Ex vivo electroporation as a potent new strategy for nonviral gene transfer into autologous vein grafts

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Yamaoka, Terutoshi, Yoshikazu Yonemitsu, Kimihiro Komori, Hiromitsu Baba, Takuya Matsumoto, Yoshihiro Onohara, and Yoshihiko Maehara. Ex vivo electroporation as a potent new strategy for nonviral gene transfer into autologous vein grafts. Am J Physiol Heart Circ Physiol 289: H1865–H1872, 2005; doi:10.1152/ajpheart.00353.2005.—Gene transfer to vein grafts has therapeutic potential to prevent late graft failure; however, certain issues, including efficacy and safety, have hindered the clinical application of this treatment modality. Here, we report the successful and efficient gene transfer of plasmid DNA via ex vivo electroporation into veins as well as into vein grafts. Two approaches were used: one involved transmural in situ gene transfer using a T-shaped electrode (the “Lu” method), and the other was an adventitial ex vivo approach using an electroporation cuvette followed by vein grafting (the “Ad” method). The Lu method was carried out at 10 V, with optimal gene transfer efficiency in the in situ jugular veins of rabbits, and transgene expression was observed primarily in endothelial cells. However, when these veins were grafted into the arterial circulation, no luciferase activity was detected; this effect was probably due to the elimination of the gene-transferred cells as a result of endothelial denudation. In contrast, optimal and satisfactory gene transfer was obtained with the vein grafts subjected to the Ad method at 30 V, and transgene expression was seen primarily in adventitial fibroblasts. Gene transfer of endothelial nitric oxide synthase cDNA to the vein graft via the Ad method successfully limited the extent of intimal hyperplasia, even under hyperlipidemic conditions, at 4 wk after grafting. We thus propose that the Ad method via ex vivo electroporation may provide a novel, safe, and clinically available technique for nonviral gene transfer to sufficiently prevent late graft failure.

endothelial cell nitric oxide synthase; adventitia; endothelial cells; hyperlipidemia; plasmid

AUTOLOGOUS VEIN GRAFTS have been shown to be a suitable conduit for surgical intervention to treat subjects with peripheral arterial occlusive diseases (9). However, neointimal hyperplasia has been a major cause of late graft failure, which has limited the rate of patency after bypass surgery (3, 8).

In the last decade, it has been hoped that gene therapy would overcome this problem; several experimental and clinical studies, including ours, have reported that gene transfer into the wall of the vein graft might have the potential to prevent late graft failure (11, 13, 25, 29, 33). A number of methods and techniques for in vivo gene transfer into the vasculature are available, including viral vector (6, 22, 27, 33, 35, 37, 41, 43), lipofection (32), viral envelope-coated liposomes (45), and naked DNA delivery with or without hydrogels (24, 38). Viral vectors are the most widely accepted gene transfer method for cardiovascular tissues in vivo, inasmuch as the efficiency of gene transfer is usually sufficient (13, 35). However, this type of vector is associated with safety issues related to immunologic and/or cytotoxic problems that remain to be resolved; these issues tend to limit the clinical application of these vectors (6, 23, 30, 42). Nonviral methods are alternatives for in vivo gene transfer into the vasculature; although such systems are considered to be safe, the efficiency of gene transfer by nonviral approaches does not tend to be sufficiently high.

Electroporation-mediated gene transfer has been shown to markedly enhance the efficiency of naked DNA transfer, and this method has been widely used to introduce genes into various types of cells in vitro (2, 5, 30). Moreover, the in vivo application of electronic pulse-mediated gene transfer, with the use of specially designed electrodes, has been demonstrated to be effective in murine skeletal muscle (1), chick embryos (31), rat liver (16), myocardium (15), and solid tumors (14, 44). Recently, we and others demonstrated that electroporation could lead to the enhanced transfer of plasmid DNA into the arterial wall in the rabbit carotid artery as well as the rat mesenteric artery (26, 28). Electroporation has a number of advantages over other methods (e.g., application of viral vectors): 1) Electroporation is theoretically applicable for use in a variety of cells and organs. 2) This method is easy to apply and simple to use, and the technique is rapidly facilitated. 3) No advance preparations are required. Therefore, electroporation-mediated gene transfer has potential for use as a novel technique to treat vascular conduits in human subjects.

Because little information is available regarding gene transfer into veins and vein grafts by electroporation, we investigated two different electric-pulse methods of plasmid DNA gene transfer: endothelial and adventitial delivery into the rabbit jugular vein. The aims of present study were as follows: 1) to determine whether electroporation is applicable for gene transfer into veins and vein grafts, 2) to determine the appropriate technique for optimal gene transfer, 3) to determine the target cell species via electric pulse-mediated gene transfer, and 4) to obtain preclinical evidence of the efficacy of this technique using plasmid DNA-expressing endothelial cell nitric oxide (NO) synthase (ecNOS), which has been shown to be efficient at restoring vasomotor function as well as limiting neointimal hyperplasia in vein grafts (29, 35, 43).

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MATERIALS AND METHODS

Plasmid Construction

The full coding region of the firefly luciferase gene was isolated from the pGL2 promoter vector (Promega, Madison, WI) by Hind III and Bam H I digestion. pcLuc was constructed by subcloning this luciferase gene into a multicloning site of pcDNA 3 (InVitrogen). A nuclear-targeted lacZ gene driven with the chicken β-actin promoter pAct-NLS-LacZ was prepared as previously described (28, 29). The plasmid pUCCAGGS-ecNOS, expressing full-length bovine ecNOS cDNA (3.7 kb; kindly provided by T. Michael, Harvard Medical School) driven by the CAG promoter [human cytomegalovirus immediate early enhance/chicken, β-actin promoter, and rabbit β-globin poly (A)], was prepared as previously described (29, 33). All the plasmids were purified by using Mega kits (Qiagen, Hilden, Germany).

Animals

A total of 95 male Japanese white rabbits (2.5–3.0 kg body wt; Kyudo, Tosu, Saga, Japan) were used in the study. The following procedures were performed by using sterile techniques and with the animals deeply anesthetized. Ketamine hydrochloride (25 mg/kg) and xylazine (10 mg/kg) were administered intramuscularly, and pentobarbital sodium (30 mg/kg) was given intravenously to maintain anesthesia. All animal experiments were carried out according to the approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals suggested by the Committees for Animal Experimentation and Recombinant DNA at Kyushu University. The studies conformed to Law No. 105 and Notification No. 6 of the Government of Japan, as well as the “Principles of Laboratory Animal Care” and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 80-23, Revised 1985).

Conditions of Electroporation

Electric pulses were delivered by using an electric pulse generator (model CUY 201 BTX) (45). The electric pulse was regulated as follows: pulse-on time (P on) = 5 ms, interval time (P off) = 95 ms, and number of pulses = 10; these optimized parameters were determined in our previous study using rabbit carotid arteries (28). The concentrations of the plasmid DNA solution used for transfection were adjusted to 200 μg/ml using balanced salt saline (in mM: 140 NaCl, 5.4 KCl, and 10 HCl, pH 7.6).

Experiment 1: Optimization and Characterization of the Gene Transfer Technique

The rabbits (n = 71) used in this series were fed a normal-cholesterol diet.

Gene transfer into in situ veins (“Lu” method). Electric pulses were delivered to the rabbit jugular vein in situ with T-shaped electrodes consisting of a pair of flat stainless steel plates (2.5 cm long, 0.7 cm wide, 2-mm fixed distance between plates; Fig. 1A). The surgical procedures were as follows. The common, internal and external jugular veins were exposed, and small branches were ligated and cut. After the distal sites of the common and internal jugular veins were clamped with clips, an 18-gauge double-lumen plastic catheter (Arrow International, Reading, PA) was inserted into the internal jugular vein through the external jugular vein. The luminal space of the isolated venous segment was rinsed with 5 ml of physiological saline containing heparin (5 U/ml) to wash out residual blood, and then the luminal space was filled with 0.5 ml of plasmid DNA solution. The internal jugular vein, which had been isolated and filled with DNA solution, was sandwiched between the electrodes, and electric pulses were delivered to the vein wall. After electroporation and flushing of the vector solution with 20 ml of physiological saline containing heparin (5 U/ml), two procedures were performed: one involved declamping the clips to restore venous circulation (for luciferase gene transfer: total n = 30; n = 5 each at 0, 5, 10, 20, 30, and 50 V, respectively; for lacZ gene transfer: n = 3), and the other involved excising the internal jugular vein for reversed vein graft and implantation into the arterial circulation of the ipsilateral common carotid artery (n = 5 for luciferase gene transfer), described as follows (33).

Ex vivo gene transfer into veins (“Ad” method). A total of 33 rabbits (for luciferase gene transfer: total n = 30, n = 5 each at 0, 5, 10, 20, 30, and 50 V, respectively; for lacZ gene transfer: n = 3) were used in the experiment. The internal jugular vein was excised and...
washed with physiological saline containing heparin (5 U/ml). The excised vein was placed in an electroporation cuvette equipped with two aluminum electrodes, and a 4-mm gap was filled with 0.3 ml of plasmid DNA solution (Fig. 1B). Electric pulses were delivered to the solution containing the vein graft, which was then implanted into the common carotid artery, as described below.

Reversed Vein Graft Implantation

A longitudinal incision was made in the left neck, and the left internal jugular vein and left common carotid artery were exposed. After the left internal jugular vein had been transfected by electroporation and excised to obtain an autologous vein graft, it was kept moistened in heparinized (5 U/ml) saline solution on ice. Heparin (1,000 U) was administered intravenously. The carotid artery was clamped at the proximal and distal ends. A proximal longitudinal arteriotomy was performed, and the vein graft was reversed and anastomosed to the artery with continuous end-to-side 8-0 polypropylene monofilament sutures under a surgical microscope. Distal anastomosis was performed in a similar manner. The left common carotid artery was ligated and divided between the anastomoses with 5-0 silk; then the wound was closed in layers.

Luciferase Assay

The luciferase activity of the rabbit jugular vein (or vein graft) was measured by a procedure described elsewhere (39). Briefly, 2 days after transfection, the jugular vein or vein graft was removed, minced, and homogenized in 500 μl of 1× cell culture lysis reagent (Promega), and 20 μl of the supernatant were examined for luciferase activity using the Promega luciferase assay system and a photoluminometer (model LB 9507, Lumat, Berthold, Germany). The total protein content for each tissue type was then determined by using a DC protein assay system (Bio-Rad Laboratories, Hercules, CA), and absorbance was counted at 750 nm using an ultraviolet-visible recording spectrophotometer. In each case, luciferase activity was expressed as relative light units (RLU) per milligram of protein.

X-Gal Staining

The internal jugular vein or vein graft was dissected gently and carefully and cannulated with a 20-gauge plastic catheter. Each sample was rinsed with normal saline for 10 min and perfused with 2% formaldehyde-0.25% glutaraldehyde (vol/vol) for 10 min at 100-mmHg positive pressure; then additional fixation was then applied for 10 min. Each sample was incubated in X-Gal staining solution [0.2% (vol/vol) 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 1 mM/l MgCl2, 150 mM/l NaCl, 3.3 mM/l K2Fe(CN)6, 3.3 mM/l K3Fe(CN)6, 60 mM/l NaH2PO4, and 40 mM/l NaH2PO4] for 6 h at room temperature. The X-Gal-stained samples were then placed in formaldehyde-glutaraldehyde fixative and embedded in paraffin. The sections (5 μm thick) were counterstained with nuclear fast red (29).

von Willebrand Factor Immunohistochemistry

Five vein grafts from five different animals treated by electrogene transfer of the luciferase gene according to the Lu method were used in the study, as were five untreated normal ipsilateral control veins from five different untreated animals fed normal chow. Rings of the veins or vein grafts (2 days after grafting) were fixed in neutralized 10% formaldehyde, embedded in paraffin, sectioned at 5-μm intervals, and deparaffinized. Sheep anti-human von Willebrand factor IgG antibody (Histofine kit, Nichirei, Tokyo, Japan) was used as the primary antibody. After the reaction was blocked with 3% skim milk for 30 min and the samples were incubated with primary antibody for 1 h, the cells were washed with 0.01 mol/l phosphate buffer and then reacted with biotinylated rabbit anti-mouse IgG + IgA + IgM antibody (Histofine kit) for 15 min at room temperature. The reaction products were developed in 3,3-diaminobenzidine tetrahydrochloride containing 0.1% H2O2. As negative controls, non-immunized mouse IgG, instead of primary antibody, and luciferase plasmid-transferred vein grafts were used.

Assessment of Intimal Hyperplasia

Eighteen rabbits were used in this study: six for ecNOS gene transfer, six for empty vector gene transfer, and six for sham operation. At 4 wk after graft implantation, the vein grafts were excised and perfusion fixed with neutralized 10% formaldehyde at 100 mmHg for 20 min. The midportion of each vein graft was sliced into four segments at 5-mm intervals, embedded in paraffin, sectioned to 5 μm, and stained with hematoxylin-eosin or Elastica von Gieson. Intimal thickness (defined according to the 8-points method) and the square of the intima were measured by MAC SCOPE software (Mitani, Fukui, Japan) as described previously (29, 35, 20).

Statistical Analysis

Values are means ± SE; n is number of rabbits. Statistical evaluation of the data was carried out by using analyses of variance. When a value was statistically significant, a post hoc test for multiple comparisons (Fisher protected least significant difference) was used to identify differences among the groups. The values were considered statistically significant at P < 0.05.

RESULTS

Experiment 1: Optimization of Voltage for Transgene Expression

To optimize the voltage of the electric pulses used for electroporation, we first assessed the luciferase activity in each vein or vein graft 2 days after electroporation at various voltages via two different gene transfer techniques (Fig. 1, A and B). In this series of experiments, the pulse length (Pem = 5 ms), number of pulses (n = 10), and DNA concentration (200 μg/ml) were fixed, because these parameters were known to be optimal for arterial gene transfer in rabbits (28).
Gene transfer into veins and vein grafts via the Lu method: a technique for endothelial gene delivery. In the case of in situ gene transfer to the jugular veins without grafting, the luciferase activity 2 days after gene transfer was optimal at ≤10 V (4.15 ± 0.83 × 10^6 RLU/mg, n = 5), and >30 V resulted in a marked decrease in gene expression (Fig. 2A, n = 5 in each group). Luciferase activity at 0 V, which represented naked DNA infiltration, was below the detection limit (n = 5). We used 15 animals to assess histological damage at 10 V (n = 5), 50 V (n = 5), and 0 V (n = 5), with sham-operated animals used as a control group. An apparent loss of cells was not found in the veins treated at 10 V; however, marked loss of hematoxylin-positive nuclei was evident at 50 V, suggesting that the dramatic decrease in transgene expression at 50 V was likely to be the result of cell death due to the electronic pulses.

We next used the lacZ gene followed by X-Gal histochemistry to examine the transfected cell species treated at 10 V via the Lu method (n = 3). Dissecting microscope analysis demonstrated frequent and diffuse blue cells on the luminal surface of the veins (Fig. 2B). Histological examination also showed that these cells were luminal endothelial cells (Fig. 2C), indicating that endothelial gene delivery was via the Lu method.

On the basis of these findings, we examined luciferase activity in the vein grafts 2 days after gene transfer at 10 V by the Lu method (n = 5). The veins were treated as described above and then implanted into the common carotid arteries; however, these grafts did not exhibit any significant luciferase activity (3.29 ± 0.42 × 10^3 RLU/mg). Because the loss of luciferase expression in vein grafts subjected to the Lu method might have been due to the denudation of endothelial cells, we used anti-von Willebrand factor antibody to immunohistochemically assess the coverage of endothelial cells in residual sections of these vein grafts (n = 5). Similar to our previous findings (33), immunohistochemical examination revealed an almost complete loss of nuclei in the graft wall (5.2 ± 3.2 and 313.8 ± 38.5 positive cells/ring in vein grafts and normal veins, respectively, n = 5; Fig. 2, D and E, arrows), suggesting that the loss of transgene expression might have been due to a loss of gene-transferred endothelial cells.

Gene transfer into vein grafts via the Ad method: a technique for adventitial gene delivery. To improve transgene expression in the vein grafts after grafting, we next examined an alternative method of gene transfer, the Ad method (Fig. 1B). In combination with this method, the jugular vein was transplanted into the arterial circulation soon after electroporation for the luciferase gene, and the grafts were subjected to luciferase assay. Under these conditions, luciferase activity increased nearly in proportion to voltage (i.e., up to 30 V), and, in turn, >50 V resulted in a significant decrease in transgene expression (Fig. 3A; n = 5 in each group). Optimal gene expression was achieved at 30 V (18.2 ± 1.7 × 10^3 RLU/mg, n = 5). Similar to the results obtained with the Lu method, no luciferase activity was observed at 0 V when the vein was placed in the electroporation cuvette for a short time and without any electric pulsing (n = 5).

![Fig. 2. Optimization of electroporation transfer to the venous wall by the Lu method. A: relative light units (RLU) in situ jugular veins measured by a luminometer 2 days after electroporation. The other parameters were fixed as follows: pulse-on time (P_on) = 5 ms, pulse-off time (P_off) = 95 ms, number of pulses = 10, plasmid DNA concentration = 200 μg/ml. Values are means ± SE (n = 5). B and C: distribution of gene-transferred cells in rabbit jugular veins detected by X-Gal staining 2 days after electroporation of the lacZ gene. B: dissecting microscope view of the luminal surface of a vein revealing diffuse and abundant blue spots. C: corresponding histopathological findings of the vein in B counterstained with nuclear fast red. Blue cells were distributed among luminal endothelial cells (thick arrows). Experiments were repeated separately in triplicate, and each experiment produced similar results. lu, lumen; ad, adventitia; m, media (thin arrows). D and E: immunohistochemical detection of von Willebrand factor (vWF, arrows) as a marker for endothelial cells of normal vein (D) or vein graft after implantation (E). Lining of untreated normal veins was frequently positive for vWF at the luminal surface (D, arrows); almost complete denudation of endothelial cells was revealed by the extremely rare observation of vWF-positive luminal cells (E, arrows) in vein grafts 2 days after implantation. Hematoxylin counterstain. Magnification ×10 (B) and ×200 (C–E).]
To determine the localization of transgene expression in the vein grafts treated with the Ad method, pAct-NLS-LacZ was then transferred to the jugular vein wall with the previously determined optimal voltage (30 V). At 2 days after gene transfer and vein grafting, dissecting microscope observation of the vein grafts stained with X-Gal solution revealed diffuse and abundant blue staining at the adventitial surface (Fig. 3B); in addition, histochemical examination revealed blue signals in the adventitial cells, whereas no blue signals were observed on the luminal surface (Fig. 3C), indicating that adventitial gene delivery was via the Ad method.

Experiment 2: Effect of ecNOS Gene Transfer Via Electroporation on Neointimal Hyperplasia in Cholesterol-Fed Rabbits

The following experiments were performed by using cholesterol-fed rabbits (total n = 24; n = 6 for immunohistochemistry and n = 18 assessment of intimal hyperplasia).

**Immunohistochemical detection of bovine ecNOS protein in rabbit vein grafts.** The distribution of bovine ecNOS protein in rabbit vein grafts was examined via immunohistochemistry using an anti-bovine ecNOS monoclonal antibody that does not cross-react with inducible or brain-type NOS. At 2 days after

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**Fig. 3. Optimization of electrogene transfer to vein grafts by the Ad method.** A: relative light units in vein grafts measured by a luminometer 2 days after electrogene transfer and vein grafting. The other parameters were fixed as follows: P_{on} = 5 ms, P_{off} = 95 ms, number of pulses = 10, plasmid DNA concentration = 200 µg/ml. Values are means ± SE (n = 5). B and C: distribution of gene-transferred cells in vein grafts detected by X-Gal staining 2 days after electrogene transfer of the lacZ gene and grafting. B: dissecting microscope view of the adventitial side of a vein graft demonstrating diffuse and abundant blue spots. C: corresponding histopathological findings of the vein graft in B counterstained with nuclear fast red. Blue cells were distributed among the adventitial cells (thick arrows). Magnification ×10 (B) and ×200 (C). Experiments were repeated separately in triplicate, and each experiment produced similar results.

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**Fig. 4. Immunohistochemical examination of protein expression of bovine endothelial cell nitric oxide synthase (ecNOS) in rabbit vein grafts with gene transfer by the Ad method at 30 V.** Experiments were repeated separately in triplicate, and each experiment produced similar results. A and B: distribution of bovine ecNOS protein in control gene (luciferase)-transferred (A) or ecNOS gene-transferred (B) vein grafts reacted with anti-bovine ecNOS antibody. Frequent positive reactions are evident in the adventitia of B (arrows), but not A. C: negative control using a serial section of B reacted with an isotype-matched antibody. No apparent positive reaction was observed. Original magnification ×400. Cells were counterstained with hematoxylin.

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**Fig. 2. Distribution of bovine ecNOS protein in control gene (luciferase)-transferred (A) or ecNOS gene-transferred (B) vein grafts reacted with anti-bovine ecNOS antibody.** Frequent positive reactions are evident in the adventitia of B (arrows), but not A. C: negative control using a serial section of B reacted with an isotype-matched antibody. No apparent positive reaction was observed. Original magnification ×400. Cells were counterstained with hematoxylin.
gene transfer in the vein grafts treated by the Ad method with optimal voltage (30 V), bovine ecNOS was frequently observed in the adventitial cells (Fig. 4B), whereas clear positive signals were not seen in serial sections treated with nonimmunized mouse IgG (Fig. 4C). Vein grafts with luciferase gene transfection carried out under the same conditions also showed no apparent positive signals in any of the tissue layers (Fig. 4A). Further immunohistochemical investigation using tissue sections of vein grafts 4 wk after gene transfer revealed no apparent ecNOS expression in any of the groups (data not shown), indicating that transgene expression was transient in the jugular vein grafts; this finding was in agreement with our previous data obtained when using rabbit carotid arteries (28).

Effect of ecNOS Gene Transfer on Neointimal Hyperplasia of Vein Grafts Under Hyperlipidemic Conditions

At the time of graft implantation and death of the animals, serum total cholesterol levels were not significantly different among the three groups: 534 ± 141, 482 ± 81, and 507 ± 60 mg/dl at graft implantation and 869 ± 99, 927 ± 171, and 916 ± 93 mg/dl at death for ecNOS, control-empty vector, and buffer treatment, respectively. At 4 wk after gene transfer, the Ad method with optimal voltage (30 V) and vein grafting was used to examine the intimal thickness, as well as the intimal area, of the vein grafts among the ecNOS-transfected group, the empty vector-transfected group, and the buffer-treatment group under a light microscope (Fig. 5, C–E). The intimal thickness in the ecNOS group (110 ± 6 μm, n = 6, P < 0.05) was significantly reduced compared with that of the empty vector-transfected and buffer-treatment groups (143 ± 7 and 142 ± 4 μm, n = 6; Fig. 5A), and similar results were obtained when using the intimal area was measured (1.51 ± 0.14 vs. 1.95 ± 0.13 and 1.96 ± 0.08 mm², P < 0.05; Fig. 5B). No apparent specific features, including sustained inflammatory reactions and any disorganized vascular structures, were observed in these hematoxylin-eosin- and Elastica van Gieson-stained tissue sections.

DISCUSSION

The present study demonstrated that a new gene transfer technique, electroporation, could be applied to vascular conduits to limit the extent of intimal hyperplasia, without raising significant safety issues, when optimized electronic pulses were used. The following key observations were made during the course of the study: 1) The Lu method resulted in endothelial gene delivery, whereas the Ad method resulted in adventitial gene delivery. 2) Adventitial delivery proved to be a better means of obtaining significant transgene expression, even when the veins were grafted under arterial circulation. 3) Adventitial, but not endothelial, cells should be targeted when the veins are to be used as bypass grafts. 4) The level of transgene expression achieved via electroporation using the Ad method might significantly limit the extent of intimal hyperplasia when ecNOS is used as a therapeutic gene. This report is the first to indicate the successful application of electroporation to vein grafts.

Vein grafts represent the most common conduit for arterial reconstruction, particularly in cases of infrainguinal bypass, because they have been shown to have better long-term patency than prosthetic grafts (9, 36). However, vascular grafts, including veins, have been associated with unresolved issues, such as late graft failure caused by intimal hyperplasia, which is a chronic structural lesion that develops after vein graft implantation and leads to luminal stenosis and occlusion. Late

Fig. 5. Effect of electrogene transfer of bovine ecNOS gene on development of neointimal formation of vein grafts in hyperlipidemic rabbits. Vein grafts were harvested 4 wk after gene transfer and grafting, and Elastica van Gieson-stained sections were evaluated under a light microscope. Sham, sham-operated group; Empty, empty vector transfection group; ecNOS, ecNOS gene-transfected group. A and B: quantitative analyses of intimal wall thickness and intimal area of rabbit vein grafts. C–E: typical light microscope findings of the midportion of sham-operated vein grafts or vein grafts transfected with empty vector or ecNOS gene. Arrows, intimal area. Original magnification ×200.
graft failure may be defined as the abnormal migration and proliferation of vascular smooth muscle cells with an associated deposition of extracellular connective tissue matrix and endothelial dysfunction (3, 8).

Several experimental studies have suggested that gene therapy may be effective for the treatment of vascular disease, including vein graft failure (13, 29, 33). Although a number of successful results have been reported involving gene therapy to treat intimal hyperplasia in animals, its application is limited in cases involving vein grafts; the main issues at stake are the efficacy and safety of this approach in the clinical setting (23).

Adenoviral vectors have commonly been used to limit intimal hyperplasia, as recently reported in studies of experimental gene therapy. The major advantage of an adenoviral vector is its high level of efficiency in transferring genetic material to the vasculature, however; the immunogenic, as well as cytotoxic, potency of this type of vector has prevented its application in the clinical setting (6, 42).

Alternatives are available, in particular nonviral vascular gene transfer methods such as hydrogel balloon delivery; however, these methods have achieved only low levels of gene transfer efficiency in vivo, suggesting that the clinical efficacy of such nonviral approaches remains limited (38). Electroporation-mediated gene transfer is a nonviral method of internalizing foreign DNA into any type of cell or tissue in a cell cycle-independent manner, inasmuch as the efficiency of electrogene transfer is regulated by physical parameters such as the strength of the electric field (28). Therefore, the application of this nonviral gene transfer technique to vascular gene transfer may be expected to overcome the limitations of nonviral gene transfer.

After implantation under arterial circulatorv, vein grafts have been shown to undergo severe damage; however, healing and vascular remodeling then take place. At the initial stage of grafting, the endothelium of the vein grafts was largely denuded (33, 40), and the walls of the grafts were extensively swollen; in addition, the medial smooth muscle cells have been shown to undergo extensive damage (17, 33). These findings, reported elsewhere, were also observed in the present study (Fig. 3C). Here, we found no transgene expression in the vein grafts on day 2, when these grafts were treated by endothelial gene delivery, i.e., the Lu method. This was due to the extensive denudation of the endothelium as a result of implantation under arterial flow. On the other hand, gene transfer from the adventitial side was successful when the Ad method was used, indicating that this approach may be a suitable means of gene transfer in cases involving vein grafts. Because our group, as well as others, demonstrated that adventitial cells, including myofibroblasts, are likely to play a significant role in graft remodeling and intimal hyperplasia (17, 39), adventitial-targeted gene transfer by the Ad method may eventually provide a meaningful approach to preventing the late failure of vein grafts.

Although a precise, direct-comparison study was not performed, the efficacy of plasmid-based gene transfer, even if coupled with electroporation, appears to be lower than that achievable by adenoviral gene transfer. However, this lower level of transfer efficacy would not necessarily indicate a less desirable outcome with respect to treatment; this is especially the case with the use of ecNOS as a therapeutic gene, because delivery of ecNOS with a highly efficient vector would cause adverse effects due to the more robust concentration of NO released into the circulation. Our present study suggests that the expression level of plasmid-based gene transfer via electroporation may be sufficient to reduce neointimal hyperplasia under conditions of hyperlipidemia.

During the chronic phase after grafting, the vein grafts underwent vascular remodeling, involving regeneration of the endothelium as well as development of the intimal hyperplasia (17, 40). In the last decade, our group demonstrated sustained impairment of the production of endothelium-derived relaxing factor, primarily composed of NO, from the regenerative endothelium of vein grafts (18, 19). We also demonstrated a close correlation between NO release and the development of intimal hyperplasia (19). Furthermore, a significant reduction of intimal hyperplasia via NO supplementation by L-arginine (34), as well as the gene transfer of ecNOS, has been achieved (29, 33).

NO has a variety of functions in vessel walls: it is an inhibitor of endothelium-leukocyte interactions (21), platelet aggregation (4), and vascular smooth muscle cell proliferation (12), as well as a vasodilator. Thus NO overproduction induced by ecNOS gene transfer may lead to a reduction in the neointimal area. In fact, ecNOS gene transfer inhibited intimal hyperplasia in a rat balloon injury model (43) and in canine vein grafts under conditions of poor runoff (29). Furthermore, perivascular delivery of NO inhibited intimal hyperplasia in hypercholesterolemic rabbits (7). These results indicate that adventitia-targeted gene transfer of ecNOS by electroporation is of potential therapeutic value in patients with vein graft failure.

In summary, we concluded that electroporation potentially offers a novel nonviral vector-based strategy that could be applied as a component of clinical gene therapy in cases involving vein graft failure. To the best of our knowledge, this is the first study to demonstrate that electroporation can be used to achieve gene transfer into vein grafts.

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