Second-Generation Adenoviral Vectors Do Not Prevent Rapid Loss of Transgene Expression and Vector DNA From the Arterial Wall

Shan Wen, Darren B. Schneider, Robert M. Driscoll, Giuseppe Vassalli, André B. Sassani, David A. Dichek

Abstract—The utility of adenoviral vectors for arterial gene transfer is limited by the brevity of their expression and by inflammatory host responses. As a step toward circumventing these difficulties, we used a rabbit model of in vivo arterial gene transfer to test 3 second-generation vectors: a vector containing a temperature-sensitive mutation in the E2A region, a vector deleted of E2A, and a vector that expresses the immunomodulatory 19-kDa glycoprotein (gp19k) from adenovirus 2. Compared with similar first-generation vectors, the second-generation vectors did not significantly prolong β-galactosidase transgene expression or decrease inflammation in the artery wall. Although cyclophosphamide ablated the immune and inflammatory responses to adenovirus infusion, it only marginally prolonged transgene expression (94% drop in expression between 3 and 14 days). In experiments performed with “null” adenoviral vectors (no transgene), loss of vector DNA from the arterial wall was also rapid (>99% decrease between 1 hour and 14 days), unrelated to dose, and only marginally blunted by cyclophosphamide. Thus, the early loss of transgene expression after adenoviral arterial gene transfer is due primarily to loss of vector DNA, not correlated with the presence of local vascular inflammation, and cannot be prevented by use of E2A-defective viruses, expression of gp19k, or cyclophosphamide-mediated immunosuppression. Adenovirus-induced vascular inflammation can be prevented by cyclophosphamide treatment or by lowering the dose of infused virus. However, stabilization of adenovirus-mediated transgene expression in the arterial wall is a more elusive goal and will require novel approaches that prevent the early loss of vector DNA. (Arterioscler Thromb Vasc Biol. 2000;20:1452-1458.)

Key Words: rabbits ■ gene therapy ■ carotid arteries ■ β-galactosidase ■ inflammation

The utility of adenoviral vectors for arterial gene transfer is limited by the brevity of transgene expression and by local inflammation.1–3 Both difficulties are believed to result from antigen-specific immune responses to adenoviral and transgene proteins.4,5 Engineering the vector genome to reduce or eliminate adenoviral protein expression (ie, production of second- and third-generation adenoviral vectors) is a commonly used strategy to circumvent destructive immune responses to adenoviral vectors. This strategy has been applied successfully in liver, lung, and skeletal muscle models.6–9 Enthusiasm for second-generation adenoviral vectors continues to be expressed in relation to cardiovascular gene transfer10; however, use of these vectors for arterial gene delivery has not yet been definitively reported. We recently reported the unanticipated result that adenovirus-mediated transgene expression declines rapidly in arteries of mice that lack antigen-specific immunity.11 This result suggested that paradigms developed to explain the loss of adenovirus-mediated transgene expression in other organs may not explain loss of transgene expression from the arterial wall.

To begin to test whether engineering of the adenoviral vector genome could improve arterial gene transfer, we constructed 2 second-generation adenoviral vectors. Results of experiments performed with these vectors, along with the above-cited data from immunodeficient mice,11 caused us to question whether antigen-specific immunity plays a major role in the early loss of adenovirus-mediated transgene expression after arterial gene transfer. We performed additional experiments with a third second-generation vector, “null” viruses (lacking a transgene), and cyclophosphamide (CTX) immunosuppression. These experiments suggested that adenovirus-mediated transgene expression in the arterial wall declines primarily due to early loss of vector DNA and that the antigen-specific immune system plays a relatively minor role in expediting this loss. Principles developed from adenoviral gene transfer studies performed in other organs do

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not appear adequate to explain the behavior of adenoviral vectors infused to the arterial wall.

Methods

For all subheadings, additional information is available in the expanded Methods section available online at http://www.atvb.ahajournals.org.

Recombinant Viruses

Of the 6 adenoviral vectors used in these experiments, 4 were generated in our laboratory: AdRSVnLacZ, Adgp19kLacZ, AdE2AαLacZ, and AdCMVNull (Figure 1). Adgp19kLacZ expresses the 19-kDa glycoprotein (gp19k) from human adenovirus type 2.13 AdE2AαLacZ contains a temperature-sensitive mutation in the E2A DNA-binding protein.14 AdCMVNull does not contain a transgene. Two additional adenoviral vectors were provided by IntroGene (Leiden, the Netherlands): AdE2A− and AdE2A+. Both IntroGene vectors were E1-deleted adenovirus 5-based vectors with a cytomegalovirus (CMV) promoter (but no transgene) inserted at the site of the E1 deletion. AdE2A− was also deleted of the E2A region.

Phenotypic Characterization of Second-Generation Vectors

Expression of gp19k protein by Adgp19kLacZ was detected by indirect immunofluorescence performed after infection of Chinese hamster ovary cells and primary rabbit smooth muscle cells (Figure I, available online at http://www.atvb.ahajournals.org). In vivo expression of gp19k was detected by reverse transcriptase–based polymerase chain reaction (RT-PCR) performed on arterial extracts.

For 2 reasons, we did not determine the affinity of gp19k for rabbit major histocompatibility class (MHC) I. First, these experiments might not be predictive of in vivo function, as gp19k appears to have immunomodulatory activity that is not explained by its affinity for MHC-I.13,15 Second, the ability of gp19k to improve vector performance is most definitively tested in vivo by direct measurement of the critical end points of duration of expression and magnitude of host immune response. We detected expression of the E2A-encoded DNA-binding protein16 by Western blotting of cell extracts by using antibodies provided by Dr G. Schouten (IntroGene; Figure II, available online at http://www.atvb.ahajournals.org).

Animal Experiments

Vector stocks (~75 μL per artery) were infused in the lumen of surgically isolated common carotid arteries of adult male, specific pathogen–free, New Zealand White rabbits (Charles River Laboratories, Montreal, Quebec, Canada; 3.0 to 3.5 kg) as previously described.17 Arteries were harvested from 1 hour to 14 days later and processed for the β-galactosidase (β-gal) activity assay, histological analysis, or DNA extraction.

AdRSVnLacZ (2 independent preparations), Adgp19kLacZ (1 preparation), and AdCMVNull (2 independent preparations) were infused at 4 to 5×10^9 plaque-forming units (pfu)/mL. (2 to 6×10^11 particles/mL). Three independent preparations of AdE2AαLacZ were required to complete the experiments. These preparations were infused either at 5×10^9 LacZ-transducing units/mL or at 5×10^10 relative pfu/mL, derived from comparison with AdRSVnLacZ.

Central venous cannulas were placed in rabbits assigned to receive either CTX or control saline infusions. CTX (30 mg · kg^-1 · d^-1, Sigma) or sterile saline was administered by catheter for 4 days preoperatively and 14 days postoperatively.

β-Gal Activity Assay

β-Gal activity in lysates of arteries was measured with a chemiluminescence assay.6

Southern Blot Analysis

DNA was extracted from cultured cells or carotid artery segments by SDS–protease K digestion. Blots were hybridized with a 32P-labeled, 2.9-kb HindIII fragment of adenovirus 5 (a probe for the E4 region). To permit quantification of adenoviral DNA in transduced tissues, each autoradiograph included a standard curve generated by electrophoresis and blotting of purified, HindIII-digested adenoviral DNA loaded in amounts corresponding to 0.1 to 300 copies per haploid genome (ie, 0.2 to 600 copies per diploid cell) in the experimental lanes. Radioactive signals on blots were quantified with a PhosphorImager.

Real-Time PCR Quantification of Adenoviral DNA

Adenoviral DNA was also quantified by real-time “Taq-Man” quantitative PCR, by using amplification and detection protocols similar to those described elsewhere.18 To aid in quantification of adenoviral DNA in experimental samples, each amplification included a standard curve in which wells were spiked with 10^2 to 10^6 copies of adenoviral DNA.

Detection of Anti-Adenoviral Antibodies

Serum antibodies to adenovirus were measured by ELISA. In brief, 96-well plates were coated with 100 μL of adenoviral vector stock. The wells were rinsed and blocked, and 100 μL of diluted rabbit serum was added to each well. Bound antibody was detected with alkaline phosphatase–conjugated mouse anti-rabbit IgG.

Histological Analysis

Arteries were embedded and sectioned, and serial sections were stained for CD5 (a T-cell antigen), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) expression, as described.2 The mean score of the 4 sections from each artery was calculated and assigned as an overall score for the artery. Individual artery scores were grouped according to treatment, and the treatment groups were compared by using the individual artery scores as experimental units.

Statistical Analysis

The effect of CTX on persistence of DNA was evaluated by unpaired t test; these data are presented as mean±SEM. Data not normally distributed (or for which variances of experimental groups were...
Figure 2. Expression of β-gal by first-generation (RSVnLacZ) and second-generation (gp19kLacZ and E2A ts LacZ) adenoviral vectors. β-Gal activity was measured in extracts of arteries harvested 3 and 14 days after vector infusion. One group of RSVnLacZ arteries was procured from rabbits treated with CTX (+CTX). Each data point represents an individual artery; bars indicate group medians. *P<0.03 vs activity at 3 days in arteries transduced with the same vector. **P=0.04 vs RSVnLacZ+saline at 14 days, P=0.05 vs RSVnLacZ at 3 days.

Results

In Vivo β-Gal Expression Is Not Prolonged by Second-Generation Vectors

We infused AdRSVnLacZ, AdE2A ts LacZ, and Adgp19kLacZ into rabbit common carotid arteries, harvested the arteries 3 and 14 days later, and measured β-gal activity in arterial extracts. Three days after gene transfer, arteries infused with the 3 vectors had similar levels of β-gal expression (Figure 2, P=0.2). At 14 days, as expected, β-gal expression in AdRSVnLacZ-transduced arteries declined by >98% (P=0.02 versus 3 days). β-Gal expression also declined significantly at 14 days in arteries transduced with the second-generation vectors (P<0.03 versus 3 days for both AdE2A ts LacZ and Adgp19kLacZ). Moreover, expression levels at 14 days were equivalent for all 3 vectors (P=0.46).

Systemic Immunosuppression Only Marginally Prolongs β-Gal Transgene Expression

The initial results with AdE2A ts LacZ and Adgp19kLacZ suggested that circumventing the immune responses to adenoviral proteins by vector engineering might not be an effective strategy for prolonging transgene expression. To test whether direct suppression of the immune response to adenovirus can prolong transgene expression in this model, we measured β-gal expression in 14-day arteries from rabbits infused with AdRSVnLacZ and treated with CTX. Treatment for 18 days with CTX (from 4 days before gene transfer until harvest) effectively immunosuppressed the rabbits (the Table and see below) and caused relative preservation of β-gal transgene expression (P=0.04 versus 14-day AdRSVnLacZ arteries without CTX; Figure 2). Nonetheless, immunosuppression did not prevent a significant decline in β-gal activity in AdRSVnLacZ arteries between 3 and 14 days (94% drop, P=0.05).

Vascular Inflammation and the Humoral Immune Response to Adenovirus Are Not Decreased by Second-Generation Vectors

To test the hypothesis that these second-generation adenoviral vectors produce less vascular inflammation, we measured T-cell infiltration and expression of ICAM-1 and VCAM-1 14 days after infusion of AdE2A ts LacZ, Adgp19kLacZ, or AdRSVnLacZ (both with and without CTX treatment). As expected, arteries infused with AdRSVnLacZ had substantial T-cell infiltration and expression of ICAM-1 and VCAM-1 (Figure 3). CTX essentially eliminated T-cell infiltrates and VCAM-1 expression (P=0.001 and 0.02, respectively, versus AdRSVnLacZ without CTX) but did not significantly alter ICAM-1 expression (P=0.16). Neither of the second-generation vectors produced significantly less inflammation than the first-generation vector AdRSVnLacZ, although there was a trend toward less T-cell infiltration and ICAM-1 expression in arteries transduced with Adgp19kLacZ (P=0.07 for both measurements versus AdRSVnLacZ without CTX). Because the second-generation vectors produced fewer antigens (AdE2A ts LacZ) or potentially decreased antigen presentation (Adgp19kLacZ), we considered the possibility that the humoral immune response to these vectors might be less. Serum anti-adenoviral IgG was measured in naïve rabbits (no known exposure to human adenovirus) and in rabbits 14 days after carotid infusion of 1 of the 3 vectors. As expected, infusion of AdRSVnLacZ provoked a robust, humoral immune response (Figure 4). This response was eliminated in rabbits treated with CTX (P<0.005 versus AdRSVnLacZ without CTX). However, anti-adenoviral IgG levels did not differ between rabbits infused with AdE2A ts LacZ or Adgp19kLacZ and rabbits infused with AdRSVnLacZ (P=0.89).

**Effects of CTX Infusion on Blood Cell Counts**

<table>
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<th>Baseline (n=8)</th>
<th>Saline (n=4)</th>
<th>CTX (n=4)</th>
<th>Saline (n=4)</th>
<th>CTX (n=4)</th>
<th>Saline (n=4)</th>
<th>CTX (n=4)</th>
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<td><strong>White blood cell count, 10^3/μL</strong></td>
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<td>Neutrophils, n/μL</td>
<td>4.8±0.8</td>
<td>5.8±0.7</td>
<td>3.5±1.1†</td>
<td>7.8±0.7</td>
<td>2.5±0.6†</td>
<td>6.0±0.6</td>
<td>1.9±0.5†</td>
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<td>Lymphocytes, n/μL</td>
<td>1553±359</td>
<td>1909±1136</td>
<td>981±478</td>
<td>3311±1504</td>
<td>379±365‡</td>
<td>1995±996</td>
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<td>1827±1077‡</td>
<td>3391±534</td>
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<td>36.5±2.6</td>
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<td>26.5±2.1‡</td>
<td>36.7±1.8</td>
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†P<0.05 vs saline control group (2-way repeated-measures ANOVA with Tukey’s test for multiple pairwise comparisons).
Adenoviral Vector DNA Is Rapidly Lost From the Arterial Wall

The substantial and significant decline of β-gal transgene expression, despite CTX treatment, suggested that loss of transgene expression was largely independent of specific immune responses to adenoviral antigens. Two alternative mechanisms for loss of transgene expression required consideration. First, β-gal expression might be lost due to a specific immune response to β-gal protein, leading to elimination of β-gal–expressing vectors solely as a result of the specific elimination of β-gal–expressing cells. Second, β-gal expression might be lost due to transcriptional shutdown despite persistence of vector DNA. To address the 2 possibilities simultaneously, we infused AdCMVNull (a first-generation vector that does not express a marker protein) and followed vector DNA persistence by quantitative Southern blotting and real-time PCR. Removing the β-gal transgene and measuring vector DNA instead of transgene expression would reveal both whether transgene immunogenicity was the critical factor leading to loss of transgene expression and whether vector DNA persisted despite loss of expression in this model.20

Arteries transduced with AdCMVNull at 4×10⁸ pfu/mL and harvested 1 hour later contained a large amount of vector DNA: ≈350 copies per arterial wall cell (Figure 5A). Vector DNA declined rapidly, with <0.5% of the initial amount of vector DNA present by 14 days. To determine whether the rate of vector DNA loss was dose related (as could occur if high concentrations of vector were directly cytotoxic), we repeated the time-course experiment at 2 lower doses: 4×10⁷ and 1×10⁷ pfu/mL. Vector DNA delivered at all 3 doses was lost from the arterial wall with identical kinetics: simple exponential decay with a half-life of ≈1 day (Figures 5B and 5C).

We next determined whether CTX immunosuppression prevented the loss of vector DNA. Twenty arteries (in 10 rabbits) were infused with AdCMVNull at 4×10⁸ pfu/mL. Beginning 4 days before gene transfer, 5 of the rabbits were treated with daily CTX and 5 were treated with saline, as described above. At 3 days after gene transfer, arteries from both groups had equivalent amounts of vector DNA (4.6±1.7 versus 3.0±1.8 copies per arterial wall cell, Figure 5D; P=0.48). At 7 days, arteries from CTX-treated rabbits had 7-fold more vector DNA than did arteries from saline-treated rabbits (0.58±0.16 versus 0.082±0.014 copies per arterial wall cell, P=0.036). Thus, CTX immunosuppression slowed the loss of viral DNA, but only modestly. Despite CTX treatment, nearly 90% of vector DNA present on day 3 is lost by day 7. These results paralleled the effects of CTX treatment on β-gal expression (Figure 2). Taken together, the data suggest that antigen-specific immune responses to adenoviral and transgene proteins play, at most, a minor role in the early loss of vector DNA and transgene expression.

Finally, to ensure that our data on vector DNA persistence were not specific to vectors generated in our laboratory, we repeated DNA persistence studies with the null vectors AdE2A+ and AdE2A−. Both vectors were infused at doses of 1 to 2×10⁹ IU/mL, and arteries were harvested either 1 or 12 days after infusion. Both vectors were lost from the arterial wall with a time course similar to that of AdCMVNull (Figure 5E).

Adenovirus-Induced Vascular Inflammation Is Dose Dependent and Occurs Independently of Vector Loss or E2A Expression

CTX immunosuppression nearly eliminated arterial wall inflammation but only modestly extended the persistence of β-gal transgene expression and AdCMVNull DNA (Figures 2 and 5D). These data suggested that loss of vector DNA from the arterial wall was largely independent of a local inflam-
also exhibited only minimal vascular inflammation (data not shown), despite rapid loss of vector DNA (Figures 5B and 5C). Thus, for all vectors tested, infusion at lower doses significantly decreased inflammation but did not increase the persistence of vector DNA.

**Discussion**

We tested whether second-generation adenoviral vectors would prolong recombinant gene expression or decrease inflammation after arterial gene transfer. Our major findings were as follows: (1) neither of 2 β-gal–expressing, second-generation vectors prolonged transgene expression, decreased the humoral immune response to adenovirus, or produced significantly less local inflammation; (2) viral DNA is rapidly lost from the arterial wall, with kinetics that parallel loss of transgene expression from the β-gal–expressing vectors; (3) essentially complete elimination of the humoral and tissue immune responses to adenovirus by CTX had relatively minor effects on the duration of transgene expression or persistence of adenoviral DNA; and (4) arterial wall inflammation after adenovirus infusion is dose dependent and does not require expression of an immunogenic transgene. Taken together, our results suggest that new paradigms are required to explain the loss of adenovirus-mediated gene expression from the artery wall and to design strategies to prevent this loss.

In contrast to previous reports, 7,21–23 inclusion of the temperature-sensitive E2A mutation did not affect either inflammation or the persistence of transgene expression. There are several potential explanations for this discrepancy. First, reversion to wild-type virus is possible. However, this is an unlikely explanation, because the E2A mutation has a low rate of spontaneous reversion14 and all of our in vivo experiments were performed with low-passage virus. Second, the preparations could have been contaminated with E1A+ virus, generated by recombination in the 293 cells. However, this is an unlikely explanation, because the E2A mutation has a low rate of spontaneous reversion14 and all of our in vivo experiments were performed with low-passage virus. Second, the preparations could have been contaminated with E1A+ virus, generated by recombination in the 293 cells. However, it is unlikely that occult E1A positivity confounded our experiments because the sensitivity of the PCR assay is such that few, if any, rabbit cells could have been transduced with an E1A+ virus. Third, animal strain and species differences can cause discrepant results in adenoviral gene transfer experiments.24 One group reported prolonged expression and decreased inflammation with E2A temperature-sensitive viruses in CBA mice, cotton rats, and rhesus monkeys.21–23
Another group found no advantage of the same E2A mutation in BALB/c mice and dogs. The absence of an effect in rabbits suggests that the applicability of this strategy is limited. Fourth, the negative results with AdE2A-LacZ might be attributable to a destructive immune response to the β-gal gene product, which is a potent antigen. However, this is an extremely unlikely explanation. The β-gal reporter gene was also used in studies in which the E2A mutation prolonged gene expression, whereas expression of a species-homologous cDNA (eg, canine factor IX in dogs) was not prolonged by the E2A mutation. In addition, DNA from AdCMVNull, AdE2A+, and AdE2A− (none of which express β-gal) was rapidly lost from the arterial wall.

Because MHC-I–restricted immune responses may play a major role in the inflammation leading to elimination of adenovirus-transduced cells, MHC-I molecules are logical targets for strategies designed to improve adenovirus-mediated gene transfer. Adenovirus gp19k protein inhibits MHC-I function by retaining MHC-I molecules in the endoplasmic reticulum. Indeed, the ability of adenovirus to establish persistent infections has been attributed, at least in part, to the properties of gp19k. Moreover, gp19k may have immunomodulatory properties that are independent of its ability to bind MHC-I.

Three previous studies have examined whether expression of gp19k could improve adenoviral vector performance. One of those studies reported positive results, whereas another failed to find a beneficial effect of gp19k expression. A third study yielded strain-specific results. In the present study, we found no effect of gp19k expression on the persistence of transgene expression or the humoral immune response. Potential explanations for these observations include the inability of gp19k to bind rabbit MHC-I alleles, unfavorable kinetics of gp19k expression (ie, expression after upregulation of MHC-I), and presentation of antigens after phagocytosis (which would uncouple gp19k expression from antigen presentation). The tendency toward decreased inflammation in Adgp19kLacZ-transduced arteries raises the intriguing prospect that gp19k may have an anti-inflammatory effect, at least in a subset of rabbits (Figure 3; note the bimodal distribution of data points in the Adgp19kLacZ groups). However, this possibility may not be terribly important in the absence of any effect of gp19k on the duration of transgene expression.

CTX blocked the humoral immune response to adenovirus and nearly eliminated T cells from the arterial wall. Although CTX increased β-gal expression at 14 days compared with first- or second-generation vectors, expression nonetheless dropped substantially (94%) below 3-day levels (Figure 2). These data suggest either that CTX-insensitive immune responses are responsible for loss of transgene expression or that expression declines because of nonimmune mechanisms. It will be difficult to discriminate between these possibilities in a rabbit model, in which strains with targeted deletions of immune system components are not available.

We recently reported that β-gal transgene expression is also rapidly lost (ie, 90% drop in β-gal expression between 3 and 14 days) after infusion of AdRSVnLacZ to carotid arteries of mice that lack antigen-specific immunity. We attributed the loss of transgene expression in mouse carotid arteries to elevated cell turnover, occurring largely as a result of surgical manipulation. We were concerned, however, that stimulation of cell turnover and loss of gene expression might be specific to mouse arteries, which are easily damaged during surgery. The present study (especially the results obtained with CTX) confirms the dissociation between effective immunosuppression and persistence of transgene expression after arterial gene transfer. Moreover, the present study also shows that vector DNA is lost from the artery wall beginning on the first day after vector infusion and continuing with the same kinetics (half-life of ∼1 day) for at least a week. If vector persistence were primarily controlled by antigen-specific immune responses, then vector DNA would be expected to persist for a few days and then decline rapidly. Alternatively, if early macrophage-mediated clearance of vector DNA were followed by antigen-specific clearance of transduced cells, elimination of adenoviral DNA would be biphasic. Neither of these models fits the observations that we have made in arterial gene transfer models. Instead, the dissociation of immune responses from transgene persistence contrasts with numerous studies that correlate persistence of adenovirus-mediated transgene expression with genetic deficiencies in the antigen-specific immune system. None of these other gene transfer studies, however, were performed in arteries.

There are at least 2 aspects of our results that bode well for the future of adenovirus-mediated arterial gene transfer. First, the amount of vector DNA present in the artery wall immediately after vector infusion is surprisingly high. At the highest dose infused (4×10^10 pfu/mL), there were >300 copies of vector DNA per arterial wall cell 1 hour after infusion, and >100 copies remained after 1 day. Even at the lowest dose tested (1×10^10 pfu/mL), 4 vector copies per artery wall cell were present at 1 day. If, as suggested by the pattern of reporter gene expression in this model, virtually all vector copies are in the endothelium, then the number of vector copies per target cell is far higher. If all of these copies are intracellular, then efficient gene delivery to the endothelium might be achieved at surprisingly low vector doses. The second positive aspect of our results is that artery wall inflammation can be nearly eliminated by lowering the dose of virus (Figure 6). Thus, optimal vector design may allow high levels of transgene expression at vector doses that do not cause local inflammation.

In summary, factors that control the early loss of arterial wall transgene expression appear to differ from factors that govern adenovirus-mediated transgene loss in other tissues. Identification of these factors, which may include cell turnover, instability of adenoviral DNA, and noncytotoxic pathways of virus elimination from host cells, is essential for developing strategies that extend the utility of adenovirus-mediated arterial gene transfer.

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EXPANDED MATERIALS AND METHODS

Recombinant Viruses

Of the six adenoviral vectors used in these experiments, four were generated in our laboratory: AdRSVnLacZ, Adgp19kLacZ, AdE2AtsLacZ, and AdCMVNull (Figure 1). To construct Adgp19kLacZ, we began by isolating the gp19k coding sequence from human adenovirus type 2 [obtained from the American Type Culture Collection (ATCC): #846-VR]. Purified genomic Ad2 DNA was used as a substrate for a PCR reaction using oligonucleotide primers 5′–GGA ATT CGC TTT TTA AAC GCT GGG GGC–3′ (Ad2 bases 28782–28801 with an EcoRI recognition sequence added to the 5′ end) and 5′–CGT CTA AGC CTG GAG CAT ATC CCA C–3′ (complementary to Ad2 bases 29332–29249 with an XbaI site added to the 5′ end). The amplification product was digested with EcoRI and XbaI and ligated to similarly digested pCI (Promega). The ligation product was digested with BglII and BamHI to release an expression cassette comprising the CMV immediate/early promoter, a synthetic intron, the gp19k coding sequence, and the SV40 polyadenylation signal. This cassette was ligated to pΔE1sp1A2 at the BamHI site: pΔE1gp19k. pΔE1gp19k was digested with BamHI and ligated to a 4.2 kb BglII fragment containing the nucleus-targeted β-gal expression cassette from AdRSVnLacZ. The ligation product (pΔE1gp19kLacZ) was linearized by digestion with NruI and cotransfected into 293 cells with the large ClaI fragment of adenovirus dl327.3

We used immunofluorescence to confirm cytoplasmic expression of gp19k in Adgp19kLacZ-transduced CHO cells and primary rabbit vascular smooth muscle cells (Figure I, and data not shown). Gp19k expression appeared equivalent in cells transduced with Adgp19kLacZ or with wild-type adenovirus 2. Expression of gp19k in arteries transduced in vivo with Adgp19kLacZ was confirmed by RT-PCR (n = 2 of 2 arteries tested; 2 control-transduced arteries did not reveal gp19k expression).
To construct AdE2A<sup>ts</sup>LacZ we first obtained adenovirus mutant ts125 (originally termed ts225) from Dr. H. Ginsberg (Columbia University, NY). Genomic DNA was extracted from ts125 and digested with SfiI, and the 6.7-kb fragment (containing the E2A region) was purified by gel electrophoresis. This fragment was ligated to SfiI-digested DNA purified from an E1/E3-deleted virus (termed dl327/pBHG11) that had been generated in our laboratory by ligation of a 6-kb EcoRI fragment of pBHG11<sup>2</sup> (containing the 3´ end of the adenovirus 5 genome with a large E3 deletion) to the 27-kb EcoRI fragment of dl327 (containing the 5´ end of the adenovirus 5 genome). When ligating the SfiI fragments, we included a four-fold molar excess of the 6.7-kb fragment of ts125, thus favoring generation of a new virus<sup>5</sup> (AdE2A<sup>ts</sup>LacZ) containing wild-type E1 and deleted E3 (from dl327/pBHG11) as well as the E2A mutation from ts125. Plaques were amplified on 293 cells grown at the permissive temperature of 32°C, and Hirt-extracted DNA was sequenced to verify the presence of the E2A mutation. The temperature-sensitive mutation in E2A is a C→T mutation in the DNA-binding protein of the adenoviral E2A region, resulting in an amino acid change (Pro→Ser), a thermally unstable protein, and decreased adenoviral late gene expression.<sup>6</sup> The presence of the anticipated mutation in the coding strand of AdE2A<sup>ts</sup>LacZ was established by sequencing of both strands of purified adenoviral genomic DNA. The temperature-sensitive phenotype of AdE2A<sup>ts</sup>LacZ was confirmed by titrations on 293 cells at 37°C which showed a plaque titer of <10<sup>6</sup> plaque-forming units (pfu)/ml and a β-gal titer of >10<sup>10</sup> β-gal transducing units/ml. In contrast, our first-generation vectors typically have plaque and β-gal titers within 2–3-fold. To guard against spurious results that might be caused by reversion to wild-type E2A, we purified DNA from all viral stocks to be used for animal experiments and sequenced the area surrounding the E2A mutation. In all cases, only the mutant nucleotide was identified.

To construct AdCMVNull, a 1.1kb HinPI fragment of plasmid pcDNA3 (Invitrogen) containing the CMV promoter and bovine growth hormone polyadenylation
signal but without an intervening transgene was ligated to *Cla*I-digested pΔE1sp1A, yielding pΔE1CMV. This plasmid was linearized by digestion with *Ssp*I and cotransfected into 293 cells with the large *Cla*I fragment of dl327.

Viral stocks were prepared, stored, and characterized as described, with the exception that AdE2AtsLacZ was propagated at 32°C. The concentration of purified stocks of all three vectors was determined in particles/ml by measuring their absorbance at 260 nm (OD260). The concentrations of AdRSVnLacZ, Adgp19kLacZ, and AdCMVNull preparations were also measured by plaque titration on 293 cells. AdE2AtsLacZ did not form easily discernable plaques at 32°C or 37°C and was therefore titered by two independent methods: 1) in LacZ-transducing units by staining transduced 293 cells to detect β-gal expression; and 2) in plaque-forming units determined indirectly by transducing 293 cells in parallel with serially diluted AdE2AtsLacZ and AdRSVnLacZ, comparing the β-gal activity in 293 cell extracts, and using the plaque titer of AdRSVnLacZ to assign a “relative” plaque titer to AdE2AtsLacZ. The absence of replication-competent virus was confirmed in all viral preparations by a PCR-based assay that can detect one E1A-containing genome per 1 × 10^6 vector genomes.

Two additional adenoviral vectors were provided by IntroGene (Leiden, the Netherlands): AdE2A+ and AdE2A−. Both IntroGene vectors were E1-deleted adenovirus 5-based vectors with a CMV promoter (but no transgene) inserted at the site of the E1 deletion. AdE2A− was also deleted of the E2A region. Use of a second-generation vector provided by others helped to clarify the general applicability of our findings and to resolve whether any shortcomings of our temperature-sensitive E2A virus might be due to leaky expression of E2A. Details of the construction of AdE2A+ and AdE2A− will be reported elsewhere. AdE2A+ was propagated on PER.C6™ cells and AdE2A− was propagated on PER.C6™/tsE2A cells, a line of PER.C6™ cells that is stably transduced with the ts125E2A gene. The absence of E2A DNA-binding protein in endothelial cells transduced with AdE2A− was confirmed by western blotting (Figure
II). In contrast, the DNA-binding protein was easily detected in endothelial cells transduced with AdE2A+.

The concentration of purified stocks of these vectors was determined by optical density (viral particles/ml) and by titration on PER.C6™ or PER.C6™/tsE2A cells [infectious units (IU)/ml]. Purified stocks (2–6 × 10^{10} IU/ml) were stored at –80 °C in PBS with 5% sucrose and were free of replication-competent virus. To permit use of equivalent doses of AdE2A+, AdE2A–, and AdCMVNull in vivo, the vectors were titered by Southern analysis performed on cultured rabbit endothelial cells transduced in parallel with each of the vectors. The titers were all within 1 log of each other as measured by equivalents of transferred vector DNA per IU (AdE2A+ and AdE2A–) or per pfu (AdCMVNull). The in vivo doses were adjusted accordingly.

**Detection of gp19k Expression**

Adenovirus gp19k protein expression by Adgp19kLacZ was detected by indirect immunofluorescence performed after infection of CHO cells and primary rabbit smooth muscle cells. CHO cells were obtained from the ATCC (# CCL-61), and rabbit smooth muscle cells were harvested by explant culture of an artery from an adult New Zealand White rabbit. Cells were grown on gelatin-coated coverslips, permeabilized with 70% ethanol, and incubated with monoclonal antibody TW1.3 (a gift of Dr. J. Yewdell, National Institutes of Health) at a concentration of 11 µg/ml. Bound antibody was detected with biotinylated horse anti-mouse IgG (Vector Labs) and Extravidin-FITC (Sigma Chemical Co.). Fluorescence microscopy was performed using standard fluorescein wavelengths. Specificity controls included omission of the primary antibody and infection with a control adenovirus. Expression of gp19k was also detected by reverse transcriptase-based polymerase chain reaction (RT-PCR), using the Superscript One-step RT-PCR System (Life Technologies) and RNA from transduced cells or arteries.
as a template. The primers were: 5’-GCT TTT TAA ACG CTG GGG GC-3’ and 5’-GCC TGG AGC ATA TCC CAC-3’.

For two reasons we did not perform in vitro experiments to determine the affinity of gp19k for rabbit MHC-I. First, these experiments might not be predictive of in vivo function as gp19k appears to have activity that is not explained by its affinity for MHC-I.\(^3,11\) Second, the ability of gp19k to improve vector performance is most definitively tested in vivo with direct measurement of the critical end points of duration of expression and magnitude of host immune response.

**Detection of E2A Expression**

We detected expression of the E2A-encoded DNA-binding protein\(^12\) by western blotting of cell extracts. Briefly, rabbit endothelial cells (a kind gift of Dr. M. Navab, UCLA) in a 24-well plate were infected for 72 h with AdE2A+ and AdE2A− at a multiplicity of infection (MOI) of 10\(^4\) particles/cell. To generate positive control samples, 293 cells were infected in parallel with AdCMVNull for 36 hr at a MOI of 20. Cell lysates were separated by SDS-PAGE, blotted onto a Hybond ECL membrane (Amersham) and probed with antibodies to E2A DNA-binding protein (provided by Dr. G. Schouten, IntroGene).

**Animal Experiments**

All animal protocols were approved by the Committee on Animal Research of the University of California, San Francisco. Vector stocks were infused in the lumen of surgically isolated common carotid arteries of adult male, specific pathogen-free, New Zealand white rabbits (Charles River Laboratories; 3.0–3.5 kg) as previously described.\(^13\) Arteries were harvested from 1 hr to 14 days later and processed for either β-gal activity assay, histologic analysis, or DNA extraction. Peripheral blood was drawn from the central ear artery or from an indwelling central venous cannula (see below) into tubes
containing 5 μM EDTA. Complete blood counts were performed by an outside laboratory (IDEXX).

AdRSVnLacZ (two independent preparations), Adgp19kLacZ (one preparation), and AdCMVNull (two independent preparations) were infused at 4–5 × 10⁹ pfu/ml (2–6 × 10¹¹ particles/ml). Because of difficulties preparing large quantities of E1A⁻AdE2A⁰LacZ, three independent preparations of AdE2A⁰LacZ were required to complete the experiments. These preparations were infused either at 5 × 10⁹ LacZ-transducing units/ml or at 5 × 10⁹ relative pfu/ml derived from comparison with AdRSVnLacZ (see above). This strategy was successful in achieving our goal of similar levels of β-gal expression at day 3 with each of the vectors (see below).

Central venous cannulas were placed in rabbits assigned to receive either CTX or control saline infusions. Rabbits were sedated with intramuscular ketamine (50 mg/kg) and xylazine (3 mg/kg). The right external jugular vein was exposed through a right anterolateral neck incision. A venotomy was performed, and a polyethylene catheter (BOLAB) was inserted and secured with two ligatures. The catheter was tunneled subcutaneously, brought out through a small skin incision between the scapulae, and placed in the pocket of a nylon vest (Alice King Chatham Medical Arts). Catheters were flushed every other day with 0.5 mL of sterile heparinized saline (100 U/mL; Abbott Labs). CTX (30 mg/kg/d; Sigma) or sterile saline was administered by catheter for 4 days preoperatively and 14 days postoperatively. This dose was chosen based on a published study¹⁴ and on pilot studies which revealed that lower doses of CTX did not cause leukopenia and higher doses were uniformly fatal (data not shown). Indeed, prolonged administration of CTX at 30 mg/kg/d was also poorly tolerated with approximately 30% mortality before the 14th postoperative day.

**β-galactosidase Activity Assay**

β-Gal activity in lysates of arteries was measured with a chemiluminescent assay.⁷
Southern Analysis

DNA was extracted from cultured cells or carotid artery segments using SDS-proteinase K digestion. Approximately 5 µg of DNA per well of cells or 3.3 µg from each artery was digested with HindIII, separated on a 1.0% agarose gel, and blotted onto Nytran. Blots were hybridized with a 32P-labeled 2.9 kb HindIII fragment of adenovirus 5 (a probe for the E4 region), washed at high stringency (0.1X SSC, 0.1% SDS), and autoradiographed. Equal loading of DNA and complete transfer to the Nytran membrane was confirmed by ethidium staining of gels before and after transfer. Selected blots of artery DNA were also rehybridized to a probe for the rabbit urokinase gene, to verify equal loading, as well as complete digestion and transfer. To permit quantitation of adenoviral DNA in transduced tissues, each autoradiograph included a standard curve generated by electrophoresis and blotting of purified, HindIII-digested adenoviral DNA loaded in amounts corresponding to 0.1–300 copies per haploid genome diploid (i.e., 0.2–600 copies per diploid cell) in the experimental lanes. Radioactive signals were quantitated with a Fuji Bas1000 bioimaging analyzer.

Real-time PCR Quantitation of Adenoviral DNA

Adenoviral DNA was also quantitated by real-time “Taq-Man” quantitative PCR. Template DNA (100 ng) was combined with primers (5´-CAT CTA CGT ATT AGT CAT CGC TAT TAC CA-3´ and 5´-TGG AAA TCC CCG TGA GTC A-3´) that amplify a 94 bp sequence in the CMV promoter. Amplified product was detected using a FAM-labeled probe to an intervening sequence: 5´-ACC GCT ATC CAC GCC CAT TGA TGT-3´. The amplification and detection protocol was similar to that described elsewhere. To aid in quantitation of adenoviral DNA in experimental samples, each amplification included a standard curve in which wells were spiked with 10²–10⁸ copies of adenoviral DNA. Amplifications in these wells generated fluorescence signals that
were linear over this range, and experimental wells always yielded signals that were within the limits of the standard curve.

**Detection of Anti-adenoviral Antibodies**

Serum antibodies to adenovirus were measured by ELISA. Briefly, 96-well plates were coated with 100 µL of adenoviral vector stock at 5 × 10^{10} particles/mL in phosphate-buffered saline. The wells were rinsed and blocked with 3% bovine serum albumin, 0.1% Tween-20 in phosphate-buffered saline. Diluted rabbit serum (100 µL) was added to each well for 90 min at 4 °C. Bound antibody was detected with alkaline phosphatase-conjugated mouse anti-rabbit IgG (Clone RG-16; Sigma) and p-nitrophenyl phosphate substrate (Sigma).

**Histological Analysis**

Arteries were embedded, sectioned, and serial sections were stained for CD5 (a T-cell antigen), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) expression, as described. Four evenly-spaced sections from each artery were stained with each of the three antibodies. Intensity of staining of each section was graded independently by two blinded observers, according to semiquantitative scales. Scores of the two observers were highly correlated (r^2 = 0.97, 0.76, and 0.83 for CD5, VCAM-1 and ICAM-1, respectively). The mean score of the four sections from each artery was calculated and assigned as an overall score for the artery. Individual artery scores were grouped according to treatment, and the treatment groups were compared by using the individual artery scores as experimental units.

**Statistical Analysis**

The effect of CTX on persistence of DNA was evaluated by unpaired t test; these data are presented as mean ± SEM. Data not normally distributed, including β-Gal expression levels, inflammation scores, and antibody levels were compared by nonparametric rank sum testing (two groups) or Kruskal-Wallis ANOVA (three groups). Not all possible
pairwise comparisons were made. The strength of correlations was assessed by Spearman rank order test, and the variability of hematologic values over time was evaluated by two-way repeated measures ANOVA with Tukey’s test for multiple pairwise comparisons.19
REFERENCES


FIGURE LEGENDS

**Figure I.** Expression of adenovirus 2 gp19k protein in CHO cells. CHO cells were infected with Adgp19kLacZ. Thirty-six hr later, cells were permeabilized with ethanol and incubated with an antibody to gp19k. Bound antibody was detected by indirect immunofluorescence. Similar results were obtained with primary rabbit vascular smooth muscle cells (not shown). No fluorescence was observed in cells infected with a control adenovirus or in Adgp19kLacZ-infected cells not incubated with primary antibody (not shown).

**Figure II.** Expression of adenovirus E2A DNA-binding protein (DBP). Western blot analysis of lysates of 293 cells transduced with the first-generation vector AdCMVNull reveals abundant expression of the 72kD DBP. No corresponding band is present in lysates of mock-transduced 293 cells or rabbit endothelial cells (EC). EC transduced with the second-generation vector AdE2A– do not express DBP; however, expression is present in EC transduced with the first generation vector AdE2A+. A non-specific band is present in all EC lysates.
Table I. Effects of cyclophosphamide infusion on blood cell counts

<table>
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<tr>
<th></th>
<th>Baseline (n = 8)</th>
<th>Day 4 Saline (n = 4)</th>
<th>Day 4 Cyclo. (n = 4)</th>
<th>Day 11 Saline (n = 4)</th>
<th>Day 11 Cyclo. (n = 4)</th>
<th>Day 18 Saline (n = 4)</th>
<th>Day 18 Cyclo. (n = 4)</th>
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</thead>
<tbody>
<tr>
<td>White blood cell</td>
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<td></td>
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<tr>
<td>count (×10³/µl)</td>
<td>4.8 ± 1.0</td>
<td>5.8 ± 0.7</td>
<td>3.5 ± 1.1‡</td>
<td>7.8 ± 0.7</td>
<td>2.5 ± 0.6‡</td>
<td>6.0 ± 0.6</td>
<td>1.9 ± 0.5‡</td>
</tr>
<tr>
<td>Neutrophils (n/µl)</td>
<td>1553 ± 359</td>
<td>1909 ± 1136</td>
<td>981 ± 478</td>
<td>3311 ± 1504</td>
<td>379 ± 365‡</td>
<td>1995 ± 996</td>
<td>351 ± 335‡</td>
</tr>
<tr>
<td>Lymphocytes (n/µl)</td>
<td>3002 ± 1097</td>
<td>3320 ± 1271</td>
<td>2106 ± 659</td>
<td>3781 ± 1847</td>
<td>1827 ± 1077‡</td>
<td>3391 ± 534</td>
<td>1313 ± 350‡</td>
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<tr>
<td>Monocytes (n/µl)</td>
<td>123 ± 147</td>
<td>217 ± 96</td>
<td>177 ± 145</td>
<td>520 ± 79</td>
<td>99 ± 102‡</td>
<td>133 ± 58</td>
<td>52 ± 52‡</td>
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<tr>
<td>Hematocrit (%)</td>
<td>38.3 ± 2.7</td>
<td>36.5 ± 2.6</td>
<td>33.7 ± 3.6</td>
<td>37.6 ± 3.4</td>
<td>26.5 ± 2.2‡</td>
<td>36.7 ± 1.8</td>
<td>19.9 ± 2.7‡</td>
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‡P < 0.05 versus saline control group (two way repeated measures ANOVA with Tukey test for multiple pairwise comparisons).