The Role of Gene Therapy for Intimal Hyperplasia of Bypass Grafts

David G. Cable, MD; James A. Caccitolo, MD; Noel Caplice, MD, PhD; Timothy O’Brien, MD; Robert D. Simari, MD; Richard C. Daly, MD; Joseph A. Dearani, MD; Charles J. Mullany, MD; Thomas A. Orszulak, MD; Hartzell V. Schaff, MD

Background—Proliferation of the intima is an early lesion of saphenous vein graft disease. Early patency rates of radial artery grafts are acceptable, but little is known about their risk of intimal hyperplasia.

Methods and Results—To develop a model of intimal hyperplasia, we incubated human saphenous veins, internal mammary arteries, and radial arteries (n=6, 8, and 10, respectively) in an organ culture with Rosewell Park Memorial Institute 1640 (30% serum) for 0, 4, 7, 10, and 14 days. Quantitative histological studies were performed, and the average intimal-to-medial (I/M) ratio was calculated for each incubation interval. After 10 and 14 days of culture, the I/M ratio increased in the saphenous veins (P<0.03, P<0.04 versus 0 day, respectively). No significant increase occurred in the I/M ratio in either the internal mammary or radial arteries. Next, the ability of adenoviral gene transfers to inhibit intimal hyperplasia in the saphenous veins was evaluated. Adenoviral-mediated gene transfer of nitric oxide synthase significantly reduced the I/M ratio at 14 days compared with vehicle (P<0.001) and virus (P<0.004) controls.

Conclusions—The human saphenous vein has a greater propensity for intimal hyperplasia than arterial grafts; the human radial artery behaves similarly to the internal mammary artery. In the future, gene therapy may augment nitric oxide synthase, limiting vein graft disease. (Circulation. 1999;100[suppl II]:II-392–II-396.)

Key Words: gene transfer • mammary arteries • nitric oxide • organ culture • radial artery • saphenous vein

Compared with saphenous vein (SV) grafts, internal mammary artery (IMA) grafts to the left anterior descending coronary artery improve the survival of patients with multivessel disease; this improved outcome correlates with better long-term patency.1 Recently, there has been renewed interest in the radial artery (RA) for coronary artery bypass in the hope that it will expand the range of arterial grafts for surgical revascularization.2 However, the long-term patency of RA conduits will not be known for nearly a decade, and little information is available about the biological behavior of these bypass conduits in regard to early thrombosis and the development of intimal hyperplasia and atherosclerosis.

The SV is the most commonly used conduit for coronary surgery, but despite improvements in methods of harvest, preservation, and early antithrombotic therapy, 12% to 27% of vein grafts become occluded in the first year, one-half of which are within the first month.2,3 The subsequent annual development of intimal hyperplasia and atherosclerosis.

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A representative section from each specimen was evaluated by trypan blue exclusion to determine endothelial cell viability. Sections were incubated at room temperature in 0.01% trypan blue in Dulbecco’s phosphate-buffered saline for 1 minute. All specimens demonstrated <10% endothelial disruption; thus, no specimens were discarded.

Organ Culture

Graft segments were sharply dissected in a laminar flow hood to remove any remaining connective tissue. The grafts were incised so the luminal surface was exposed and divided into 1-cm lengths. The segments were cultured by a modification of the method described by Pederson and Bowyer. Vessels were divided into matched specimens; each portion was then mounted (with the adventitial surface adjacent to P500 polyester cloth) to sterile Sylgard resin (Dow Corning) with minute pins. After fixation, the specimens were immediately overlaid with 6 mL of a culture medium of the following composition: RPMI 1640 medium containing 2 g/L sodium bicarbonate, 30% (v/v) fetal bovine serum, 100 U/mL (final) penicillin, 100 U/mL (final) streptomycin, and 0.8 mmol/L L-glutamine (temperature, 37°C; pH 7.4).

Cultures were performed in a humidified, 5% CO2 incubator at 37°C (Forma Scientific, Inc). The culture medium was aspirated under sterile conditions and replaced every other morning. The same lot of fetal bovine serum was used for the entire study.

Histologic Examination

Segments were removed from the organ culture, fixed in buffered formalin, embedded in paraffin, and cut transversely into 3-μm-thick sections, which were stained with Verhoef–van Gieson stain for elastic fibers. The areas of the intima and media were averaged over the entire length of each specimen using an image analysis system consisting of a microscope (Nikon, OPTIPHOT-2) with a solid-state charge-coupled device color video camera mounted on the phototube. The video signal underwent 24-bit digitalization by a video frame grabber. The signal was then digitized using a Scion color digitizer board incorporated in an ImagePro Plus image analysis system. Color images were stored and analyzed as 24-bit files with a 640 (horizontal) and 480 (vertical) pixel resolution.

Separate samples were snap-frozen (−70°C) in OCT compound, and 5-μm-thick sections were stained for eNOS and proliferating nuclear cell antigen, as previously described. The number of positively stained cells was counted with standard light microscopy and normalized to the product of the intimal and medial areas for the entire specimen.

Gene Transfer

Paired human SVs were randomly assigned to 3 groups. The first group was placed in phosphate-buffered saline with 0.1% albumin (PBSA, dilution vehicle) alone. The second group was exposed to recombinant adenovirus-encoding *E. coli 2β-galactosidase (Ad.CMVLacZ) for 1 hour at 37°C, as previously described. The third group was exposed to adenovirus-encoding bovine eNOS (Ad.CMVeNOS) at the same titer. The vein segments were incised so the luminal surface was exposed; they were then divided into 1-cm lengths. The segments were cultured as described above.

Data Analysis

Results are expressed as mean±SEM. In all experiments, n is the number of patients from whom vessels were taken. Vessels incubated for each period were obtained from the same patient; analyses between incubation durations used the 2-way t test for paired samples. The SV, IMA, and RA samples were obtained from different patients, and analysis between graft types used the 2-way test for unpaired samples. SVs exposed to recombinant adenoviruses were obtained from the same patients and, thus, analysis used the 2-way t test for paired samples. Values of P<0.05 were considered significant.

Organ culture (days)

**Figure 1.** I/M ratio (averaged over entire length of each specimen using image analysis system) for SV, IMA, and RA by days in organ culture. Note progressive increase in ratio in SV but not in IMA or RA. Values are mean±SEM. n refers to number of vessels.

**Results**

Mean intimal and medial thicknesses of human SVs before culture were 36.5±12.4 and 310.0±32.4 μm, respectively; mean intimal and medial thicknesses of human IMAs before culture were 13.2±3.8 and 137.5±36.4 μm, respectively; and mean intimal and medial thicknesses of human RAs before culture were 16.9±7.0 and 213.1±73.7 μm, respectively. The control intima-to-media (I/M) ratios for SVs, IMAs, and RAs were 0.12±0.04, 0.09±0.01, and 0.07±0.01, respectively.

**Intimal Hyperplasia With Organ Culture**

Human SVs demonstrated progressively greater I/M ratios with extended culture, which were first significant after 10 days (Figure 1). The control I/M ratio of SV segments was 0.12±0.04, and this increased to 0.28±0.05 after 10 days (P=0.03) and to 0.36±0.11 after 14 days (P=0.04). In contrast, after 14 days, the IMA I/M ratio increased to only 0.15±0.04 (P=0.20 versus control) and the RA I/M ratio, to 0.09±0.01 (P=0.15 versus control).

Previous studies suggested that the resulting intimal hyperplasia in extended organ culture is derived predominantly from the cut edges. The I/M ratios reported above represent the composite average of the entire specimen to eliminate sampling error. A subgroup analysis was performed of the I/M ratios within 1 optical field of the cut surface and the sample middle. For 7-day culture of SVs, the cut (0.25±0.05) and middle (0.22±0.10) I/M ratios were similar (P=0.96); these ratios were not significantly different (0.43±0.10 versus 0.67±0.23; P=0.65) at 14 days. Although some specimens had greater intimal hyperplasia at the cut surface, the subgroup analysis did not support the hypothesis of predominant growth in this region.

**Adenoviral-Mediated Gene Transfer and Intimal Hyperplasia**

Initial studies (n=6) were performed at a viral titer of 10^{10} plaque-forming units per milliliter (pfu/mL). After 14 days of incubation, intimal hyperplasia was reduced in Ad.CMVeNOS veins compared with PBSA controls. However, a concomitant reduction occurred in the I/M ratio of
Ad.CMVLacZ veins, suggesting that overexpression of a recombinant gene indiscriminately reduced intimal hyperplasia in human SV. Vehicle PBSA controls had an I/M ratio of 0.20±0.08 at 7 days and 0.30±0.07 at 14 days. Viral Ad.CMVLacZ controls had an I/M ratio of 0.12±0.03 at 7 days and 0.15±0.04 at 14 days, and the ratio for Ad.CMVeNOS veins was 0.11±0.04 at 7 days and 0.13±0.02 at 14 days.

Therefore, additional studies (n=7) were performed at a lower viral titer (10⁹ pfu/mL). Vehicle PBSA controls had an I/M ratio of 0.23±0.06 at 7 days of incubation and 0.39±0.09 at 14 days (Figure 2), and the ratio for viral Ad.CMVLacZ controls was 0.24±0.09 at 7 days and 0.30±0.03 at 14 days. The difference between vehicle and viral controls for either period of incubation was not significant (P=0.95 and 0.13, respectively). Human SVs exposed to Ad.CMVeNOS had an I/M ratio of 0.22±0.10 at 7 days of incubation (P=0.85 versus PBSA and P=0.83 versus Ad.CMVLacZ). However, the I/M ratio of eNOS veins decreased to 0.13±0.06 after 14 days (P=0.001 versus PBSA and P=0.004 versus Ad.CMVLacZ).

Immunohistochemical studies with a monoclonal antibody specific for eNOS demonstrated discrete, high-level expression of eNOS in the endothelium and adventitia of Ad.CMVeNOS veins, which was similar to the findings in previous reports. Minimal eNOS immunostaining was noted in PBSA and Ad.CMVLacZ veins, which correlated with the known low-level endothelial expression.

Immunohistochemical studies for proliferating nuclear cell antigen and Ki67 demonstrated low-level expression of each proliferation index after extended organ culture. This correlates with the results of previous reports.

**Discussion**

The major findings of the present study are that (1) the human SV, but not the IMA, generates intimal hyperplasia during extended organ culture; (2) the human RA does not produce significant intimal hyperplasia during extended organ culture; and (3) adenoviral-mediated gene transfer of eNOS inhibits intimal hyperplasia in the human SV.

The original study of extended organ culture used rabbit aortas to examine the endothelial response to injury. This model consistently produces neointimal overgrowth by smooth muscle cell migration, and it is devoid of fibroblast contamination. Intimal proliferation of human SV in organ culture is dependent on surgical preparation, production of vasoactive substances, and presence of growth factors. The advantages of this preparation include the manner in which human vessels can be maintained in controlled and reproducible conditions to analyze specific interventions. Human tissue can be manipulated ex vivo in a manner that would require animal studies for an in vivo setting.

However, as with all models, limitations exist. Although the human tissue underwent the usual surgical manipulation, the preparation does not duplicate the clinical setting because the system is without immunologic, cytokine, and hemodynamic influences. Despite these shortcomings, the model seems to be a good alternative to in vivo studies.

The specimens harvested in the present study correlate well with previously published morphometric data. The mean intimal and medial thicknesses of SV were similar to the 100 consecutive veins examined by Varty et al. The greater wall thickness in RAs than in IMAs correlated well with the results of van Son et al., except that the RA specimens in our series showed less of a differential.

**RA Grafts**

Reasonable early and midterm patency rates have been reported recently for RA grafts. Rates have ranged from 87.8% to 95.7% during the first 18 months. The reported 5-year patency rates range from 83% to 92%. Although these values are favorable compared with those for saphenous vein grafts, skepticism is still justified on the basis of the earlier history of RA grafts.

Although areas of preexisting disease have previously been noted, the average I/M ratio of RAs in our study was not different from that of other grafts. Organ culture of up to 14 days was not associated with significant intimal hyperplasia. This is in contrast to the behavior of SVs; the I/M ratio in veins nearly tripled during the same duration of incubation. The behavior of the RA in the current study, which was similar to that of the IMA, supports the continued, cautious, clinical use of this conduit.

**Gene Therapy for SV Graft Disease**

Neointimal hyperplasia is the ubiquitous response of the vasculature to injury; it results in a lesion that can be flow-limiting and consists of secretory vascular smooth muscle cells and extracellular matrix. The pathophysiology of neointimal hyperplasia after vascular injury has been studied extensively. In brief, endothelial injury and denudation permit platelet adhesion and aggregation during harvest or surgical exposure. The adherence of platelets to the endothelial surface is also likely due to decreased production of prostacyclin and nitric oxide concomitant with endothelial dysfunction. This is followed by stimulation of vascular smooth muscle cells to proliferate and to migrate to the intima. The smooth muscle cells undergo a phenotypic
change, from contractile to secretory cells, and extracellular matrix accumulates. Platelet activation provokes the release of platelet-derived growth factor and basic fibroblast growth factor. Both factors, present in the fetal calf serum used for our culture preparation, stimulate the migration of vascular smooth muscle cells from the media into the intima and the synthesis of extracellular matrix, collagen, and proteoglycans.23

Our preparation of human SVs consistently produced intimal hyperplasia during 2 weeks of culture. The viral LacZ controls had a slightly less robust intimal hyperplasia than vehicle controls. Newman et al24 noted that intimal hyperplasia was markedly stimulated with an adenoviral vector encoding for LacZ in rabbit arteries. However, it seems that gene therapy elicits an immunologic response to either transgene expression or low-level viral protein expression on the cell membrane.25 In extended organ culture, no immunologic response was permitted; the culture media was acellular. Therefore, an intriguing suggestion from the present study is that highly promoted overexpression of a transgene, in and of itself, may tend to inhibit intimal hyperplasia.

Augmented nitric oxide levels inhibit the intimal hyperplasia of grafts. Von der Leyen et al26 first demonstrated that gene transfer of eNOS inhibited intimal thickening in rat carotid arteries after angioplasty. Immunohistochemical examination demonstrated increased expression of eNOS with adenoviral-mediated gene transfer; this is similar to findings we have reported previously in animal and human tissue.8,9,27 That eNOS overexpression inhibited SV hyperplasia is not entirely unexpected. Soyombo et al16 demonstrated that the 8-bromocyclic guanosine monophosphate analogue inhibited SV neointimal hyperplasia by 40%. What is remarkable is that the I/M ratio was similar for all groups at 7 days of incubation. It was not until after 14 days that a significant difference was observed between the groups. This may be that the I/M ratio was similar for all groups at 7 days of culture. It was not until after 14 days that a significant difference was observed between the groups. This may be explained partly by the kinetics of guanosine triphosphate during organ culture.15 Nitric oxide stimulates the conversion of guanosine triphosphate to cyclic guanosine monophosphate by guanylate cyclase. Immediately after surgical preparation, the level of guanosine triphosphate decreases significantly and slowly begins to increase during the subsequent 14 days of incubation.

Recently, others have demonstrated reduced intimal hyperplasia in human SV in organ culture with gene therapy.28 Adenovirus-mediated gene transfer of the tissue inhibitor metalloproteinase 1 inhibited neointimal formation by 54% and smooth muscle cell migration by 78%. The viability of human SV in extended organ culture after exposure to the current generation of adenoviral vectors supports the role of this model in preclinical testing of coronary artery bypass conduits.

Gene therapy has attracted attention for its potential to treat several cardiovascular disease processes. However, adenoviral vectors can elicit an immunologic response as a result of a viral product or transgene expression, and this immunologic response may increase the intimal hyperplastic reaction above baseline levels. Despite these possible deficiencies, gene therapy may be useful in the prevention of coronary artery bypass graft disease.

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References


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