Background  The efficacy of aorto-coronary vein grafting is limited by early graft thrombosis and accelerated graft atherosclerosis. Direct adenovirus-mediated transfer of genes encoding inhibitory proteins may prevent or slow progression of vein graft disease.

Methods and Results  Recombinant adenoviruses containing the cDNA for the marker gene lacZ (Ad.CMVlacZ) or soluble vascular cell adhesion molecule (sVCAM) (Ad.CBSVCAM) were used to infect segments of porcine jugular vein or human saphenous vein. Ex vivo testing showed expression of the introduced genes after incubation with Ad.CMVlacZ or Ad.CBSVCAM for periods from 1 to 24 hours, with an increase in transfection efficiency with increasing incubation time. Porcine jugular veins were then interposed as vascular grafts in the carotid arteries of four juvenile farm pigs after ex vivo gene transfer by incubation for 90 to 120 minutes with Ad.CMVlacZ or Ad.CBSVCAM. sVCAM-transfected carotid vein grafts were placed on one side and lacZ transfected veins were placed contralaterally as controls. Three days later, the vein graft segments were resected. Expression of the lacZ gene was confirmed by X-Gal chromagen staining and visualization by light and transmission electron microscopy. Gene expression was apparent in all layers of the vein graft wall, with prominent staining in the adventitia. sVCAM expression was confirmed by immunohistochemistry and in situ hybridization.

Conclusions  We conclude that ex vivo gene transfer before vein grafting is feasible using a replication-deficient recombinant adenovirus and results in a high level of gene expression in vivo. The potential for this approach to prevent early vein graft thrombosis or accelerated vein graft atherosclerosis requires further evaluation. (Circulation. 1994;89:1922-1928.)

Key Words  • gene transfer • adenoviruses • bypass grafts • atherosclerosis • Brief Communications

The long-term efficacy of aorto-coronary vein graft surgery remains limited by early graft thrombosis and the almost universal development of accelerated atherosclerosis and graft occlusion.1,3 Histological studies of resected arterialized vein grafts have suggested that intimal thickening occurs early in the postoperative period4,5 and may be preceded by the infiltration of mononuclear cells into the intima and media as early as the fourth postoperative day.6 These observations suggest that local inhibition of mononuclear cell recruitment may be an effective means of preventing foam cell formation, cytokine release, and late vein graft attrition from atherosclerosis. One potential means of achieving this is to block the binding of circulating monocytes to venous endothelium, a process mediated by the interaction of specific leukocyte receptors and adhesion molecules expressed at the endothelial cell surface.7 These molecules include vascular cell adhesion molecule (VCAM-1),7,8 a protein that is not expressed constitutively on cultured endothelial cells or on uninjured endothelium in vivo but that can be induced by a number of cytokines including tumor necrosis factor-α (TNF), interleukin-1, and bacterial lipopolysaccharide.9 VCAM-1 is also expressed by smooth muscle cells and macrophages in human atherosclerotic plaques.10 The leukocyte receptor for VCAM-1 is the integen very late activation antigen 4 (VLA-4) (or CD49d/CD29).11-14

We have characterized a soluble, secreted form of VCAM-1 that inhibits mononuclear cell binding to endothelial cells in vitro by competitive inhibition of the interaction of wild-type, cell surface VCAM-1, and the VLA-4 ligand of circulating monocytes and lymphocytes (unpublished data). Accordingly, this study was undertaken to determine whether genes encoding the soluble form of VCAM-1 (sVCAM), or the marker gene lacZ, could be introduced into the cells of a segment of vein before its placement as a graft and be expressed in vivo as a potential treatment for the prevention of vein graft disease. The findings of the study suggest that this approach is feasible using a recombinant adenovirus with high levels of gene expression in the early postoperative period.

Methods

Site-Directed Mutagenesis to Generate Recombinant sVCAM

VCAM cDNA was kindly provided by Dr L. Osborn (BioGen).8 An oligonucleotide (GACTATTTTCTCCCTTAAGTCTCTGTGC) spanning 2189-2213 of VCAM cDNA was synthesized; it encodes an inframe stop codon at the conjunction of the transmembrane and extracellular domains and a unique restriction enzyme site. Site-directed mutagenesis was performed using the Promega system. The mutation was confirmed by plasmid sequencing.
above. Two days after infection, the cells were metabolically labeled with \( ^{35}S \)-methionine (NEN) in 10% dialyzed FCS, methionine-free RPMI 1640 for 2 hours at 37°C. The conditioned medium and cell lyse were prepared and subjected to immunoprecipitation by incubation with mouse monoclonal anti-human VCAM-1 antibody (2 μg, 4B9) kindly provided by Dr. J. Harlan (University of Washington) and with sheep anti-mouse IgG antibody (3 μg) as secondary antibody, followed by protein A–agarose. The protein A–agarose immunocomplex was harvested by centrifugation, washed, resuspended in SDS-PAGE sample buffer with 0.1 mol/L DTT, and then separated with 8% SDS-PAGE. The photointensity of \( ^{35}S \) on the gel was enhanced by autorfluor (Amersham) according to the instructions provided by the manufacturer, and results were determined by autoradiography.

Ex Vivo Transfection of Human Saphenous and Porcine Jugular Veins

To determine the feasibility of direct gene transfer to cells of the vein wall and the incubation time before engraftment for optimal gene expression, segments of human saphenous vein obtained at the time of coronary artery bypass graft surgery and segments of porcine jugular veins were infected with Ad.CMVlacZ or Ad.CBsvCMV at 2 \times 10^{10} \text{ particles/mL} for 1 hour, 2 hours, or 24 hours. After infection, each vein segment was transferred to 10% FCS, 2.5 μg/mL amphotericin B, 10 U/mL penicillin, 10 mg/mL streptomycin, and RPMI 1640, incubated for 24 hours, and en face X-Gal staining was performed as described previously.

Immunohistochemical Staining

Fresh frozen sections (6 μm) of the infected vein segments were mounted on poly (L-lysine)-coated slides and fixed with cold acetone for 10 minutes. They were then rehydrated in PBS and incubated with 20% goat serum in PBS for 30 minutes to block nonspecific binding. Sections were incubated with 10 μg/mL 4B9 antibody for 45 minutes in 2% goat serum/PBS after three washes with PBS. Alkaline phosphatase-conjugated goat anti-mouse IgG antibody was applied and incubated for 45 minutes and was followed by three washes with PBS. Immunocomplex was detected by reaction with alkaline phosphatase substrate, nitro blue tetrazolium (NBT from BMB), bromo-chloro-indolyl-phosphate (BCIP) in 100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl₂, and 40 mmol/L levamisole (Sigma) to block endogenous enzyme activity. The reaction was terminated by the addition of 20 mmol/L EDTA in PBS after 10 minutes of incubation.

In Situ Hybridization

Riboprobe was synthesized with an in vitro transcription system from Promega using \( ^{35}S \)-CTP and \( ^{35}S \)-UTP to label the probe initiated from the T7 RNA promoters of a 500-bp \( HincII \)-EcoRI (nt 1104-1584) fragment of human VCAM cDNA subcloned into pGEM3 vector. Pretreatment of frozen tissue sections was performed as previously described. Hybridization was performed at 54°C for 17 hours by addition of 8 \times 10^{-6} \text{ cmol/mL} of riboprobe with prehybridization solution (10 mmol/L Tris, pH 8.0, 50% formaldehyde, 2.5×Denhardt's, 0.6 mol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 500 μg/mL Escherichia coli RNA, and 10 mmol/L DTT) and 10% dextran sulfate onto the tissue sections. Subsequently, the slides were rinsed to remove unbound probe and dehydrated gradually with ethanol as previously described before dipping in photoemulsion (Kodak). The results were analyzed by autoradiography with bright- and dark-field microscopy (Nikon Microphot-FXA microscope).

In Vivo Gene Expression of Transfected Vein Grafts

All in vivo studies were performed with the approval of the University of Michigan Committee on the Use and Care of

Adenoviral Gene Transfer to Jugular Vein Grafts

Cell Culture and In Vitro Gene Transfer

Low-passage (P3-P8) human umbilical vein endothelial cells (HUVEC), kindly provided by Dr. R. Marks (University of Michigan) were grown in 20% fetal calf serum (FCS), 100 μg/mL heparin (Sigma, H9133, bovine lung), 50 μg/mL endothelial cell growth supplement (Collaborative Biomedical Products), and M199 (Gibco). The cells were either stimulated with 10 ng/mL TNF (Biogen) for 8 hours to upregulate wild-type VCAM expression or infected with Ad.CMVlacZ and Ad.CBsvCMV at 1 \times 10^{11} \text{ particles/mL} in 2% FCS, DMEM for 90 minutes and then grown in the medium described.
Animals. After sedation with telazol (6 mg/kg body wt) and xylazine (2.2 mg/kg), juvenile farm pigs (mean weight, 16 kg) were intubated and anesthetized with 1% halothane; both carotid arteries and jugular veins were isolated through a midline ventral cervical incision. The jugular veins were ligated proximally and distally, and a 2- to 3-cm-long segment was resected and used as an interposition graft. The vein segments were exposed to Ad.CMVlacZ or Ad.CBsvCAM at 5 × 10⁷ particles/mL in 2% FCS, DMEM for 90 to 120 minutes at room temperature as described above. The lumen of the vein segment was held open during this period using a metallic rod to allow access of the solution to the luminal surface of the vein segment. Before implantation, the lumen of the vein was also flushed with transfection solution for 15 to 20 minutes. Each carotid artery was then clamped proximally and distally and was divided transversely. On one side, the vein segment exposed to Ad.CMVlacZ was placed as an interposition graft by end-to-end anastomosis with the cut ends of the carotid artery using 6-0 Prolene suture material. On the contralateral side, the vein exposed to Ad.CBsvCAM was placed in a similar manner, using a new set of instruments to avoid cross contamination. After ensuring adequate hemostasis, the cervical wound was closed in layers. Each animal received 150 mg oral aspirin for at least 3 days before surgery and daily during the postoperative period. Intravenous heparin (300 U/kg) was given intraoperatively. Three days after surgery, the animals were killed under general anesthesia, and the vein graft segments were resected and fresh frozen or fixed in 0.5% gluteraldehyde, 4% paraformaldehyde, or cold acetone for immunohistochemistry, immunofluorescence, in situ hybridization, transmission electron microscopy, and X-Gal staining as described above. Tissues were also obtained to determine the presence of introduced genes in other organ systems including the liver, spleen, myocardium, lung, kidneys, and gonads.

Results
Analysis of Recombinant Adenovirus-Infected Cells for Evidence of Secretion of sVCAM
To demonstrate the capability of Ad.CBsvCAM-infected HUVEC to secrete mutant protein, conditioned medium and lysate were prepared from adenovirus-infected cells that were labeled with ³⁵S-methionine. Expression of the introduced gene was detected by immunoprecipitation as presented in Fig 1. No VCAM gene product was detected in either lysate or conditioned medium isolated from unstimulated Ad.CMV-LacZ-infected cells. However, the mature, fully glycosylated forms of VCAM (MW, 110 and 100 kDa) were detected in the lysates but not in conditioned medium of HUVEC preincubated with TNF. High quantities of a form of VCAM with molecular weight of 90 kDa were detected in both lysates and medium of HUVEC cells infected with Ad.CBsvCAM.

Effect of Incubation Time on Efficiency of Ex Vivo Gene Transfer
To evaluate factors influencing the efficiency of infection of the recombinant adenovirus, segments of human saphenous and porcine jugular veins were infected with sVCAM or β-galactosidase-encoding adenovirus at
1 \times 10^{11} \text{ pfu/mL} \) for time courses ranging from 1 hour to overnight. Histochemical staining was performed at least 24 hours after infection to allow the transgene to be fully expressed. As shown in Figs 2A and D, no β-galactosidase activity (blue staining) was detected in the vein segments infected with Ad.CBsvCAM. In contrast, significant staining of the luminal surface and adventitia was apparent in the vessels infected with Ad.CMVlacZ for 1 hour (Figs 2B and E) or overnight (Figs 2C and 2F). The staining intensity was not significantly different between veins infected between 1 and 2 hours (data not shown) but was substantially higher in vessels incubated with virus overnight.

### In Vivo Expression of Transgene in Porcine Vein Grafts

The efficiency of gene transfer was evaluated by en block histochemical analysis of the vein grafts explanted 3 days after ex vivo transfection with Ad.CBsvCAM or Ad.CMVlacZ. No background X-Gal staining was apparent in the veins infected with Ad.CBsvCAM (Fig 3A). On the other hand, in Ad.CMVlacZ-transfected veins, a striped, unidirectional pattern of blue staining of the luminal surface and uniform blue discoloration of the adventitial surface were apparent in the en block tissue (Fig 3B). Positive signal was detected in cells of each layer of the vein wall, with a predominance in cells lining the luminal surface (Fig 3D) and in the adventitia (data not shown); no chromagen was apparent in the cells of veins infected with Ad.CBsvCAM (Fig 3C). Examination of infected vein segments by transmission electron microscopy confirmed the presence of X-Gal chromagen in the cytoplasm of endothelial cells and medial smooth muscle cells of veins infected with Ad.CMVlacZ (data not shown).

### Detection of sVCAM Gene Expression by Immunohistochemical Staining and In Situ Hybridization

Cells expressing the mutant soluble adhesion molecule were detected by immunohistochemical staining. The immunofluorescence staining used in the in vitro studies was unsuitable for in vivo studies because of interference by autofluorescence from elastin in the extracellular matrix. Tissue sections were therefore probed with anti-VCAM antibody followed by alkaline phosphatase-conjugated secondary antibody. Gene expression was recognized by the presence of the characteristic brown staining of the enzyme reaction product. Whereas only a uniform background staining was observed in the control vein infected with Ad.CMVlacZ (Fig 4A), vein graft segments infected with Ad.CBsvCAM clearly stained positively, particularly in cells at the luminal surface (Fig 4B). Because of the background of endogenous alkaline phosphatase staining (even in the presence of levamisole), it is difficult to determine whether sVCAM-expressing cells were present in the media or adventitia. To overcome this...
Problem and to confirm the results of immunohistochemical studies, in situ hybridization was performed using a VCAM-specific riboprobe. These studies showed that VCAM mRNA was not detectable in the control vein (Fig 4C) but that a high level of expression of VCAM transcript was observed in some of the cells at the luminal surface (Fig 4E) and in a large proportion of cells in the adventitia of the vein transduced with Ad.CBsVCAM (Fig 4G). Sections probed with sense RNA probe displayed no significant signal over background (data not shown). Tissues were also obtained at the time of sacrifice for analysis of lacZ gene expression in the liver and spleen, myocardium, lung, kidneys, and gonads. No evidence of expression of introduced gene was apparent in any of the nonvascular tissues analyzed. Similarly, at the site of engraftment, gene expression was confined to the venous segment, with no evidence of expression in the adjacent arterial wall.

**Discussion**

Somatic gene therapy is a potentially valuable approach for the treatment of certain inherited disease processes. Some acquired diseases such as atherosclerosis in arterialized vein grafts also might be amenable to gene transfer using genes encoding proteins that inhibit thrombus formation, lipid accumulation, or smooth muscle cell proliferation. Accordingly, this study was performed to determine the feasibility of gene transfer at the time of vein resection and engraftment. The findings of the study suggest that transfer of exogenous DNA into cells of the vein graft wall can be achieved with short incubation times at high efficiency levels using a replication-deficient adenovirus.

Adenoviruses have been used to transfect a variety of cell types both in vitro and in vivo and offer several advantages over other vectors. In contrast to retroviruses, adenoviruses do not require host cell replication for efficient genomic expression and may therefore be more suitable for treatment of the normal, uninjured vein wall in which cell turnover is extremely low. Adenoviral gene constructs can be produced in very high titers (10^12 pfu/mL), thus allowing even higher transfection efficiency. Relatively large foreign genes (up to 6-8 kb size) can be accommodated in the adenoviral genome. Since the adenoviral DNA does not appear to integrate into the host cell genome but remains episomal, there is minimal risk of insertional mutagenesis.

One principal limitation of adenoviruses may be the relatively short duration of expression of the introduced gene. Although not evaluated in this study, some investigators have noted a loss of gene expression within 4 weeks of gene transfer; others have noted stable expression for at least 12 months. In this study, efficient gene transfer was achieved using recombinant adenoviruses. In cell culture, endothelial cells incubated with Ad.CBsVCAM expressed sVCAM with a greater intensity than was achieved with TNF stimulation of wild-type VCAM expression, with an efficiency of almost 100%. Secretion of sVCAM by Ad.CBsVCAM-transduced cells was confirmed by analysis of conditioned medium. The feasibility of transfecting cells of a vein graft wall by ex vivo incubation was demonstrated using both Ad.CBsVCAM and Ad.CMVlacZ. Gene transfer and expression (ex vivo and in vivo) were achieved in porcine and human vein segments with incubation periods of as little as 1 hour, and very high levels of gene transfer were achieved with overnight incubation.

Cells expressing the introduced genes at the time of graft resorption were predominantly those lining the internal and external surfaces of the vein segment, with relatively low levels of gene expression apparent in the media. By transmission electron microscopy, the endothelium appeared intact and well preserved. In vein segments infected with ad.CMVlacZ, X-Gal staining was evident in the cytoplasm of both endothelial cells and medial smooth cells adjacent to the lumen. Considerably greater gene transfer to the luminal surface might be possible by continuous irrigation and dispersion of the vein segment at physiological pressures. Importantly, expression of the introduced genes was not apparent in any organ system remote from the vein graft site.

The genes selected for the study were the bacterial lacZ gene, used as a control marker gene, and a mutant form of the human VCAM gene encoding a soluble form of the molecule. Previous studies in our laboratory have shown that expression of the latter gene inhibits mononuclear cell binding to endothelial cells in vitro by competitive inhibition of the interaction between endothelial cell surface VCAM and the VLA-4 ligand of leukocytes (unpublished data). Conceivably, chronic local expression of the gene may inhibit mural lipid accumulation by blocking macrophage infiltration or transplant arteritis by blocking lymphocyte binding to arterial allografts. Whether the duration of gene expression after adenovirus-mediated gene transfer is sufficient to inhibit these chronic processes remains to be evaluated. Clearly, however, introduction of genes encoding proteins that inhibit thrombin formation or platelet adhesion might be effective in preventing early thrombotic graft closure even if only expressed for a period of several weeks.

In addition to the duration of gene expression and the selection of potentially therapeutic genes, several other questions must be answered before adenoviral gene transfer can be used routinely in the clinical arena. For example, does expression by the E1, E3-deleted adenovirus of virus proteins stimulate an immune response, and can it be rescued? Studies addressing these issues are currently ongoing. We believe, however, that the strategy of ex vivo gene therapy of interposition vein

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**Fig 4.** Facing page. Soluble vascular cell adhesion molecule (sVCAM) gene expression determined by immunohistochemical staining with VCAM antibody (A and B) and in situ hybridization (C, D, E, F, G, and H) with VCAM-specific probe on frozen sections 3 days after ex vivo infection and vein grafting. Intense enzyme reaction product (brown staining, arrow points toward the surface of the lumen) was detected in cells facing the lumen (indicated by L in the figure) of veins infected with VCAM-encoded virus (B) but not with the β-Gal-encoded virus (A). VCAM transcript was identified in the cells (probably endothelial cells) at the luminal surface (indicated by the arrow pointing toward the surface of the lumen) and in cells in the adventitia (shown as open arrows) in VCAM-virus-transduced veins (E, F, G, and H) but not in the contralateral control vein sections (C and D). The results were evaluated by both bright and dark field microscopy. M and A indicate media and adventitia, respectively, of the vessel wall. Bar represents 50 μm in A and B and 200 μm in C, D, E, F, G, and H.
grants outlined in this article is an attractive approach because gene transfer is accomplished in a specific vascular segment, away from the patient, in a setting that is clinically practical.

References

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