Genetic Modification of the Vessel Wall
Comparison of Surgical and Catheter-Based Techniques for Delivery of Recombinant Adenovirus

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**Background** Gene transfer can potentially alter vessel wall biology and intervene in the pathogenesis of human disease. Although several methods for vector delivery have been described, systematic comparisons of these methods are unavailable. Therefore, this study compared three catheter-based strategies and a surgical technique to assess efficient and selective gene transfer to the vascular wall.

**Methods and Results** The common carotid arteries and internal jugular veins of New Zealand White rabbits were infected with recombinant adenovirus encoding either firefly luciferase or a nuclear-localizing variant of β-galactosidase.

Delivery of recombinant virus was achieved by one of four methods: (1) instillation within a surgically isolated vessel segment (dwell), (2) a double-balloon catheter, (3) a perforated balloon catheter (Wolinsky), or (4) an angioplasty balloon catheter coated with a hydrophilic adsorbent polymer (Hydrogel).

Vessel segments were analyzed 4 days after infection for luciferase and β-galactosidase activity and for the extent of injury to the vessel wall. Luciferase activity in vessels infected using the double-balloon method was substantially greater than that achieved by catheter-based methods (*P* < .05). The dwell and double-balloon methods yielded selective expression in intimal cells, whereas arteries infected using perforated or Hydrogel-coated balloon catheters demonstrated expression primarily in medial cells. Tissue injury was most pronounced with the perforated balloon catheter.

**Conclusions** Prototype catheters permit relatively efficient direct gene transfer to vascular endothelium; however, delivery methods for targeting the medial cells are inefficient. Modifications are needed to optimize direct gene transfer and minimize tissue injury. (*Circulation*. 1994;89:2190-2197.)

**Key Words** • gene transfer • gene therapy • vessel wall • endothelium • catheters
Methods

Recombinant Adenovirus

Generation of the recombinant, replication-defective adenoviruses AdCMVLuc and AdCMVβGal has been described previously.12 These recombinant adenoviruses contain polymeric genes in which cDNAs encoding firefly luciferase13 or a nuclear-localizing variant of Escherichia coli β-galactosidase14 are expressed from the human cytomegalovirus immediate-early promoter.15 These recombinant adenoviruses lack the EIA gene and are therefore replication defective unless supplied with the EIA gene product in trans.

High-titer stocks of recombinant viruses were prepared by a modification of the method described previously.12 Confluent monolayers of transformed human embryonic kidney (293) cells16 were infected by exposure to primary recombinant viral stocks for 1 hour at a multiplicity of infection of 0.01 and incubated in Dulbecco’s modified Eagle’s medium containing 4.5 g/L glucose and supplemented with 2% fetal bovine serum until extensive cytopathic effects were observed. Infected monolayers were lysed by the addition of 0.1% NP-40 to their culture medium, and cellular debris was removed by centrifugation. Recombinant virions were precipitated from the supernatant by the addition of 0.5 vol 20% polyethylene glycol 8000, 2.5 mol/L NaCl at 4°C for 1 hour and then centrifuged at 20000 rpm for 15 minutes. The viral pellet was resuspended in ρ=1.10 g/mL CsCl in 20 mmol/L Tris HCl, pH 8.0, applied to a p=1.30, p=1.40 discontinuous CsCl density gradient and centrifuged at 20000 rpm for 2 hours at 20°C in a Sorvall TH641 rotor. Virus particles were recovered from the 1.30- to 1.40 interface and desalted on a Sepharose CL-4B (Pharmacia) column equilibrated with isocratic buffer (10 mmol/L Tris HCl, pH 7.4, 137 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2). Purified virus was stabilized by supplementation with sterile 0.1 mg/mL bovine serum albumin and stored frozen at −80°C in aliquots until used. Titers of purified virus preparations were determined by plaque assay on monolayers of 293 cells.

Surgical Procedures

The protocols for instrumentation of experimental animals were performed in accordance with American Association for Accreditation of Laboratory Animal Care guidelines and approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. Adult New Zealand White (NZW) rabbits (3 to 4 kg) were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg), intubated, and ventilated with room air. Supplemental anesthesia was provided by inhaled enflurane as necessary. The anterior cervical neck was shaved and steriley prepared for surgery. Following a longitudinal midline anterior cervical incision, the common carotid arteries and internal jugular veins were bluntly dissected free of surrounding tissue and tagged with a silk tie for later use. Rabbits were systemically heparinized with intravenous heparin (100 U/kg) before administration of recombinant virus.

Recombinant adenoviruses were delivered to vessels by one of four techniques. In six rabbits, recombinant virus stock was instilled through a 24-gauge angiocath (Baxter Healthcare Corp) into a 1-cm segment of carotid artery or internal jugular vein temporarily isolated by silk ligatures (dwell technique). After 30 minutes, the ligatures and angiography were removed, and hemostasis was obtained by direct pressure; occasionally primary closure with 8.0 nonabsorbable suture was necessary. A silk tie was placed in the adjacent tissue to identify the vessel segment to be harvested.

Latex double-balloon catheters (USCI Division, CR Bard, Inc, and Mansfield, Boston Scientific Corp) were introduced into the external jugular veins or carotid arteries of eight rabbits and advanced proximally. The proximal balloon was inflated first, and after decompression of the vessel lumen, the distal balloon was inflated, creating a 1-cm segment of isolated vessel for infusion of recombinant virus stock (0.3 mL of 3 to 4×10^10 pfu/mL). Balloons were deflated after 30 minutes, the catheters were removed, external jugular veins were ligated, and the carotid arteriotomies were repaired with nonabsorbable suture. Due to their large profile, most double-balloon procedures were performed in internal jugular veins rather than carotid arteries.

Similarly, after carotid arteriotomy, 2.5-mm-diameter perforated balloon infusion catheters (Wolinsky, USCI Division, CR Bard, Inc) with 28, 100, or 300 pores (25-μm perforations) per balloon were inserted into 15 rabbits. Balloons were positioned under direct visualization, inflated to 2 atm during infusion of recombinant adenovirus stock (1.0 mL of 3 to 4×10^10 pfu/mL) into the vascular wall, and then removed after balloon deflation. A primary repair of the carotid arteriotomy was performed using 8-0 nonabsorbable suture.

In 12 rabbits, 2.5-mm Hydrogel-coated balloon catheters (Mansfield, Boston Scientific Corporation) were used for local delivery of recombinant adenovirus. To prepare the balloons for delivery of virus, they were inflated to 2 atm, submersed in high-titer viral stock (8 to 30×10^10 pfu/mL) for 3 minutes, deflated, and loaded into a 6F vascular sheath. After a stable intravascular position was obtained, the balloon was advanced under direct visualization just beyond the end of the sheath and immediately inflated to 2 atm. After 30 minutes, the balloon was deflated, and after catheter withdrawal, the arteriotomy was closed with 8-0 nonabsorbable suture.

After instrumentation, all animals were observed under heat lamps until fully awake. They subsequently received chloramphenicol 10 mg/kg subcutaneously twice daily and were provided with a regular diet. Four days after infection, the rabbits were anesthetized, 1 cm of the genetically modified vessel was harvested, and the animals were euthanized with pentobarbital (100 mg/kg). Vessel segments infected with a mixture of both AdCMVLuc and AdCMVβGal were equally divided: One half (0.5 cm) was assayed for luciferase activity and the other for β-galactosidase activity (luciferase activity for vessels divided and assayed for both luciferase and β-galactosidase activity was corrected by multiplying ×2).

Determination of the Volume of Virus Delivered by the Hydrogel-Coated Balloon

To estimate the efficiency with which recombinant adenovirus would be adsorbed into the Hydrogel coating and the volume of recombinant virus stock that could be delivered using these coated-balloon catheters, balloons were inflated to 2 atm and submersed in high-titer virus stock (8×10^10 pfu/mL AdCMVLuc) for 1 to 15 minutes. The balloons were quickly rinsed by dipping them in sterile isotonic saline to remove virus that might be passively residing on the balloon surface. They were then deployed as described above. After 30 minutes, the balloons were deflated, and 1-cm vessel segments were harvested, homogenized, and used to infect African green monkey kidney (CV-1) cells.17 The volume of adenovirus stock delivered by the Hydrogel-coated balloon catheters was calculated by comparing plaque assays in 293 cells and luciferase activity in CV-1 cell lysates 48 hours after infection with dilutions of high-titer stock and vessel homogenate.

Luciferase Assay

Tissue specimens were harvested and immediately placed in 1 mL of extraction buffer (70 mmol/L potassium phosphate, 55 mmol/L Tris HCl, 2 mmol/L MgCl2, 0.7 mmol/L DTT, pH 7.8, containing 250 μg/mL soybean trypsin inhibitor and 20 μg/mL aprotinin) on ice. Specimens were homogenized in a Brinkmann polytron, and extracts were microfuged for 5 minutes at 4°C. Aliquots of the supernatant were immediately assayed for luciferase activity as previously described.13 A 50-μL sample of extract was added to 250 μL of assay buffer (45.2 mmol/L glycyl-glycine, pH 7.8, 22 mmol/L MgSO4, 2.4 mmol/L EDTA, 7.4 mmol/L ATP, 1 mmol/L DTT, and 0.4 mg/mL bovine
serum albumin) in a test tube that was placed in a Biotemp LB9500C luminometer (Berthold Analytical Instruments, Inc) set at 25°C. The reaction was initiated by the injection of 100 μL of 0.13 mg/mL luciferin, and activity was determined by integration of the light emitted over 10 seconds. Each sample was run in duplicate and diluted as necessary to yield luciferase activities within the linear range of the assay. Luciferase activity (light units) for each sample was determined by correcting for background activity and sample dilution.

**Histochemical Staining for β-Galactosidase Activity**

Harvested vessel segments were fixed in 0.25% gluteraldehyde in Dulbecco's phosphate-buffered saline for 2 hours at room temperature and then washed extensively in PBS to remove the fixative. The specimens were then submersed in X-Gal staining solution (35 mmol/L K₄Fe[CN]₆, 35 mmol/L K₃Fe[CN]₄, 1 mmol/L MgCl₂, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (X-Gal, GIBCO-BRL) for 16 to 24 hours at room temperature. Stained tissue sections were then dehydrated through graded alcohol to xylene washes and embedded in paraffin for sectioning. Sections were lightly counterstained with eosin and examined for the presence and location of nuclear-localizing β-galactosidase activity, as indicated by blue-staining cell nuclei. Histological sections were qualitatively evaluated for β-galactosidase activity in cells residing in the intima and media. Histological sections were examined by an experienced pathologist who was blinded to the type of delivery method used.

**Statistics**

Mean luciferase activity of vessel segments infected with AdCMVLuc by the dwell method and the double-balloon, Hydrogel-coated, and Wolfinsky catheter techniques were compared using ANOVA. Pairwise post hoc analysis, using Scheffé's method, was used to determine significant differences between groups.19 The luciferase activities in vessel segments infected with adenovirus by each of the four delivery methods were compared both before and after normalization for the total "administered" dose of recombinant adenovirus. Luciferase activity of vessel segments instrumented with perforated balloons containing 28, 100, or 300 pores were compared by ANOVA. For all analyses, a value of P<.05 was considered significant.

**Results**

**Volume of Virus Delivered by Hydrogel-Coated Balloons**

Luciferase activity from CV-1 cells infected with 0.1 mL of vessel homogenate (one tenth of the vessel infected with AdCMVLuc via the Hydrogel-coated balloon) ranged from 1.07x10⁶ to 2.27x10⁹ for balloon incubations of 1 minute to 2.27x10⁹ for 15-minute incubations compared with 1.25x10⁶ for CV-1 cells infected with 20 μL of recombinant adenovirus stock (7.7x10⁶ pfu/mL). The volume of virus adsorbed into the Hydrogel matrix is therefore approximately 2 μL for a 3-minute incubation (range, 1.5 to 4 μL for incubations of 1 and 15 minutes, respectively) and appears to be time dependent. All balloons used for the experiments comparing the four methods of virus delivery were incubated in virus for 3 minutes.

**Luciferase Activity**

A total of 60 vessel segments were infected with AdCMVLuc and analyzed for expression of luciferase activity 4 days after infection. Because the number of recombinant virions administered by the four methods of delivery varied (differing volumes and titers), luciferase activities were normalized (to provide an index of the intrinsic efficiency of the delivery system) and are reported as luciferase activity per 10⁴ virions. In addition, total luciferase activity (luciferase activity that is uncorrected for differences in the dose of virus administered, thus providing an estimate of the achievable efficiency of gene transfer using each technique) is reported. Mean luciferase activity in vessels infected using the dwell technique after normalization was 1.3±1.46x10⁴, substantially greater (range, 3- to 26-fold) than that observed in vessels infected by any of the catheter-based methods (Fig 1): 4.16±4.76x10⁴.
1.24±1.08×10^4, and 0.53±1.70×10^4 for the Hydrogel-coated, double-balloon, and perforated balloon catheters, respectively (P<.05). In contrast, uncorrected (total) luciferase activity for vessels infected using the double-balloon method was 2.67±2.72×10^7, substantially greater (range, 2 to 13) than the perforated (0.60±1.51×10^7) and Hydrogel-coated (0.20±2.84×10^7) balloons (P<.05) and similar to the dwell method (1.12±1.55×10^7) (Fig 2). Luciferase activity in vessels infected with perforated balloon catheters with 28, 100, or 300 pores was similar (P=.67).

**Histological Analysis of Infected Vessels**

The methods used to deliver recombinant adenovirus resulted in genetic modification of different populations of cells in the vessel wall. Vessel segments infected with AdCMVβGal by either the dwell or the double-balloon technique showed selective expression of β-galactosidase activity in endothelial cells (Fig 3A and 3B, respectively). In contrast, vessels infected using either the Hydrogel-coated or the perforated balloon catheter demonstrated expression of β-galactosidase activity primarily in medial cells (Fig 3C and 3D, respectively). Vessels infected with perforated balloon catheters revealed foci of cells staining for β-galactosidase activity in the media that demonstrated characteristic morphology of vascular smooth muscle cells. The β-galactosidase activity was limited to sites of focal disruption of the internal elastic lamina, presumably reflecting local injury produced by the high-pressure jet exiting the balloon pores. This pattern did not differ between arteries infected using 28-, 100-, or 300-pore catheters. In comparison to vessels instrumented with other catheters, use of the perforated balloon catheter resulted in more extensive subintimal injury and hemorrhage and more frequent dissection of the vessel wall (Table).

**Discussion**

Ready accessibility and involvement by important human disease processes make the vessel wall an attractive target for gene therapy. Several catheter systems designed to facilitate direct delivery of biologically active molecules to the vessel wall have been developed. These systems facilitate gene transfer by either filling the lumen of an isolated vessel segment with the vector and allowing passive contact between the two or pressurized diffusion/infusion of the vector into the vessel wall.

Several prior studies have demonstrated successful introduction and expression of foreign genes in isolated vascular segments after catheter delivery of gene transfer vectors. Nabel et al. used a double-balloon catheter to infect pig iliofemoral arteries with a recombinant retrovirus encoding β-galactosidase and demonstrated direct gene transfer to endothelial cells. Flugelman et al. used a perforated balloon catheter (Wolinsky) to deliver a similar retrovirus to rabbit aorta. Chapman et al. used a modified perfusion balloon catheter with six laser-created holes (40 to 60 μm in diameter) to deliver a luciferase expression vector to canine coronary arteries and observed luciferase activity in infected vessels 10-fold higher than background. More recently, an angioplasty balloon catheter coated with a hydrophilic polymer (Hydrogel) has been described as highly efficient for local delivery of various adsorbed agents to the vascular wall.20,21 While these catheter-based delivery devices have been used successfully to deliver foreign genes to selected vessel segments and achieve biological effects in some cases,22 overall, the efficiency of genetic modification observed in these studies has been disappointingly low. In the Flugelman study,8 polymerase chain reaction analysis of infected tissues demonstrated genetic modification of fewer than 100 cells in a 2-cm segment of rabbit aorta. In part, this low efficiency reflects limitations of recombinant retrovirus as a gene transfer vector.23 In contrast, preliminary results of direct gene transfer into vascular tissue using recombinant adenoviral vectors have demonstrated high efficiency.10,11 Lemarchand et al. reported that direct intraluminal instillation of recombinant adenovirus encoding a nuclear-localizing variant of β-galactosidase into isolated vessel segments resulted in expression in essentially all exposed endothelial cells. Expression of human α1-antitrypsin by endothelial cells infected in situ in isolated vessel segments with a recombinant adenovirus also has been reported by these investigators.24

Comparison of the efficiency and selectivity of genetic modification achieved with available catheter-based delivery strategies from previous studies is difficult because diverse vectors and experimental conditions have been used. Because catheter-based delivery is obviously attractive for eventual clinical application of vascular gene transfer, we attempted to quantitatively and qualitatively evaluate gene expression after delivery of the same adenoviral constructs using four techniques. Two of these methods (the dwell and double-balloon techniques) were designed to evaluate delivery to isolated vessel segments; the Hydrogel-coated and perforated balloon catheters evaluated pressure-driven delivery techniques.

Our comparison of a surgical dwell technique and catheter-based techniques for local delivery of gene therapy demonstrates that mean luciferase activity in vessels infected by the double-balloon technique are substantially greater than that observed in vessels infected by any other catheter-based method (P<.05). These experiments also demonstrate that the strategies used to facilitate vascular gene transfer produce qualitatively different patterns of foreign gene expression. Vessels infected by the dwell or double-balloon techniques show selective genetic modification of vascular endothelium, whereas introduction of virus via high-pressure balloon systems resulted in gene expression primarily in medial smooth muscle cells. In view of the impermeability of the endothelial layer to particles the size of adenovirus (70 to 80 nm), mechanical disruption of the intima may be necessary for gene transfer targeting medial smooth muscle cells. Furthermore, since the clinical application of localized delivery of vascular gene therapy will most likely target sites of therapeutic angioplasty, the importance of vessel trauma related to local delivery techniques remains controversial.

The quantitative comparisons of gene expression presented here should not be interpreted in absolute terms. Rather, these experiments allow for objective comparison of techniques available for local delivery and assessment of their relative efficiencies in facilitating foreign gene expression in the vessel wall. For example, although the Hydrogel-coated balloon yielded efficient
gene transfer and expression (Fig 1), its clinical utility may be limited because it can only adsorb a small quantity of adenovirus. In contrast, the double balloon is capable of delivering large quantities of virus to boost the total level of foreign gene expression into a range that may have clinical application (Fig 2). While this system seems very promising, the potential for injury to the vessel wall at the points contacted by the inflated proximal and distal balloons may limit the clinical utility of this approach. Direct gene transfer using the perforated balloon unfortunately yielded a low level of foreign gene expression despite infusion of high doses of virus, suggesting that the duration of virus-to-cell contact is important for highly efficient foreign gene expression. In addition, the degree of tissue injury resulting from this method of delivery also may play a role in impairing foreign gene expression. Based on the pattern of β-galactosidase activity (isolated to areas of pore jet tissue penetration) observed in vessels infected using the perforated balloon catheter, we anticipated that balloons with more pores would result in more efficient gene transfer. However, quantitative assessment of foreign gene expression did not support this hypothesis. Comparison of luciferase activity of vessels infected with AdCMVLuc delivered by perforated balloon catheters containing 28, 100, or 300 pores revealed no difference in levels of foreign gene expression. Therefore, simply increasing the area of virus distribution does not seem to improve the efficiency of gene transfer when using the perforated balloon technique.

In aggregate, our observations imply that the efficiency and selectivity of gene transfer into the vessel wall depend on the delivery system used. Specifically, while available approaches appear sufficient for relatively efficient targeting of endothelium in normal vessels, the delivery strategies evaluated for targeting cells residing in the media result in less efficient genetic modification. The effects of local vessel trauma (e.g., angioplasty) on the pattern and efficiency of gene transfer are unknown. In fact, the barotrauma associated with angioplasty may actually complement local

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<th>Histological Variables Observed for Each Method of Local Delivery</th>
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<td><strong>Method of Virus Delivery</strong></td>
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delivery by techniques based on vessel isolation, allowing greater access to the media for gene transfer.

**Study Limitations**

This study has a number of potentially important limitations. Since we only used a single gene transfer vector, our results may not apply to other vectors. Quantitation of viral DNA in the vessel wall for direct correlation of expression levels with the number of genetically modified cells was not performed. We did not systematically assess the effects of balloon size, inflation pressure, exposure time, or viral titer for each delivery technique. For approaches in which the virus is allowed to dwell in contact with the endothelial monolayer, the efficiency of gene transfer should be directly related to both the concentration of infectious virus particles and the time of exposure. In preliminary experiments in which recombinant adenovirus was introduced into the lumen of explanted vein segments for 10 to 40 minutes before surgical reimplantation, we observed a time-dependent increase in the number of genetically modified cells in the intimal layer (unpublished observations). The protocols that we used in the current study were intended to compare the various delivery techniques using high viral titers (10⁶ pfu/mL) and prolonged virus to cell contact (30 minutes), variables previously identified as important for efficient gene transfer. While an effort was made to optimize the number of pores in perforated balloons and the loading time for Hydrogel balloons, it is possible that modifications to the methods used here might improve upon the observed efficiency of gene transfer. The balloon-to-artery ratio (≤1:3:1), balloon inflation pressure (2 atm), and volume of infusate (1.0 mL, perforated balloon) used in these experiments were selected in an effort to limit vascular wall injury. Only normal vessel segments were instrumented in an effort to assess the pattern and relative efficiency of the various delivery strategies independent of the inherent variability introduced by preexisting vascular disease. Therefore, we are uncertain which cell populations could be efficiently targeted using these techniques after therapeutic angioplasty. Finally, these experiments were not intended to elucidate the mechanism(s) for one technique being more efficient than another. Therefore, we can only speculate as to why the efficiencies of foreign gene transfer by the double-balloon and dwell techniques are disparate. The two most likely explanations for the difference in efficiency between the dwell and double-balloon techniques are (1) the external diameter of the double-balloon catheter is large relative to the internal diameter of rabbit vessels and may dislodge some of the virus from the vascular wall during catheter removal and (2) since the vessels were completely dissected free from surrounding tissue for the dwell but not for the double-balloon deliveries, small branches may have existed undetected from the balloon-isolated vessel segment, allowing for siphoning off of virus. Nonetheless, these experiments provide insight into the pattern and relative efficiency of gene transfer achievable using a highly efficient gene transfer vector under experimental conditions that might approximate therapeutic vascular gene transfer. This study also provides the first formal comparison of catheter-based methods for direct gene transfer to the vessel wall.

**Summary**

Among the catheter-based local delivery systems tested, the double balloon yielded the greatest level of foreign gene expression, in part, by virtue of the larger dose of virus that can be administered by this technique. In order to target cells residing in the media with foreign gene transfer, disruption of the intima and internal elastic lamina or delivery of the vector under pressure appears to be necessary. Finally, although several prototype catheters for direct gene transfer are available, additional modifications are needed to optimize direct gene transfer and reduce tissue injury.

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**References**


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