Autogenous veins are frequently used for bypassing coronary and peripheral vascular lesions. However, long-term patency of vein bypass grafts is compromised by intimal hyperplasia and accelerated atherosclerosis, and up to 30% of grafts require intervention within the first 2 years. \(^1\)

Nitric oxide (NO) has emerged as the local factor controlling vascular tone and structure, and it increases in vein grafts in response to the rising pressure and shear stress of the arterial circulation. \(^2\)-\(^4\) Oral administration of L-arginine, the substrate for NO synthase (NOS), reduces vein graft intimal hyperplasia and preserves NO-mediated relaxation in

**BASIC RESEARCH STUDIES**

**Adventitial versus intimal liposome-mediated ex vivo transfection of canine saphenous vein grafts with endothelial nitric oxide synthase gene**

Manju Kalra, MD, Corey J. Jost, MD, Sandra R. Severson, AA, and Virginia M. Miller, PhD

Rochester, Minn

*Purpose:* Experiments were designed (1) to evaluate liposome-mediated endothelial constitutive nitric oxide synthase (ecNOS) transfection in vein grafts and (2) to compare intimal and adventitial routes of transfection.

*Methods:* Male mongrel dogs (N = 36) underwent bilateral femoral artery bypass grafting with the lateral saphenous vein. In each animal one vein was transfected with plasmid (pVR1012) containing the ecNOS gene, and another vein was transfected with plasmid alone (control). Gene transfer was performed from either the intimal surface (Group I, n = 18) or the adventitial surface (Group II, n = 18). In each group there were three transfection subgroups (n = 6 each): (a) 10 µg/mL naked plasmid DNA, (b) 10 µg/mL plasmid DNA + liposome (LipofectAMINE PLUS), and (c) 100 µg/mL plasmid DNA + LipofectAMINE PLUS. Grafts were harvested on the third postoperative day, and the transfection was assessed with molecular techniques and enzyme assay for activity of NOS by conversion of tritiated L-arginine to tritiated L-citrulline. Proliferating cells were quantified with a digital analysis of histologic sections after nuclear antigen Ki-67 (MIB1) immunohistochemistry.

*Results:* Transgene was identified with polymerase chain reaction in all ecNOS-transfected grafts, regardless of transfection modality. However, significant transcription of the ecNOS transgene was observed only in Group IIc (mean ecNOS messenger RNA, 8.7 ± 1.7 vs 3.1 ± 0.7 × 10\(^{-2}\) attomole/µL, in transfected compared with control grafts, respectively, \(P = .01\)). NOS activity increased approximately twofold in this group (11.58 ± 2.1 and 6.3 ± 1.0 pmol tritiated L-citrulline per milligram protein per hour in transfected and control grafts, respectively, \(P = .05\)). Numbers of proliferating cells did not differ among ecNOS-transfected and control grafts in any transfection group.

*Conclusion:* These results suggest that ecNOS transfection of vein grafts is feasible through intimal and adventitial routes with naked DNA or a liposomal vector. However, efficient transcription of the transgene is evident at postoperative day 3 only after adventitial transfection of 100 µg/mL of the gene. (J Vasc Surg 2000;32:1190-200.)
rabbits. However, vein grafts explanted from humans and animals have reduced focal expression of endothelial cell NOS (ecNOS) specific to atherosclerotic sites. The prospect of upregulating NOS activity by transfer of the ecNOS gene into veins before grafting to limit subsequent intimal hyperplasia is an attractive one.

The highly successful transfer of genes into normal and balloon-denuded arteries and veins has been accomplished with adenoviral vectors. Transfection of the ecNOS gene into the arteries of animals results in the functional expression of NO and the reduction of neointimal hyperplasia. However, viral vectors may promote undesirable inflammatory responses. Liposome-mediated gene transfer is devoid of the cytotoxic and immune reactions associated with viral vectors, making it more attractive for use in the clinical setting. Liposome vectors have a lower transfection efficiency than adenoviral vectors under comparable conditions. However, in spite of limited gene expression with lipofection (< 1% of cells), significantly increased levels of the transgene product can be detected for up to 3 weeks in arteries in organ culture. Successful gene transfection of human saphenous veins has been performed in vitro with naked DNA alone and with viral and liposomal vectors; however, these veins have not been subsequently grafted into the arterial circulation.

There are no systematic evaluations of liposomal transfection of veins used for arterial reconstruction where the pressure flow characteristics and subsequent remodeling may cause an impact on transgene expression and function. The aim of this study was to assess the feasibility and efficiency of liposome-mediated ex vivo ecNOS gene transfer into saphenous vein grafts with the use of a third-generation liposome vector and to compare transfection through intimal and adventitial routes.

**MATERIALS AND METHODS**

**Construction of plasmid.** The complementary DNA (cDNA) for ecNOS cloned from bovine aortic endothelial cells (4.1 kilobase [kb]; plasmid cDNA-ecNOS; gift from Dr William C. Sessa, Yale University School of Medicine) was ligated into the EcoRI site of the plasmid (pVR1012, 4.9 kb; Vical Inc, San Diego, Calif), which contains a cytomegalovirus promoter and translation-enhancer intron sequence. The plasmids (with and without ecNOS) were propagated in Escherichia coli XLI-Blue grown in Luria-Bertani medium containing 50 µg/mL of kanamycin. Plasmid DNA was purified by anion-exchange chromatography integrated with endotoxin removal (QIAGEN Endofree Mega Plasmid Kit; QIAGEN GmbH, Hilden, Germany). The amount of endotoxin was measured (Limulus Amebocyte Lysate, QCL-1000; Biowhittaker Inc, Walkersville, Md) in pVR1012 alone (0.02 EQ/μg of DNA) and pVR1012 + ecNOS (0.07 EQ/μg of DNA). ecNOS fragment size was verified by restriction digestion, and plasmid DNA was quantified by the measurement of absorbance at a 260-nm wavelength.

**Gene transfection and vein grafting.** Adult male mongrel dogs (weight, 20-30 kg; N = 36) were intravenously anesthetized with methohexital (Brevital), 12.5 mg/kg, and were intramuscularly given 1.2 mouse unit of procaine penicillin (Durapen). After endotracheal intubation, the ani-
animals were ventilated with halothane (1%) and oxygen to maintain anesthesia. Lateral saphenous veins were harvested bilaterally and flushed with reduced serum medium (OPTI-MEM; Life Technologies, Rockville, Md) to remove blood, and then they were emptied. One vein was transfected with plasmid alone (without the ecNOS gene), and another vein was transfected with plasmid containing the ecNOS gene. For the transfection solution to be made, plasmid DNA and LipofectAMINE PLUS (2,3-dioleoyloxy-N-2sperminecarboxamido-ethyl-N,N-dimethyl-1-propanaminium trifluoro-acetate/dioleoylphosphatidylethanolamine 3:1 wt/wt + Plus Reagent, Life Technologies) were diluted separately in OPTI-MEM, then mixed together in a ratio 1:6 wt/wt, and incubated at room temperature for 15 minutes to allow liposome-DNA complexes to form. For intimal transfection, the vein was filled with transfection solution to a pressure of 150 mm Hg with a pressure lock syringe (Medtronic Inc, Minneapolis, Minn) clamped at both ends and incubated in OPTI-MEM at 37°C. For adventitial transfection, the vein was ligated at either end and immersed in transfection solution. All veins were transported immediately to an incubator maintained at 37°C for 1 hour. Veins were rotated every 15 minutes during the incubation period. At the end of the hour, transfected veins were anastomosed end-to-side as reversed grafts to bypass a ligated segment of the femoral artery. Blood flow (milliliters per minute) was measured in each graft with an ultrasonic flow meter (T206; Transonic Systems Inc, Ithaca, NY). After the closure of all wounds, a Doppler ultrasound scan examination (Apogee 800; ATL, Ambler, Pa) was performed to record peak and mean blood flow velocities (meters per second) and vein graft cross-sectional area (square centimeters) in the proximal and distal sections of the graft. Postoperatively, all animals were intramuscularly given analgesics (Torbugesic), 10 mg, and were given antibiotics (cefadroxil), 20 mg/kg twice a day.

Experimental groups. Animals were divided into two groups \((n = 18\) in each group) according to the route of transfection: Group I (intimal transfection) and Group II (adventitial transfection). In each group the transfection modalities studied were \((a)\) 10 \(\mu\)g/mL naked ecNOS plasmid DNA \((n = 6)\), \((b)\) 10 \(\mu\)g/mL ecNOS plasmid DNA + LipofectAMINE PLUS \((n = 6)\), and \((c)\) 100 \(\mu\)g/mL ecNOS plasmid DNA + LipofectAMINE PLUS \((n = 6)\). Each animal served as its own control, with the contralateral vein graft transfected similarly with plasmid without ecNOS gene. All animals received humane care according to guidelines issued by the National...
Institutes of Health and contained in the Guide for the Care and Use of Laboratory Animals, compiled by the Institute of Laboratory Animal Resources, National Research Council.

Experimental measurements. The grafts were removed on the third postoperative day after collection of blood for plasma NO (NO) measurement, Doppler ultrasound scan examination, and blood flow measurement. Segments (0.5-1.0 cm) of graft tissue were frozen in liquid nitrogen, and three segments (proximal, mid, and distal graft) were fixed in 10% neutral buffered formalin (4% formaldehyde wt/vol, pH 6.8-7.2) for histologic examination.

Polymerase chain reaction. Polymerase chain reaction (PCR) was performed on DNA extracted from the vein grafts to confirm successful transfection. Overnight, 0.5-cm segments from each graft were digested at 55°C in 500 μL proteinase K (0.6 mg/mL) and dissolved in Tris-HCl buffer (Tris-HCl 50 mmol/L, sodium chloride 50 mmol/L, EDTA 100 mmol/L, and 1% sodium dodecyl sulfate). Digested tissue was mixed with 1 mL of phenol: chloroform premixed with isoamyl alcohol (25:24:1; Amresco, Solon, Ohio) and centrifuged at room temperature for 5 minutes. The aqueous phase was transferred to a fresh tube, and total DNA was extracted with 1 mL of chloroform. This step was performed twice. The DNA was precipitated with two volumes of 95% ethanol and then washed in 1 mL of 70% ethanol. The DNA pellet was briefly dried in a Speed Vac (SC110; Savant, Holbrook, NY) and reconstituted in diethyl pyrocarbonate–treated water.

Total RNA was then extracted with 0.2 mL of chloroform and precipitated with 0.5 mL of isopropanol. The supernatant was removed, and the RNA pellet was washed with 1 mL of 75% ethanol, air dried, and reconstituted in diethyl pyrocarbonate–treated water. RNA concentration was determined by measuring absorbance at 260 nm in a spectrophotometer (DU 640; Beckman). Deoxyribonuclease (DNase) treatment of 1 μg of total RNA was carried out with 1 μL of DNase buffer (200 mmol/L Tris-HCl at pH 8.4, 500 mmol/L potassium chloride, 20 mmol/L MgCl₂) + 2 μL of amplification grade DNase I (Life Technologies) for 15 minutes at room temperature. DNase I was then inactivated by heating to 65°C after addition of 1 μL of 25 mmol/L of EDTA.

First-strand cDNA synthesis was next performed (Superscript Preamplification System; Life Technologies) with sequential reactions after the addition of 1 μL of oligo(dT)₁₂₋₁₈ primers to hybridize to 3′ poly(A) tails on messenger RNA (mRNA) (70°C for 10 minutes), then 7 μL of reaction mixture (12 μL 10× PCR buffer, 2 μL 25 mmol/L magnesium chloride, 1 μL deoxyribonucleoside triphosphate mix, and 2 μL 0.1 mol/L DTT) 42°C for 5 minutes), and 1 μL Superscript II reverse transcriptase (42°C for 50 minutes). The reaction was terminated by heating to 70°C for 15 minutes, which was followed by incubation with ribonuclease H for 20 minutes at 37°C. Target cDNA was next amplified with PCR: 38 cycles of denaturation (94°C for 45 seconds), annealing (60°C for 45 seconds), and polymerization (72°C for 60 seconds). The primers used detected ecNOS (transfected and endogenous): 5′ primer (TCA ACC AGT ACT ACA GCT CC) and 3′ primer (GTG GTT GCA GAT GTA GGT GA). A 251 bp product was visualized on 2% agarose gel electrophoresis. GAPDH primers were added to verify the efficiency of cDNA synthesis. Control reactions performed in the absence of reverse transcriptase were negative for genomic DNA.

The cDNA was next quantified with the PCR-MIMIC technique (Clontech Labs, Palo Alto, Calif). A MIMIC DNA was constructed by performing two rounds of PCR amplification. In the first reaction two composite primers were used: 5′ primer (TCA ACC AGT ACT ACA GCT CCC GCA AGT GAA ATC TCC TCC G) and 3′ primer (GTG GTT GCA GAT GTA GGT CAT GTG TCA ATG CAG TTT GTA G). Each contained the target gene primer sequence attached to a nucleotide strand designed to hybridize with opposite strands of a MIMIC DNA fragment. A dilution of this reaction was then amplified again with only the gene-specific primers. The
Absolute values of diameter, blood flow, NO, and cell proliferation in ecNOS-transfected and control vein grafts

<table>
<thead>
<tr>
<th>Group I—intimal</th>
<th>Tx graft/Ctrl graft*</th>
<th>Tx graft/Ctrl graft†</th>
<th>Tx graft/Ctrl graft‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSA (cm²)</td>
<td>0.18 ± 0.01/0.17 ± 0.01</td>
<td>0.19 ± 0.02/0.17 ± 0.01</td>
<td>0.17 ± 0.01/0.17 ± 0.01</td>
</tr>
<tr>
<td>Blood flow (mL/min)</td>
<td>166.0 ± 20.9/156 ± 35.4</td>
<td>211.5 ± 44.0/232.2 ± 27.7</td>
<td>157.2 ± 29.0/120.7 ± 36.5</td>
</tr>
<tr>
<td>Arterial NO&lt;sub&gt;x&lt;/sub&gt; (×10&lt;sup&gt;-1&lt;/sup&gt; nmol/mL)</td>
<td>11.7 ± 1.4/11.8 ± 1.6</td>
<td>10.3 ± 1.5/10.1 ± 1.6</td>
<td>9.9 ± 1.3/10.0 ± 1.2</td>
</tr>
<tr>
<td>mRNA level (×10&lt;sup&gt;-2&lt;/sup&gt; attomole/µL)</td>
<td>3.3 ± 0.7/5.0 ± 1.8</td>
<td>5.9 ± 0.7/5.5 ± 1.3</td>
<td>5.6 ± 1.0/5.7 ± 2.0</td>
</tr>
<tr>
<td>NOS activity (pmol)</td>
<td>11.7 ± 4.0/14.8 ± 5.5</td>
<td>18.6 ± 6.5/20.9 ± 6.8</td>
<td>5.9 ± 1.0/7.4 ± 1.5</td>
</tr>
<tr>
<td>MIB1 index (%)</td>
<td>15.8 ± 3.0/15.1 ± 3.4</td>
<td>16.0 ± 4.5/14.4 ± 2.4</td>
<td>19.4 ± 3.4/20.2 ± 3.4</td>
</tr>
</tbody>
</table>

All values in mean ± SEM, n = 6 in all groups.
*Modality was 10 µg/mL, naked plasmid DNA.
†Modality was 10 µg/mL plasmid DNA + LipofectAMINE PLUS.
‡Modality was 100 µg/mL plasmid DNA + LipofectAMINE PLUS.
§P = .01 compared with control grafts.
¶P = .05 compared with control grafts.
Venous NO<sub>x</sub> (×10<sup>-1</sup> nmol/mL) | 11.8 ± 1.2/9.5 ± 1.1 | 10.5 ± 1.5/11.6 ± 2/2 | 10.0 ± 1.1/12.4 ± 0.8

MIMIC DNA was designed to be approximately 200 bp larger in size than the target ecNOS PCR product. The MIMIC DNA was next purified by a passage through CHROMA SPIN+TE-100 columns; the yield was calculated and diluted to 100 attomole/µL. Competitive PCR amplification was next performed by titrating 1 µL of the target cDNA against serial 10-fold dilutions of the MIMIC DNA, using the eNOS 5′ primer (TCA ACC AGT ACT ACA GCT CC) and eNOS 3′ primer (GTG GTT GCA GAT GTA GGT GA), and 35 cycles of denaturation (94°C for 30 seconds), annealing (63°C for 30 seconds), and polymerization (72°C for 30 seconds). The PCR products were analyzed with ethidium bromide–stained 1.6% agarose gel electrophoresis, and bands of cDNA and MIMIC DNA of equal intensity were identified by visual inspection. Fine-tuned competitive PCR was next performed by titrating 1 µL of the target cDNA against serial twofold dilutions of the identified MIMIC DNA dilution in the same manner as described above. The mRNA concentration (attomole/microliter) was determined from the known concentration in the MIMIC DNA band of equal intensity.

Activity of NOS. NOS activity was determined in the vein grafts by measuring the conversion of tritiated L-arginine to tritiated L-citrulline with methods previously described.24 Sections of vein grafts were frozen in liquid nitrogen and homogenized on ice for 30 seconds in five volumes of homogenization buffer (50 mmol/L Tris, 320 mmol/L sucrose, 0.1 mmol/L EDTA, 100 µg/mL phenylmethylsulfonyl fluoride, and protease inhibitor cocktail: two tablets/50 mL [Complete; Boehringer Mannheim, Indianapolis, Ind]). The homogenates were centrifuged at 2000 g for 15 minutes at 4°C, and the supernatant was passed onto an equilibrated 10-DG desalting column (Bio-Rad, Hercules, Calif) and eluted. A small aliquot was used to determine protein concentration with bicinchonicic acid protein assay reagent (Pierce, Rockford, Ill).

For the quantitation of NOS activity, reactions were carried out in duplicate to measure (a) total activity, in the presence of calcium; (b) calcium-independent activity, in the absence of both calcium and EGTA; and (c) nonspecific activity, in the absence of calcium and EGTA, in the presence of NG-monomethyl-L-arginine (L-NMMA). Reactions were performed in a shaker bath at 27°C for 1 hour, with 150 µL of protein homogenate and 150 µL of cofactor mix (14.7 nmol/L tritiated L-arginine [0.3 µCi specific activity at 68 Ci/mmol], 5 µmol/L L-arginine, 54 mmol/L L-valine, 1.2 mmol/L magnesium chloride, 1 mmol/L NADPH, 50 U/mL calmodulin, 2 µmol/L flavin adenine dinucleotide, 10 µmol/L tetrahydrobiopterin, with or without 0.83 mmol/L calcium chloride, 1 mmol/L EGTA, and 2 mmol/L L-NMMA) and were terminated by...
adding ice-cold stop buffer (20 mmol/L Hepes, 8 mmol/L EDTA, pH 5.5). Tritiated l-citrulline was separated from tritiated l-arginine by passing the assay mixture over Poly-Prep chromatography columns (Bio-Rad) loaded with 1 mL of equilibrated AG 50W-X8 Na⁺ form 200 to 400 mesh molecular biology grade resin (BioRad), and then washing the column with 2 mL of water. The elution was collected in 18 mL of Opti-Fluor (Packard, Meriden, Conn) scintillation fluid and tritiated l-citrulline activity determined with a Beckman 6800 liquid scintillation counter. Blanks consisting of homogenization buffer that passed through a 10-mL bed volume, desalting grade column were counted as part of each experiment, and calculations were made to account for scintillation counting efficiency and the ratio of tritiated l-arginine to nonradioactive l-arginine in the incubation mixture. NO produced by NOS was presumed to be in a 1:1 molar ratio with l-citrulline, and NOS activity was expressed as picomoles of tritiated l-citrulline produced per milligram of protein per hour. Calcium-dependent activity attributable to eNOS was expressed as total activity minus calcium-independent activity after correcting for nonspecific activity.

**Measurement of NOx.** NOₓ (×10⁻¹ nmol/mL) was measured in peripheral venous blood preoperatively and venous and arterial blood on postoperative day 3. NO and its oxidation products NO₂⁻ and NO₃⁻ were performed each time and were found to be linear over the range 50 pmol to 2 nmol of NOₓ. All measured values fell within this range.

**Immunohistochemistry.** Immunohistochemistry was performed on paraffin-embedded sections of vein grafts with the Vectastain Elite ABC system (Vector Laboratories, Burlingame, Calif). Sections were deparaffinized and hydrated, and endogenous peroxidase activity was quenched by incubating in 0.3% hydrogen peroxide in methanol (1:1) for 10 minutes. After it was washed in phosphate-buffered saline, antigen was retrieved by steaming the slides in EDTA 1 mmol/L, pH 8.0 (Sigma Chemicals, St Louis, Mo) for 30 minutes. The sections were next incubated with 5% horse serum for 20 minutes to block nonspecific staining. Excess serum was blotted off, and the sections were incubated with primary mouse monoclonal antibody against nuclear antigen Ki-67 (MIB1, 1:50; Immunotech, Westbrook, Me) for 1 hour at room temperature. Slides were then washed in phosphate-buffered solution and incubated in turn with biotinylated secondary antibody for 30 minutes (horse antimouse immunoglobulin G, 1:2000; Vector Laboratories) and Vectastain elite ABC reagent for 30 minutes; they were stained with diaminobenzidine-tetrahydrochloride substrate kit (DAB; Vector Laboratories) and counterstained with Gill’s hematoxylin.

**Quantitative image analysis.** Sections immunostained for nuclear antigen Ki-67 (MIB1) were viewed under an Axioscan 2 microscope (Carl Zeiss, Inc, Oberkothen, Germany) equipped with a Plan-Apochromat 10X/0.45 objective and digitized to 768 × 580 pixels with a ProgRes 3012 camera (Jenoptik Laser; Optik Systems, Eching, Germany). Images cov-

<table>
<thead>
<tr>
<th>Group II—adventitial</th>
<th>Tg graft/Ctrl graft*</th>
<th>Tg graft/Ctrl graft†</th>
<th>Tg graft/Ctrl graft‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.17 ± 0.02/0.18 ± 0.02</td>
<td>0.18 ± 0.01/0.17 ± 0.01</td>
<td>0.18 ± 0.02/0.17 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>160.7 ± 13.3/187.5 ± 29.3</td>
<td>225.7 ± 37.1/210.0 ± 28.9</td>
<td>249.2 ± 33.0/247.2 ± 40.0</td>
<td></td>
</tr>
<tr>
<td>17.0 ± 2.3/15.9 ± 2.0</td>
<td>9.8 ± 1.4/9.8 ± 1.5</td>
<td>11.1 ± 2.6/11.3 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>4.2 ± 0.5/3.4 ± 0.4</td>
<td>2.6 ± 0.5/2.3 ± 0.4</td>
<td>8.7 ± 1.7/3.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>14.5 ± 2.0/11.6 ± 2.2</td>
<td>13.6 ± 2.5/13.8 ± 2.9</td>
<td>11.6 ± 2.1/6.4 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>21.1 ± 3.5/21.6 ± 2.4</td>
<td>19.5 ± 3.4/20.4 ± 3.1</td>
<td>14.5 ± 3.1/15.0 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>POD 3/preop</td>
<td>POD 3/preop</td>
<td>POD 3/preop</td>
<td></td>
</tr>
<tr>
<td>17.0 ± 2.7%/10.6 ± 1.1</td>
<td>9.8 ± 1.7/10.5 ± 1.7</td>
<td>11.1 ± 2.5/10.4 ± 1.2</td>
<td></td>
</tr>
</tbody>
</table>
Fig 3. Quantification of ecNOS mRNA (total ecNOS mRNA – transfected + endogenous) by RT-PCR followed by PCR MIMIC in canine saphenous vein grafts transfected with ecNOS. Results are presented as mean ± SEM of values in ecNOS-transfected grafts normalized to contralateral control grafts (transfected with plasmid alone). Dotted line represents mRNA level in control vein grafts (100%; absolute values shown in Table). *P < .01 versus all other transfection groups (n = 6 in each group).

Fig 4. Calcium-dependent activity of NOS in canine vein grafts transfected with ecNOS. Results are presented as mean ± SEM of levels in ecNOS-transfected grafts normalized to contralateral control grafts (transfected with plasmid alone). Dotted line represents calcium-dependent NOS activity in control vein grafts (100%; absolute values shown in Table) (n = 6 in each group).

Fig 5. NOx levels in dogs with ecNOS transfection of saphenous vein grafts. Results are presented as mean ± SEM. A, Peripheral venous NOx levels on day 3 normalized to preoperative baseline levels (represented by the dotted line). B, Arterial NOx levels in ecNOS-transfected grafts on day 3 at explant, normalized to contralateral control grafts (transfected with plasmid alone). Dotted line represents NOx level in control vein grafts (100%; absolute values shown in Table) (n = 6 in each group).

MIB1 positive (brown) and total nuclei (brown + blue) were highlighted in turn by selecting the appropriate red-green-blue color intensity range and were counted. The MIB1 proliferative index (positive cells/total cells × 100) was calculated for each section, as well as separately in the medial and adventitial layers. The consistency of the computer analysis was maintained by repeated analysis of a control slide to ensure less than 5% variation.

Statistical analysis. All values are expressed as mean ± SEM. The Student t test was used to compare transfected and control grafts in each transfection modality. One-way analysis of variance with Newman–Keuls multiple comparison posttests was used to analyze data and compare groups after normalization of values in transfected grafts with their
corresponding contralateral control grafts. A P value less than .05 was considered significant.

RESULTS

Doppler ultrasound scan examination and transfection

Cross-sectional area (square centimeter), blood flow (milliliter/minute), and peak and mean flow velocities (cm/s, data not shown) did not differ significantly between grafts transfected with plasmid containing the ecNOS gene or plasmid alone, or among the various transfection groups (Table, Fig 1). Successful transfection of the ecNOS gene was confirmed by a positive 321 bp signal with PCR in all vein grafts transfected with plasmid + ecNOS. No PCR signal was observed in any graft transfected with plasmid alone (Fig 2). The PCR signal was strongest in the group with liposome-mediated transfection at 100 µg/mL.

Efficiency of gene transfection

Quantification of ecNOS mRNA. Intimal transfection (Group I) did not result in increased ecNOS mRNA levels in grafts transfected with plasmid + ecNOS compared with controls (transfected with plasmid alone) regardless of the transfection modality (Table, Fig 3). With adventitial transfection (Group II), the ecNOS mRNA level was significantly higher compared with control grafts only with 100 µg/mL ecNOS DNA and LipofectAMINE PLUS (P = .01).

Activity of NOS. Mean calcium-dependent NOS activity was increased to nearly twofold compared with control grafts (P = .05) in Group II after liposome-mediated transfection with 100 µg/mL ecNOS DNA (Table, Fig 4). There was no significant increase in NOS activity with any other transfection modality (Table, Fig 4). There were no significant differences in calcium-independent NOS activity between grafts transfected with plasmid plus ecNOS gene or plasmid alone (data not shown; n = 6 per group).

Plasma nitric oxide

Peripheral venous NOx levels (×10⁻¹ nmol/mL) increased postoperatively in 15 of 36 dogs presumably as a response to surgery and inflammation, and

Fig 6. Representative light micrographs (magnification ×100) showing cell proliferation (brown nuclei) identified by nuclear antigen Ki-67 (MIB1) immunohistochemistry in canine saphenous vein graft transfected through the adventitia with liposome and 100 µg/mL plasmid containing ecNOS gene (A). B, Control graft transfected with liposome and plasmid alone.
this increase from preoperative levels reached significance only in Group IIa (P = .04, Table). NOx levels at day 3 were similar in peripheral venous blood and arterial blood from grafts transfected with plasmid + ecNOS gene or plasmid alone in all transfec-
tion groups (Table, Fig 5).

Distribution and quantitation of proliferating cells

Proliferating cells were localized to the media and inner adventitia of the vein wall at day 3 on graft removal (Fig 6). The distribution of proliferating cells was similar in all plasmid + ecNOS transfected grafts and grafts transfected with plasmid alone (control), regardless of the modality of transfection. The MIB1 proliferative indices (positive cells/total cells × 100) were calculated for each section (Table), as well as separately in the medial (dark) and adventitial (shaded) layers (Fig 7). No significant differences in total cell proliferation or in the distribution of proliferating cells within medial and adventitial layers were observed between grafts transfected with plasmid + ecNOS gene or plasmid alone in any transfection group.

DISCUSSION

Results of this study (1) demonstrate the feasibility of ex vivo liposome-mediated ecNOS gene trans-
fer in vein grafts, (2) compare liposome-mediated transfection at two doses of DNA with transfection with naked DNA, and (3) compare the efficiency of gene transfer through the intimal and adventitial routes. NO participates in several physiologic func-
tions in a variety of different cell types (eg, regulation of vascular tone, antiplatelet and antileukocyte prop-
erties, and modulation of cell growth [proliferation and apoptosis]).25 These properties make it an attractive molecule for use as a therapeutic modality to influence changes in vascular architecture that occur with vein grafting. Increasing the amount of locally available NO with arginine feeding,5,6 application of NO donors,26,27 and ecNOS or inducible NOS gene
transfer have been shown in animal studies to limit the formation of intimal hyperplasia in balloon-
injured arteries, vein grafts, and segments of human saphenous vein.13,28,29

To date, gene transfer in vein grafts has been accomplished with replication-deficient adenoviruses and Sendai viruses in small animals.10,23,30-33 The most widely studied adenoviral gene transfer is efficient, but short-lived, and accompanied by significant infiltration of inflammatory cells. In addition, exposure of normal arteries to adenovirus vectors has been shown to produce pronounced infiltration with T cells, upregulation of adhesion molecules, and, ultimately, neointimal hyperplasia.34 Liposome-mediated gene transfer has been shown to be effective and safe in animal experiments and clinical trials.35 Furthermore, cationic liposomes are nonimmunogenic and therefore safe for repeated therapy, if necessary.

Results of the current study demonstrate that liposomal gene transfer is feasible in vein grafts, in which the intimal or adventitial route of gene deliv-
ery is used. However, intimal transfection did not result in efficient gene transcription; there was no increase in mRNA in ecNOS-transfected grafts over that in control grafts on day 3. On the other hand, adventitial transfection with liposome-mediated transfection of 100 µg/mL of ecNOS DNA resulted in significantly elevated mRNA levels compared with control grafts and all other transfection modal-
ities studied.

The feasibility of gene transfer through the adventitia has been demonstrated in uninjured rab-
bit carotid arteries in vivo and canine basilar arteries in vitro.36,37 The superior transfection efficiency after adventitial transfection seen in the current study may be due in part to loss of endothelial cells on exposure to arterial blood flow as a result of grafting. In a study of in vivo intimal transfection of
rabbit jugular veins with adenovirus expressing \textit{E. coli} \(\beta\)-galactosidase, the vein reversed and grafted into the carotid artery and expressed half the amount of transgene product at day 4, as compared with the contralateral vein left in situ.\(^3\)\(^2\) Functional effect with enhanced NO-mediated relaxations has been demonstrated 4 days after in vivo adenoviral ecNOS gene transfer in uninjured rabbit carotid arteries transfected from within or without\(^3\)\(^6\),\(^3\)\(^8\) as well as after ex vivo transfection in large-animal coronary and basilar arteries.\(^1\)\(^3\),\(^3\)\(^7\) Diminished cell proliferation has been demonstrated in vitro in adenoviral ecNOS-transduced porcine coronary vascular smooth muscle cells\(^3\)\(^9\) and retroviral-mediated ecNOS transfection of rat aortic smooth muscle cells,\(^4\)\(^0\) as well as 4 days after balloon injury and Sendai virus (hemagglutinating virus of Japan) mediated in vivo gene transfer of ecNOS in rat carotid arteries.\(^1\)\(^3\) These findings have not so far been confirmed in vivo in large animals.

A significant reduction in cell proliferation was not observed on day 3 in spite of elevation in ecNOS mRNA levels and NOS activity. Preliminary experiments in our laboratory with nontransfected vein grafts in dogs demonstrated maximal cell proliferation on day 5. This is consistent with maximal cell proliferation observed in canine femoral vein grafts.\(^4\)\(^1\) Differences in cell proliferation with NOS transfection may be observed at other time points. Alternatively, functional transgene products in grafts in situ may be limited by posttranscriptional regulation of the enzyme by either substrate limitation or cofactor availability. The ability of ecNOS to produce NO depends on the availability of calcium, calmodulin, and tetrahydrobiopterin.\(^4\)\(^2\),\(^4\)\(^3\) The aim of this study was to assess feasibility and efficiency of gene transfer to vein grafts in a large experimental animal by means of a clinically relevant vein for graft material. Evaluation of the functional effects of ecNOS transfection in vein grafts may be more relevantly performed at later time points, with morphometric analysis of neointimal hyperplasia. For example, the migration of cells from the adventitia to the intima\(^4\)\(^4\) rather than proliferation may be a more relevant end point for determining transfection efficacy.

In summary, this study provides evidence for the feasibility of successful nonviral gene transfer in clinically relevant vein bypass grafts in large experimental animals. Ex vivo liposomal gene transfection of vein grafts is feasible and is more efficiently performed from the adventitia, which results in a significant increase in message and a near twofold increase in enzyme activity.

**REFERENCES**


Submitted Jul 16, 1999; accepted Apr 17, 2000.