In Vivo Adenoviral Vector–Mediated Gene Transfer Into Balloon-Injured Rat Carotid Arteries

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We studied the ability of adenoviral vectors to achieve gene transfer into injured arteries. A recombinant adenoviral vector expressing a nuclear-targeted β-galactosidase gene was constructed and infused into balloon-injured rat carotid arteries. Three days after gene transfer, recombinant gene expression was assessed quantitatively by (1) measuring β-galactosidase antigen and activity in tissue extracts and (2) histochemical staining and counting of cells expressing β-galactosidase. Exposure of injured carotid arteries to increasing concentrations of the vector (10^8 to 10^10 plaque-forming units per milliliter) resulted in a dose-responsive increase in β-galactosidase expression, with peak expression of approximately 43 mU or 25 ng β-galactosidase per vessel. Microscopic examination of histochemically stained arteries demonstrated gene transfer limited to the vascular media; transduced cells were identified immunohistochemically as smooth muscle cells. Counting of both histochemically stained and total nuclei in the media revealed that approximately 30% of the cells in the media of the injured vessels were transduced. Calculations based on both counting cells and on the level of β-galactosidase expression in tissue extracts suggested the presence of 5000 to 10 000 transduced cells per 10 mm of vessel. Arteries infused with either vehicle only, a control adenoviral vector, or liposomes combined with the vector plasmid contained little or no evidence of β-galactosidase expression. High levels of in vivo β-galactosidase expression persisted for at least 7 days after gene transfer but declined significantly by day 14. We conclude that adenoviral vector–mediated gene transfer into the injured rat carotid artery results in efficient gene transfer into the vascular media, with levels of recombinant protein production significantly higher than any previously reported in arterial gene transfer studies. Adenoviral vectors appear to be particularly useful agents for in vivo arterial gene transfer. (Circ Res. 1993;73:797-807.)

KEY WORDS: • gene therapy • β-galactosidase • smooth muscle cells • gene expression

Direct in vivo transfer of genetic material into the arterial wall offers an experimental approach to (1) the study of the role of individual genes in vascular pathophysiology and (2) the development of localized gene therapies for vascular disorders.1,2 Optimal features for a system of in vivo arterial gene transfer would include high efficiency (as measured by the percentage of target cells into which gene transfer occurred), significant levels of gene expression (as measured by the mass of recombinant gene product present in arterial tissue extracts), and an ability to identify specifically in tissue sections the cells into which gene transfer had occurred. A model gene transfer system that includes all of these features would facilitate the performance of both pathophysiological and therapeutic experiments. Several groups have reported data generated in animal models of in vivo arterial gene transfer, using either viral or plasmid vectors.3-9 None of these model systems, however, incorporate all of the three features listed above: high efficiency, significant mass of recombinant protein, and specific cellular localization.

Low efficiency has been a recurrent problem in arterial gene transfer studies. In a previous study, we estimated the efficiency of retroviral vector–mediated arterial gene transfer at fewer than 100 cells per vessel.5 Leclerc et al10 reported gene transfer into fewer than 1 in 103 cells, using a liposome-assisted technique of plasmid transfection. No definitive study has yet presented quantitative data that support an in vivo arterial gene transfer efficiency higher than that reported in these two studies.

Three groups have reported calculations of the mass of recombinant protein produced after arterial gene transfer.4,7,8,11 These calculations have all been based on measurements of luciferase activity, with conversion to luciferase mass by reference to a luciferase preparation of known specific activity. Reported levels of recombinant luciferase have been low, in the range of femtograms to picograms per vessel, and have varied widely between studies.4,7,8 No direct measurement of luciferase mass has yet been reported, and no histological identification of recombinant luciferase protein has yet been demonstrated.
Specific identification in tissue sections of arterial cells expressing a recombinant protein has been reported by three groups, all working with genes other than luciferase.\textsuperscript{3,9,12,13} Histological identification was accomplished by histochemical staining for \(\beta\)-galactosidase expression\textsuperscript{3,9} or by immunohistochemistry for the HLA-B7 or Simian virus-40 (SV40) T antigen.\textsuperscript{12,13} None of these groups has presented quantitative data that document the existence of high-efficiency in vivo gene transfer, and in none of these studies was recombinant protein expression specifically quantitated.

Recent reports of the use of recombinant adenoviral vectors led us to attempt to use these vectors for in vivo arterial gene transfer. These reports have provided evidence that adenoviral vectors are capable of high-efficiency gene transfer both in vivo and ex vivo.\textsuperscript{14-17} Incapacitating in a easy, detectable levels of recombinant protein production.\textsuperscript{15,18} In addition to the potential for adenoviral vectors to effect efficient gene transfer and high levels of recombinant gene expression, many of these studies incorporated a nuclear-targeted \(\beta\)-galactosidase marker gene to allow specific histological identification of transduced cells, thereby demonstrating simultaneous achievement of high-efficiency significant gene expression and histological localization.\textsuperscript{15,18}

In the present study, we used an adenoviral vector containing a nuclear-targeted \(\beta\)-galactosidase gene to perform direct in vivo gene transfer into the balloon-injured rat carotid artery. The purpose of the study was to determine the following in this well-recognized model of arterial injury: (1) the efficiency of in vivo adenoviral vector-mediated gene transfer, (2) the level of recombinant protein production consequent to gene transfer, (3) the cellular targets of gene transfer, and (4) the duration of recombinant gene expression. The results demonstrate that adenoviral vector-mediated gene transfer into the injured rat carotid artery results in efficient gene transfer into the vascular media, with levels of in vivo recombinant protein production significantly higher than any previously reported in arterial gene transfer studies.

Materials and Methods

Construction of Recombinant Adenoviral Vectors

A recombinant adenoviral vector (Av1LacZ4, Fig 1) expressing a nuclear-targeted \(\beta\)-galactosidase gene was constructed in several steps, consisting of routine prokaryotic cloning and a homologous recombination procedure in the cell line 293 (American Type Culture Collection, Rockville, Md). Adenovirus sequences were obtained from Ad-d1327, an adenovirus serotype 5 (Ad5) derivative.\textsuperscript{19} A plasmid was built that contained the following elements inserted between the Not I and Xba I sites of pSKII (Stratagene Inc, La Jolla, Calif): (1) the Ad5 left-inverted terminal repeat and encapsidation signal/E1A enhancer (bases 1 to 392 of Ad5; Genbank accession No. M73260 applies to this and all other Ad5 sequences), (2) an Asc I restriction site, (3) the Rous sarcoma virus LTR promoter (bases 209 to 605 from plasmid pRC/RSV; Invitrogen, San Diego, Calif), (4) an Sfi I restriction site, and (5) the tripartite leader sequence from the Ad5 major late transcriptional unit (exon sequences from bases 6049 to 9730). The following DNA sequences were ligated into this first plasmid:

(1) A nuclear-targeted \(\beta\)-galactosidase (also termed LacZ) gene (see below for construction of this gene) was inserted into the EcoRV site. (2) An Hpa I–BamHI fragment of SV40 DNA (BRL, Gaithersburg, Md) containing a polyadenylation site was inserted at the Sal I site. (3) An Ad5 fragment (bases 3328 to 6241) was inserted into the Xho I site. The nuclear-targeted \(\beta\)-galactosidase gene was constructed from the first four codons of the SV40 large T antigen, codons 127 to 147 encoding the SV40 large T antigen nuclear-targeting signal (Genbank accession No. V01380), and codons 6 to 1021 of the Escherichia coli LacZ gene (bases 1302 to 4358, Genbank accession No. J01636). The final plasmid resulting from these steps was termed pAvS6nLacZ.

To construct the infectious replication-deficient adenovirus Av1LacZ4, the pAvS6nLacZ plasmid was linearized and cotransfected into 293 cells along with the 35-kb Cla I fragment of Ad-d1327.\textsuperscript{19} The use of this cotransfection technique to promote homologous recombination between the regions of each molecule corresponding to Ad5 sequences 3328 to 6241 with resultant generation of infectious virus has been described previously.\textsuperscript{20} Recombinant plaques were picked and propagated further in 293 cells. The Av1LC2 vector was constructed by similar methods using a plasmid that contained a normal human cystic fibrosis transmembrane conductance regulator cDNA (Genbank acces-
sion No. M28668) in place of the nuclear-targeted lacZ gene that was present in pAv6nLacZ. Purified DNA from both recombinant vectors was evaluated by Southern analysis to confirm structural integrity (data not shown).

Production of High-Titer Adenoviral Vectors

Highly purified Av1LacZ4 and Av1C12 viruses were produced, stored, and titered as previously described. Briefly, lysates of 293 cells infected with the recombinant virus were centrifuged through cesium chloride gradients. Purified virus was dialyzed and then stored at -80°C until use. To expedite the use of the viral preparations, titer of purified viral stocks was determined by a "quick titer" method based on evaluation of the virus preparations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie blue staining. The intensity of the 110-kDa band corresponding to the viral hexon protein was compared with the intensity of a band produced by electrophoresis of a standard virus preparation of known biologic titer (as determined by formation of plaques on 293 cells). This quick titer method routinely provides titers that are within twofold to fourfold of titers determined by inoculation of 293 cells with dilutions of the same viral stocks and counting plaques. This relation has held true both in general and specifically for the viral stocks used in the present study (data not shown). The titers of the purified viral stocks available for use in these experiments were approximately 10^7 plaque-forming units (pfu) per milliliter, as determined by the quick titer method. All experiments were performed using dilutions of virus based on these quick titers.

Rat Aortic Smooth Muscle Harvest, Culture, and In Vitro Adenoviral Vector–Mediated Gene Transfer

Rat aortic smooth muscle cells (SMCs) were harvested from a segment of abdominal aorta of a male Fisher rat by enzymatic digestion with collagenase type I (Worthington Biochemical Corp, Freehold, NJ), as previously described. The cells were characterized by positive indirect immunofluorescent staining for muscle actin (HHF35, Dako, Carpinteria, Calif) and negative staining for von Willebrand's factor (Boehringer Mannheim Corp, Indianapolis, Ind). Cells were grown in M-199 medium (Biofluids, Inc, Rockville, Md) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah) at 37°C under 5% CO₂ and were passaged using trypsin digestion.

For in vitro gene transfer, SMCs of passages 4 to 6 were plated in a 12-well plate at 2 x 10⁶ cells per well. One to 2 days after plating, SMCs were transduced by incubation for 90 minutes with adenoviral vectors diluted in M-199 supplemented with 2% heat-inactivated fetal calf serum. After this incubation, virus-containing culture medium was removed and replaced with fresh virus-free medium.

Determination of Transduction Efficiency In Vitro

The percentage of cultured SMCs transduced by the Av1LacZ4 vector was estimated by staining transduced cultures with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal), as previously described. X-Gal staining was performed 3 days after transduction. To eliminate selection bias that might have resulted from counting transduced cells in situ, three wells of SMCs were trypsinized, fixed in 2% formaldehyde and 0.2% glutaraldehyde for 5 minutes, and then resuspended and stained with X-Gal for 2 hours. Stained cell suspensions were loaded into a hemacytometer, and only cells present in the counting chamber were counted. Total cells as well as cells with nuclear-localized blue staining were counted. Fixation and X-Gal staining of SMCs was also performed on cultured cells in situ, without trypsinization, to obtain an additional visual assessment of the percentage of transduced cells.

The mean amount of β-galactosidase expressed per transduced cell was measured by determination of both β-galactosidase antigen and activity. Transduced populations of SMCs in three wells were trypsinized, and the total amount of cells per well was determined by counting aliquots in a hemacytometer. The remaining cells were lysed with buffer containing 0.2% Triton X-100 and 100 mmol/L potassium phosphate, pH 7.8. The total volume of cell lysate was 25 to 200 µL.

Aliquots of cell lysate were assayed for β-galactosidase activity with the substrate 3-[(4-methoxyxypir[1,2-dioxetane-3,2′-tricyclo-[3.3.1.1³⁷]decane]-4-yl)phenyl]-β-d-galactopyranoside (AMPGD, Galacto-Light, Tropix, Inc, Bedford, Mass). Light emission was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, Calif) calibrated with a standard curve generated by using purified E. coli β-galactosidase (specific activity, 300 U/mg; Boehringer Mannheim). One unit of β-galactosidase releases 1 µmol of 2-nitrophenol from the substrate 2-nitrophenyl-β-d-galactoside in 1 minute at 37°C, pH 7.8. The activity assay gave a linear response to levels of the β-galactosidase standard ranging from 1.5 to 1500 µU, with the amount of light emission at 1.5 µU approximately two to five times background. All assays were done in duplicate on 2- to 13-fold dilutions of the cell lysate. Assay samples with light emission below that generated by the 1.5-µU standard were read as "undetectable."

Additional aliquots of cell lysate were assayed for β-galactosidase antigen with a β-galactosidase enzyme-linked immunosorbent assay (ELISA) kit (5 Prime 3 Prime, Inc, Boulder, Colo). This ELISA gave a linear response to β-galactosidase concentrations between 100 and 2000 pg/mL. Samples of cell lysate were assayed at dilutions of 1:40 to 1:500 to obtain readings within the range of the standard curve.

In Vivo Transduction With Adenoviral Vectors

All animal procedures were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. A total of 66 male Fisher rats (Taconic Farms, Inc, Germantown, NY) weighing 350 to 450 g were used for adenoviral vector inoculation, lipofection (see below), or control studies. Rats were anesthetized with intramuscular injections of ketamine (66.7 mg/kg, Fort Dodge Laboratories, Inc, Fort Dodge, Iowa) and xylazine (6.7 mg/kg, Mobay Corp, Shawnee, Kan). Further anesthesia was administered by means of inhaled methoxyflurane (Pitman-Moore, Mundelein, Ill). The left carotid artery was surgically exposed, and an arteriotomy was performed in the external carotid artery. Injury of a segment of the left common carotid artery was achieved by insertion and passage of a 2F Fogarty balloon catheter, as previously described.
ensure adequate injury, the inflated catheter was passed a total of three times. After balloon injury, a segment of common carotid artery approximately 1 cm in length was isolated by placement of vascular clamps on the proximal common carotid artery and the proximal internal carotid artery. A plastic catheter was introduced through the external carotid arteriotomy, and the isolated vessel segment was flushed with M-199 medium before introduction of either adenoviral vectors or control infusate.

Adenoviral vector stocks were thawed, diluted, and maintained on wet ice until use. All stocks were used within 2 hours of thawing. For each rat, a total of 50 μL diluted adenoviral vector was instilled into the isolated common carotid segment by means of a catheter placed in the external carotid artery. During infusion of the vector-containing medium, the isolated segment became distended and remained so for the duration of the instillation period. After 20 minutes of incubation, the vector-containing medium was withdrawn, the external carotid was ligated, and blood flow through the common and internal carotid arteries was reestablished. Rats were allowed to recover from anesthesia and were returned to their cages. At varying time intervals after gene transfer, rats were killed by CO2 asphyxiation, and the left carotid artery was removed for analysis.

In Vivo Liposome-Mediated Gene Transfer

Liposome-mediated gene transfer was attempted in 3 of the 66 animals. Surgical procedures were performed in the same manner as described above for instillation of adenoviral vectors. Briefly, 2 μg plasmid DNA in 10 μL M-199 medium was added to 10 μL M-199 medium containing 6 μg Lipofectin reagent (BRL). After a 15-minute incubation at room temperature, the DNA-liposome mixture was further diluted with 80 μL M-199 medium. The diluted DNA-liposome mixture (50 μL) was instilled into an isolated segment of left common carotid artery for 20 minutes, as described above for adenoviral vector-mediated gene transfer.

Measurement of β-Galactosidase Activity and Antigen After In Vivo Gene Transfer

The adventitia of the excised carotid artery was carefully removed. The vessel was then minced in lysis buffer containing 0.2% Triton X-100 and 100 mmol/L potassium phosphate, pH 7.8. Tissue debris was cleared by centrifugation at 14,000g for 10 minutes, leaving a lysate volume of approximately 200 μL. β-Galactosidase activity in the tissue extract was measured using the Galacto-Light assay kit as described above for extracts of cultured cells. All measurements were performed in duplicate with twofold and fourfold dilutions of the lysate. The mean of the two measurements was used to calculate total β-galactosidase activity per vessel. These duplicate measurements typically varied by no more than 20% and, as described above for the cultured cell lysates, were considered to be “undetectable” if the luminometer readings were below those obtained with the 1.5-μU standard. Total protein concentration in the tissue lysate was measured with the BCA protein assay (Pierce Chemical Co, Rockford, Ill). Additional aliquots of the tissue extracts were assayed for β-galactosidase antigen. Antigen concentration was measured using an ELISA kit, as described above. The tissue lysate from experimental vessels required dilutions of 4- to 400-fold to obtain readings within the range of the standard curve.

Duration of Recombinant Gene Expression After Adenoviral Vector-Mediated Gene Transfer

To determine the duration of expression of the β-galactosidase transgene after in vivo gene transfer, 31 of the total of 66 rats were included in a time-course study. Twenty-one carotid arteries were injured and infused with the AviLacZ4 vector at 1010 pfu/mL, as described above; 10 were injured and infused with vehicle only. AviLacZ4-transduced vessels were harvested at 3 days (n=4), 7 days (n=4), 14 days (n=5), 28 days (n=5), and 42 days (n=3) after gene transfer. Two vehicle-only control vessels were harvested at each of these five time points. β-Galactosidase antigen and activity were measured in tissue extracts, as described above.

Histological, Histochemical, and Immunohistochemical Analysis

A separate series of experiments, including 10 rats, was performed to obtain samples for histological analysis. Six carotid arteries were exposed to the AviLacZ4 vector at a concentration of 1010 pfu/mL, and four arteries were exposed to vehicle only. On day 3 after gene transfer, the rats were killed, and the arteries were harvested. Four of the arteries that were exposed to the AviLacZ4 vector and two of the arteries that were exposed to vehicle alone were removed and divided longitudinally. One half of each vessel was processed for histochemical examination; the other half was used for extraction and measurement of β-galactosidase, as described above. For the additional (two experimental and two control) arteries, the entire vessel was processed for histology, without longitudinal division and with no tissue extraction performed. Before histological analysis, all vessel segments were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 20 minutes, rinsed in phosphate-buffered saline, and placed in X-Gal staining solution for 2 hours at 37°C. After 2 hours, the vessel segments were rinsed in phosphate-buffered saline and returned to the formaldehyde/glutaraldehyde fixative. A relatively short period of staining (2 hours) was used since a longer duration of staining occasionally resulted in a nonspecific background staining (diffuse blue). Our use of a short period of staining could potentially result in underestimation of the number of transduced cells because of the lack of staining of cells expressing low amounts of β-galactosidase. The estimated transduction efficiency therefore may represent a lower limit of transduction efficiency using the adenoviral vector.

X-Gal-stained arteries (both half- and whole-vessel segments) were examined and photographed through a Zeiss dissecting microscope and then were sectioned into three or four serial segments each approximately 2 to 3 mm in length. These serial segments were embedded alongside each other in a single paraffin block. Microtome sections (5 μm thick) were cut from the blocks at two levels spaced 50 μm apart, placed on slides, and stained with either hematoxylin and eosin, nuclear fast red, Masson’s trichrome, or Movat’s stain. In all cases, the observed pattern of staining confirmed
that the 50-μm spacing effectively separated the sections so as to avoid counting the same cells twice.

Stained sections were examined by light microscopy for the presence of deep blue nuclei indicative of β-galactosidase expression. The number of X-Gal-stained cells and the total number of cells in the vascular media were determined by counting nuclei on sections stained with either nuclear fast red (for X-Gal-positive nuclei) or hematoxylin and eosin (for total nuclei). For the half-vessel segments, the counted numbers of X-Gal-stained and total cells were multiplied by two in order to determine the number of cells per section of whole vessel. In this manner, for each vessel both transduced and total medial cells were counted from a total of six to eight sections per vessel, and the percentage of medial cell transduction was calculated. In addition, for the vessels receiving the AvlLacZ4 vector, the number of transduced cells in one half of a vessel segment (as determined by X-Gal staining) could be compared with the levels of β-galactosidase antigen and activity in the lysates obtained from the opposite half of the same vessel.

Sections of whole vessels that had been stained with X-Gal were also processed for immunohistochemical detection of smooth muscle cell actin. Staining was performed using an antibody to smooth muscle cell actin (Sigma Chemical Co, St Louis, Mo) at a dilution of 1:20,000. Bound antibody was detected with a secondary antibody conjugated to horseradish peroxidase.

**Results**

**In Vitro Transduction and Expression of β-Galactosidase**

In preliminary experiments, cultured rat SMCs were transduced with dilutions of the AvlLacZ4 vector, resulting in a multiplicity of infection (MOI) ranging from $5 \times 10^3$ to $5 \times 10^4$ pfu per cell. Cells were fixed and stained with X-Gal 3 days after transduction. Based on a visual assessment of the percentage of X-Gal-stained cells, transduction efficiency increased with increasing MOI over this entire range, with over 80% transduced cells resulting in an MOI of $5 \times 10^4$. However, significant cellular toxicity was observed at an MOI of $5 \times 10^4$, as determined by detachment of a majority of cells from the dish after exposure to the virions. For this reason, further in vitro experiments were carried out at an MOI of $5 \times 10^3$ pfu per cell.

To obtain a more quantitative assessment of gene transfer, 3 days after transduction SMCs were trypsinized, stained with X-Gal, and counted. The mean number of cells staining positively for nuclear-localized β-galactosidase expression was $30.4 \pm 5.6\%$ (mean±SD, $n=7$ from two separate experiments). This calculated transduction efficiency corresponded well to gross examination of wells transduced in parallel with the AvlLacZ4 adenoviral vector and stained with X-Gal in situ (Fig 2).

The quantity of recombinant gene product expressed from cultured rat SMCs transduced by the AvlLacZ4 adenoviral vector was determined by assay of both β-galactosidase antigen and activity. Activity and antigen were measured in extracts prepared from cell populations that had been transduced at an MOI of $5 \times 10^3$. As determined by the ELISA, the extracts contained $184 \pm 35$ pg β-galactosidase antigen per $10^6$ total cells (mean±SD, $n=4$). The activity assay revealed the presence of $162 \pm 48$ μU β-galactosidase activity per $10^6$ total cells (mean±SD, $n=5$ from two separate experiments). With an assumed transduction efficiency of 30%, as estimated by the nuclear X-Gal staining results described above, and the results of the activity and antigen assays performed on lysates of total cells, transduced SMCs were determined to express approximately 610 pg or 540 μU β-galactosidase per $10^3$ transduced cells.

**In Vivo Gene Transfer and Expression of β-Galactosidase**

In initial studies to optimize in vivo gene transfer, 22 rat carotid arteries were injured and exposed to either the AvlLacZ4 adenoviral vector ($n=16$), the AvlCf2 adenoviral vector ($n=3$), or vehicle alone ($n=3$). Three
additional rat carotid arteries were injured and exposed to Lipofectin and the pAVS6nLacZ plasmid. Three days after transduction, arteries were harvested, and β-galactosidase antigen was measured in tissue extracts (Fig 3). β-Galactosidase antigen was undetectable in extracts from arteries that were exposed to either vehicle alone or to the AV1CF2 adenoviral vector. In extracts from arteries exposed to the AV1LacZ4 adenoviral vector, β-galactosidase antigen was undetectable in arteries exposed to $10^9$ pfu/mL of vector. β-Galactosidase antigen was measurable in extracts from three of seven arteries (43%) exposed to $10^9$ pfu/mL and six of six arteries (100%) exposed to $10^{10}$ pfu/mL. Within this range of vector concentration, the level of β-galactosidase antigen in the tissue extracts increased in a dose-responsive manner.

Extracts from carotid arteries were also assayed for β-galactosidase activity (Fig 4). As with the results from the β-galactosidase antigen assay, activity was below the level of detection in extracts from arteries that were exposed to either vehicle alone or to an adenoviral vector that contains a cDNA for the human cystic fibrosis transmembrane conductance regulator. No β-galactosidase activity was detectable in extracts prepared from arteries exposed to $10^9$ pfu/mL of vector. β-Galactosidase activity was measurable in four of seven arteries (57%) exposed to $10^9$ pfu/mL and six of six arteries (100%) exposed to $10^{10}$ pfu/mL. As for the antigen measurements, levels of β-galactosidase activity in extracts prepared from arteries that were exposed to the AV1LacZ4 adenoviral vector also increased in a dose-responsive manner.

The levels of both β-galactosidase activity and antigen in extracts prepared from arteries exposed to identical concentrations of the AV1LacZ4 vector were quite variable. For example, both antigen and activity measurements from extracts of arteries exposed to $10^9$ pfu/mL varied by up to one to two logs (Figs 3 and 4). Part of the variability (in results expressed as antigen or activity per milligram of total protein) derived from differences in the amount of total protein measured in the tissue extracts (data not shown). This component of variability most likely results from differences in the extent of removal of the adventitia, which is a technical issue in the assay.

![Graph showing β-galactosidase antigen expression after in vivo gene transfer.](Fig 3)

Injured rat carotid arteries were exposed to increasing concentrations of a β-galactosidase–containing adenoviral vector (AV1LacZ4), an adenoviral vector (AV1CF2) expressing the human cystic fibrosis transmembrane conductance regulator (at a concentration of $10^{10}$ pfu/mL), vehicle alone, or Lipofectin-plasmid DNA (Lipofectin/pAVS6nLacZ) complex. Three days after transduction, β-galactosidase expression was measured from tissue lysate using an enzyme-linked immunosorbent assay. Each data point represents a single animal, bars indicate the mean ± SD of β-galactosidase antigen in each group, and pfu indicates plaque-forming units. In calculating means, samples with antigen below the level of detection were arbitrarily assigned an antigen level of 3.2 ng/mg (the lower limit of detection in this experiment).

![Graph showing β-galactosidase activity in vivo.](Fig 4)

Aliquots (5 and 10 µL) of tissue lysate from the same injured rat carotid arteries shown in Fig 3 were used to measure β-galactosidase activity. Injured rat carotid arteries were exposed to increasing concentrations of a β-galactosidase–containing adenoviral vector (AV1LacZ4), an adenoviral vector (AV1CF2) expressing the human cystic fibrosis transmembrane conductance regulator (at a concentration of $10^{10}$ pfu/mL), vehicle alone, or Lipofectin-plasmid DNA (Lipofectin/pAVS6nLacZ) complex. Each data point represents mean values for a single animal, bars indicate the mean ± SD of β-galactosidase activity for each group, and pfu indicates plaque-forming units. In calculating group means, samples with activity below the level of detection were arbitrarily assigned an activity of 2.2 mU/mg (the lower limit of detection in this experiment).
cally difficult aspect of the protocol to control quantitatively. However, even when calculations were done on a per vessel basis, which eliminates the variable of adventitial removal, there remained significant variations in both antigen and activity. In vessels exposed to $10^{10}$ pfu/mL, $\beta$-galactosidase antigen values ranged from 3.4 to 25 ng per 10-mm vessel, and $\beta$-galactosidase activity ranged from 1 to 43 mU per 10-mm vessel.

To compare the level of $\beta$-galactosidase expression achieved after adenoviral vector–mediated gene transfer with that obtained with liposome-mediated gene transfer, the parent plasmid used to generate the $\beta$-galactosidase transcriptional unit of the AvlLacZ4 vector was introduced into the carotid arteries of three rats along with the Lipofectin reagent. In all animals, both $\beta$-galactosidase antigen and activity were below the level of detection of the assays (less than 3.2 ng of antigen per milligram of total protein and fewer than 2.2 mU of activity per milligram of protein, Figs 3 and 4). The level of expression resulting from liposome-mediated gene transfer is therefore at least one to two orders of magnitude below that detected after adenoviral vector–mediated gene transfer.

**Duration of $\beta$-Galactosidase Expression In Vivo**

Thirty-one rats were included in a time-course study; 21 received the AvlLacZ4 vector, and 10 received “vehicle only” control infusions. Carotid arteries were harvested at 3, 7, 14, 28, and 42 days after injury and adenoviral vector–mediated gene transfer (Fig 5A). $\beta$-Galactosidase activity in AvlLacZ4-transduced vessels at 3 days was $210 \pm 45$ mU/mg protein, similar to that found in the initial in vivo studies (Fig 4). The corresponding values at 7, 14, 28, and 42 days were $340 \pm 155$, $3.9 \pm 3.7$, $2.8 \pm 0.94$, and $2.2 \pm 1$ mU/mg protein. Therefore, the high level of initial transient expression persisted for at least 7 days but declined dramatically by day 14 after gene transfer. $\beta$-Galactosidase antigen levels in the AvlLacZ4-transduced vessels followed a similar pattern with initially high levels at 3 and 7 days (109 ± 28 and 215 ± 72 ng/mg protein), followed by a steep decrease such that in the day-14 and -28 samples antigen levels were only $2.6 \pm 4.1$ and $1.3 \pm 1.5$ ng/mg protein, respectively (Fig 5B). By day 42, $\beta$-galactosidase antigen was undetectable. For all control vessels, both $\beta$-galactosidase antigen and activity were below the limit of detection.

**Histological and Histochemical Analysis of Transduced Vessels**

For purposes of histological and histochemical analysis, an additional 10 rat carotid arteries were injured, exposed to the AvlLacZ4 vector (n=6) or vehicle alone (n=4), excised 3 days later, and stained with X-Gal. Gross observation of the luminal surface of X-Gal-stained arterial segments that were exposed to the AvlLacZ4 vector showed distinct blue dots as well as confluent dark blue areas (Fig 6A). Gross examination of the X-Gal–stained arterial segments that were exposed to the vehicle alone showed occasional diffuse light blue staining on the abluminal surface (not shown). However, these control vessels showed absolutely no dark blue or punctate staining on either the abluminal or the luminal surface (Fig 6B).

Microscopic examination of tissue sections obtained from vessel segments exposed to AvlLacZ4 and stained with X-Gal revealed focal dark blue nuclear staining, suggestive of expression of nuclear-targeted $\beta$-galactosidase (Fig 7A). Blue-staining cells were limited to the media of the vessel wall (Fig 7C) and were identified as smooth muscle cells by immunohistochemistry (Fig 7D). Sections of experimental arteries showed extensive injury in the media, as evidenced by the fairly widespread coagulation necrosis of smooth muscle cells and absence of nuclei (Fig 7F). In some areas, focal loss of cells between elastic laminas was observed (Fig 7C). In addition, many sections revealed the presence of intra-
mural hemorrhage within the vascular media (Fig 7E). Substantial medial injury including hemorrhage and necrosis was also present in control vessels that were balloon-injured but into which only vehicle had been infused. Microscopic examination of sections of the control vessels revealed evidence of vascular injury but with total absence of deep blue staining in any of the layers of the vessel wall (Fig 7B).

Estimation of Number of Transduced Cells per Vessel

The histological sections were used to obtain an estimate of the number of transduced cells per vessel and of the percentage of total medial cells that were transduced. The number of X-Gal-stained cells per histological section of whole vessel was 20±10 (mean±SEM; n=44 sections from six animals; range, 0 to 100 cells), and the number of total medial nuclei per histological section of whole vessel was 76±10 (mean±SEM; n=44 sections from six animals; range, 0 to 240 cells). The percentage of X-Gal-stained cells in the media of vessels taken from the six animals was 29±5% (mean±SEM; range, 15% to 43%). Counting of transduced and total cells was repeated by a second observer, with findings differing by less than 10% from those cited (data not shown). Assuming an SMC diameter of 20 μm, there are 500 layers of SMCs in a 10-mm length of carotid artery. With a mean of 20 transduced cells per layer, we calculated a total of 10,000 transduced cells per 10 mm of vessel.

As an additional means of determining the number of transduced cells per vessel segment, β-galactosidase antigen and activity were measured in tissue extracts from the other half of four of the six vessel segments that were exposed to the Av1LacZ4 vector. Measured β-galactosidase antigen and activity in tissue extracts were converted to number of transduced cells using the level of β-galactosidase expression determined per transduced cell in vitro (see above). Extrapolation of the level of expression of β-galactosidase by cultured smooth muscle cells to that present in vivo rests on the untested assumption that the expression of β-galactosidase per cell in vivo and in vitro is similar.

Tissue extracts of the four vessel segments contained 6.2±3.0 ng β-galactosidase antigen per 10-mm vessel (mean±SD). The same extracts contained 2.9±1.2 mU (mean±SD) of β-galactosidase per 10-mm vessel. Assuming 610 pg or 540 μU of β-galactosidase per 10⁶ transduced cells (as calculated from the transduced cultured SMCs), the measured antigen and activity in vessel extracts suggest the presence of approximately 5000 to 10,000 transduced cells in a 10-mm length of carotid artery. This calculated number of cells is very close to that obtained by the method of counting X-Gal-stained cells in the tissue sections that were made from
the other halves of the same vessels (see above). Of note, in this experiment (in contrast to the results of the experiment illustrated in Fig 4), a small amount of endogenous β-galactosidase activity was detectable in the tissue extracts from the two arteries infused with vehicle only (data not shown). The level of β-galactosidase activity in these control vessels was only slightly above the limit of detection and was 30-fold below that present in extracts from the four experimental arteries. No β-galactosidase antigen was detectable in these control extracts.

Discussion

In the present study, we demonstrate that, after balloon injury, adenoviral vectors can be used to achieve in vivo gene transfer into a significant percentage of cells in the arterial media. On day 3 after balloon injury and gene transfer, approximately 30% of the medial smooth muscle cells express the recombinant gene, as determined by histochemical staining. In addition, gene transfer is accompanied by the production of recombinant protein at levels that are easily detectable both by activity and antigen assays of tissue extracts. Both the efficiency of gene transfer and the levels of recombinant protein production that we report are far above any previously reported after in vivo arterial gene transfer.

All previous reports of arterial gene transfer in which a recombinant gene product was quantitated have involved liposome-mediated gene transfer of a luciferase cDNA. Our finding of approximately 6 ng β-galactosidase antigen per 10-mm vessel compares favorably with these previous reports, in which measured luciferase activity was calculated to reflect the presence of luciferase mass ranging from approximately 60 fg to 30 pg per arterial segment. Given that these masses of recombinant protein are expressed on a per-vessel basis from arteries harvested from dogs, rabbits, and pigs, the relative levels of β-galactosidase that we report from extracts of the far smaller carotid arteries of rats compare quite favorably with those obtained from the larger animals.

Fig 7. Microscopic views of sections from rat carotid arteries. All vessels were stained with 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal) to detect β-galactosidase expression before sectioning. In all panels, LU indicates the vessel lumen. A shows a β-galactosidase-containing adenoviral vector (AvLacZ4)-transduced artery. Dark blue staining in the arterial media provides evidence of nuclear-targeted β-galactosidase expression (nuclear fast red stain, original magnification ×50). B shows artery exposed to vehicle alone. No blue staining is present (nuclear fast red stain, original magnification ×50). C shows an AvLacZ4-transduced artery. β-Galactosidase expression is confined to the vascular media. Arrow indicates focal loss of cells between sequential elastic laminae, which appear as black bands (Movat’s stain, original magnification ×200). D shows an AvLacZ4-transduced artery with an immunohistochemical stain for α-smooth muscle actin. Horseradish peroxidase reaction product colocalizes with X-Gal staining (original magnification ×400). E shows an AvLacZ4-transduced artery. The arrow indicates one of several areas of intramural hemorrhage (hematoxylin and eosin stain, original magnification ×200). F shows an AvLacZ4-transduced artery. Only a small number of cells in this section stain positively for β-galactosidase expression (arrow). Within the media, only very few nuclei are present, indicating extensive intramural necrosis (hematoxylin and eosin stain, original magnification ×50).
As a direct means of comparing lipofection and adenoviral vector–mediated gene transfer, we attempted liposome–mediated transfer of a plasmid expressing the same transcriptional unit present in the AvlLacZ4 vector. As expected from the historical comparisons made above, the level of gene transfer resulting from lipofection of the plasmid was far below that obtained with the AvlLacZ4 adenoviral vector. Although we did not make any attempts to further optimize the conditions for liposome–mediated gene transfer into the injured rat carotid, the concentrations of plasmid DNA and Lipofectin reagent used in the present study (20 μg/mL DNA with a ratio of DNA to Lipofectin of 1:3) are similar to those used by other investigators in optimized protocols, suggesting that the low efficiency of gene transfer is not a result of technical factors. We did not attempt retroviral vector–mediated gene transfer, given the very low efficiency previously reported both by our group and by others.

The utility of in vivo arterial gene transfer as an experimental system has been limited, to date, by the low levels of recombinant gene expression obtainable with either plasmids or retroviral vectors. Nabel et al reported the induction of vasculitis after arterial gene transfer of a cDNA encoding HLA-B7, although levels of HLA-B7 protein were not quantitated. Although a low level of expression of a particularly immunogenic protein may be sufficient to produce a biologic response, experiments designed to elucidate the in vivo role of other, less potent molecules may require higher levels of expression. Experiments designed to test the therapeutic effect of recombinant gene products when expressed in the vessel wall are also likely to require higher levels of expression than are obtainable with previously described arterial gene transfer techniques. Finally, it is possible that in individual systems modulation of the level of gene transfer may be desirable. The dose-responsive behavior described herein (Figs 3 and 4) may permit gene expression to be titrated over a broad range.

We compared the mass of recombinant β-galactosidase measured in the present study to levels of other proteins reported in tissue extracts from rat carotid arteries. The concentration of β-galactosidase in tissue extracts of carotid arteries (13 to 550 ng/mg protein for vessels transduced with AvlLacZ4 at viral concentrations of 10^10 PFU/mL) was higher than that reported for the endogenous enzyme tissue plasminogen activator (2 ng/mg protein) or the endogenous cytokine basic fibroblast growth factor (0.02 ng/mg protein). The level of β-galactosidase was below the reported concentrations of the cytoskeletal and contractile proteins vimentin, desmin, actin, and tropomyosin (10 to 250 μg/mg protein in rat carotid artery extracts). These data suggest that adenovirus–mediated gene transfer may result in levels of transgene expression that are in many cases sufficient to produce a local biologic effect.

Cultured Fisher rat smooth muscle cells were relatively resistant to transduction, as evidenced by the apparent transduction (based on 2 hours of X-Gal staining) of only 30% of cultured cells at an MOI of 5000 pfu per cell. This seemingly low efficiency of gene transfer compares with previous reports of significantly higher efficiencies of in vitro adenovirus–mediated gene transfer at a lower MOI. This low efficiency of in vitro adenovirus–mediated gene transfer may be a characteristic of rat smooth muscle cells, since transduction of sheep arterial smooth muscle cells using the same viral stocks resulted in an efficiency of approximately 80% at an MOI of 50 pfu per cell (data not shown). Finally, as mentioned above, our use of a brief (2-hour) X-Gal staining protocol may have resulted in an underestimation of the number of transduced cells, both in vitro and in vivo.

The confining of gene transfer to the vascular media was an unanticipated finding. Nabel et al reported both plasmid- and retroviral vector–mediated gene transfer in all layers of the normal pig iliofemoral artery wall. Leelerc et al reported that successful liposome–mediated gene transfer was confined to the neointima of atherosclerotic rabbit arteries. In a previous study in normal sheep carotid arteries, we found gene transfer essentially limited to the endothelium and the vasa vasorum of the adventitia with virtually no gene transfer into the media (J.J. Rome, V. Shagani, M.Y. Flugelman, K.D. Newman, A. Farb, R. Virmani, D.A. Dichek, manuscript submitted). It is difficult to reconcile all of these data produced in different laboratories with different techniques. We believe that the discrepancy between our findings in the sheep and those in the rat are due to two factors: (1) the presence of balloon denudation and (2) variability between the two species in the presence and distribution of vasa vasorum. In the rat carotid model, the passage of an inflated balloon removes the intima and causes significant damage to the media, thereby allowing relatively large viral particles (80 to 100 nm) to have access to the medial layers. Indeed, the barrier function of the intima is sufficiently disturbed by balloon injury to permit access of erythrocytes (7 μm in diameter) into the media (Fig 7E). Lack of transduction in the adventitia of the rat carotid artery is most likely due to the absence of ostia of vasa vasorum within the injured segment. Without these ostia, virions have no direct access to the adventitia and are otherwise unable to penetrate through the full thickness of the media.

Although the present model of gene delivery into an injured artery may be useful both for the study of gene expression in vivo and for the development of gene therapies for proliferative arterial disease, there are potential problems with the model that merit consideration. First, the level of gene transfer was substantially variable both within and between vessels (Figs 3 through 6). In vessels infused with equal numbers of virions, the expression level of β-galactosidase varied over more than one order of magnitude. The number of transduced cells per histological section also varied widely (see “Results”). One likely cause for the observed range of gene transfer efficiencies is variability in the degree of vessel wall necrosis secondary to balloon injury. When cell necrosis is extensive (Fig 7F), little or no transduction can occur; when cell viability is preserved, significant levels of gene transfer are possible (Fig 7A). Efforts to improve the uniformity of arterial injury may prove helpful in decreasing experimental variability.

An additional potential difficulty in the present model is the possibility that some of the observed cytopathic effects may result from exposure of cells to high-titer adenoviral vectors. Akli et al reported significant cytopathogenicity after injection of a high-titer (3 × 10^10 pfu/mL) adenoviral vector suspension into rat brains. These authors found no evidence of cytopathogenicity after injections of suspensions of lower titer. In the present study, we reviewed histological sections...
taken from a small number of balloon-injured carotids both with and without exposure to high-titer adenoviral vectors (10^10 pfu/mL). There appeared to be additional cell loss attributable to virion exposure (data not shown). A more extensive and systematic study of cytopathogenicity in blood vessels exposed to adenoviral vectors should be informative.

We documented high levels of gene expression for up to 7 days after gene transfer. Previous in vivo studies using adenoviral vectors have demonstrated long-term expression (over 1 year) in certain systems but not in others.30,31 Despite the limited duration of expression of the β-galactosidase adenoviral vector in the injured rat carotid, we anticipate that this system will be useful for both pathophysiological and therapeutic studies. Informative in vivo gene expression studies can be carried out within several days after gene transfer,32 and therapy effective in preventing arterial proliferation has been successful when delivered as a single bolus dose immediately after the injury.33

In summary, direct in vivo gene transfer into the vascular media of injured arteries can be achieved at high efficiency using adenovector-mediated gene transfer. The level of transgene expression is easily detectable and is as high or higher than expression of certain endogenous arterial proteins. In vivo adenovector-mediated gene transfer has the potential to be a useful technique for the study of vascular pathophysiology and for vascular gene therapy.

References
In vivo adenoviral vector-mediated gene transfer into balloon-injured rat carotid arteries.
S W Lee, B C Trapnell, J J Rade, R Virmani and D A Dichek

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