロエチレン(ePTFE，Gore-Tex)が合成素材として用いられている．口径の大きな血管では開存率も向上しており広く臨床の場に普及している．しかし冠動脈など小口径(<5mm)の血管への臨床応用は血管閉塞が高率に生じるため困難なのが現状である．また小児の先天性血管疾患に対する治療に人工血管を用いる場合には時間の経過に伴い成長することがないという問題点がある．こういった状況の中でtissue engineeringを用い自己の細胞を使った血管の再構築・移植は，抗血栓性，成長，感染などの諸問題を解決し得る治療法として期待されている．血管の組織再生では，生

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