Adeno-associated virus vector transduction of vascular smooth muscle cells in vivo

MATTHIAS RICHTER,1 AKIKO IWATA,2 JOHN NYHUIS,2 YOSHIKO NITTA,2 A. DUSTY MILLER,3 CHRISTINE L. HALBERT,3 AND MARGARET D. ALLEN2
1Division of Cardiothoracic Surgery, Department of Surgery, University of Washington; the 2Fred Hutchinson Cancer Research Center, Seattle, Washington; and the 3Humboldt Universität zu Berlin, Berlin, Germany

**Richter, Matthias, Akiko Iwata, John Nyhuis, Yoshio Nitta, A. Dusty Miller, Christine L. Halbert, and Margaret D. Allen.** Adeno-associated virus vector transduction of vascular smooth muscle cells in vivo. Physiol Genomics 2: 117–127, 2000.—Adeno-associated virus (AAV) vectors might offer solutions for restenosis and angiogenesis by transducing nondividing cells and providing long-term gene expression. We investigated the feasibility of vascular cell transduction by AAV vectors in an in vivo rabbit carotid artery model. Time course of gene expression, inflammatory reaction to the vector, and effects of varying viral titer, exposure time, and intraluminal pressures on gene expression were examined. Recombinant AAV vectors with an Rous sarcoma virus promoter and alkaline phosphatase reporter gene were injected intraluminally into transiently isolated carotid segments. Following transduction, gene expression increased significantly over 14 days and then remained stable to 28 days, the last time point examined. Medial vascular smooth muscle cells were the main cell type transduced even with an intact endothelial layer. Increasing the viral titer and intraluminal pressure both enhanced transduction efficiency to achieve a mean of 34 ± 7% of the subintimal layer of smooth muscle cells expressing gene product. A mild inflammatory reaction, composed of T cells with only rare macrophages, with minimal intimal thickening was demonstrated in 40% of transduced vessels; inflammatory cells were not detected in sham-operated control arteries. These findings demonstrate that AAV is a promising vector for intravascular applications in coronary and peripheral vascular diseases.

**GENE THERAPY COULD PROVIDE new treatment options for genetic and acquired diseases but is currently limited by vector technology. An ideal vector would have high transduction efficiency, transfer genes into quiescent as well as dividing cells, and provide long-term gene expression. Additionally, the ideal vector should not create inflammatory or immune responses nor direct cytopathic effects, and it should not be pathogenic for humans.**

Adeno-associated virus (AAV) vectors provide relatively high transduction efficiency and are able to transduce nondividing as well as dividing cells (1, 10, 15, 16, 18–21, 36, 37, 40). The structure of their single-stranded DNA can be reduced to two terminal repeats, providing for packaging and integration (34), while leaving most of the 4.5-kb DNA to accommodate a promoter to enhance gene expression and a reporter or functional gene. Finally, AAV appears to be nonpathogenic for humans.

Given these advantages, AAV vectors would seem to be suitable for vascular transduction, given the quiescent state and slow turnover of vascular cells and the desirability of long-term gene expression for the treatment of such vascular diseases as atherosclerosis, posttransplant arteriopathy, and fibromuscular hyperplasia. Since the objective in treating arterial pathology is to provide local delivery of gene product, our aim in this study is to maximize the prevalence of gene expression in the vascular wall rather than to produce systemic levels of secreted gene products.

AAV vectors have been previously studied in airway, heart, liver, brain, and skeletal and cardiac muscle (1, 10, 15, 16, 18–21, 36, 37, 40), but fewer investigations have examined the potential for in vivo AAV vector transduction of the vascular wall (3, 13, 23, 24, 30). Controversy exists as to whether intraluminal delivery results in transduction of just endothelial cells in the vaso vasorum (23) or whether these small vectors can traverse the internal elastic lamina (IEL) and transduce medial smooth muscle cells (3, 13, 30). Compared with previous studies, these experiments utilize the high viral titers now available, a different promoter, varying intraluminal pressures, a surgical technique, and quantitative assessments of the prevalence of gene expression over the entire arterial luminal surface on whole vessel preparations as well as on cross sections.

In addition, host immune reaction was specifically sought by immunocytochemical identification of infiltrating leukocytes. The result of optimizing several variables in this model is that we have arrived at a method of achieving reporter gene expression in approximately one third or more of the subintimal vascular smooth muscle cells (VSMCs) in a rabbit carotid artery model with an intact endothelium. These techniques would be suitable for clinical applications in either intraoperative intra-arterial delivery or catheter-based percutaneous interventions for a number of vascular pathologies where alteration of smooth muscle cell targets would be advantageous.
MATERIALS AND METHODS

Animals. New Zealand White and inbred Stauffland rabbits (R and R Rabbitry, Stanwood, WA), ranging in weight from 2.2 to 3.6 kg, were used for all experiments. All experiments were performed in accordance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research, and the “Guide for the Care and Use of Laboratory Animals” (DHHS Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892).

Operative technique for in vivo AAV vector transduction of rabbit carotid arteries. Rabbits were sedated with a mixture of ketamine (35 mg/kg), xylazine (5 mg/kg), and atropine (0.032 mg/kg) given intramuscularly. After endotracheal intubation, animals were mechanically ventilated with halothane in 100% oxygen to maintain general anesthesia. Each rabbit was heparinized systemically with 300 U/kg of heparin sodium prior to carotid artery manipulation. The left carotid artery was surgically exposed, and vascular spasm was averted with topical lidocaine. A 3.0-cm segment of the carotid was isolated between atraumatic vascular clamps. A 25-gauge butterfly needle was introduced into the proximal end of the isolated segment. The distal clamp was opened briefly, and blood was flushed out of the vessel by injecting Ringer solution through the butterfly needle; then the distal clamp was again closed. A second needle for later vector instillation was then inserted into the distal end of the isolated segment and fixed in place with a pursestring to ensure that no leakage could occur. The Ringer solution was withdrawn up into the proximal needle to empty the vascular lumen, and a third clamp was placed just distal to the “Ringer needle” tip so that the site of needle entry would be excluded from the surface area exposed to the vector. Into this isolated and now-empty vascular segment, the AAV vector was injected with a volume between 35 and 80 µl, distending the artery to decrease endothelial infolding and to enhance contact of the AAV vector with all portions of the luminal endothelium (Fig. 1).

For intraluminal pressure measurements, a pressure line was connected to the proximal (Ringer) needle with a three-way stopcock. The proximal (Ringer) and distal (vector) needle insertion sites were surrounded with pursestring sutures to provide a closed system in which the intraluminal pressure could be measured. As before, Ringer solution was withdrawn into the proximal needle after the lumen was flushed free of blood; then, the proximal stopcock was opened to the pressure-monitoring line. Now the vessel was filled with vector from the distal needle, and the intraluminal pressure was continuously recorded. Both injection sites were repaired with single adventitial sutures of 7–0 prolene after the injection needles were withdrawn. The clamps were removed after a 30- or 60-min exposure time, and blood flow was reestablished. The cervical incision was closed in layers, and the rabbits were returned to their cages for recovery.

Sham operations were performed in eight animals, using exactly the same operative technique, but injecting Ringer solution instead of AAV vector. These served as controls for the degree of inflammatory reaction that might be incurred by surgical manipulation alone, unrelated to gene transduction and viral vector exposure. The contralateral carotid arteries in all rabbits were also harvested and examined as normal, untreated control arteries.

In the interests of both vector and animal conservation, data from 19 of the 58 animals in various treatment groups were utilized in more than one experiment. The data from the 11 animals killed at 14 days in the time course experiment were used as a basis of comparison for the effects of higher viral titer. Data from the eight animals transduced with higher viral titers in the titer experiments were used as controls for the effects of exposure time, but this time the titer was kept constant.

AAV vectors. The AAV vector CWRAP (11) contains the cDNA encoding human placental alkaline phosphatase (AP) driven by a Rous sarcoma virus (RSV) promoter and enhancer sequence (15). AAV vector stocks were generated as previously described (16). Briefly, cells were infected with adenovirus 5, and then vector plasmid (4 µg) and the AAV packaging plasmid pMTrep-CMV cap (2) were cotransfected by using the calcium phosphate transfection method. Vector stocks were purified by cesium chloride centrifugation and stored at –80°C. Vector titers were determined by using HT-1080 cells as targets for transduction and were equal to $5 \times 10^8$ or $2 \times 10^9$ AP-positive (AP+) focus-forming units (FFU) per milliliter (ml). The ratio of genome-containing vector particles to FFU for the CWRAP vector under these production conditions ranges from 100 to 700, and for the vector stock with a titer of $5 \times 10^8$ AP+ FFU/ml, the ratio was 100. Vector stocks were tested for the presence of infectious adenovirus by plaque assay (16), and none were detected ($<100$ plaque-forming units (PFU) per ml). Vector stocks were also assayed for contamination by replication-competent AAV by infectious center assays, and contained no detectable replication-competent AAV ($<100$ infectious units/ml).

Histochemical AP staining of carotid arteries. Animals were killed at 3, 7, 10, 14, and 28 days following...
vector instillation. Rabbits were anesthetized (as described above) and systemically heparinized. Both the transduced and the contralateral native carotid arteries were surgically exposed, isolated proximally and distally, then ligated and excised. The rabbits were then killed with concentrated pentobarbital given intravenously at 120 mg/kg.

Each carotid specimen was divided into three parts: the central part was fixed in zinc fixative (6) or 4% neutral buffered formalin (NBF) (pH 7.2) for histological cross sectioning. The remaining vessel ends were opened longitudinally, pinned open to expose the endothelial surface, and fixed in NBF to produce two en face specimens, or whole tissue mounts of the entire vessel wall. En face tissue specimens were warmed up in a microwave for 40 s to flatten the fixed specimen, then washed in PBS and 0.1% Triton X-100 for 10 min. To heat-inactivate endogenous AP, specimens were placed in a water bath at 68.5°C for 1 h. To assess AP enzymatic activity, specimens were then placed in AP substrate solution (Zymed Laboratories, San Francisco, CA) for 4 h at 37°C. The contralateral carotid artery was similarly processed.

Quantification of the prevalence of gene product expression. By viewing the arterial luminal surface on the en face preparations, the prevalence of gene expression was quantified as the percentage of the arterial surface expressing the transduced human placental AP enzyme, using the Optimas computerized image analysis program (Optimas, Bothell, WA). After enzymatic visualization of alkaline phosphatase, the margins of the transduced area were traced and the stained area was measured. This AP-stained area was then calculated as a percentage of the entire luminal surface that had been exposed to the vector to determine the prevalence of transduction by area. The smooth muscle cells that were transduced were found predominantly just underneath the luminal endothelium and could be visualized on these en face preparations and quantified as the percent of gene expression in the subendothelial, sub intimatal layer.

Cross sections were used to examine which cell types were transduced and, on these cross sections, the number of transduced cells were also counted manually, and the data were compared with the quantitative results from the en face preparations. At least two cross sections were evaluated for gene expression in 20 of the 50 transduced arteries in which gene expression was calculated by the en face method. The entire circumference of each arterial cross section was examined in 8–12 low-powered microscopic fields. In each field, the number of transduced cells in the media was compared with the total number of medial cell nuclei. The sum of the percentage of transduced cells for all fields was calculated for each vessel cross section, and the results of two to three cross sections were averaged for each carotid artery. In the intima, the number of transduced cells, although rare, was also counted manually. Because of the considerable variation in gene expression from one part of the vessel to another, we felt that the best overview of the prevalence of gene product expression was provided by the en face preparations, with data substantiated in selected experiments by analysis of the cross sections.

Immunocytochemistry. To determine cell type specificity on the cross sections, antibody to α-actin (BoehringerMannheim, Germany) was used to identify smooth muscle cells; antibody to CD-31, PECAM-1 (DAKO, Carpinteria, CA), was used to identify endothelial cells. Immunocytochemistry utilizing antibodies specifically directed against rabbit T lymphocytes, MAb Tib 188 (American Type Culture Collection, Rockville, MD), and rabbit macrophages, Ram-11 (DAKO), was used to determine the cell-type specificity of leukocytes infiltrating the intima, media, and adventitial compartments of the vascular wall. MAb Tib 188 is an L11/135 hybridoma that recognizes a cell surface determinant on all rabbit thymocytes and peripheral blood T cells, but not rabbit B cells (17). Splenocytes marked by this antibody include all that respond to T cell mitogens, both resting and activated, and alloantigenic stimuli (17). Ram-11, developed against a cytoplasmic component of rabbit alveolar macrophages, binds broadly to all tissue macrophages (39).

Briefly, histological cross sections of the arteries were embedded in paraffin. These cross sections included en face specimens that had previously been stained for AP activity. The specimens were then deparaffinized and hydrated through xylene and alcohol series. Endogenous peroxidase was blocked with 1.5% H2O2 in methanol for 10 min. Nonspecific binding was controlled for by treating with a blocking solution for 30 min (1% BSA/PBS, 1% horse serum, and 0.1% Triton X-100). The tissue was then incubated with primary antibody diluted in BSA/PBS at room temperature for an hour (MAb Tib at 1:200, MAb Ram-11 at 1:400, MAb to α-actin at 1:1,000, and MAB to CD31 at 1:100 dilutions). After washing with PBS, sections were incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted in PBS (1:100) for 30 min at room temperature, then washed again and treated with the avidin-biotin peroxidase technique (ABC Elite Kit, Vector Laboratories) for 30 min. The reaction product was developed using diaminobenzidine (DAB) substrate (Vector Laboratories) for 4 to 10 min, resulting in the deposition of a brown reaction product, which visualizes bound antibodies. Nuclei were counterstained with hematoxylin.

On the cross sections from 42 arteries (35 transduced and 7 sham operated), the presence or absence of intimal lesions and the extent of T lymphocyte and macrophage infiltration were assessed semiquantitatively. By and large, these specimens represented those in the high-titer and high-pressure groups, the “at risk” groups for lesion development, and their controls. In 23 specimens in which lesions were identified, including both transduced and untransduced specimens, the intimal lesions were further characterized by assessing

http://physiolgenomics.physiology.org
the percentage of the luminal circumference involved with lesion development and by noting the number of cell layers present between the endothelium and the IEL.

Furthermore, in 11 arteries exposed to high titers of vector and 6 sham-operated arteries, the absolute number of T lymphocytes in the media were manually counted, averaging results from at least two cross sections per artery. As above, each arterial cross section comprised a summation of 8–12 low-powered microscopic fields. In these specimens, intimal areas were also quantified by computerized image analysis.

Statistical analysis. Data were compared between groups using Student’s t-test. Differences were considered to be statistically significant at a P < 0.05. Standard errors of the mean follow the mean values.

RESULTS

Time course of expression within 28 days. First, the time course of gene expression after AAV vector transduction was investigated (Fig. 2). We examined AP gene expression at 3, 7, 10, 14, and 28 days, using between 35 and 80 µl of vector stock with a viral titer of $5 \times 10^8$ AP FFU/ml, in 25 rabbits. Minimal gene expression was evident as early as days 3 and 7 after treatment (mean 0.1% and 0.9 ± 0.4% of the luminal surface area with AP expression, respectively). The prevalence of gene expression increased significantly between day 10 and day 14 (from a mean of 5 ± 2% at day 10 to a mean of 16 ± 3% at day 14) and decreased somewhat, but without statistical significance, by day 28.

No AP activity was seen in the sham-operated controls nor in the contralateral normal arteries, indicating that the histological techniques effectively eliminated any endogenous cross-reacting AP.

Cell types transduced. In these en face preparations of the entire luminal surface, gene expression was not uniform: areas of high enzyme activity were seen as well as areas with little or no expression. Cross sections revealed that, despite an intact endothelial layer, VSMCs in the media were the main cell type transduced as evidenced by AP histochemical staining (Fig. 3). Specifically, gene expression was most commonly seen in the layer of smooth muscle cells immediately below the IEL. Immunocytochemistry using antibodies to smooth muscle cells, T lymphocytes, and macrophages confirmed that the medial cells with histochemical staining for AP were smooth muscle cells and not T lymphocytes or macrophages. AP staining was occasionally detected in rare endothelial cells in the intima.
Correlation between VSMC gene expression on en face preps and cross sections.
In 29 of the 50 arteries examined, gene expression was also quantified on at least two cross sections for each artery. On the cross sections, gene expression was calculated as the percentage of medial cells with positive AP staining divided by the total number of cells in the media, defined by the number of nuclei. Across all specimens examined, gene expression on the en face preparations, representing the surface area transduced, correlated well with gene expression on the cross sections, measured as a percent of cells transduced ($R^2 = 0.69$). However, in the specimens from the groups transduced with high titers of vector and those transduced at high pressures, the correlation between transduced areas on the en face preps and gene expression on the cross sections was even more striking ($R^2 = 0.82$). The maximum transduced area on the en face preparations was 58%, which compared well with 55% of medial smooth muscle cells transduced on the cross sections of the same specimen.

Effect of viral titer. The percentage of the subluminal area expressing gene product on the en face preparations varied with the viral titer (Fig. 4). A comparison was made between the AP-stained subluminal area using a viral titer of $5 \times 10^8$ AP FFU/ml ($n = 11$) vs. a viral titer of $2 \times 10^9$ AP FFU/ml ($n = 8$) at 14 days following transduction (volumes for both groups were between 35 and 80 µl of vector stock). A significantly higher subluminal area of gene expression was seen at the higher titer ($P < 0.05$). The mean percentage area expressing gene product on the en face preparations was $34 \pm 7\%$ for the higher titer vs. $15 \pm 3\%$ for the vessels transduced with the lower viral titer. Among the specimens transduced with the higher viral titer, the highest percentage of intra-arterial area expressing gene product on the en face preparations was 58%, demonstrating that widespread transduction of the subintimal VSMC layer is feasible (Fig. 5).

Similarly, on the cross sections, the highest percentage of total medial smooth muscle cells expressing gene product in any specimen was 55% (Fig. 3). When gene product expression was calculated across the 8–12 fields comprising an entire arterial circumference and averaged over the arteries comprising each group, the average percentage of medial smooth muscle cells expressing gene product was $26 \pm 6\%$ for the specimens transduced with the higher-titer vector compared with...
Volume between 35 and 80 µl of vector stock and a viral titer at 2 × 10^9 AP FFU/ml for both groups. A doubling of the exposure time increased the area of gene expression (Fig. 6); however, this did not reach statistical significance with the numbers of carotids we examined (mean area expressing gene product was 21 ± 4% for 30-min exposures compared with a mean area of 34 ± 7% for 60-min exposures).

Effect of intraluminal pressure. In this in vivo system, it was not possible to keep intraluminal pressure absolutely constant over an hour’s time, so animals were divided into two treatment groups (n = 5 per group) where the vector was introduced under “high” or “low” intraluminal pressure, using a constant viral titer of 5 × 10^9 FFU/ml. In the “high-pressure” group, systolic peak pressures ranged from 30 to 40 mmHg. The percentage of the vascular wall surface expressing gene product was higher in arteries transduced under high (mean area expressing gene product was 28 ± 2%) vs. low peak systolic pressures (mean area of 12 ± 6%) (P < 0.05) (Fig. 7).

Effect of exposure time. Because gene expression could also vary with the time that the vessel lumen was exposed to the viral vector, we compared exposure times of 30 min (n = 7) and 60 min (n = 8), with a volume between 35 and 80 µl of vector stock and a viral titer at 2 × 10^9 AP FFU/ml for both groups. A doubling of the exposure time increased the area of gene expression (Fig. 6); however, this did not reach statistical significance with the numbers of carotids we examined (mean area expressing gene product was 21 ± 4% for 30-min exposures compared with a mean area of 34 ± 7% for 60-min exposures).

On the cross sections (n = 4 per group), the average percentage of medial cells expressing gene product was 25 ± 5% for the high-pressure group vs. 6 ± 3% for the low-pressure group, also a significant difference (P < 0.05). Gene expression ranged from 14% to 38% in the high-pressure group compared with 0 to 11% in the low-pressure group.

Lesion development. Small but identifiable intimal lesions, consisting of one or more cell layers beneath an intact endothelium, were seen in 15 of 35 transduced carotids examined (Fig. 8). Immunocytochemical analysis of the cell types involved revealed that the intimal lesions were composed of α-actin-positive VSMCs. Similar mild degrees of intimal thickening were also seen in 6 of 7 sham-operated and in 2 of 18 native unoperated vessels. Intimal lesions were not circumferential in any of the specimens, and, overall, the observed lesions would be classified as mild degrees of intimal thickening. In the 23 arteries with lesions, the lesions encompassed an average of 35% of the luminal circumference in both the transduced and sham-operated vessels. Similarly, in most of the involved arteries, the lesions were only 1–2 cell layers deep. Only three of the transduced vessels and two of the sham-operated arteries had lesions that were 2–4 cell layers in depth. Although the majority of observations were made at 14 days following transduction, equivalent lesions were seen in both a 10-day specimen (without gene expression) and a 28-day specimen (with gene expression).

Intimal areas were calculated by computerized image analysis for 11 “at risk” arteries transduced with high-titer vectors and for 6 sham-operated control arteries, and no significant difference was found between the transduced arteries and the shams controls. When specimens were separated out by exposure to high viral titers and/or elevated intraluminal pres-
sures, lesion development could not be tied to viral titer (1/9 low-titer and 2/8 high-titer specimens had lesions). In the transduced arteries, lesions were seen in arteries both with and without gene expression. However, in this sample, there were more lesions among the transduced specimens in the high-pressure group (4/5 specimens) than in the low-pressure group (2/5 specimens). In two sham-operated arteries also exposed to high intraluminal pressures, one of the two developed an intimal lesion.

Inflammatory/immune reactivity. What differentiated the lesions in the transduced vessels from those in the sham-operated arteries was the presence of T lymphocytes in the transduced vessels (Fig. 8). Of 35 transduced vessels examined by immunocytochemistry, T lymphocytes were detected in the intima in 8, in the media in 14 (or 40%), and in the adventitia in 24. In contrast, only rare T lymphocytes were seen in the adventitia and surrounding fibrinous tissue of sham-operated controls and the unoperated contralateral carotid arteries, and no T lymphocytes were seen in either the intima or media of these control specimens (7 sham-operated arteries and 15 native carotids were examined).

Although adventitial infiltration of transduced arteries could be extensive, this was usually seen in vessels in which a lesser degree of T lymphocytes were also present in the media. Intimal T cells were much less common and virtually always coexisted with T lymphocyte infiltration of the media.

In a sample of “at risk” arteries, we counted the absolute number of T lymphocytes in the media, as being the most accurate representation of vessel wall infiltration. When the mean number of T lymphocytes in the arterial media was compared between 11 arteries exposed to high-titer vector (8 for 60 min, 3 for 30 min) and 6 sham-operated arteries, the infiltration of T lymphocytes was 100-fold higher in the transduced vessels than in the sham controls (25 ± 14 vs. 0.25 ± 0.2 T cells/total medial area, P < 0.05).

T lymphocyte infiltration of the media appeared to correlate with gene expression, but, with this sample size, clear differences related to intraluminal pressure, viral titer, exposure time, or lesion development could not be determined. Among the examined specimens, T lymphocyte infiltration was seen exclusively in those specimens with gene expression. Three of five specimens exposed to high intraluminal transduction pressures exhibited T cell infiltration compared with one of four specimens in the low-pressure group. T lymphocyte infiltration was found in 5/8 specimens transduced with high titers of virus and 4/9 low-titer specimens. Among the high-titer specimens, T lymphocytes were present in 5/7 specimens with 60-min exposure times and 3/5 specimens with 30-min exposure times. Intimal lesions were seen both in specimens with and without T lymphocyte infiltration.

Macrophages were also examined by immunocytochemistry in 31 transduced and 3 sham-operated control specimens. Compared with T lymphocytes, macrophages were much less commonly detected, being present in the media in only 7 (23%) of transduced specimens. Intimal macrophages were present as more than a single cell in only two transduced specimens. Like the T lymphocytes, macrophages were not present in the sham-operated controls.

Also like the T lymphocytes, macrophages were found only in specimens with overt gene expression. However, among the examined specimens with gene expression, only 25% had medial macrophages. Also, although five of the seven specimens with medial macrophages also had intimal lesions, among all examined specimens with intimal lesions, only 31% had macrophages present in the media.

Taken together, intimal lesion formation was found with equal frequency and extent among transduced
arteries and sham-operated controls, whereas T lymphocyte infiltration and, to a much lesser extent, macrophage infiltration were unique to the transduced arteries. Leukocyte infiltration appeared to relate to gene expression.

**DISCUSSION**

This study demonstrates that, with optimization of viral titer, exposure time, and intraluminal pressure, AAV vectors can transduce approximately one-third of the subendothelial layer of VSMCs in this rabbit carotid artery model of intra-arterial gene transduction, with a maximum transduction rate of 58%. One of the most important findings of this study is that, despite an intact endothelial layer, VSMCs were preferentially transduced by this AAV vector. This preferential transduction could be an advantage therapeutically, since VSMC transduction could be an important gene therapy target in the treatment of postangioplasty restenosis, atherosclerosis, and posttransplant arteriopathy. Importantly, those smooth muscle cells that immediately underlie the IEL are the ones most likely to express adhesion molecules (8, 28) and synthetic phenotypes (12, 22, 29) in several pathological processes and therefore, could be the preferred target cells for therapy (4, 25).

Previous in vitro work has shown that VSMCs can be transduced by AAV more readily than endothelial cells (23), and that, in cultured VSMCs, gene expression can be sustained for at least 8 wk (25). It may be that there is an inherent difference in relevant receptor density on VSMCs as opposed to endothelial cells or that the conversion rate from single to double-stranded DNA may differ between cell types.

In vivo studies to date, however, have given conflicting results. Although skeletal and cardiac muscle is readily transduced by AAV vector in vivo (10, 26, 37, 40), it has not been clear that the same is true for vascular smooth muscle. Using the same AAV vector as in our studies, Halbert et al. (16) did report VSMC gene expression in murine pulmonary blood vessels after AAV vector delivery by nasal aspiration, supporting a potential predilection for VSMC transduction. However, among previous studies utilizing direct AAV vector transduction of arteries, only two (3, 13, 30) of four research teams (3, 13, 23, 24, 30) detected gene expression in the media of the arterial wall. The Bahou laboratory (3) found evidence of transferred DNA by in situ PCR in up to 90% of both endothelial and VSMCs following intra-arterial catheter-based AAV transduction of rat carotid arteries. Interestingly, using higher intraluminal pressures and lower viral titers than in our studies, they found a pattern of transduction similar to our findings, with preferential transduction of the layer of VSMCs just under the IEL in a vessel with an intact endothelium. The low levels of recombinant protein expression, which could not be detected histochemically, may well be related to the considerably lower viral titers (2–5 × 10^4 I.U./ml) and total viral dose utilized for these studies. Rolling et al. (30) also found transduction of both medial VSMCs and adventitial cells of rat carotid arteries utilizing a cytomegalovirus (CMV) promoter, a viral titer and dose near the range of our low-titer group, and a 20-min exposure time. However, in their study, as in Lynch et al. (23), balloon-injured carotids demonstrated significantly greater transduction than uninjured arteries, whereas in our work, we see effective gene transfer through an intact endothelium.

The preferential VSMC transduction we found is in contrast to the findings of Lynch et al. (23), who found that endothelial cells in the adventitial vaso vasorum were preferentially transduced in hypercholesteremic primates. In our rabbit carotid model, microvessels surrounding the adventitia are dissected off prior to transduction, thus limiting the opportunities for transduction of adventitial vessels, which may explain some of the differences between our findings and those of Lynch et al. (23); vaso vasorum may also be more prominent in primates. Maeda et al. (24) also found transduction of only endothelial and adventitial cells when they exposed cross sections of rat aorta to AAV vectors. The viral titer used by Lynch et al. (23) is probably equivalent to our low range, and that used by Maeda et al. (24) is probably equivalent to our high range. However, both of these laboratories were examining gene expression at very early time points, 1–3 days posttransduction. In our studies, we saw only minimal gene expression at 3 days, and our cross sections to evaluate the cell types expressing gene product were performed at 14 days, which may explain some of the variance in findings.

The choice of promoter is also known to influence cell type-specific and organ-specific expression. A CMV promoter, known to provide efficient gene expression in arterial endothelial cells, was used by both Lynch et al. (23) and Maeda et al. (24). At much lower viral titers, the Bahou laboratory (3) found functional recombinant protein could be detected only with a collagen α1(i) promoter and not with a CMV promoter, although similarly efficient in vivo transgene delivery into both endothelial cells and VSMCs was demonstrated with both promoters (3). The RSV promoter in the construct we used may have facilitated gene expression in VSMCs vs. endothelial cells. We did observe limited gene expression in endothelial cells in the intima in some of our rat carotid specimens, so we know that in vivo AAV vector transduction of endothelial cells is possible with the vector construct we used.

Using this same AAV vector construct, RSV promoter, and human AP reporter gene in vitro, we found surprisingly efficient transduction of nonconfluent rabbit vena caval endothelial cells (14) with an actual viral dose of 5 × 10^5 AP FFU/ml (data not shown). However, our in vitro results were not predictive of the low incidence of endothelial cell transduction in vivo. One reason for this difference between in vitro and in vivo gene expression might be the proliferating state of the cultured cells compared with the quiescent state of endothelial cells in vivo, given that AAV vectors may preferentially transduce cells in S phase (32). Alternatively, it seems likely that the endothelial cells in the artery walls in the in vivo experiments may have been...
in fact, transduced, but that gene expression might have been at a lower level than what we could detect histologically.

AAV belongs to the parvovirus family and, with a particle size of 20 nm, is one of the smallest viruses. However, its small size, although generally a limiting factor for gene packaging, might also enable AAV vectors to gain access to cells and portions of the vessel wall that are not accessible to larger vectors. It has been proposed (31) that, during intravascular gene delivery, anatomical barriers, in particular the IEL, may prevent larger vectors from penetrating into the media. Rome et al. (31) have even suggested that the media of the vessel wall is virtually inaccessible to vectors with a size around 70 to 100 nm such as adenoviruses or the larger liposome vectors. In contrast, smaller particles such as low-density lipoproteins with a diameter of 22–24 nm, could pass, although to a limited degree, into the media beyond the IEL (38). In our studies, the AAV vector, smaller than even the above-mentioned low-density lipoproteins, might reach the tunica media simply due to its small size. Similarly, in the lung (16), the AAV vector must have been able to circumvent physical barriers such as the basement membrane of the airway epithelium to gain access to VSMCs. Dzau et al. (9), using HVJ-liposome vectors, have also demonstrated that factors other than size alone may affect vector penetration of tissue (9).

Although we did not create a demonstrable arterial injury to the endothelium or disrupt the IEL, it is also possible that, in our surgical model, simply occluding the blood flow through the vessel for 30–60 min might result in some ischemia of the vascular wall itself, which could, theoretically, alter the permeability of the endothelial layer and facilitate penetration of the vector through endothelial gap junctions to deeper layers of the vessel wall. In comparison, other investigators (3, 13, 23, 30) have introduced the AAV vectors through intraluminal catheters without subjecting the vessel wall to this mild degree of ischemia. Differences in endothelial cell permeability may thus also account for some differences in results.

Our findings show that AAV vectors can transduce VSMCs in the media in vivo with an apparent transduction efficiency of up to 58%. This was achieved with intraluminal pressures up to 110 mmHg and with prior injury of the vessel and, thus an intact endothelial layer. Although Nabel et al. (27) have proposed that pressures in the range of 150–200 mmHg might be needed for effective DNA-liposome transfection, we chose pressures around 100 mmHg to avoid barotrauma to the artery. In our comparisons between arteries transduced with pressures between 67 and 110 mmHg and those transduced at lower pressures, we found an increase in the prevalence of gene expression at the higher pressures. In both groups, however, VSMCs were clearly the main cell type transduced as detected by the AP histochemical staining: the higher pressures did not increase the number of endothelial cells expressing gene product.

In these studies, we first detected appreciable gene expression histochemically on day 10, and expression increased significantly by day 14. Similarly, Rolling et al. (3) found that gene expression in AAV-transduced rat carotid arteries accelerated between days 10 and 20, persisted for 30 days, and, in one animal, was still detectable at 6 mo (30). Although we also show here that gene expression can be sustained for at least 4 wk, we did not examine later time points, and, certainly, gene expression might have been demonstrable for a much longer period of time in these vessels. In murine studies, gene expression in transduced skeletal muscle has been reported for up to 1.5 years after AAV vector gene delivery (40).

An unexpected finding was an apparent mild inflammatory response to AAV vector transduction with small intimal lesions in some arteries. AAV vectors are generated by excluding viral regulatory and structural genes to reduce host cellular inflammatory response. However, in 14 of 35 transduced specimens examined, we observed mild T lymphocyte infiltration into at least two of the three layers of the vessel wall. This inflammatory cell migration into the media was clearly different in the transduced vessels compared with sham-operated carotid arteries. This inflammatory response might be triggered by the AAV vector capsid, since Halbert et al. (16) have found that a humoral immune response can be generated to AAV vector proteins, and, therefore, a cellular response might also be possible. If so, other AAV vector serotypes (33) may be found to be less immunogenic. Alternatively, this could be a reaction to the expressed protein (27), in our case the human placental AP gene product, although, by and large, AP is not known to engender an inflammatory reaction.

Brockstedt et al. (7) have recently reported that high-titer intravenous, but not intramuscular, AAV transduction can induce a cytotoxic T lymphocyte response as well as a humoral response against the foreign protein. Their intravenous delivery model is likely to be more immunogenic than ours, because the intravenous vector-DNA constructs could traffic through the spleen and lymphoid organs, whereas, in our experiments, the vector is isolated in an intra-arterial section. However, even plasmid DNA encoding an endogenous protein has been shown capable of inducing a host immune response (35).

Finally, vascular endothelial injury and repair due to a needle tip abrasion or surgical dissection of the adventitia (5) might explain the small intimal lesions in sham-operated vessels, but this does not explain the greater prevalence of T cell infiltration in the transduced vessels. Certainly, among experimental animals, rabbits are more prone to vasculopathy following any stimulus than other experimental animals; the question is whether such sensitive vascular reactivity in the rabbit would actually mimic expected vascular responses in humans. Further investigations are needed to confirm whether these preliminary findings represent a cellular inflammatory response to the viral vector or to the foreign protein.
In summary, this AAV vector transduced vascular tissue and, in particular, VSMCs with an AP reporter gene for up to 28 days. The prevalence of gene expression, which varied with viral titer, exposure time, and intraluminal pressure, could be as high as 58% by area. These studies of rabbit carotid artery AAV vector transduction also demonstrated that a mild cellular inflammatory response could be elicited, which appeared to occur only in the specimens with gene product expression.

This study shows that AAV vectors have the potential for becoming very promising vectors for clinical applications in cardiovascular disease because of their relatively high transduction efficiency, potential for long-term gene expression, and selective targeting of VSMCs.

We acknowledge the contributions of Edward Sien in the review and preparation of this report. Portions of this project were supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-47754 and by Cystic Fibrosis Foundation Grant CFFR565.

Address for reprint requests and other correspondence: M. D. Allen, Division of Cardiothoracic Surgery, Mailstop 356310, 1959 NE Pacific St., Seattle, WA 98195 (E-mail: mdallen@u.washington.edu).

REFERENCES


