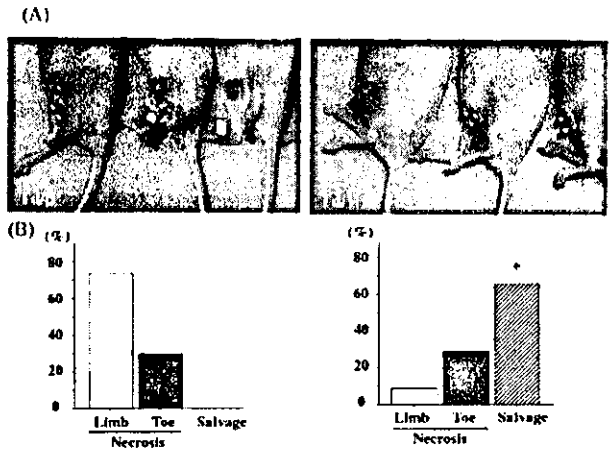

Figure 6. Administration of hEPCs leads to reduced limb loss and increased limb salvage. A, Representative macroscopic photographs of mice show two different outcomes observed in the study. Left panel, Td/β-EPC–treated animals; right panel, Td/V-EPC–treated animals at day 28. B, Percent distribution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

mice receiving Td/β-EPCs (421±15 versus 230±14/mm^2, $P<0.05$) or nontransduced mice EPCs (183±16/mm^2, $P<0.05$) (Figure 8). Similarly, the capillary/muscle fiber ratios in the Ad/VEGF transplanted mice were significantly higher than in Td/β-EPCs or non-Td/EPCs transplanted mice (0.62±0.02 versus 0.39±0.03 versus 0.35±0.05, $P<0.01$).

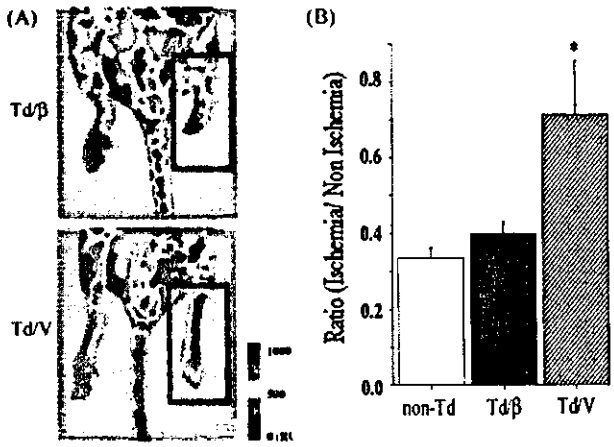

Figure 7. LDPI performed at day 28. A, LDPI was used to record serial blood flow measurements immediately before and 28 days after administration of transduced EPCs. In these digital, color-coded images, red hue indicates regions with maximum perfusion; medium perfusion values are shown in yellow; lowest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

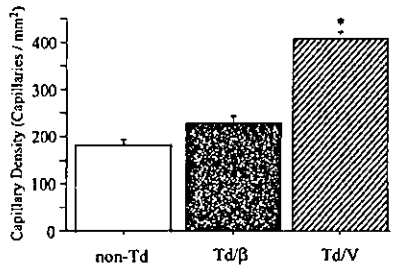

Figure 8. Histological evidence of neovascularization in ischemic hindlimb. Extent of neovascularization was assessed by measuring capillary density in light microscopic sections prepared from muscles of ischemic hindlimbs 28 days after administration of Td/β-EPCs, Td/V-EPCs and non-Td/EPCs. *$P<0.05$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

Discussion

The finding that circulating EPCs may home to sites of neovascularization and differentiate into ECs in situ is consistent with "vasculogenesis,"[24] a critical paradigm for establishment of the primordial vascular network in the embryo. Although the proportional contributions of angiogenesis and vasculogenesis to postnatal neovascularization remain to be clarified, our findings together with the recent reports from other investigators[7–13] suggest that growth and development of new blood vessels in the adult are not restricted to angiogenesis but encompass both embryonic mechanisms. As a corollary, augmented or retarded neovascularization—whether endogenous or iatrogenic—probably includes enhancement or impairment of vasculogenesis.

Therapeutic Vasculogenesis

We therefore considered a novel strategy of EPC transplantation to provide a source of robust ECs that might supplement fully differentiated ECs thought to migrate and proliferate from preexisting blood vessels according to the classic paradigm of angiogenesis developed by Folkman and colleagues.[16] Our studies indicated that ex vivo cell therapy, consisting of culture-expanded EPC transplantation, successfully promotes neovascularization of ischemic tissues, even when administered as "sole therapy," for example, in the absence of angiogenic growth factors.

Such a "supply side" version of therapeutic neovascularization in which the substrate rather than ligand comprises the therapeutic agent was first demonstrated in a immune deficient murine model of hindlimb ischemia, with the use of donor cells from human volunteers.[14] These findings provided novel evidence that exogenously administered EPCs augment naturally impaired neovascularization in an animal model of experimentally induced critical limb ischemia. Not only did heterologous cell transplantation improve neovascularization and blood flow recovery, but important biological consequences—notably limb necrosis and autoamputation—were reduced by 50% in comparison with mice receiving differentiated ECs or control mice receiving media in which harvested cells were expanded ex vivo before transplantation.

More recently, this same strategy has been used successfully to enhance myocardial function after myocardial infarc-

tion in experimental animal models as well.[15] Peripheral blood mononuclear cells obtained from healthy human adults were cultured in EPC medium, harvested 7 days later, and administered intravenously to Hsd:RH-rnu (athymic nude) rats with myocardial ischemia induced by ligation of the left anterior descending coronary artery. Fluorescent microscopy of DiI-labeled EPCs revealed that transplanted EPCs accumulated to the ischemic area and incorporated into foci of myocardial neovascularization. Echocardiography disclosed ventricular dimensions that were significantly smaller and fractional shortening that was significantly greater in the EPC versus control animals. Necropsy examination disclosed that capillary density was significantly greater and the extent of left ventricular scarring was significantly less in rats receiving EPCs versus control animals.

Logistics of Primary EPC Transplantation

Despite promising potential for regenerative applications, the fundamental scarcity of EPC populations in the hematopoietic system, combined with possible functional impairment of EPCs associated with a variety of phenotypes such as aging, diabetes, hypercholesterolemia, and homocysteinemia,[17–20] constitute important limitations of primary EPC transplantation. Ex vivo expansion of EPCs cultured from the peripheral blood of healthy human volunteers typically yields $\approx 5.0\times10^6$ cells per 100 mL of blood. Our animal studies[14] suggest that heterologous transplantation requires $0.5\approx2.0\times10^4$ human EPCs/grams of weight (of the recipient mouse) to achieve satisfactory reperfusion of the ischemic hindlimb.

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

Advantage of VEGF EPC Gene Transfer

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

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

The results of our in vitro studies provide potential insights into the mechanisms responsible for the in vivo outcomes. First, VEGF gene transfer augmented EPC proliferative activity. Second, VEGF gene transfer enhanced adhesion and incorporation of EPCs into a quiescent endothelial cell monolayer as well as ECs activated by pretreatment with TNF-α. These findings suggest that modulation of adhesion molecule expression after VEGF gene transfer may promote homing of EPCs to foci of ischemia and are consistent with previous studies demonstrating VEGF-induced upregulation of certain EC integrins and matrix proteins.[25] Given that EPCs by definition express VEGF receptors, the potential for autocrine effects—demonstrated previously by our laboratory for ECs[26]—on proliferation, migration, and possibly survival of EPCs probably contributed to the reduced requirement of harvested EPCs.

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

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

Vector-Specific Issues

Transient gene expression is characteristic of adenoviral vectors.[27] For purposes of therapeutic neovascularization, this feature is unlikely to constitute a liability, given the plethora of previous preclinical and clinical studies suggesting that VEGF overexpression for 4 weeks or less, whether achieved by transfer of naked plasmid DNA[28–31] or use of an adenoviral vector,[32,33] is sufficient to augment angiogenesis. Indeed, previous work by others has demonstrated that protracted VEGF overexpression may result in hemangioma formation in normal skeletal muscle[34] as well as myocardi-

um.[35] This potential complication was not observed in the experiments described herein.

The potential for immunologic complications remains a concern attached to the use of adenoviral vectors, despite certain reports to the contrary.[33] In the current application, however, the ex vivo transduction strategy used may preclude exposure of the adenovirus to the immune system of the transplant recipients. Thus, administration of the transgene by ex vivo viral gene transfer may not detract from the safety of this application for clinical gene therapy.

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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.).

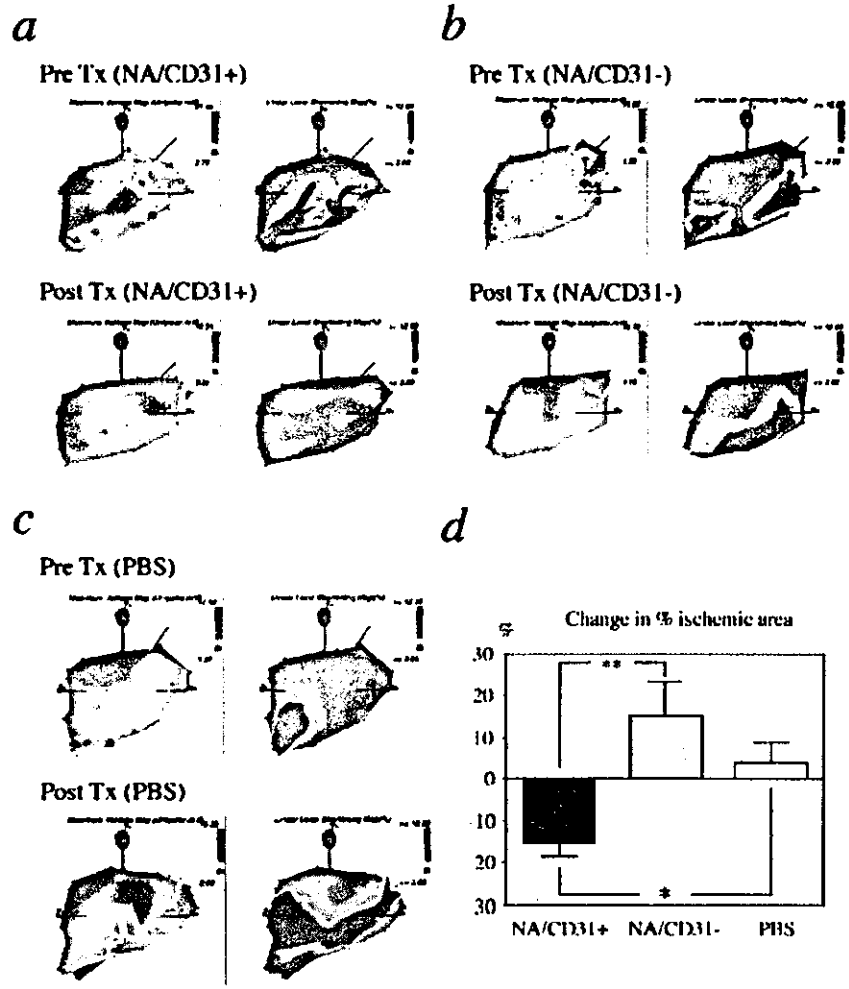

Figure 1. a, Representative findings of NOGA electromechanical mapping before (top) and 4 weeks after (bottom) NA/CD31+ MNC transplantation. Brown dots in pretreatment map show sites of cell transplantation. Red area on pretreatment linear local shortening map (top right) indicates area of decreased 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

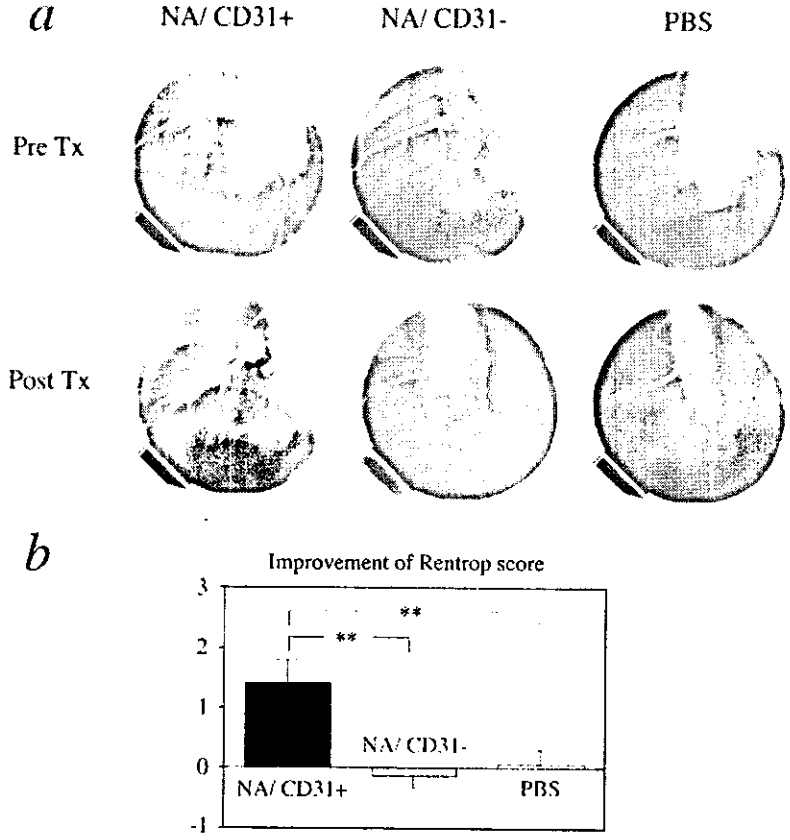

Figure 2. a, Representative left coronary angiographic findings in swine before and 4 weeks after cell transplantation. Well-developed collaterals (arrows) to LCx were observed in NA/CD31+ MNC group, resulting in complete opacification of LCx and its branches. b, Improvement of Rentrop angiographic score 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).

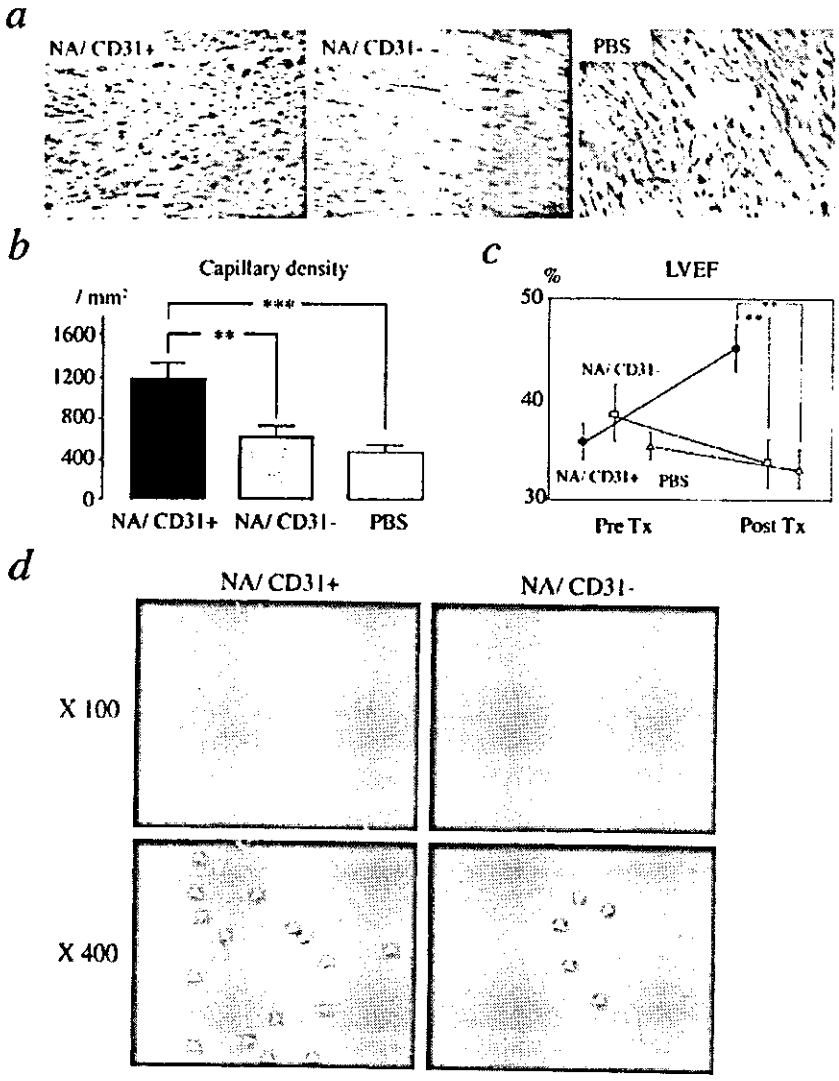

Figure 3. a, Representative immunohistochemical findings for isolectin B4 in swine ischemic myocardium 4 weeks after cell transplantation. b, Capillary density in swine ischemic myocardium 4 weeks after transplantation. $^{**}P<0.01$; $^{***}P<0.001$. c, Echocardiographic LV ejection fraction (EF) before 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

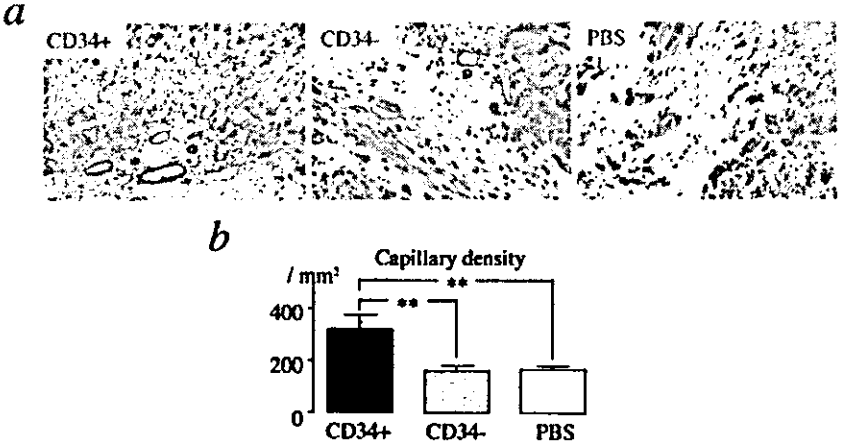

Figure 4. a, Representative immunohistochemical findings for isolectin B4 in ischemic myocardium of nude rats 4 weeks after cell transplantation. b, Capillary density in rat ischemic myocardium 4 weeks after cell transplantation. $^{**}P<0.01$.

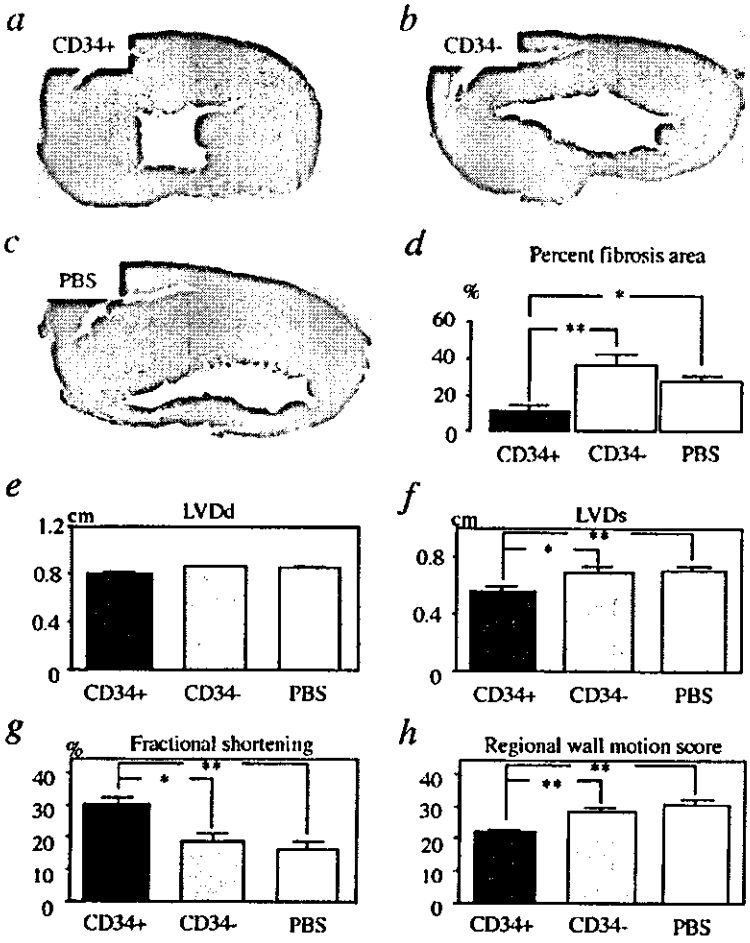

Figure 5. a through c, Representative elastic tissue–trichrome-stained sections from nude rats 4 weeks after receiving CD34+ MNCs (a), CD34− MNCs (b), and PBS (c). d, LV fibrosis was significantly reduced in CD34+ MNC-treated group compared with CD34− MNCs (*$P<0.05$) or PBS group (**$P<0.01$). e through 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-I 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

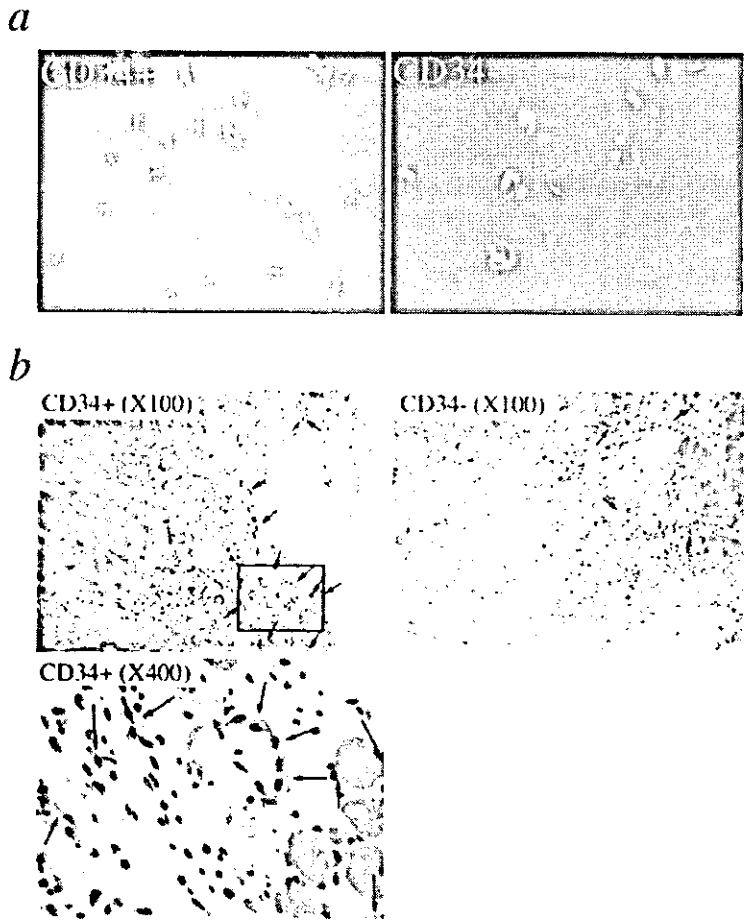

Figure 6. a, Representative fluorescence microscopic findings of ischemic myocardium of nude rats 10 days after cell transplantation. Red fluorescence indicates DiI labeling of transplanted human cells, and green fluorescence indicates BS-1 lectin, a marker for rat ECs. Transplanted cells with tube-like 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.

Acknowledgments

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血管再生療法の未来と画像評価法

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1. はじめに

動脈硬化性疾患の増加，高齢化に伴って，重症の冠動脈疾患や末梢動脈閉塞症が増加している．これらの疾患に対しては，通常，カテーテル治療やバイパス手術などの血行再建術が施行されている．しかしながら，びまん性病変や末梢性病変に対しては，血行再建術は困難である．近年，血行再建術が困難な重症の虚血性循環器疾患に対して血管再生療法が試みられている．まずまずの臨床効果が報告されているが，評価法が確立されておらず，再生血管を描出できる微小循環撮影装置が望まれている．そこで，本稿においては，血管再生の機序，血管再生療法の現況と展望，微小循環造影法の開発と意義について，臨床応用の観点から基本的なことを中心に述べたい．

2. 虚血から血管再生へ

2.1 虚血によって生じる血管の変化

組織が虚血（低酸素）に陥ると，HIF (Hypoxia Induced Factor) などの転写因子やVEGF (Vascular Endothelial Growth Factor)，Angiopoietinなどの血管内皮増殖因子とよばれるサイトカインが産生される[1]．これらのサイトカインの中で，特にAngiopoietin 2の働きにより血管の平滑筋細胞が脱落する．VEGFはこのAngiopoietin 2の作用を増強させ，さらに血管内皮細胞の結合を緩やかにし，血管の透過性を亢進させる．また，マクロファージを介して，MMP (Matrix Metalloproteinase) などの組織分解酵素が産生される（図1）．

2.2 血管内皮細胞による血管再生（狭義のAngiogenesisとVasculogenesis）

血管平滑筋細胞の脱落，血管透過性の亢進，血管周囲組織の変成の後，血管内皮細胞による血管再生が生じる．血管内皮細胞による血管再生には，血管内皮細胞が増殖して，再生血管が進展する（狭義のAngiogenesis）と血管内皮前駆細胞が集合して，あらたに血管が形成される（Vasculogenesis）の二つがある．

狭義のAngiogenesisは，既存の血管から，VEGFなどの血管内皮増殖因子により血管内皮細胞が血管の外側に増殖し，血管側枝として再生血管を形成していく現象である（図2a）．以前から考えられていた血管再生の機序であるが，近年，これに関与する因子が多数発見されており，そのメカニズムが明らかにされつつある．

Vasculogenesisは主に胎生期に血管が形成される時に生じる血管再生であるが，近年，成人においても血管内皮前駆細胞が骨髄または末梢血液中に存在することが報告された[2]．Vasculogenesisにおいては，血管内皮前駆細胞がVEGFなどのサイトカインの影響下に，虚血部に遊走，集合して毛細血管網を形成する（図2b）．毛細血管網は再構築を繰り返しながら，しだいに小血管へと成長していく．基礎および臨床

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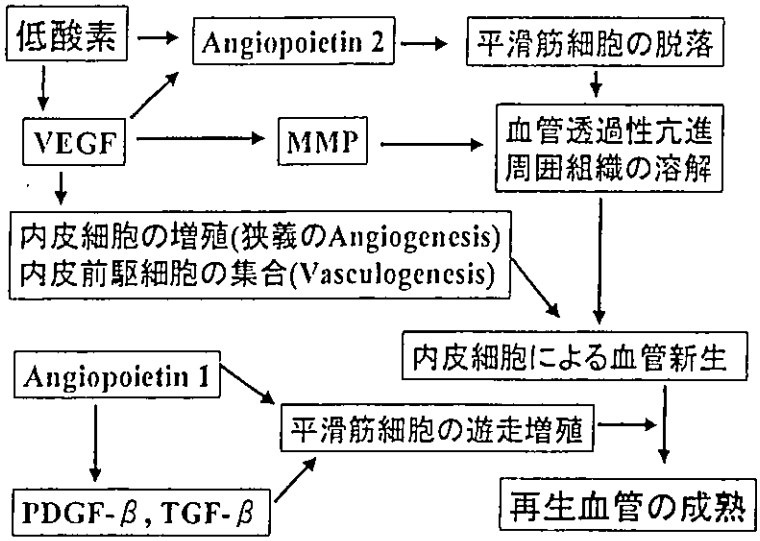

図1　虚血から血管再生までの機序
VEGF: Vascular Endothelial Growth Factor, MMP: Matrix Metalloproteinase, PDGF: Platelet Derived Growth Factor, TGF: Transforming Growth Factor.

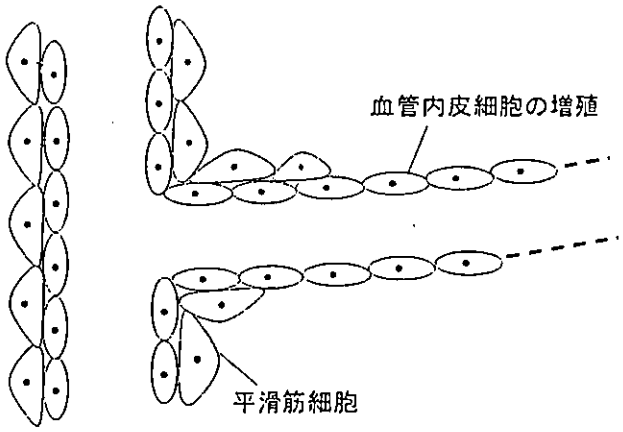

図2a　狭義の Angiogenesis の模式図

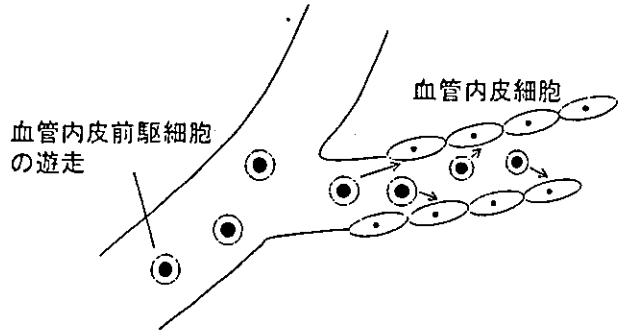

図2b　Vasculogenesis の模式図

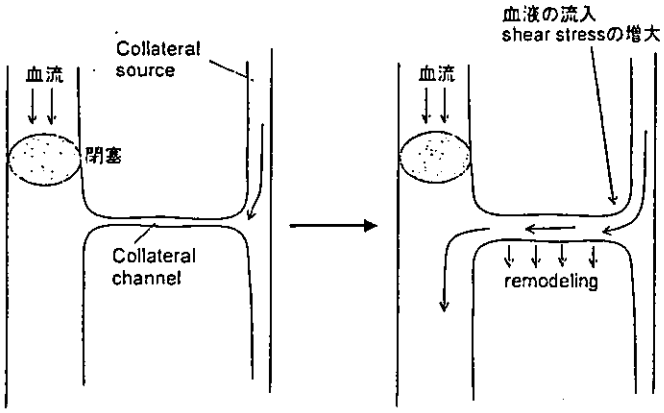

図2c　Arteriogenesis の模式図

検討において，血管内皮前駆細胞が含まれる骨髄単核球を虚血部位の筋肉内に投与することにより，血管再生がみられることが報告されている[3]．

2.3　再生血管の成熟

血管内皮細胞のみで形成された血管は，脆弱で破綻しやすい．例えば，糖尿病性網膜症でみられる再生血管は，血管内皮細胞のみで形成されており，瘤状に拡大したり，破綻出血したりする．このため，血管再生の最終過程において，Angiopoietin 1，TGF (Transforming Growth Factor)，PDGF (Platelet Derived Growth Factor)

などのサイトカインにより血管平滑筋細胞が遊走，増殖し，血管内皮細胞で形成された血管の外側を覆い，成熟した再生血管へと変化する（図1）．

2.4 その他の血管再生（Arteriogenesis）

血管再生には，狭義のAngiogenesisとVasculogenesisの他に，平静時は可視化できない側副路（いわゆるcollateral channel）が，虚血時にcollateral sourceからの血液の流入，remodelingによって拡大し，血管造影で新しい血管としてみとめられることがある．これはArteriogenesisと呼ばれている．Arteriogenesisには，collateral sourceや他の新生血管から血液供給をうけることによるshear stressの増大，MMPなどのサイトカインによる周囲組織の変性や血管のremodeling，血管内皮細胞や平滑筋細胞の発達増殖などが関与している（図2c）．

3. 血管再生療法の現状

3.1 血管内皮増殖因子による血管再生療法

血管再生においては，上述したようにVEGFが大きな役割を担っている．Isnerらは重症の下肢虚血患者においてVEGF遺伝子を虚血肢に筋注することにより，虚血症状の改善を報告している[9]．VEGFを直接，筋肉内または動脈内に投与することも動物実験レベルでは考えられたが，局所で持続的に血管内皮増殖因子が効果発現するために，遺伝子導入による方法が選択された．ただ，遺伝子導入には当初，ウイルスがベクターとして用いられたが，ウイルス感染に伴う副作用が問題となった．現在，プラスミドDNAでの遺伝子導入が行われている．しかしながら，この遺伝子導入法は，安全性は高いが，DNA分解酵素により代謝され，導入効率が低いことが指摘されている．その他の臨床試験としては，欧米ではFGF（Fibroblast Growth Factor）を用いた試験が，本邦では大阪大学においてHGF（Hepatocyte Growth Factor）を用いた試験が開始されている．

3.2 骨髄細胞移植による血管再生療法

Vasculogenesisを目的に，骨髄細胞移植による血管再生療法が本邦を中心に開始されている．血管内皮前駆細胞は骨髄単核球の中に含まれており，骨髄液を採取し，遠心分離により単核球を選別する．これを虚血部に筋注することで，血管再生を試みる．閉塞性動脈硬化症やバージャー病などの末梢動脈閉塞症の患者を対象に臨床治療が開始されているが，虚血性心疾患の患者に対しても，一部，試みられている．

3.3 血管再生療法の臨床効果

血管内皮増殖因子による遺伝子治療と骨髄細胞移植による血管再生療法の臨床試験においては，いずれも症状の改善は8割程度に認められており，皮膚所見を含め自覚症状的には良好な効果が示されている．しかしながら，血管造影上などの他覚的な検査での改善は少ない．自覚症状と他覚的な検査との結果で乖離がみられている．また，血管再生療法の長期的な効果については，現在の所，不明である．

4. 微小循環造影法による再生血管の評価

4.1 再生血管と血管造影

血管再生療法で再生される血管は主に100 μm以下の小血管が主体と考えられている．一般の血管造影装置では200 μm以下の血管は描出困難であるため，再生血管を評価することは困難と思われる．これが，前述した血管再生療法における血管造影での有意な改善がみられない理由と考えられる．時に，血管再生療法により血管造影で側副路の発達がみられることがあるが，この新しく描出された側副路は再生血管そのものではなく，再生血管により側副路の血流が増加したいわゆるArteriogenesisと考えられている．血管再生療法による再生血管を直接描出しうる微小循環の造影装置が望まれている．

4.2 放射光を用いた微小循環撮影装置の開発

前述したように再生血管の評価には，100 μm以下の小血管の描出が必要となる．著者らは，より微量の造影剤を検出可能とするため，放射光を

用いて微小循環造影装置の開発を行った[5,6].

放射光は大型のシンクロトロン加速器で作成される強い光（電磁波）である．これは，紫外部から硬X線までの広いスペクトルを持っている．さらに，これをシリコン結晶に通過させることで，単色放射光（一定のエネルギーレベルを持つX線）に変換することができる．ヨード剤は，質量吸収係数が不連続に上昇するX線エネルギーレベルが存在する．したがって，そのエネルギーレベルの単色放射光を用いることで，ヨードと周囲組織とのX線吸収差を最大にし，造影剤のコントラスト効果を最良とすることができる．ここでは，ヨードのK吸収端直上のエネルギーレベル（33.30 keV）に放射光を単色化し，この単色放射光をX線源として用いた．

撮像系として，高解像度高感度蛍光板（FOS, Gd_2O_2S: Tb, 浜松ホトニクス）を用いた．撮影系として，アバランシェ型ハイビジョンモノクロ新Super-HARPカメラ（NHK）を用いた[7~9]．放射光微小循環撮影装置はこれらから構成され，25 μm以下の空間分解能を可能とした．

4.3 放射光微小循環造影法による再生血管の描出と機能評価

著者らは，ウサギの虚血肢モデルを作成して，プラスミドFGF4投与による血管再生療法を行い，放射光微小循環造影法を用いて再生血管の描出を試みた．図3で示すような，200 μm以下の微小血管も描出されている．

また，放射光微小循環造影法は再生血管の機能評価も可能である．通常の血管は血管内皮細胞と血管平滑筋細胞の両者によって構成されているが，血管再生療法においては，血管内皮細胞の増殖のみが注目され，血管平滑筋細胞に対しては十分な配慮はなされてこなかった．また，再生血管の数のみが問題とされ，質的，機能的な検討は，十分にはなされていない．

著者らは，DNA分解酵素から遮断されるゼラチンハイドロゲル（GHG）とDNAの複合体を考案し，その徐放性から，より成熟した再生血管の新生を試みた．ウサギの虚血肢モデルを用いて，FGF4遺伝子単独投与とGHG-FGF4遺伝子複合体投与の血管再生療法を行い，GHG-LacZ複合体投与のコントロールを含めて3群において，放射光微小循環造影法により再生血管の機能評価を行った．アデノシン投与による再生血管の拡張反応は，GHG-FGF4遺伝子複合体群でのみ認められ，FGF4遺伝子単独群とコントロール群ではみられなかった（図3）．GHG-FGF4遺伝子複合体を用いた血管再生療法は，成熟した再生血管の形成に有効であると判断された．

放射光微小循環造影法は，再生血管の機能評価，成熟度の判定に有用であると考えられた．

4.4 普及型微小循環造影装置

放射光は，大型のシンクロトロン加速器が必要

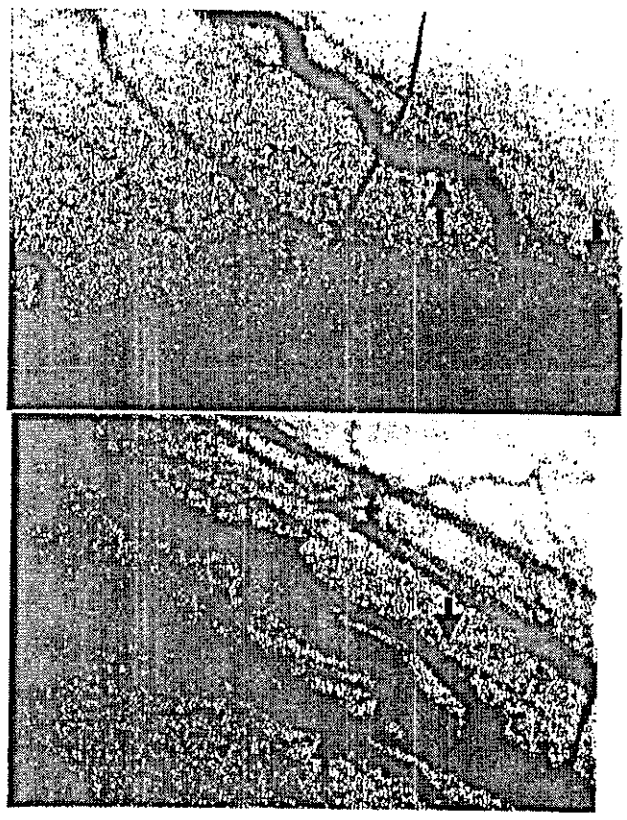

図3 放射光微小血管造影法を用いた再生血管の描出と血管機能評価
上段：Baseline，下段：アデノシン投与．ゼラチンハイドロゲルFibroblast Growth Factor遺伝子複合体投与による再生血管（蛇行した100 μm以下の小血管）が描出されている．アデノシン投与により血管の拡張がみられ（矢印），描出される血管数も増えている．

であり，一般の臨床医療用として使用することは困難である．したがって，血管再生療法の臨床評価においては，小型で医療用X線源を利用した装置が望まれる．新エネルギー産業技術総合開発機構（NEDO）の支援を受けて，次世代単色X線診断・治療システム開発グループは，このような観点から，臨床で使用可能な微小循環のX線診断装置を開発している．X線源として高出力の医療用X線源装置を用い，X線フィルターにより高域と低域のエネルギーをカットして，擬似的に単色線（ヨードのK吸収域）を得る．さらに，X線コリメータにより疑似単色線を平行化して照射する．これにより，コントラスト効果の高い微小循環造影画像が得られる．また，超高感度ハイビジョンカメラシステムを搭載することで，高分解能の撮像が可能となる．この診断装置の開発により，血管再生療法の臨床応用における評価系の確立が期待される．

5. 血管再生療法の新たな展開

今後，血管再生療法の関心は，再生された血管の数から質へと展開していくと考えられる．すなわち，より成熟した再生血管の形成のために，種々の試みがなされるであろう[10]．

5.1 上流の血管内皮増殖因子の選択

VEGFは血管内皮細胞に直接作用し，多くの血管内皮増殖因子の中で下流に位置する因子である．一方，他の血管内皮増殖因子は，VEGFの増加作用により，またVEGFを介して二次的に血管内皮細胞への作用を有するものが多く，他の血管構築細胞への影響を有するものも多い．虚血による生理的な血管再生では，低酸素により活性化するHIF-1α等の転写因子や上流の血管内皮増殖因子がまず活性化し，引き続いてVEGFを始めとする下流の血管内皮増殖因子が発現する．血管再生は下流の血管内皮増殖因子によって可能であるが，より生理的な血管再生を考慮すれば，HIF-1αなどの転写因子やFGFなどのより上流の血管内皮増殖因子が望まれる．

5.2 複数の血管内皮増殖因子の投与

VEGFの作用は，血管内皮細胞に比較的限定している．そのため血管平滑筋に覆われた再生血管の形成には，さらにPDGFやAngiopoietin 1などの平滑筋細胞に作用するサイトカインとの併用療法が望まれる．したがって，成熟した再生血管の形成のためには，上記のような作用部位の異なる複数のサイトカイン投与が，効果的と予想される．

5.3 効率的で持続性を有する安全な遺伝子導入法

VEGFの単独投与でも，一定期間，適切な発現濃度が保たれれば，2次的にAngiopoietinやPDGFなどのサイトカインが上昇し，成熟した再生血管が期待できる．これには，遺伝子導入法が重要な鍵となる．ウイルスベクターを用いた方法は，持続的な血管内皮増殖因子の発現が可能であるが，その持続性が逆に，血管腫や悪性腫瘍の発生につながる可能性も否定できない．また，ウイルス感染に伴う副作用も大きい．一方，naked DNAによる遺伝子導入は，安全性は高いものの，DNA分解酵素により代謝され，導入効率が低く，持続性も期待できない．著者らが開発したGHG-DNA複合体は，徐放性により効果の持続が期待できる．マクロファージ等に貪食されて，細胞内で遺伝子を発現することも，遺伝子治療の効果を高めるのに役立っていると考えられる．成熟した再生血管の形成に効果的な遺伝子導入法の一つとして期待される．

5.4 血管内皮前駆細胞移植と血管内皮増殖因子との併用療法

Vasculogenesisを主体とする治療が血管内皮前駆細胞移植であり，狭義のAngiogenesisを主体とする治療が血管内皮増殖因子を用いた遺伝子治療である．血管内皮前駆細胞移植では，採取できる細胞数に限りがあり，その機能が血管再生に十分なものであるかどうかが問題となっている．その意味から，血管内皮前駆細胞移植に血管内皮増殖因子の遺伝子治療を併用すれば，上記の問題点を克服する可能性が考えられる．また，血管内

皮前駆細胞を投与する際，あらかじめex vivoで血管内皮増殖因子の遺伝子導入を行い，機能強化した後に生体に投与すれば，限られた血管内皮前駆細胞で効果的な血管再生療法となることも考えられる．GHGは血管内皮前駆細胞に貪食される性質を有しているため，血管内皮増殖因子の遺伝子導入による血管内皮前駆細胞の機能強化法にも有用と思われる．血管内皮前駆細胞移植と血管内皮増殖因子の遺伝子治療の併用療法は，今後，期待される血管再生療法の一つである．

6. おわりに

血管再生療法は，虚血性循環器疾患の次世代の治療として期待されており，基礎及び臨床で多数の研究が行われている．しかしながら，未だ評価法が定まっていない中で，血管再生療法の効果を判断することは困難である．実際，多くの血管再生療法が報告されているが，その効果については，今の所，明らかな結論は得られていない．微小循環造影法は，血管再生療法の評価法として大きな役割をはたすものと期待される．放射光微小循環造影法に続く臨床普及型の微小循環造影法の開発は，安全で効果的な血管再生療法の確立に貢献できる．

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生分解性ゼラチンを用いた遺伝子治療法

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遺伝子治療において，安全かつ高効率な遺伝子導入法の開発は重要な課題である．そこでわれわれは遺伝子を格子構造の生分解性ゼラチンに結合させて生体へ導入する方法を開発した．ゼラチンと結合した遺伝子は核酸分解酵素等の影響を受けにくく生体内での残存期間が延長し，血管新生療法においては遺伝子の単独投与時と比べて新生血管の機能的な成熟を促した．また細胞移植による遺伝子治療においてもゼラチン-遺伝子複合体を用いて機能を強化または補間した細胞を移植し，良好な治療効果を得つつある．次世代遺伝子治療法の開発への足がかりとなることを期待する．

Key Words
非ウィルス性ベクター，生分解性ゼラチン，遺伝子治療，
血管新生：angiogenesis，血管形成：vasculogenesis，放射光微小循環造影，
VEGF: vascular endothelial growth factor（血管内皮成長因子），
FGF: fibroblast growth factor（線維芽細胞成長因子），血管内皮前駆細胞，マクロファージ．

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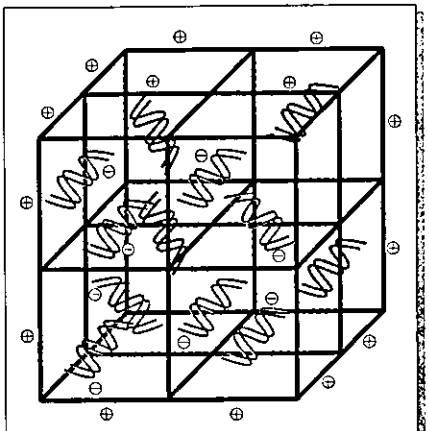
はじめに

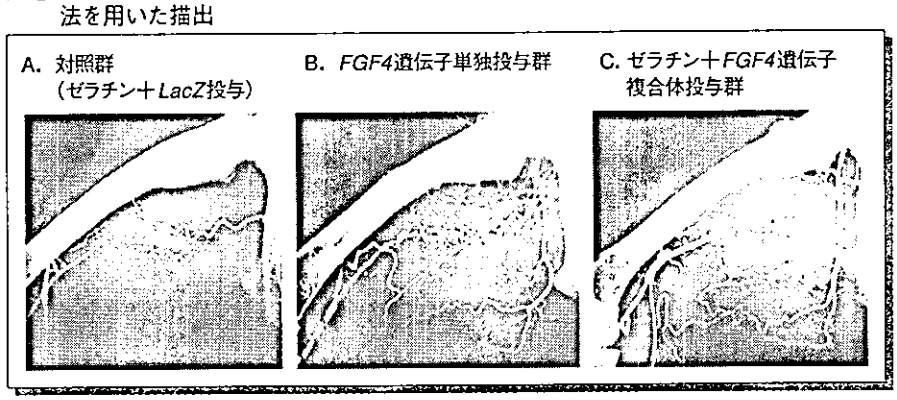
遺伝子治療は本来，ある特定の遺伝子の欠損または変異に起因する疾患に対して必要な遺伝子を補充する治療法である．ところが近年の遺伝子治療に関する技術の進展は目覚ましく，遺伝子の欠損または変異の有無に関わらず，生体の治癒力を補う目的で遺伝子治療が行われ始めている．なかでも虚血性疾患における血管新生療法に対する遺伝子治療の有効性が期待されており，実際に重度の末梢動脈閉塞性疾患に対してVEGF（vascular

図❶ 生分解性ゼラチンの格子構造に結合する遺伝子の模式図



生分解性ゼラチンの表面電荷を陽性とし格子状にすることで，陰性荷電した遺伝子とイオン結合させた．

図❷ 下肢虚血モデル家兎の遺伝子治療後38日目における新生血管の放射光微小循環造影法を用いた描出



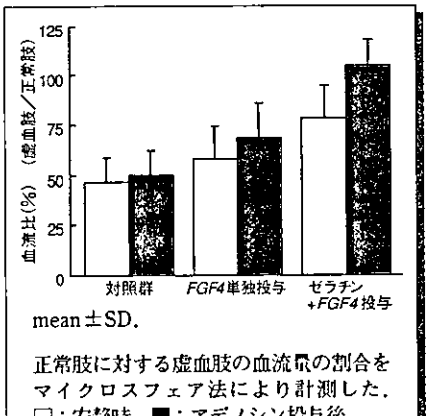
endothelial growth factor）のプラスミドを動脈内投与することにより，著明な治療効果が報告されている[1]．このように遺伝子治療は21世紀においてその臨床応用に大きな期待が寄せられている治療法の一つである．

現在行われている遺伝子導入法は，ウィルスベクターを用いる方法（トランスダクション）と，物理学的または化学的な導入法（トランスフェクション）の二つに大別される．トランスダクション法は，遺伝子導入効率はよいもののウィルスによる感染の問題が指摘されている[2)3)]．一方，トランスフェクション法では導入したい遺伝子を標的組織へ直接または経血管的に投与する手法が取られてきた．この方法はウィルスベクターを用いないので感染の危険性は低いものの，投与したプラスミドDNAが細胞へ導入される前に生体内に存在する様々な核酸分解酵素によって分解され，または組織内へ拡散してしまう可能性が指摘されている．従って有効な治療効果を得るには大量の遺伝子が必要となり，非常に効率が悪い[4)5)]．従って導入したい遺伝子を標的の細胞へいかに効率よくかつ安全に導入するかが最重要課題となっている．そこでわれわれは，投与した遺伝子をできるだけ長く生体内に残存させる手段として生分解性ゼラチンを用いた新たな遺伝子導入法を考案し，血管新生に対するその有用性を報告した[6]．本稿では，生分解性ゼラチンを用いた新しい遺伝子導入方法に関するわれわれの最近の知見を中心に，次世代の遺伝子治療の可能性について概説する．

Ⅰ．ゼラチン-遺伝子複合体による遺伝子発現強化

ゼラチンはその構造や表面電荷を変えることが容易であることから[7]，われわれはゼラチンを遺伝子の担体として利用することを着想した．そこでゼラチンの構造を格子状にしてその表面電荷を陽性とすることで陰性荷電した遺伝子とイオン結合させ，ゼラチン-遺伝子複合体を作成した（図❶）．遺伝子をあらかじめゼラチンの格子構造内へ封入して生体内へ投与することで核酸分解酵素による遺伝子の代謝が緩徐となり，結果として安全かつ高効率に遺伝子を導入することができると考えられる．本法は安全性の問題が指摘されているウィルスベクターを用いずに済むということに加えて，ゼラチン自体も生体内でプロテアーゼにより分解されるという点で優れた方法であると考えられる．実際に遺伝子をゼラチンと結合させて投与したところ，生体内における遺伝子の残存期間を飛躍的に延長させることに成功し，遺伝子の発現率も従来の遺伝子の単独投与と比較して約10倍の増加が認められた[6]．そこでわれわれは，家兎の下肢虚血モデルを用いてこのゼラチン-遺伝子複合体の治療効果を調べた．大腿動脈摘除後10日目に血管新生因子である*pFGF4*や*pVEGF165*を虚血部位へ筋肉内投与したところ，動脈摘除後38日目において遺伝子非投与群（図❷A）と比較して通常の血管造影上有意な血管新生（図❷C）と，これに伴う血流量の有意な増加が観察された（図

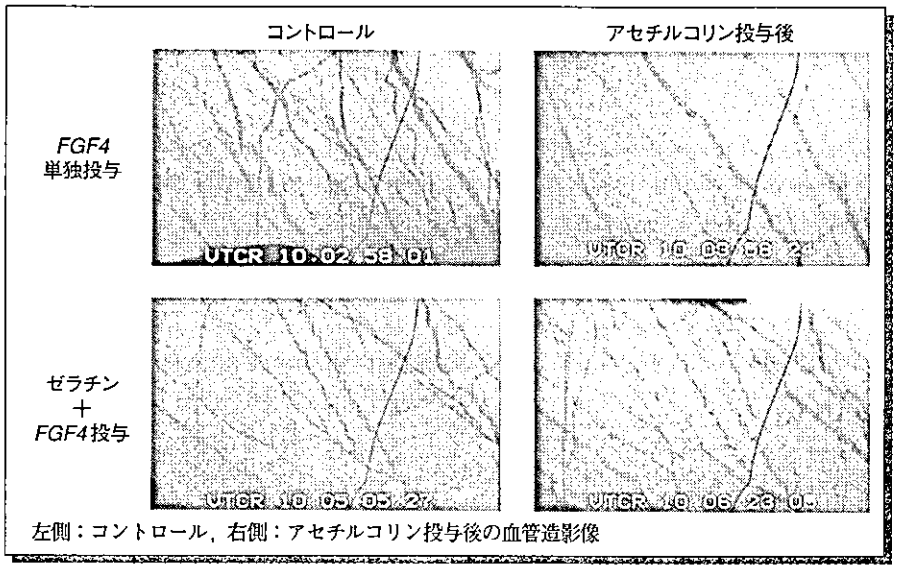
図❸ 虚血モデル家兎の下肢筋血流量に対する各種遺伝子導入法の治療効果



mean±SD.

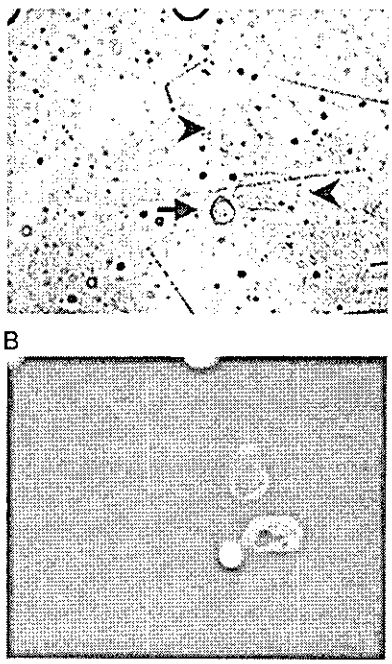
正常肢に対する虚血肢の血流量の割合をマイクロスフェア法により計測した．
□：安静時，■：アデノシン投与後

図❹ アセチルコリンに対する新生血管の血管拡張能の放射光微小循環造影法による評価



左側：コントロール，右側：アセチルコリン投与後の血管造影像

図❺ 生分解性ゼラチンを用いてGFP遺伝子を導入したマクロファージ



A：*GFP*遺伝子を結合させたゼラチンとマクロファージの光学顕微鏡画像（矢印頭ーマクロファージ；矢印ーゼラチン）．
B：*GFP*遺伝子を発現したマクロファージの蛍光顕微鏡画像．

❸）．遺伝子単独投与群（図❷B）とゼラチン-遺伝子複合体投与群の比較においては既存の血管造影法による差異は認められなかったものの，放射光による微小循環造影法とマイクロスフェアー法を用いた血流計測法で両群の新生血管の血管拡張物質に対する反応性に有意な差異が確認された（図❸）（図❹）．遺伝子非投与群および遺伝子単独投与群ではアデノシンあるいはアセチルコリンの投与による血管陰影（図❸）および血流量（図❹）の増加は有意ではなかったが，ゼラチン-遺伝子複合体投与群ではアデノシンまたはアセチルコリン投与に伴う血管陰影（図❸）および血流量（図❹）の明らかな増大が観察された．この事実は，ゼラチン-遺伝子複合体による治療群では血流制御能を伴う血管床の再生が実現されていることを示唆している．遺伝子単独投与群とゼラチン-遺伝子複合体投与群を比較すると安静時血流量に有意な差異は認められなかった．このことから，遺伝子導入後の安静時筋血流量の増加は新生血管の血流制御能よりもむしろ新生血管の数を反映していると考えられ，一方アデノシンやアセチルコリンといった血管拡張物質による増加はおそらく新生血管の機能的成熟度を示しているのであろう．以上の結果から，ゼラチン-遺伝子複合体の投与による遺伝子治療は虚血部位における機能的な血管新生に対して有効な治療法となり得る可能性が示された．さらに導入する遺伝子を変えることで本法は悪性疾患の治療などにも応用できるものと考えられる．

Ⅱ. 遺伝子導入した貪食細胞を用いた血管新生療法

遺伝子治療において残された問題は遺伝子の投与方法である．上述の方法を含め，これまで行われてきた遺伝子治療による血管新生療法では目的の遺伝子を標的組織へ直接投与する必要があり，例えば虚血心筋への遺伝子導入の場合には開胸または心臓カテーテルといった外科的手術による侵襲を伴う．これに対し最近の血管新生療法においては，血管内皮細胞やその前駆細胞を経血管的に移植する方法が試みられており，細胞移植による血管新生療法の可能性が示唆されている[8]．この治療法は自己の細胞を用いるため感染の危険性が低いこと，並びに用いる細胞の遊走能を利用することで傷害部位特異的な治療が行えるという利点を有する．しかしながら現在の細胞移植法の場合，十分な治療効果を得るためには大量の細胞を必要とするため，効率という点では大きな課題を残している．そこでわれわれは生分解性ゼラチンによる遺伝子導入法と細胞移植の長所を組み合わせたハイブリッドな遺伝子治療法の開発を試みた．マクロファージや単球は貪食能によりゼラチンを自身の体内へ取り込むことが報告されている[9]．しかもこれらの細胞は走化性により虚血部位や癌組織に集まることから，患部への遺伝子の担体としてふさわしいと考えた．そこで貪食