

Circulation

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Graft Permeabilization Facilitates Gene Therapy of Transplant Arteriosclerosis in a Rabbit Model

Mark D. Rekhter, Nikhil Shah, Robert D. Simari, Carolyn Work, Jong-Seung Kim, Gary J. Nabel, Elizabeth G. Nabel and David Gordon

Circulation 1998;98;1335-1341

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214
Copyright © 1998 American Heart Association. All rights reserved. Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circ.ahajournals.org/cgi/content/full/98/13/1335>

Subscriptions: Information about subscribing to *Circulation* is online at
<http://circ.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
<http://www.lww.com/reprints>

Graft Permeabilization Facilitates Gene Therapy of Transplant Arteriosclerosis in a Rabbit Model

Mark D. Rekhter, MD, PhD; Nikhil Shah, BS; Robert D. Simari, MD; Carolyn Work, BS; Jong-Seung Kim, DDS; Gary J. Nabel, MD, PhD; Elizabeth G. Nabel, MD; David Gordon, MD

Background—Smooth muscle cell (SMC) replication plays a central role in the pathogenesis of transplant arteriosclerosis. One strategy to eliminate dividing cells is to express a herpesvirus thymidine kinase (*tk*) gene that phosphorylates the nucleoside analogue ganciclovir into a toxic form leading to cell killing. However, medial SMCs are resistant to gene transfer unless the artery undergoes deendothelialization. We hypothesized that manipulations that increase the “porosity” of the artery can make SMCs prone to gene transfer without denudation.

Methods and Results—In organ culture of rabbit aorta, longitudinal stretch and supraphysiological pressure applied for 3 hours during incubation with adenoviral vector facilitated gene transfer into medial SMCs without denudation. Of the SMCs, $10.2 \pm 3.8\%$ expressed a reporter gene of human placental alkaline phosphatase (hpAP), whereas SMCs in control arteries did not express hpAP. To evaluate the feasibility of transgene expression in arterial grafts, we performed such permeabilization-assisted reporter gene transfer into aortas of donor Dutch Belted rabbits and transplanted them into carotid arteries of recipient New Zealand White rabbits. Unstretched transfected grafts were used as a control. SMCs expressed hpAP ($7.3 \pm 2.4\%$ of cells in 2 days and $4.2 \pm 1.9\%$ in 2 weeks) in stretched grafts only. In the next series of experiments, we transfected stretched grafts with ADV-*tk* and combined transplantation with systemic administration of ganciclovir. Stretched ADV-hpAP grafts were used as a control. In 2 weeks, the formation of intimal thickening in *tk*-expressing grafts was significantly reduced ($P < 0.01$) because of a decrease in proliferating SMCs.

Conclusions—Manipulations within target tissues can enhance the efficiency of gene transfer into SMCs. Although mechanical permeabilization is clinically problematic, in principle, targeting SMC replication may provide a genetic approach to the treatment of transplant arteriosclerosis. (*Circulation*. 1998;98:1335-1341.)

Key Words: arteriosclerosis ■ genes ■ muscle, smooth ■ transplantation

Transplant arteriosclerosis is a thickening of the intima that develops in the arteries of transplanted organs.^{1,2} Smooth muscle cell (SMC) replication plays a central role in its pathogenesis.³⁻⁵ One strategy to selectively eliminate dividing cells is to express a herpesvirus thymidine kinase (*tk*) gene, which phosphorylates the nucleoside analogue ganciclovir into a toxic form that leads to cell killing.^{6,7}

The *tk* gene, as well as the other genes being used to inhibit in vivo SMC proliferation, encodes a protein with nuclear localization, which normally cannot be secreted.⁸ Therefore, such therapy might work only if the transgene is expressed in SMCs. However, most of the medial SMCs are resistant to gene transfer unless the artery wall undergoes mechanical injury, such as occurs with balloon angioplasty.⁹⁻¹² At the same time, preservation of vascular endothelium is required for successful organ transplantation, and therefore, gene transfer into medial SMCs should not entail deliberate endothelial denudation.

We hypothesized that the endothelium and/or extracellular connective tissue may limit viral vector penetration to medial

SMCs and that manipulations that increase the porosity of the artery can make those cells prone to gene transfer. Accordingly, the purpose of this study was 2-fold: to achieve transgene expression in arterial SMCs without significant or irreversible endothelial damage and to investigate whether the proliferative response to allogenic arterial transplantation can be inhibited by local expression of a *tk* gene followed by ganciclovir administration.

Methods

Recombinant Adenoviral Vectors

Three replication-deficient, recombinant adenoviral vectors were constructed and purified as previously described. These vectors were prepared from the adenovirus 5 serotype and contain deletions in the E1 and E3 regions, rendering them replication-deficient. The 3 adenoviral vectors (ADV) included a vector encoding herpesvirus thymidine kinase (ADV-*tk*), driven by a polyoma virus promoter and enhancer and containing an SV40 polyadenylation sequence. An adenoviral vector lacking a cDNA insert, ADV- Δ E1, was used for control experiments. A third adenoviral vector, ADV-hpAP, encodes for a human placental alkaline phosphatase (hpAP) reporter gene

Received January 21, 1998; revision received April 7, 1998; accepted April 16, 1998.

From the Departments of Pathology (M.D.R., N.S., R.D.S., C.W., J.-S.K., D.G.), Internal Medicine (R.D.S., G.J.N., E.G.N.), and Physiology (E.G.N.), and Howard Hughes Medical Institute (G.J.N.), University of Michigan, Ann Arbor.

Correspondence to David Gordon, MD, Vascular and Cardiac Diseases, Parke-Davis Pharmaceutical Research Division, 2800 Plymouth Rd, Ann Arbor, MI 48105. E-mail gordond@aa.wl.com

© 1998 American Heart Association, Inc.

driven by a β -actin promoter and CMV enhancer with an SV40 nsf in Organ Culture of Rabbit Aorta

Abdominal aortas ($n=9$) were obtained from intact New Zealand White rabbits fed a regular rabbit chow. Before the animals were euthanized, intravenous heparin was infused (300 U/kg). The abdominal aorta was exposed, and both iliac and renal arteries were cannulated. All other side branches were ligated. Through these ports, the aorta was perfused *in situ* with culture medium and ligated. Then 2 ligatures were placed around the central part of the abdominal aorta (distal to the renal arteries) to divide it into 2 halves. The ends of the isolated segments were tied to surgical retractors (to prevent longitudinal contraction of aortic tissue). Every segment was attached to its own retractor. Later, the aorta was cut into 2 halves between retractors. Both segments were then excised and transferred with the retractors into separate Petri dishes containing culture media. To increase the porosity of arterial wall, longitudinal stretch was applied to the aortic segments. The retractors were spread to 1.5 times the original length of aortic segment, and arteries were kept stretched for 3 hours. ADV-hpAP solution (2 mL; 2×10^9 pfu/mL) was infused into the lumen via the cannula, and the solution dwelled for 3 hours (dwell time) under a pressure of 150 mm Hg. Control aortic segments were pressurized and incubated with the vector as described above but did not undergo longitudinal stretch. To control for the specificity of gene expression, 3 aortas were transfected with ADV- $\Delta E1$. The aortic segments were washed with media and cut into rings 3 to 4 mm thick. Aortic rings were then transferred into 24-well plastic dishes, maintained in the culture medium (medium 199, 10% FCS, 1% glutamine, and 1% penicillin-streptomycin) for 2 days, and fixed in 10% formalin.

Transplantation of Transfected Rabbit Aorta

Transplantation of aortic segments from Dutch Belted donors to carotid arteries of New Zealand White rabbits was performed.

Diet

Cholesterol feeding was added for the recipient rabbits (0.5% cholesterol diet supplemented with 2% peanut oil, provided in 4-oz/d aliquots). It was started 1 week before the transplantation and continued until euthanasia. Before surgery and at euthanasia, serum cholesterol and triglyceride levels were measured (Roche Biomedical Laboratories).

Gene Transfer

Dutch Belted rabbits were anesthetized with ketamine (35 mg/kg IM) and xylazine (5 mg/kg IM). The abdominal aorta was harvested as described above. The group 1 experiments (Table 1) were designed to study the fate of the cells transfected with reporter gene in a transplantation environment *in vivo*. To eliminate variability potentially introduced by systemic factors, we harvested the abdominal aorta from a donor rabbit, split it into 2 halves, and transfected 1 half with ADV-hpAP without longitudinal stretch (group 1A). The other half was transfected with the same vector, but longitudinal mechanical stretch was applied during incubation with adenovirus (group 1B). Then these transfected grafts were transplanted into left and right common carotid arteries, respectively, of the same recipient rabbit. The only difference between the grafts was the presence or absence of longitudinal stretch during incubation with ADV-hpAP.

The group 2 experiment (Table 1) was designed to explore whether the *tk*-ganciclovir treatment can inhibit the development of

transplant arteriosclerosis. The abdominal aorta of a donor rabbit was attached to wound retractors and split into 2 halves. In short-term organ culture, the retractors were spread to 1.5 times the original length of aortic segment, and arteries were kept stretched for 3 hours. One half of the aortic graft was mock-transfected with ADV-hpAP (group 2A), and the other half was infected with the ADV-*tk* (group 2B). Viral solution (2 mL; 2×10^9 pfu/mL) was infused into the lumen of the stretched arteries at a pressure of 150 mm Hg.

Transplantation surgery was performed similarly to the method of Bowyer and Reidy.¹⁵ Recipient animals were anesthetized with ketamine and xylazine as described above and heparinized. A midline cervical skin incision was performed, strap muscles were bluntly dissected, and both left and right common carotid arteries were exposed. Aortic grafts were placed into both carotid arteries of the recipient by a cuff anastomosis method (sterile Teflon cuffs 4 mm long over which the arteries are stretched and joined with a single circumferential suture).

Drug Treatment

Immune rejection of the aortic grafts (in both groups 1 and 2) was suppressed by the administration of cyclosporine ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ SC) beginning at the time of transplantation. To inhibit cell proliferation in the transplant (group 2 only), ganciclovir ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) was administered for 7 days beginning at day 7 after the transplantation. This therapeutic window was chosen because according to Tanaka et al¹⁴ and our own pilot studies (data not shown), proliferation of medial SMCs in arterial grafts starts in 1 week after the transplantation, and in 2 weeks intimal thickening is detectable.

Animal Euthanasia

To analyze reporter gene expression in transplanted arteries, animals of group 1 were euthanized by administration of intravenous sodium pentobarbital (120 mg/kg) 2 days and 14 days after surgery (Table 1). To assess the therapeutic efficiency of a *tk* gene, animals of group 2 were euthanized 14 days after transplantation (Table 1). To label proliferating cells, these rabbits received an intravenous infusion of 5-bromo-2'-deoxycytosine (BrdC, Sigma) 1 hour before death. Aortic transplants were harvested, and each graft was cut into 6 rings ≈ 3 mm wide. Rings 1, 3, and 5 were fixed in 10% formalin, and rings 2, 4, and 6 were fixed in methacarn (methanol:chloroform:acetic acid). Formalin-fixed material was used for the histochemistry, and methacarn-fixed samples were collected for immunocytochemistry.

Histochemistry

Expression of recombinant hpAP protein was detected by histochemical analysis. The slides were incubated in PBS at 65°C for 30 minutes to inactivate endogenous alkaline phosphatase and then in PBS containing a chromogenic substrate of 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine (1 mg/mL, Gibco BRL) and nitro blue tetrazolium chloride (1 mg/mL, Gibco BRL) for 19 hours. This substrate yields a dark purple-to-brown stain in the presence of alkaline phosphatase.

Triple-label immunocytochemistry was used to analyze proliferation among different cell types by techniques that we have described earlier in detail.¹⁵ To visualize BrdU-positive cells, the cell proliferation kit (Amersham) was used. The cell type-specific antibodies used on these tissues were (1) an anti-smooth muscle α -actin antibody (Boehringer Mannheim) to identify SMCs and (2) RAM11 (DAKO) to identify macrophages.

TABLE 1. Design of Transplantation Experiments

Group	n	ADV-hpAP	ADV- <i>tk</i>	Longitudinal Stretch	Cholesterol Feeding	Cyclosporine	Ganciclovir	2 d	14 d
1A	10	+	-	-	+	+	-	+	+
1B	10	+	-	+	+	+	-	+	+
2A	6	+	-	+	+	+	+	-	+
2B	6	-	+	+	+	+	+	-	+

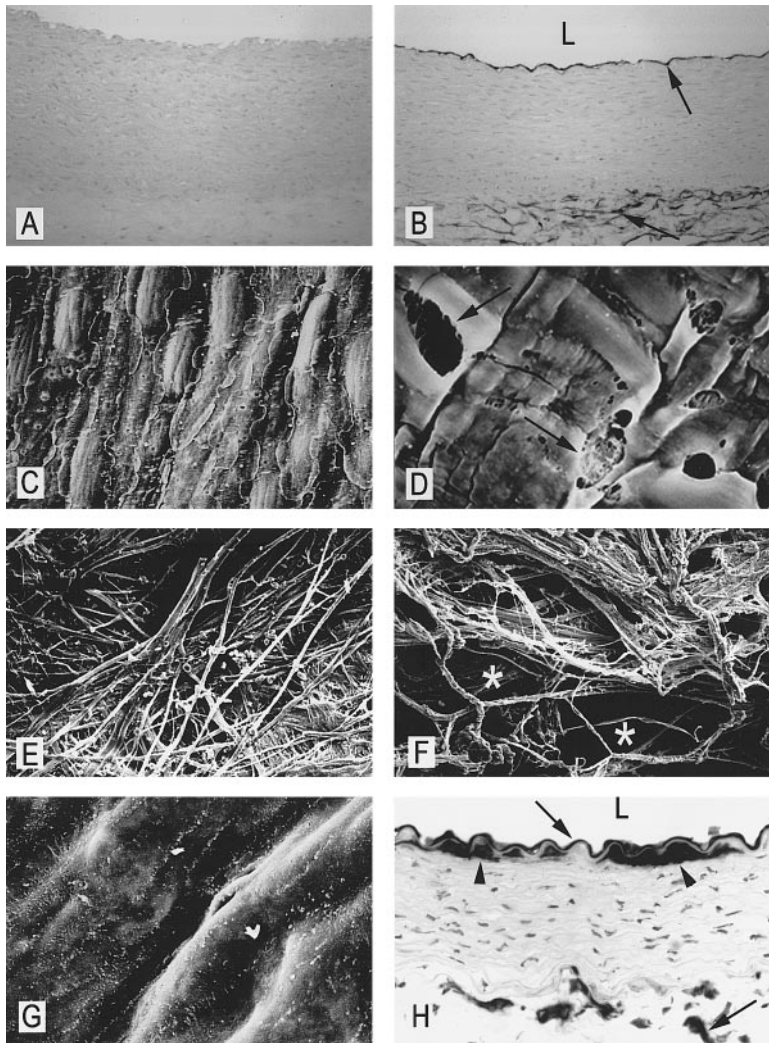


Figure 1. Optimization of gene transfer conditions in organ culture of rabbit aorta. A, Alkaline phosphatase histochemical reaction shows no staining in mock-transfected (ADV- Δ E1, 2×10^9 pfu/mL for 3 hours) rabbit aorta, thus demonstrating specificity of assay. B, Pattern of hpAP expression in organ culture of normal aorta incubated with ADV-hpAP at optimal titer (2×10^9 pfu/mL) for 3 hours. Luminal endothelial cells and adventitial cells express transgenic hpAP (dark product of histochemical reaction, arrows). C, Normal rabbit aorta in organ culture. Scanning electron microscopy of luminal surface shows tightly packed, overlapping endothelial cells. Magnification $\times 1500$. D, Longitudinally stretched segment of rabbit aorta. Scanning electron microscopy reveals formation of gaps between luminal endothelial cells (arrows). Magnification $\times 1900$. E, Tight meshwork of connective tissue fibers in subendothelium of normal rabbit aorta in organ culture. Scanning electron microscopy, magnification $\times 400$. F, Formation of wide gaps (*) between subendothelial connective tissue fibers in longitudinally stretched rabbit aorta. Scanning electron microscopy, magnification $\times 400$. G, Luminal endothelium restores its integrity after release of stretch and pressure. Scanning electron microscopy, magnification $\times 1500$. H, Pattern of transgene hpAP expression in organ culture of rabbit aorta incubated with ADV-hpAP for 3 hours under stretch and pressure. Luminal endothelial cells and adventitial cells express transgenic hpAP (dark product of histochemical reaction, arrows), as in intact aortas. In addition, medial SMCs show reporter gene expression (arrowheads). Alkaline phosphatase histochemical reaction. Methyl green nuclear counterstain. Objective $\times 20$. L indicates lumen.

Scanning electron microscopy was used to evaluate intimal porosity in the stretched arteries. Cultured aortic segments were perfused with PBS and then with 2.5% glutaraldehyde. Specimens were dehydrated and air-dried from hexamethyldisilazane (Sigma) and sputter-coated with gold. To visualize subendothelial connective tissue structures, fixed samples were incubated in potassium ethoxide (30% KOH and 96% ethanol, 1:1) until endothelial cells detached, as we described previously.¹⁶

Morphometric Analysis

To evaluate the efficiency of gene transfer into rabbit aorta, the specimens stained for alkaline phosphatase were studied by light microscopy at $\times 400$ magnification with the Image 1 system of computer image analysis (Universal Imaging Corp). Contiguous, nonoverlapping microscopic fields covering the whole tissue on the slide were analyzed. Each field was scored for the total number of nuclei and the number of alkaline phosphatase-positive cells. The average index per sample was then used to calculate the statistical comparisons between different groups.

Measurements of intimal and medial cross-sectional area 14 days after transplantation were determined by 2 independent reviewers in a blinded fashion on 4 sections from each aortic graft with the image analysis system. Intimal boundaries were determined by digital planimetry. The entire circumference of neointima and media was measured at $\times 40$ magnification. Average intimal and medial areas were obtained from the 4 measurements per observer and then between the 2 observers. In addition, intima/media ratios were

calculated for each individual measurement and then averaged in the same fashion.

To evaluate proliferation of different cell types, the ratio of double-labeled cells (BrdU-incorporated SMCs and macrophages) to the total number of cells was calculated. Contiguous, nonoverlapping microscopic fields covering the whole section were analyzed.

Statistical Analysis

All values are expressed as mean \pm SEM. Average percentages of hpAP-positive cells in each group of blood vessels in organ culture were compared by 2-tailed, unpaired Student's *t* test. Comparisons of percentages of hpAP-positive cells, as well as intimal areas and BrdU labeling indices in aortic transplants, were made by 2-tailed, paired *t* test, because pairs of experimental and control grafts were always obtained from the same donor and transplanted into the same recipient.

Results

Optimization of Gene Transfer Into Normal Rabbit Aorta in Organ Culture

The alkaline phosphatase histochemical reaction showed no staining in the mock-transfected (ADV- Δ E1) aortas (Figure 1A). Staining of the specimens incubated with ADV-hpAP for 3 hours revealed that all endothelial cells and variable numbers of adventitial cells expressed hpAP. However, no

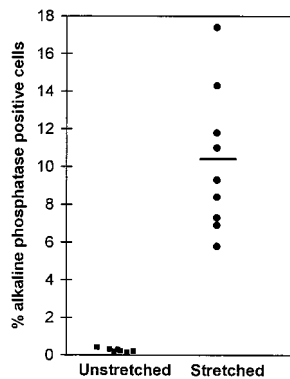


Figure 2. Percentage of hpAP-expressing medial SMCs in longitudinally stretched and pressurized vs untreated rabbit aortas, 2 days in organ culture.

medial SMCs (located centrally) showed alkaline phosphatase positivity (Figures 1B and 2).

Most likely, luminal endothelium and/or underlying connective tissue structures limited direct interaction between the vector and the target SMCs. We hypothesized that longitudinal mechanical stretch would induce the formation of transient holes between endothelial cells as well as between any connective tissue fibers, and intraluminal pressure would propel viral particles through these holes. To test this hypothesis, aortic segments were attached to surgical wound retractors, the retractors were spread to 1.5 times the original length of the aortic segment, and arteries were kept stretched for 3 hours. ADV-hpAP was infused into the lumen of stretched segments under a pressure of 150 mm Hg. After the incubation, the stretch was slowly released, and aortic tissue was cut, maintained in culture, and fixed. Indeed, scanning electron microscopy demonstrated the development of holes between luminal endothelial cells (Figure 1C and 1D) and between extracellular connective tissue fibers (Figure 1E and 1F). Normal morphology was restored after the release of mechanical stretch (Figure 1G). Corresponding to these acute alterations of vascular structure, we found that 2 days after stretch-assisted gene transfer, $10.2 \pm 3.8\%$ of medial SMCs expressed the transgene (Figures 1H and 2). Thus, it was concluded that mechanically enhanced porosity of the arterial wall facilitated gene transfer into medial SMCs without endothelial denudation. This stretch-and-pressure approach was subsequently used in our in vivo transplantation experiments.

Fate of Transgene-Expressing Cells in a Transplantation Environment

To analyze reporter gene expression over time after transplantation, we performed ex vivo hpAP gene transfer into aortas derived from Dutch Belted rabbits and transplanted them into the carotid artery position of recipient New Zealand White rabbits. Control grafts were infected without longitudinal stretch (group 1A), whereas experimental grafts underwent mechanical treatment during incubation with ADV-hpAP (group 1B).

Two days after transplantation, the pattern of hpAP expression was reminiscent of that seen in organ culture. In both groups, $\approx 100\%$ of luminal endothelial cells and $\approx 22\%$ of

adventitial cells were alkaline phosphatase-positive (Figure 3A and 3B, Table 2). In the control group, there was no transgene expression in medial SMCs, whereas $7.3 \pm 2.4\%$ of medial SMCs in previously stretched grafts demonstrated recombinant protein activity (Figure 3A and 3B, Table 2). Two weeks after surgery, transplantation-associated intimal thickening had developed in both stretched and unstretched grafts (intimal area, 1.2 ± 0.2 and 1.1 ± 0.3 mm², respectively, $P = \text{NS}$). These values were not different from a baseline group that was transplanted but not subjected to gene transfer (1.0 ± 0.3 mm²). Almost no endothelial cells covering intimal thickening or adventitial cells expressed the transgene in either group (Figure 3C and 3D, Table 2). However, $4.2 \pm 1.9\%$ of SMCs in the experimental group demonstrated persistent hpAP expression (Figure 3D, Table 2). These cells were located both in the graft media and, less frequently, in the intima. Thus, ex vivo-transduced SMCs, unlike endothelial cells and fibroblasts, are capable of relatively long-term transgene expression in a transplantation environment in vivo. Longitudinal mechanical stretch and pressure applied during the gene transfer procedure facilitated transgene expression in SMCs without additional stimulation of intimal thickening.

Expression of *tk* and Ganciclovir Administration Inhibits Development of Transplant Arteriosclerosis

To explore whether the cytotoxic effects of *tk*-ganciclovir treatment can inhibit the development of transplant arteriosclerosis, we obtained abdominal aortas from Dutch Belted rabbits, split each aorta into halves, and stretched and pressurized both of them to ensure effective vector delivery to the target SMCs. In each pair, 1 segment was infected with ADV-hpAP (control, group 2A) and another segment with ADV-*tk* (group 2B). Every pair of grafts was then transplanted into carotid arteries of New Zealand White rabbits, as described for group 1. Recipient animals were kept on a cholesterol diet (started a week before surgery) and received cyclosporine (started immediately after surgery and continued until euthanasia) exactly as described for the group 1 experiments. In addition, recipient animals in the group 2 experiment received daily injections of ganciclovir for 7 days (days 7 through 14 after transplantation).

Fourteen days after transplantation, the thickness of the neointima and intima/media ratio in *tk*-transfected grafts were significantly reduced ($P < 0.01$, paired *t* test) compared with their hpAP-transfected counterparts subjected to the same systemic factors (Figures 3E, 3F, 4A, and 4B). This reduction in neointimal area was accompanied by a significant decrease in SMC proliferation, measured by triple-label studies of BrdU, α -actin, and RAM11 (Figure 3G and 3H). SMC proliferation, expressed as a ratio of BrdU-positive, α -actin-positive cells to the total cell number (intima + media) dropped from $2.3 \pm 0.2\%$ in hpAP-expressing (control) grafts to $1.1 \pm 0.2\%$ in *tk*-expressing (experimental) grafts ($P < 0.01$, paired *t* test). In this animal model, a decrease in macrophage proliferation was not observed (Figure 3G and 3H). Moreover, the percentage of BrdU-positive, RAM11-positive cells in *tk*-expressing grafts increased ($2.5 \pm 0.9\%$) compared with

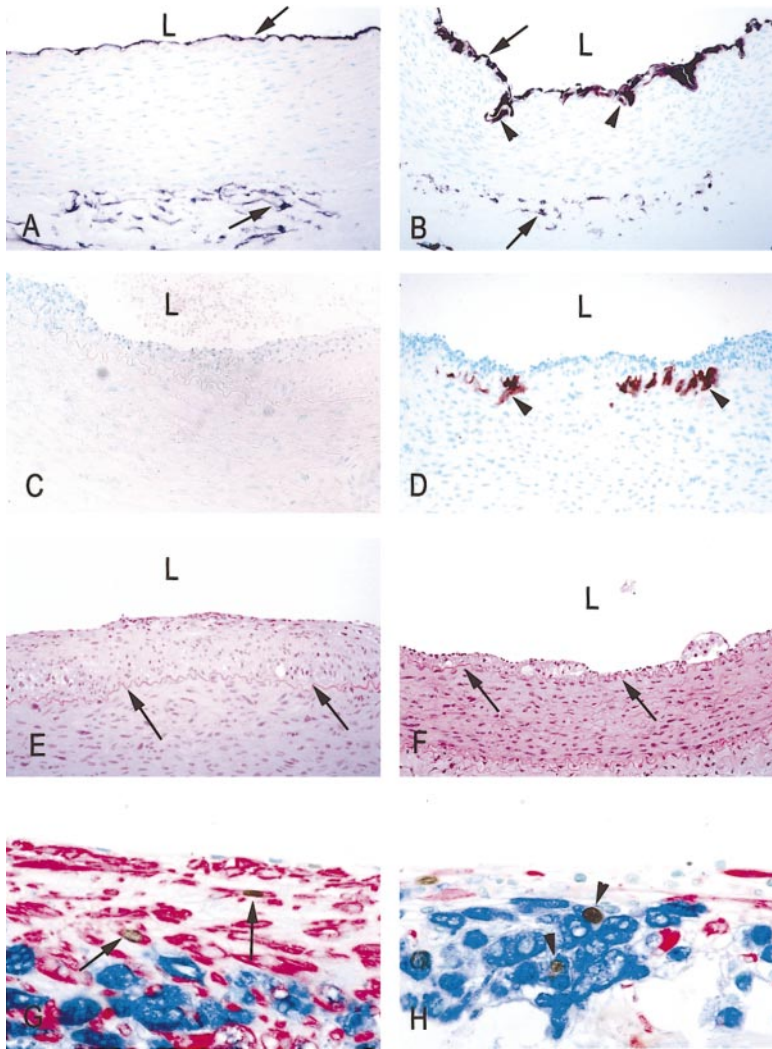


Figure 3. Gene transfer and transplant arteriosclerosis. A and B, Pattern of transgenic hpAP expression in rabbit aortic allografts 2 days after transplantation. Grafts were incubated with ADV-hpAP for 3 hours ex vivo before transplantation, without (A) and with (B) longitudinal stretch. Recipient rabbits underwent cholesterol feeding and cyclosporine treatment. In both cases, all luminal endothelial cells and some adventitial cells express transgenic hpAP (purple product of histochemical reaction, arrows). In addition, medial SMCs in a previously stretched graft (B) show reporter gene expression (arrowheads). L indicates lumen. Alkaline phosphatase histochemical reaction. Methyl green nuclear counterstain. Objective $\times 20$. C and D, Pattern of transgenic hpAP expression in rabbit aortic allografts 14 days after transplantation. Grafts were incubated with ADV-hpAP for 3 hours ex vivo before transplantation, without (C) and with (D) longitudinal stretch. Recipient rabbits underwent cholesterol feeding and cyclosporine treatment. In both cases, luminal endothelial cells and adventitial cells do not express transgenic hpAP. However, medial SMCs in a previously stretched graft (D) maintain reporter gene expression (arrowheads). Alkaline phosphatase histochemical reaction. Methyl green nuclear counterstain. Objective $\times 20$. E and F, Inhibition of intimal thickening in rabbit aortic allografts by ADV-*tk*+ganciclovir 14 days after transplantation. Control graft (E) was infected with ADV-hpAP and experimental graft (F) with ADV-*tk* before transplantation (both were stretched and pressurized during incubation with vector). Recipient rabbits underwent cholesterol feeding, cyclosporine, and ganciclovir treatment. Arrows indicate internal elastic lamina. Hematoxylin and eosin staining. Objective $\times 20$. G and H, Proliferation of different cell types in rabbit aortic allografts, 14 days after transplantation. Control graft (G) was infected with ADV-hpAP, and experimental graft (H) with ADV-*tk* before transplantation (both were stretched and pressurized during incubation with vector). Recipient rabbits underwent cholesterol feeding and cyclosporine and ganciclovir treatment. Triple immunolabeling for BrdU

(black product of peroxidase reaction), smooth muscle-specific α -actin (red product of alkaline phosphatase reaction), and macrophage-specific antigen RAM11 (blue product of alkaline phosphatase reaction) demonstrates predominantly proliferating SMCs (black nuclei colocalized with red cytoplasm, arrows) in ADV-hpAP infected grafts (G), and predominantly proliferating macrophages (black nuclei associated with blue cytoplasm, arrowheads) in ADV-*tk* infected grafts (H). Methyl green nuclear counterstain. Objective $\times 100$.

hpAP-expressing grafts ($1.0 \pm 0.3\%$; $P < 0.01$, paired *t* test). However, triple-label studies suggest that SMCs, rather than macrophages, contribute to the majority of intimal volume (Figure 3G and 3H).

Discussion

Gene therapy is a reasonable approach to transplant arteriosclerosis, because the disease is limited to graft arteries and the graft itself is available for ex vivo manipulations before transplantation. SMC replication plays a central role in the pathogenesis of transplant arteriosclerosis.³⁻⁵ To design rational gene therapy targeting SMC proliferation, 3 major questions should be addressed: (1) How should a vector be delivered specifically to target medial SMCs? (2) What is the fate of transduced cells in the transplantation environment? (3) Does gene therapy inhibit SMC proliferation, and does this result in a reduction of graft intimal thickening?

In the organ culture experiments, we found that medial SMCs of the rabbit aorta did not express a transgenic reporter

after incubation with adenoviral vector. These data confirm previous in vivo observations indicating that in normal arteries, gene transfer was limited to luminal endothelium and adventitia.⁹⁻¹¹ It has also been demonstrated that medial SMCs are able to express a transgene when adenoviral particles are delivered either under very high pressure of 5 atm¹⁰ or after vigorous mechanical injury^{6,12} permitting penetration of the vector. However, the goal of this study was to inhibit SMC proliferation in arterial transplants, and denudation of the graft before transplantation would be counterproductive. We hypothesized that transient graft permeabilization might facilitate gene transfer into medial SMCs. To increase the porosity of the arterial wall, we applied longitudinal mechanical stretch to the sealed segment of aorta. The combination of longitudinal stretch with supraphysiological intraluminal pressure allowed us to perform efficient gene transfer into medial SMCs without endothelial denudation.

It was unknown, however, whether these results were directly applicable to the in vivo situation. Therefore, specific

TABLE 2. Percentage of hpAP-Expressing Cells in Rabbit Aortic Grafts

Time After Transplantation, d	Luminal Endothelium		SMCs		Adventitia	
	Unstretched	Stretched	Unstretched	Stretched	Unstretched	Stretched
2	97.8±1.4	96.4±1.7	0.03±0.06	7.3±2.4	22.1±8.7	21.2±7.1
14	0	0	0.02±0.04	4.2±1.9	0.7±1.5	0.8±1.8

experiments were designed to study the patterns of reporter gene expression in the aortic grafts that had been transfected *ex vivo* and then transplanted into recipient carotid arteries. We found that 2 days after transplantation, luminal endothelial cells and various adventitial cells were alkaline phosphatase-positive in both stretched and unstretched grafts. However, only stretched grafts contained hpAP-expressing SMCs, thus reproducing the pattern observed in organ culture. Fourteen days after transplantation, comparable numbers of SMCs were still alkaline phosphatase-positive, whereas hpAP gene expression was barely detectable in the luminal endothelium and adventitia. Disappearance of transgenic alkaline phosphatase activity might be attributed to a cell-mediated immune response induced by adenovirus-mediated gene transfer¹⁷ and/or allogeneic transplantation⁴ and is not surprising. The apparently selective preservation of transgene expression by the SMCs is more remarkable. The mechanisms of such selectivity are poorly understood, because little is known about cell kinetics in this extremely complex combination of allogeneic transplantation, immunosuppression, hypercholesterolemia, and gene transfer. We think that the same anatomic barriers that did not allow adenoviral particles to penetrate to aortic media could retard or prevent the main participants of cell-mediated immune response (T cells and macrophages) from gaining direct

contact with at least the majority of medial SMCs. As a result of such compartmentalization, exposed “external” cells like endothelial and adventitial cells could be preferentially killed while “hidden” medial SMCs could be spared from the immune attack and maintain a transgene expression, at least initially. Regardless of the mechanisms of SMC protection, this phenomenon enabled us to proceed with gene therapy experiments and, in particular, to determine a “therapeutic window” for ganciclovir administration (day 7 to 14) when SMC proliferation and transgene expression in SMCs coincided.

We realize that mechanical permeabilization of the arteries is far from an ideal gene transfer-facilitating technique. Although complete restoration of endothelial structural integrity was demonstrated, it is possible that endothelium-dependent responsiveness of the stretched vessels could be impaired. It is also obvious that longitudinal stretch is clinically impractical for solid-organ transplants, as opposed to the use of allograft artery segments as bypass grafts. However, the important point is that medial SMCs are not intrinsically resistant to gene transfer but rather are hidden behind anatomic barriers. Our observations suggest that any technique that would allow direct contact between the vector and target SMCs would significantly increase the efficiency of gene therapy. We hope that other more clinically relevant protocols will be developed on the basis of this paradigm.

Currently, as a proof of the principle, we demonstrated that stretch-facilitated *tk* gene expression and systemic ganciclovir treatment reduced intimal formation in allogeneic arterial transplants. Our control experiments showed that mechanical stretch and pressure *per se* did not stimulate intimal growth. Mehra et al¹⁸ reported that adenovirus-mediated reporter gene transfer also did not increase intimal thickening in a similar rabbit model. These data suggest that gene therapy targeting SMC proliferation may be used for a treatment of transplant arteriosclerosis.

The effects on SMC proliferation are probably a result of inhibition of DNA replication after phosphorylation of ganciclovir by the enzyme thymidine kinase. The lethal effects of phosphorylated ganciclovir probably are not limited to transduced cells; inhibition of cell proliferation can be achieved by a paracrine mechanism, the so-called “bystander effect.”¹⁸ We suggest that metabolic cooperation may explain why in our experiments, ≈50% reduction of intimal thickness was observed but only ≈10% of SMCs were originally transduced. It is unclear whether the bystander effect is functional between dissimilar cell types like SMCs and macrophages.

Recently, we applied the same treatment to an *in vivo* model of restenosis in rabbit atherosclerotic arteries.¹⁹ It was demonstrated that *tk*-ganciclovir treatment of preexisting atherosclerotic lesions significantly inhibited the proliferation

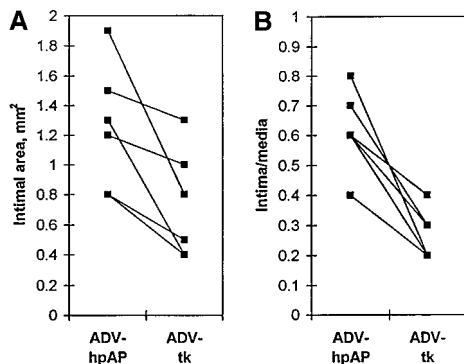


Figure 4. Effects of ADV-*tk*+ganciclovir on intimal thickening 14 days after transplantation. Control grafts were infected with ADV-hpAP, and experimental grafts with ADV-*tk* before transplantation (both were stretched and pressurized during incubation with vector). Recipient rabbits underwent cholesterol feeding and cyclosporine and ganciclovir treatment. Each recipient hosted 2 grafts (1 infected with ADV-hpAP and 1 infected with ADV-*tk*). Data are presented as matched pairs of samples (control and experimental grafts), whereby each pair belongs to same recipient. Each point in chart represents average intimal area of individual graft. Connected points represent pairs of grafts transplanted into same recipient animal. Points on left show average intimal areas of ADV-hpAP transfected grafts, and points on right, average intimal areas of ADV-*tk* transfected grafts. A, Intimal area. Two-tailed, paired *t* test: $P < 0.01$. B, Intima/media ratio. Two-tailed, paired *t* test: $P < 0.01$.

of both SMCs and macrophages after angioplasty. In the present study, the overall reduction of neointimal thickening was accompanied by an inhibition of SMCs but not macrophage proliferation. We suggest that the major reason for such a disparity lies in the difference between cellular targets in various experimental systems. In preexisting atherosclerotic lesions, both SMCs and macrophages were successfully transduced.¹⁹ In our present experimental system, only SMCs were originally transduced *ex vivo*, and macrophages infiltrated the artery later after transplantation. Nevertheless, because SMCs but not macrophages compose the bulk of intimal volume in transplant arteriosclerotic lesions³ and because SMCs, unlike macrophages, synthesize extracellular matrix components, thus indirectly contributing to the growth of neointima, inhibition of SMC proliferation is sufficient for significant reduction of intimal thickness.

Thus, our results suggest that certain treatments can facilitate gene transfer into elusive SMCs and that, in principle, targeting SMC replication may provide a genetic approach to the treatment of transplant arteriosclerosis.

Acknowledgments

This study was supported in part by grants from the Michigan Affiliate of the American Heart Association (Drs Gordon and Rekhter) and from the National Institutes of Health (HL-43757 to Dr E.G. Nabel). Dr E.G. Nabel is an Established Investigator of the American Heart Association. We gratefully acknowledge Dawn Lutz and Dr Val Kolpakov for technical expertise.

References

- Weis M, von Schneidt W. Cardiac allograft vasculopathy: a review. *Circulation*. 1997;96:2069–2077.
- Billingham ME. Cardiac transplant atherosclerosis. *Transplant Proc*. 1987;19:19–25.
- Gordon D. Transplant arteriosclerosis. In: Fuster V, Ross R, Topol EJ, eds. *Atherosclerosis and Coronary Disease*. Philadelphia, Pa: Lippincott-Raven Press; 1996:715–726.
- Libby P, Tanaka H. The pathogenesis of coronary arteriosclerosis (“chronic rejection”) in transplanted hearts. *Clin Transplant*. 1994;8:313–318.
- Hayry P, Mennander A, Yilmaz S, Ustinov J, Raisanen A, Miettinen A, Lautenschlager I, Lemstrom K, Bruggeman CA, Paavonen T. Towards understanding the pathophysiology of chronic rejection. *Clin Invest*. 1992;70:780–790.
- Ohno T, Gordon D, San H, Pompili VJ, Imperiale MJ, Nabel GJ, Nabel EG. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. *Science*. 1994;265:781–784.
- Guzman RJ, Hirschowitz EA, Brody SL, Crystal RG, Epstein SE, Finkel T. In vivo suppression of injury-induced vascular smooth muscle cell accumulation using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. *Proc Natl Acad Sci U S A*. 1994;91:10732–10736.
- Borrelli E, Heyman R, Hsi M, Evans RM. Targeting of an inducible toxic phenotype in animal cells. *Proc Natl Acad Sci U S A*. 1988;85:7572–7576.
- Schulick AH, Dong G, Newman KD, Virmani R, Dichek DA. Endothelium-specific *in vivo* gene transfer. *Circ Res*. 1995;77:475–485.
- Rome JJ, Shayani V, Flugelman MY, Newman KD, Farb A, Virmani R, Dichek DA. Anatomic barriers influence the distribution of *in vivo* gene transfer into the arterial wall: modeling with microscopic tracer particles and verification with a recombinant adenoviral vector. *Arterioscler Thromb*. 1994;14:148–161.
- Lemarchand P, Jones M, Yamada I, Crystal RG. In vivo gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. *Circ Res*. 1993;72:1132–1138.
- Steg PG, Feldman LJ, Scazecz JY, Tahlil O, Barry JJ, Boulechfar S, Ragot T, Isner JM, Perricaudet M. Arterial gene transfer to rabbit endothelial and smooth muscle cells using percutaneous delivery of an adenoviral vector. *Circulation*. 1994;90:1648–1656.
- Bowyer DE, Reidy MA. Scanning electron-microscope studies of the endothelium of aortic allografts in the rabbit: morphological observations. *J Pathol*. 1977;123:237–243.
- Tanaka H, Swanson SJ, Sukhova G, Schoen FJ, Libby P. Early proliferation of medial smooth muscle cells in coronary arteries of rabbit cardiac allografts during immunosuppression with cyclosporine A. *Transplant Proc*. 1995;27:2062–2065.
- Rekhter MD, Gordon D. Does platelet-derived growth factor-A chain stimulate proliferation of arterial mesenchymal cells in human atherosclerotic plaques? *Circ Res*. 1994;75:410–417.
- Rekhter MD, Tertov VV, Andreeva ER, Kolpakov V, Mironov AA, Orekhov AN. Lipid accumulation in the subendothelial cells of human aortic intima impairs cell-to-cell contacts: a comparative study *in situ* and *in vitro*. *Cardiovasc Pathol*. 1993;2:53–62.
- Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A*. 1994;91:4407–4411.
- Mehra MR, Stapleton DD, Cook JL, Zhang T, Ventura HO, Huang C, Maldonado B, Smart FW, Re RN, Murgo JP, Barbee RW. Adenovirus-mediated *in vivo* gene transfer in a rabbit model of allograft vasculopathy. *J Heart Lung Transplant*. 1996;15:51–57.
- Simari RD, San H, Rekhter M, Ohno T, Gordon D, Nabel GJ, Nabel EG. Regulation of cellular proliferation and intimal formation following balloon injury in atherosclerotic rabbit arteries. *J Clin Invest*. 1996;98:225–235.