Efficient and Selective Adenovirus-Mediated Gene Transfer Into Vascular Neointima

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Background. Previous attempts to target arterial smooth muscle cells (SMCs) for gene delivery using liposomal or retroviral methods were limited by low transfection efficiency. We therefore evaluated the efficiency of adenovirus-mediated gene delivery in cultured vascular SMCs and in an in vivo model of balloon injury–induced SMC cell proliferation.

Methods and Results. We used a recombinant adenovirus, Ad.RSVβgal, which contained the β-galactosidase (β-gal) histochemical marker gene. For in vitro studies, rat aortic SMCs were incubated in media containing Ad.RSVβgal for 5 to 120 minutes. The proportion of SMCs expressing the β-gal gene product increased from 25% (5-minute exposure) to 80% (120-minute exposure). For in vivo studies, uninjured and injured rat carotid segments were incubated with 0.5 to 1.0×10⁶ pfu Ad.RSVβgal for 45 minutes. Uninjured arteries showed adenovirus-mediated gene transfer limited to the endothelium. Injured arteries were exposed to adenovirus 0, 3, 7, or 12 days after injury. In these segments, β-gal expression was minimal with infection at 0 or 3 days after injury but marked when infection was delayed until 7 or 12 days after injury. Neointimal cells constituted the dominant target of adenovirus gene transfer, with efficiency of gene transfer ranging from 10% to >75%. Medial SMCs, whether covered or uncovered by neointimal cells, were minimally infected. Infection with a control adenovirus vector showed no β-gal staining.

Conclusions. Recombinant adenovirus selectively targets neointimal cells with high-efficiency gene transfer. This suggests that adenovirus vectors should be useful in targeting cells for the delivery of genes whose products may be relevant to the treatment of restenosis. (Circulation. 1993;88:2838-2848.)

Key Words • adenovirus • neointima

The smooth muscle cell (SMC) proliferation associated with arterial injury remains a major obstacle to the long-term success of coronary angioplasty. Angioplasty failure rates of 25% to 50% within 6 months have been reported and confirmed by several authors. Previous attempts to modulate this cellular proliferation have included various mechanical and pharmacological therapies that have been the subject of several reviews. More recently, many efforts have been directed against various growth factors, their receptors, or cellular proto-oncogenes thought to play an important role in SMC proliferation. Although several of these methods have shown encouraging results in vitro and, more recently, in vivo, all approaches have both practical and theoretical drawbacks. Hence, while one or more of these therapeutic strategies may ultimately show clinical efficacy, the need for more powerful and specific approaches is compelling.

Gene therapeutic techniques offer the promise of efficiently transferring genes, whose products may convey therapeutic benefit, to specific groups of cells. One potential application in the treatment of restenosis would be to transduce neointimal cells with genes whose products inhibit cell growth or increase their susceptibility to cytotoxic agents. Previous efforts to directly transduce arterial segments in vivo have used liposomal or retroviral methods to transfer marker genes into endothelial cells or SMCs. The feasibility of such efforts, however, has been limited by a low transfection efficiency. In in vivo models, estimates of gene transfer into arterial segments range from fewer than 1 in 10 000 cells transduced with retroviral methods to fewer than 1 in 1000 cells using liposomes.

Replication-deficient recombinant adenoviral vectors have previously been shown to be efficient for transferring exogenous genes to a wide variety of cells in vivo. Such vectors can be manipulated to encode for recombinant gene products up to 7.5 kilobases (kb) in length. The recombinant virus can be propagated in certain mammalian cell lines that serve to complement the growth of replication-defective adenovirus. This allows for the potential production of viral stocks of 1×10¹¹ plaque-forming units (pfu)/mL or greater. Additionally, transduction by adenovirus as opposed to retrovirus does not depend on active replication of the host cell and therefore could be used to target both quiescent and actively replicating cells. With this knowledge, we have studied the feasibility of using adenovirus-mediated gene transfer to target medial SMCs of normal arterial segments and both medial and neointimal SMCs after vascular injury.
Methods

Adenovirus Vectors

The replication-deficient recombinant adenovirus (Ad.RSVβgal) has previously been described. Briefly, the recombinant virus encodes for the histochemical marker gene β-galactosidase (β-gal). The gene, derived from Escherichia coli, has been modified by a eukaryotic nuclear translocation signal and placed under the control of the Rous Sarcoma Virus (RSV) long terminal repeat. Expression of the β-gal gene results in a nuclear-dominant blue staining pattern when cells are exposed to the chromogen 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal). A similar adenovirus containing the human cystic fibrosis transmembrane conductance regulator cDNA (Ad-CFTR), whose recombinant gene product does not react with the X-Gal chromogen, was used as a control. Viral stocks (1 x 10^9 pfu/mL) were prepared by passaging recombinant adenovirus in 293 cells.

Cell Culture

Vascular SMCs were isolated from a rat thoracic aorta by enzymatic digestion as previously described. Cells were maintained in M199 medium (Biofluids, Gaithersburg, Md) containing 10% fetal bovine serum (Biofluids) and 1x penicillin/streptomycin in humidified air containing 5% CO₂ at 37°C. Cells were routinely passaged just before reaching confluence by brief exposure to Puck’s saline solution containing 0.5% trypsin (Biofluids) and 0.5 mmol/L EDTA. At passage 3, cells were plated on 8-well microcarrier slides and stained for α-smooth muscle actin (Sigma Immunochemicals Co, St Louis, Mo) to confirm their identity.

In Vitro Gene Transfer

Early passage (P4) SMCs were plated in duplicate on T-25 culture flasks and grown to 30% confluency. They were then placed in media containing 0.5% serum and allowed to become quiescent over 36 hours. The cells were then washed twice in phosphate-buffered saline (PBS) and incubated for 5, 15, 30, 60, or 120 minutes in 2 mL of media containing 2.5 x 10^7 pfu/mL, which was equivalent to approximately 100 pfu of Ad.RSVβgal per cell. After the given interval, the cells were washed five times in PBS and incubated for 24 hours in 5 mL of media containing 0.5% serum. Evidence for β-gal gene product was therefore obtained a minimum of 24 hours after exposure to the adenovirus. Before staining in X-Gal solution, cells were washed and fixed for 5 minutes in 2% formaldehyde and 0.2% glutaraldehyde in PBS pH 7.4. The percent of infected cells was calculated by counting 500 cells in each of two duplicate flasks and noting the number of cells with nuclear-dominant blue staining. Percentages shown represent an average for two flasks at each time point.

Animals

All animals were studied under protocols approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute and in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services publication No. [NIH] 86-23, revised 1985).

Adult Sprague-Dawley rats weighing 350 to 450 g (Taconic Farms, Germantown, NY) were used for these experiments. All procedures were performed under general anesthesia and using sterile technique. General anesthesia was administered using ketamine 150 mg/kg and xylazine 15 mg/kg IM and supplemental ketamine/xylazine IP as necessary. Beef lung heparin (Upjohn, Kalamazoo, Mich) was routinely given intravenously (100 U/kg) before cross-clamping the vessel for virus incubation. Upon completion of each procedure, animals were allowed to recover with free access to food and water.

In Vivo Gene Transfer Into Arterial Segments

Adenovirus-mediated gene transfer was first evaluated in uninjured arterial segments. In each of these animals, the left carotid artery was exposed, proximal and distal control was obtained, and an arteriotomy was made in the external carotid artery. A solution containing either Ad.RSVβgal or Ad.CFTR was instilled through the external carotid using a 24-gauge catheter (Criticon Inc, Tampa, Fla) that was secured in place with a 4-0 silk tie. Viral incubations consisted of instilling a solution containing 0.5 to 1.0 x 10^9 pfu of adenovirus in a total volume of 100 μL for 45 minutes. Medium M199 (Biofluids) was used to dilute stock viral solution to the appropriate concentration. The solution was then evacuated, the external carotid was ligated, and the incision was closed. All rats in this group were killed at 3 days after infection, and the carotid was subsequently harvested, fixed, and stained as described below.

To assess gene transfer to areas of vascular injury, the left carotid artery was exposed and injured as previously described. Briefly, the common carotid and its external branch were exposed and encircled using 4-0 silk ties. An arteriotomy was made in the external carotid artery. A 2F Fogarty embolectomy catheter was then passed into the common carotid, filled with 0.2 mL of air, and passed back and forth three times in order to denude the endothelial lining of the vessel. The carotid artery of one group of rats was exposed to virus immediately after injury. Adenoviral solution consisting of 0.5 to 1.0 x 10^9 pfu adenovirus in 100 μL of M199 was gently delivered into the common carotid lumen via the external carotid artery. After the 45-minute incubation, the external carotid was ligated, the common carotid was reperfused, and the animal was allowed to recover as previously described.

In three other groups of animals, carotid arteries were exposed to virus 3, 7, or 12 days after injury. These animals were reanesthetized at the prescribed time interval after injury. The common carotid artery was exposed through the previous incision, and a portion of the artery measuring approximately 1.3 cm was isolated between 1-mm microvascular clamps. An arteriotomy was then made in the common carotid artery using a 24-gauge catheter, and the vessel was irrigated with 50 to 100 μL of normal saline. Adenoviral solution containing 0.5 x 10^9 pfu Ad.RSVβgal or control virus in 100 μL of M199 was delivered directly into the carotid lumen via the 24-gauge needle catheter. Sufficient solution was instilled to gently distend the vessel. At the conclusion of the viral incubation, the catheter was removed and the arteriotomy was closed using 3 interrupted 10-0 Ethilon stitches (Ethicon Inc, Somerville, NJ) under 17x magnification.
Fig 1. This page and facing page. Time dependence of adenovirus-mediated gene transfer into cultured vascular smooth muscle cells (SMCs). Quiescent SMCs were exposed to media containing Ad.RSV/βgal for varying time intervals as described in the text: A, Five-minute exposure; B, 60-minute exposure; C, mock-infected; D, percent of cells that are nuclear-dominant blue staining as a function of time of viral incubation. Five hundred cells were counted in duplicate flasks that had been stained for β-gal activity. Averages from duplicate flasks varied less than 5% from the mean. Data shown are from one experiment but are representative of three different experiments.
The incision was closed, and the animal was allowed to recover as described above.

**Evaluation of In Vivo Gene Transfer**

Gene transfer was assessed 3 days after exposure to adenovirus. Animals were killed by overdosing with pentobarbital. The carotid artery was harvested and cut longitudinally to expose the lumen. It was then washed in 2 mL of PBS and fixed for 5 minutes in a solution containing 2% formaldehyde and 0.2% glutaraldehyde in PBS. The artery was washed in PBS several times to remove any excess fixative and placed into X-Gal solution (5 mmol/L K₄Fe(CN)₆, 5 mmol/L K₂Fe(CN)₃, 1 mmol/L MgCl₂, and 1 mg/mL X-Gal in PBS) for 4 hours. For histological examination, carotid arteries were cut into 2-mm segments, embedded in paraffin, cut into 5-μm sections, and counterstained with nuclear fast red. To estimate gene transfer efficiency in histological sections, neointimal cells were counted in at least three high-power (400×) fields from three or more histological sections of each test artery, and the percentage of nuclear-dominant blue cells was calculated. At least 300 cells were counted in each section. In 3 of the animals that were infected 3 days after injury and killed 3 days later, the brain, heart, and liver were also harvested to determine if gene expression in distal organs was detectable. These specimens were washed in PBS, fixed in 2% formaldehyde, 0.2% glutaraldehyde for 20 minutes, and stained in X-Gal solution for 6 hours. Histological sections were counterstained using nuclear fast red.

In one group of animals that underwent infection 7 days after injury, persistence of gene expression was assessed 2 weeks after infection. These animals were treated similarly to animals killed 3 days after infection, with the sole exception that killing and harvest were performed 14 days after viral infection.

In two separate sections containing neointima, immunohistochemical staining with an antibody to α-smooth muscle actin was performed. This analysis demonstrated cytoplasmic staining of most neointimal and medial cells, a finding in agreement with previous studies that identified neointimal cells as being primarily of SMC origin.41

A total of 35 rats were used in this study. Two rats died presumably as a result of anesthesia within 24 hours of injury and were excluded from further analysis. The remaining 33 rats were used as follows: For uninjured arteries, 4 rats were used and either exposed to Ad.RSVβgal (n=2) or AdCFTR (n=2). For injured arteries, 29 rats were used. Infection was carried out either immediately, 3, 7, or 12 days after injury. A total of 7 rats were infected immediately after injury with Ad.RSVβgal (n=5) or AdCFTR (n=2). Nine rats were infected 3 days after injury with Ad.RSVβgal (n=7) or AdCFTR (n=2). For the 7-day time point, a total of 9 rats were used. Six of these rats infected with Ad.RSVβgal (n=3) or AdCFTR (n=3) were killed at the usual 3 days after infection, whereas an additional 3 rats, all infected with Ad.RSVβgal, were killed 2 weeks after infection to assess persistence of gene expression. Finally, a total of 4 rats were infected 12 days after injury with Ad.RSVβgal (n=2) or AdCFTR (n=2).

**Results**

**In Vitro Gene Transfer**

We first sought to assess the efficiency of adenovirus-mediated gene transfer in cultured primary rat aortic SMCs. In SMCs exposed to adenovirus-containing media for 5 minutes and returned to low-serum media for 24 hours, β-gal expression was detected in approximately 25% of cells (Figs 1A and 1D). With increasing time of incubation in adenovirus-containing media, a higher percentage of cells stained positive. With cells exposed to adenovirus-containing media for 1 hour and returned to low-serum media for 24 hours before staining, close to 70% of cells appeared positive (Figs 1B and 1D). As expected, the predominant staining was intranuclear, since the β-gal gene was modified by the addition of a nuclear localizing sequence. A nuclear-dominant blue stain was not seen in mock-infected cells (Fig 1C). As noted by others,21'24 however, we noted occasional faint blue cytoplasmic staining in both infected and control cells of vascular origin. Although these results were obtained on quiescent cells, qualitatively similar results were obtained in three different experiments performed on cells maintained in normal growth media (data not shown).

**In Vivo Gene Transfer Into Uninjured Arterial Segments**

Extrapolating from our in vitro data, we chose to expose the vessel wall to adenoviral solution for 45 minutes. With this incubation period, gene transfer efficiency appeared to be within the plateau region of the curve (Fig 1D). As shown in Fig 2A, β-gal gene expression in uninjured arterial segments (n=2) could be detected on gross examination when assessed 3 days after infection. Histological examination showed β-gal staining of scattered endothelial cells throughout the region incubated with adenovirus (Fig 2B). Staining was also occasionally noted in cells of the adventitia. No medial SMCs stained for β-galactosidase activity. This suggests that the endothelium or the internal elastic lamina may form a barrier to adenoviral infection of
medial cells. We observed no β-gal staining in arteries infected with the AdCFTR control virus (n=2).

**In Vivo Gene Transfer Into Injured Arterial Segments**

To assess the efficiency of gene transfer in injured arterial segments and to determine whether neointimal cells have different susceptibility to gene transfer than medial SMCs, the rat carotid injury model was used. In this model, balloon abrasion removes the endothelial layer and disrupts one or more layers of internal elastic lamina. The injury activates medial SMCs, which begin to migrate and proliferate to form a neointima between 3 and 5 days after injury. A well-formed neointima is routinely present at 12 days after injury. For this reason, we assessed gene transfer immediately, 3 days, 7 days, and 12 days after balloon injury.

In carotid segments where virus was instilled immediately after injury, only 3 of 5 segments showed gross evidence of β-gal expression. The staining was in general limited to less than 5% of the vessel surface. Histological examination confirmed that no endothelial layer existed. Despite this, only occasional medial SMCs positively stained for β-gal (data not shown). Similar results were obtained when infection was delayed to 3 days after injury. In this group, only 4 of 7 carotid vessels incubated with Ad.RSVβgal showed gross evidence of β-gal staining (Fig 3A). This was again limited to less than 5% of the vessel surface. Histology revealed only occasional neointimal or medial SMC staining (Fig 3B). Control segments treated with AdCFTR either immediately (n=2) or 3 days after injury (n=2) showed no staining for β-gal activity.

In contrast, markedly increased β-gal activity was observed in segments in which infection was delayed until 7 days after injury (n=3). At this time, we found that neointima covered most but not all of the arterial surface. Intense staining, indicative of gene transfer and expression, was evident on gross examination in each of the arterial segments obtained from these animals exposed to β-gal-containing adenovirus (Fig 4C). Histological examination showed staining limited almost exclusively to the neointima (Fig 4D). We examined
multiple histological sections containing neointima from each of the animals infected 7 days after injury. The efficiency of gene transfer was noted to vary from approximately 10% to over 75% of neointimal cells (Figs 4D and 4E). In the majority of sections, over 50% of neointimal cells expressed β-gal. Interestingly, neointimal cells appeared to selectively express β-gal, as we observed that very few cells located deep to the first layer of internal elastic lamina stained positive for β-gal whether or not overlying neointima was present (Fig 4F).

Arterial segments infected 7 days after injury with control AdCFTR virus (n=3) showed no visible staining on gross or microscopic examination (Figs 4A and 4B).

In the final group in this series of experiments, we exposed arteries to Ad.RSVβgal at 12 days after injury and harvested the vessels 3 days later (n=2). At this point, the neointima is thicker and covers most of the arterial surface. Each of these segments showed uniform staining of their luminal surface (Fig 5A). Histology again showed efficient gene transfer that was selective for neointimal cells (Fig 5B). In general, the cells located in the more superficial portions of the neointima appeared to have a higher efficiency of gene transfer. Carotid segments in which animals were infected 12 days after injury with the control adenovirus AdCFTR showed no evidence of staining (n=2).

In the 3 animals in which distal organs were harvested in order to evaluate gene expression at distal sites, no β-gal staining could be visualized in any area by gross or histological evaluation.

Persistence of Gene Expression

In a separate group consisting of 3 animals, we made a preliminary assessment of the persistence of gene expression. Rats were infected 7 days after injury, and their carotid arteries were harvested 2 weeks later. Analysis of β-gal expression in these segments showed persistence of β-gal activity in each of the 3 animals. However, relative to segments harvested 3 days after infection, expression was markedly diminished as assessed by counting the number of cells with nuclear-dominant blue stain per high power field (data not shown).
Fig 4. This page and facing page.
Evaluation of in vivo gene transfer into injured arterial segments. Ad-CFTR (A and B) or Ad.RSVβgal (C through F) was instilled into injured carotid segments 7 days after balloon injury and harvested 3 days later. A, Gross pathology, ×10 magnification of a control infected artery shows no staining; B, histology, ×400 magnification of control infected segment shows no β-gal staining; C, gross pathology, ×10 magnification shows areas of β-gal staining; D, histology, ×400 magnification shows area of high-efficiency (>75%) gene transfer (neointimal cells appear to be specifically targeted, with little staining of underlying medial smooth muscle cells); E, histology, ×400 magnification shows an example of an area from another animal exhibiting a region of lower-efficiency gene transfer; F, histology, ×200 magnification shows variation in the presence and thickness of neointima. Staining is only evident in areas where neointima is present. N indicates neointima; M, media; and A, adventitia.
Discussion

Previous attempts to test the potential efficacy of gene transfer into the vascular wall, using either retrovirus- or liposome-mediated techniques, have been hampered by low efficiency of gene transfer.20-24 Recently it has been shown that replication-defective adenovirus vectors can efficiently transfer genes to the endothelium of uninjured veins and arteries both ex vivo and in vivo.36,43 Based on these observations, we evaluated the efficiency of adenovirus-mediated gene transfer in vivo to cells involved in the vascular response to injury. Using the rat carotid injury model, the data demonstrate that this can be achieved efficiently and selectively.

In uninjured vessels, gene transfer and expression were confined predominantly to endothelial cells and adventitial cells. Only rare medial SMCs expressed the β-gal gene product. Adventitial staining was often found around the vasa vasorum, which suggests that the virus may have entered the vasa vasorum and thereby gained access to and infected the surrounding cells. Another possibility is that minute quantities of viral solution could have been inadvertently spilled on the adventitial surface during the procedure. The efficiency of staining in uninjured vessels appeared to be considerably less impressive than was previously seen in sheep carotid segments.36 The basis for this difference is unclear but may reflect species variability to adenoviral infection.

In injured vessels, we noted a marked difference in gene transfer efficiencies, depending on when after injury the artery was exposed to virus. At early time points after injury (3 days or less), when endothelial cells were absent and there were few neointimal cells, efficiency of gene transfer was low and expression was confined to scattered medial and neointimal cells. Exposing vessels to adenovirus 7 days or 12 days after injury, during which time significant neointima had formed, we found a markedly increased efficiency of β-gal gene transfer and expression. Arteries treated identically but given a control virus (AdCFTR) showed no comparable β-gal staining. Previous studies have
indicated that injury itself may induce endogenous β-gal activity in cells.21,24 However, this injury induced, endogenous β-gal activity contrasts with that seen with Ad.RSVβgal (which contains a nuclear localization signal) in that it is generally weaker and thereby requires prolonged exposure to X-Gal solutions. We noted that control arteries left in X-Gal solution overnight, rather than the usual 4 hours that we used in this study, developed a faint blue staining pattern. Of importance, however, is the fact that this weak staining was cytoplasmic rather than nuclear.

In injured arteries, expression of the β-gal gene product was confined almost exclusively to neointimal cells. The mechanism responsible for this difference in susceptibility between neointimal and medial SMCs to adenovirus-mediated gene transfer and expression is unclear. One probable explanation is that a physical barrier, most likely the internal elastic lamina, prevents diffusion of virus into medial layers. Thus, neointimal cells that reside on the luminal side of the internal elastic lamina are able to be infected, whereas medial SMCs, which reside deep to the internal elastic lamina, are not. Alternatively, the process of injury-induced neointimal proliferation may affect the expression of the as yet uncharacterized adenoviral cell surface receptor. Increased expression of the target viral receptor by proliferating neointimal cells could explain their efficient and selective uptake of adenovirus. A third possibility is that both medial and neointimal cells were equally infected but that only neointimal cells possess those endogenous transcription factors needed for efficient expression of RSV-driven transcripts. Of note, however, in our in vitro experiments we found no appreciable difference in adenoviral infection between quiescent and proliferating SMCs. These in vitro experiments argue against large differences in viral receptor number or in RSV-dependent transcription between quiescent and proliferating SMCs and therefore support the concept of a physical barrier to infectivity.

In this study, we routinely evaluated gene expression 3 days after infection. In three segments, however, we assessed β-gal staining 2 weeks after infection. These segments showed continued but diminished expression of the marker gene. Previous studies in endothelial cells in vitro have shown that gene expression peaks around 7 days after infection and persists for at least 14 days.45 The same is true in endothelial cells in vivo.36 Long-term gene expression would not be expected from adenoviral gene transfer since the virus does not stably integrate into the genome of the host cell. Such a time frame of gene expression, while a potential drawback in the treatment of inherited genetic diseases, might actually be an advantage for the treatment of a temporally discrete event such as restenosis.

Any treatment strategy that uses recombinant adenovirus must address the question of safety. As mentioned above, the general lack of stable integration of adenovirus, as opposed to retroviruses, is a significant advantage in that issues of insertional mutagenesis are of less concern.37 The safety of adenovirus in humans has been tested in vaccine trials in the past44-47 and presently in ongoing clinical trials in the treatment of cystic fibrosis.48 Although we made no concerted effort to evaluate distal organs in all animals exposed to Ad.RSVβgal, we could not detect β-gal expression in the brain, liver, or heart of each of 3 animals in which these organs were harvested. This suggests that adenoviral gene transfer can in large part be limited to discrete vascular segments without subjecting distal organs to infection.

Although the present study uses only the histochemical marker gene β-gal, it suggests that other recombinant adenoviral vectors, with genes whose products have cytotoxic or antiproliferative effects, could be targeted to neointimal cells. Such viral constructs would be expected to have similarly high efficiency and selectivity of expression in neointimal cells. If the expression of such recombinant molecules inhibits the proliferation of SMCs, adenovirus-mediated gene transfer may be a useful strategy in the treatment of restenosis.

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