Synthetic Vascular Prosthesis Impregnated With Mesenchymal Stem Cells Overexpressing Endothelial Nitric Oxide Synthase

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Background—Endothelial dysfunction is known to exaggerate coronary artery disease, sometimes leading to irreversible myocardial damage. In such cases, repetitive coronary revascularization including coronary artery bypass grafting is needed, which may cause a shortage of graft conduits. On the other hand, endothelial nitric oxide synthase (eNOS) is an attractive target of cardiovascular gene therapy. The vascular prostheses, of which the inner surfaces are covered with mesenchymal stem cells (MSCs) overexpressing eNOS, are expected to offer feasible effects of NO and angiogenic effects of MSCs on the native coronary arterial beds, as well as improvement of self-patency. Herein, we attempted to develop small caliber vascular prostheses generating the bioactive proteins. Also, we attempted to transduce eNOS cDNA into MSCs.

Methods and Results—The MSCs were isolated from rat bone marrow and transduced with each adenovirus harboring rat eNOS cDNA and β-galactosidase (β-gal) (eNOS/MSCs and β-gal/MSCs). The β-gal/MSCs were impregnated into vascular prostheses, then the expressions of β-gal on the inner surfaces of them were evaluated by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining. The NOS activity of eNOS/MSCs was assayed by monitoring the conversion of 3H-arginine to 3H-citrulline. The inner surfaces of the vascular prostheses were covered with MSCs expressing β-gal. The amount of the 3H-citrulline increased, and eNOS/MSCs were determined to generate enzymatic activity of eNOS. This activity was completely inhibited by Nω-nitro-L-arginine methyl ester.

Conclusions—The inner surface of expanded polytetrafluoroethylene vascular prostheses seeded with lacZ gene-transduced MSCs exhibited recombinant proteins. Development of eNOS/MSC-seeded vascular prostheses would promise much longer graft patency and vasculoprotective effects.

Key Words: mesenchymal stem cells • endothelial nitric oxide synthase • gene-transduction • adenovirus • small caliber expanded polytetrafluoroethylene (ePTFE) vascular prosthesis

Impaired endothelial function induces several cardiovasculardiseases, including atherosclerosis, hypertension, heart failure, arteriosclerosis obliterans, and coronary heart disease. Endothelial dysfunction also causes NO insufficiency, resulting in the limitation of NO-mediated signal transduction and excretion of bioactive hormone-like products. The attenuated production of NO and the hormone-like products exaggerates these cardiovascular diseases further. Coronary artery disease is one of the most critical diseases enhanced by endothelial dysfunction, sometimes leading to irreversible myocardial damage. Some patients require repetitive coronary revascularization, including coronary artery bypass grafting, which may lead to a shortage of graft materials.

Small caliber vascular prostheses, which would be suitable for graft conduits for coronary artery bypass grafting or arteriosclerosis obliterans below the knee, have an extremely high failure rate that is attributed to thrombus formation and occlusion. Many attempts have been made to increase the patency of small-caliber vascular prostheses over the years. Recently, vascular grafts releasing NO infer possibilities preventing thrombosis and stenosis. However, they are far from practical use, because the material requires more improvement.

On the other hand, impregnation of autologous bone marrow cells (BMCs) into the vascular prostheses differentiates into endothelium exerting autocrine functioning of basic fibroblast growth factor. Among BMCs, mesenchymal stem or stromal cells (MSCs) have feasible capacities of differentiating into the vascular endothelium and exerting the paracrine mechanism with a broad spectrum of arteriogenic cytokines. They are also considered to be potent vehicles of gene transduction for drug targeting or delivery systems.
speculate that small caliber vascular prostheses, which are impregnated with eNOS gene-transduced MSCs, might prevent thrombosis and stenosis on the anastomoses sites and attenuate atherosclerotic changes of the distal arteries by NO and the arteriogenic cytokines.

In this study, therefore, we attempted to develop small caliber expanded polytetrafluoroethylene (ePTFE) vascular prostheses, of which inner surfaces are covered with eNOS gene–transduced MSCs. This hybrid vascular prosthesis is expected to provide a therapeutic advantage by extended production of NO from the inner surfaces.

Methods

Expansion of Rat MSCs
Male Sprague–Dawley rats weighing 160 to 180 g (6 weeks old, Japan SL C) were used in this study. All of the animals were maintained in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Preparation of MSCs was described previously.7–9 Briefly, BMCs were isolated by flushing the cavity of femurs and tibias and transferred to a humidified CO2. All of the culture experiments in this study were performed at 37°C and in 5% CO2. The isolated BMCs were then cultured in minimal essential medium supplemented with 10% FBS (BD Biosciences Clontech), 100 μg/mL of streptomycin, and 100 U/mL of penicillin (Sigma). When adherent cells were confluent (defined as passage 0), they were continuously cultured as MSCs until passage 3 to 5 using 0.25% trypsin and 1 mmol/L EDTA (Sigma) for 5 minutes. All of the MSCs were harvested using the same method of harvesting as that of lacZ/Adeno-X.

Construction of MSCs With LacZ/Adeno-X Viruses
Replication-incompetent Adeno-X vectors (BD Biosciences Clontech) were used according to the supplier’s instructions. LacZ/Adeno-X vectors, harboring β-galactosidase (β-gal) gene, were packaged into infectious adenoviruses by transduction HEK293 cells. LacZ/Adeno-X virus particles were purified and concentrated with Virakit (Virapure). The titer of lacZ/Adeno-X was determined by plaque assay and was packaged into infectious adenoviruses by transduction HEK293 cells. LacZ/Adeno-X virus particles were purified and concentrated with Virakit (Virapure). The titer of lacZ/Adeno-X was determined by plaque assay and was packaged into infectious adenoviruses by transduction HEK293 cells. LacZ/Adeno-X virus particles were purified and concentrated with Virakit (Virapure).

Construction of MSCs With eNOS/Adeno-X Tet-Off Viruses
Polymerase chain reaction (PCR) was performed using oligonucleotides 5'-tctgaagtctggtggacactg-3' (XbaI sense primer) and 5'-cctttgatgtcctggaac-3' (AflI antisense primer) with rat heart cDNA as a template to amplify a fragment consisting of the full-length eNOS cDNA flanked by the XbaI and AflI sites. The PCR product was cloned into the XbaI/AflI sites of pShuttle (BD Biosciences Clontech) and sequenced by the dideoxynucleotide chain termination method using the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences). The full-length eNOS cDNA was inserted into the multicloning site of pDNR-CMV in Adeno-X Tet-Off Expression System 2 (BD Biosciences Clontech) to construct eNOS/pDNR-CMV. The eNOS/Adeno-X Tet-Off vector was then generated from the eNOS/pDNR-CMV and pLP-Adeno-X-TRE (BD Biosciences Clontech) by the Cre-loxP site-specific recombination method and was packaged into infectious adenoviruses by transduction of HEK293 cells. The eNOS/Adeno-X Tet-Off virus particles were purified and concentrated with Virakit. The titer of the eNOS/Adeno-X Tet-Off virus was determined by cytopathic changes in infection of HEK293 cells by the serial dilution method. The cultured MSCs were exposed to the eNOS/Adeno-X Tet-Off viruses and the regulatory viruses, which had a tetracycline-controlled activator, at MOI of 2000 in 2 mL of the fresh culture medium containing 2% FBS for 1 hour. Then, the MSCs that had been exposed to the eNOS/Adeno-X Tet-Off viruses were cultured for 48 hours in 5 mL of the complete culture medium. The MSCs were harvested using the same method of harvesting as that of lacZ/Adeno-X.

Impregnation of Gene-Transduced MSCs into Vascular Prostheses
Small-caliber ePTFE vascular prostheses with 2-mm internal diameter, 4-cm length, and 90-μm fibril length were provided from W.L. Gore and Associates, Inc. The ePTFE vascular prostheses used in this study were flexible and, therefore, never snapped, even when looped. An 18-gauge cannula with a 14-gauge diameter (Terumo) was inserted after clamping the other side of the ePTFE graft in the culture dish. The MSCs infected with lacZ/Adeno-X viruses were impregnated into the ePTFE using a syringe via the cannula. The ePTFEs were soaked in the culture medium and incubated for 48 hours. They were fixed with 0.25% glutaraldehyde and incubated in the X-Gal staining solution for 3 hours as described previously.10 Expressions of β-gal were evaluated by the light microscopy.

Measurements of eNOS Activity
NO synthase (NOS) activity was measured from the conversion rate of L-[^3H]arginine to L-[^3H] citrulline in homogenates of MSCs. The MSCs were detached from tissue culture dishes using rubber scrapers, and the cell suspension in PBS was centrifuged at 15 000 rpm at 4°C for 5 minutes. The pellet was suspended in homogenization buffer containing 25 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, and 1 mmol/L EGTA. The reaction mixture contained 10 mmol/L reduced nicotinamide adenine dinucleotide phosphate, 0.6 mmol/L CaCl2, 1 mmol/L flavin adenine dinucleotide, 1 μmol/L flavin mononucleotide, 3 μmol/L tetrahydrobiopterin, 0.1 μmol/L calmodulin, 25 mmol/L Tris-HCl (pH 7.4), and L-[^3H]arginine. The reaction was initiated by the addition of the homogenates and carried out for 8 minutes at 37°C. The reaction was quenched with 0.4 mL of ice-cold stop buffer containing 50 mmol/L HEPES (pH 5.5) and 5 mmol/L EDTA. Experiments were also performed in the presence of N^-nitro-L-arginine methyl ester, hydrochloride (L-NAME) 1 mmol/L as an inhibitor of NOS. The amount of L-[^3H]citrulline content was determined by liquid scintillation counting after separation from the reaction mixture by passage through a column of the cation exchange resin Dowex 50WX-8 (100 μL of Na+ form). As the negative control, MSCs were exposed to the eNOS/Adeno-X Tet-Off viruses and the regulatory viruses in tetracycline-containing medium, resulting in the inhibition of eNOS gene transduction. We had full access to the data and take responsibility for its integrity. We have read and agree to the article as written.

Results

NOS Activity of eNOS Gene-Transduced MSCs
Most of the gene-transduced MSCs were spindle-like in shape and adhered to the plastic culture dishes. They were
stretched into monolayer structures. The transduction efficiency was ≈90% at MOI of 2000, which was determined with lacZ gene-transduction and X-Gal staining.

The titer of the reconstructed eNOS/Adeno-X Tet-Off was roughly determined to be $5.5 \times 10^{10}$ infection-forming units/mL, which was determined with the cytopathic morphological change of HEK 293 cells by the serial dilution method.

The NOS activity of eNOS gene-transduced MSCs, which is measured by the converted amount of L-[3H]arginine, was observed to increase in proportion to time at 37°C for 8 minutes (Figure 1). This activity was thoroughly inhibited by the addition of L-NAME (Figure 2). For the negative control, the addition of tetracycline completely quenched the gene transduction of eNOS in MSCs (Figure 2).

**Impregnated ePTFE With Transduced MSCs**

The ePTFE impregnated and infiltrated with the gene-transduced MSCs were longitudinally incised, and the luminal surface was covered with X-Gal-positive blue cells (Figure 3a). In the microscopic observation, X-Gal-positive MSCs were distributed in 2 forms; one form included large cells stretching over the luminal surface and colonized, and another form included small cells scattering over the luminal surface (Figure 3b and 3c).

**Discussion**

One of major findings in the current study is that the inner surface of ePTFE vascular prostheses was seeded with lacZ gene–transduced MSCs exhibited recombinant proteins. The ePTFE grafts are made of an expanded polymer of PTFE that is a porous material with a characteristic structure having solid nodes interconnected by fine fibrils. To seed BMCs in ePTFE vascular prostheses stably, high porosity (60 to 90 μm and more; fibril length) is thought to be advantageous for endothelialization in vivo, because the anchoring of seeded cells and the capillary ingrowth from perigraft tissue are likely to occur.\(^{11-13}\) Hence, in this study, we chose high-porosity ePTFE (90 μm; fibril length) as a scaffold and were able to succeed in expressing recombinant proteins. This result may be consistent with the work of Noishiki et al\(^4\) demonstrating that BMC-seeded high-porosity vascular prosthesis exhibited autocrine function of angiogenic factor.

It has been suggested that MSC separation and amplification in vivo can be easily performed. MSCs are also known to be rapidly amplified and tough enough to survive even under the low-serum conditions.\(^9\) Those natures of MSCs may promise a potential use as a vehicle of gene therapy. In this study, therefore, we used MSCs as a cell source. In fact, lacZ gene-transduced MSCs seeded on the inner surface of ePTFE vascular prostheses showed X-Gal-positive responses.

These results raise a hypothesis that this model of biohybrid vascular prosthesis may hold therapeutic ability in addition to the role of graft conduits. In the clinical sphere, many of patients, who have undergone replacement of vascular prostheses, have systemic vascular disorders. If vascular prostheses have a function of secreting vasculoprotective agents such as NO, it may be beneficial not only in preventing thrombosis and/or inhibiting in situ neointimal hyperplasia\(^14\) but also in treating peripheral vascular diseases.

As a next step, therefore, we attempted to investigate whether the eNOS gene can be transduced into MSCs. As shown in the results of NOS activity assays, eNOS-transduced MSCs exhibited significant NOS activity. This is another major finding of the current study. L-NAME inhibited this NOS activity. This strongly suggests that elevated NOS activity was attributed to eNOS genes that had been transduced into MSCs.

Accordingly, if eNOS-transduced MSCs are seeded into the inner surface of ePTFE vascular prostheses, therapeutic advantages of this hybrid vascular prosthesis would be dramatically extended. Because NO has vasodilatation effects, replacement of this prosthesis may increase peripheral blood flow, which eventually may lead to improvement of graft patency. Also, this prosthesis may be useful as a pulmonary arterial graft for a patient with severe pulmonary hypertension. Persistent NO release in the pulmonary artery may rescue the patients with severe hypertension.

Although results of the current study revealed that this biohybrid vascular prosthesis might provide a new insight to overcome the limitations of the small caliber vascular pro-

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**Figure 2.** The MSC/eNOS produced NOS activity, and this was inhibited by the addition of L-NAME. For the negative control, MSCs that did not undergo eNOS gene transduction produced almost the same amount of NOS activity as MSCs that did undergo eNOS gene transduction and L-NAME.

**Figure 3.** a, The luminal surface of ePTFE impregnated and infiltrated with the gene-transduced MSCs was covered with X-Gal-positive blue cells. b and c, X-Gal-positive MSCs were distributed in 2 forms: large cells stretching over the luminal surface and colonized, small cells scattering over the luminal surface. Magnification of objective lenses were (b) ×4 and (c) ×10.
theses, several problems remain to be resolved. First, this model was not subjected to blood flow in vivo. Bloodstream produces several dynamic alterations (ie, shear stress), which may cause harmful effects on stability and survival of seeded cells. Because the shear stress is known to induce expression of angiogenic factors and eNOS, seeded MSCs may also express such agents under the shear stress. Second, duration of pharmacological effects by recombinant proteins has not been elucidated. Gene expression mediated by adenovirus is known to persist for 2 weeks. It must be determined whether the duration of 2 weeks may long enough for clinical application. Furthermore, MSCs are the progenitors of multiple lineages, including bone, cartilage, muscle, fat, cardiomyocytes, and vascular endothelial cells.9 Theoretically, seeded MSCs may be expected to differentiate into endothelium. However, overexpression of eNOS may change the direction of cell differentiation. Further investigations must be needed to clarify these points.

Conclusions
We presented genetically modified MSC-seeded small caliber vascular prostheses that express recombinant proteins and succeeded in recombination of bioactive NOS on MSCs. This may lead to a development of vasculoprotective hybrid vascular prosthesis.

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Disclosures
None.

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