Inclusion of the E3 Region in an Adenoviral Vector Decreases Inflammation and Neointima Formation After Arterial Gene Transfer

Shan Wen, Robert M. Driscoll, Darren B. Schneider, David A. Dichek

Abstract—Adenoviral vectors are promising agents for vascular gene transfer. Their use, however, is limited by inflammatory host responses, neointima formation, and brevity of transgene expression. Inclusion of the immunomodulatory adenoviral E3 genes in a vector might prevent inflammation and neointima formation and prolong transgene expression. We compared 2 adenoviral vectors in a model of in vivo gene transfer to rabbit arteries. Both vectors expressed a luciferase reporter gene. One vector (AdE3Luc) contained the adenovirus early 3 (E3) region and the other (AdRSVLuc) lacked E3. Expression of E3 genes by AdE3Luc was confirmed in vitro and in vivo. Arteries transduced with AdE3Luc had substantially and significantly less inflammation (fewer T cells and lower levels of vascular cell adhesion molecule-1 and intercellular adhesion molecule 1 expression) and decreased neointima formation 14 days after gene transfer. Luciferase expression from the 2 vectors was equivalent, however, at both 3 and 14 days after gene transfer. Expression of E3 had no systemic immunosuppressive effects, as measured by peripheral blood counts and by assays for serum antibodies to adenovirus. We conclude that expression of E3 significantly decreases adenovirus-induced arterial wall inflammation and neointima formation. Because inflammation and neointima formation are major barriers to the clinical application of adenoviral vectors, use of E3-containing vectors improves the promise of adenovirus-mediated arterial gene transfer. (Arterioscler Thromb Vasc Biol. 2001;21:1777-1782.)

Key Words: adenovirus ■ carotid arteries ■ cell adhesion molecules ■ gene therapy ■ inflammation

Adenoviral vectors are useful agents for studies of arterial biology and potentially for gene therapy of human vascular disease. Despite their promise, the utility of adenoviral vectors is limited by brevity of expression and inflammatory responses in arteries of both small animals and primates.1-3 Both of these limitations are believed to result from low-level expression of adenoviral genes. Expression of adenoviral genes in vivo causes local inflammation and provokes a cytotoxic T-cell response that, in some settings, can eliminate transduced cells and extinguish transgene expression.4 On the basis of this model, several groups have modified adenoviral vectors by further disabling or deleting the viral genes.5-7 In liver and lung gene transfer models, these second- and third-generation vectors have reduced inflammation and prolonged transgene expression. These advances, however, have not yet been extended to vascular gene transfer.

We previously reported initial experiments testing the promise of second-generation vectors for arterial gene transfer.8 Surprisingly, none of 3 second-generation vectors significantly decreased vascular inflammation or prolonged transgene expression. For one of the vectors, however, in which the immunomodulatory adenovirus 2 gp19K protein was expressed as a transgene, we found a trend toward decreased local inflammation. The gp19K protein is one of several proteins expressed by the E3 region of adenovirus. The E3 proteins have a common function in modulating host antiviral and inflammatory responses8; therefore, inclusion of the entire E3 region in an adenoviral vector might be a more effective strategy to decrease inflammation. Indeed, in a mouse model of adenovirus-mediated hepatic gene transfer, expression of E3 genes nearly eliminated the host immune response and significantly prolonged gene expression, as would be expected if the two were linked.10

Here, we report the results of investigations that determine whether incorporation of an E3 expression cassette in an adenoviral vector decreases inflammation and neointima formation and prolongs transgene expression after arterial gene transfer.

Methods

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Adenoviral Vectors
We constructed 2 adenoviral vectors, AdRSVLuc and AdE3Luc (Figure 1). Both vectors have the native E3 region deleted, and both express a luciferase cDNA from the Rous sarcoma virus (RSV) LTR promoter. We used luciferase instead of β-galactosidase as a reporter because luciferase is a smaller gene and there is insufficient space in the vector backbone to accommodate both E3 and β-galactosidase. AdE3Luc also expresses the E3 region of adenovirus 2, with expression driven by the cytomegalovirus (CMV) immediate-early promoter. The remainder of the adenoviral sequences in both vectors are from adenovirus 5. Because expression from the native E3 promoter is dependent on the presence of E1 proteins and because replication-defective vectors are necessarily E1-deleted, we used a heterologous promoter (from CMV) to ensure high-level, constitutive expression of E3 gene products. In preliminary experiments during vector construction, we placed the luciferase and E3 expression cassettes in 4 different orientations in the left-end shuttle plasmid and measured expression of the cassettes after transfection into Chinese hamster ovary (CHO) cells. The orientation chosen for final vector construction (Figure 1) showed robust expression from both promoters, suggesting that promoter interference was not present. Details of vector construction, preparation, and characterization can be accessed online at http://atvb.ahajournals.org. The AdNull vector, another Ad5-based E1, E3–deleted vector that does not express a transgene,11 was used as a control vector in in vitro experiments.

In Vivo Gene Transfer
Vectors were infused into surgically isolated common carotid arteries of New Zealand White rabbits.12 We chose rabbits for this study because they are an informative model for human vascular disease13 and share with nonhuman primates a propensity to develop inflammatory vascular lesions after intra-arterial infusion of adenovirus.1,3 All animal protocols were approved by the Committee on Animal Research of the University of California, San Francisco. AdRSVLuc and AdE3Luc were both infused at a concentration of 108 particles/mL (~108 to 109 pfu/mL, or 1010 particles/artery). This is a relatively low dose of virus; however, this dose is associated with both inflammation and neointima formation. For several reasons, we compared the vectors on the basis of particle, not plaque, titers. First, particle titers are measured more reproducibly and objectively than plaque titers.14 Second, nonspecific toxic effects of vector infusion are immediate15 and are therefore most likely related to total exposure to adenoviral particles. Third, our decision to use particle titers to compare the vectors was supported by experiments showing that luciferase expression by the 2 vectors was equivalent when measured in relative light units (RLU) generated per particle both in vitro and in vivo.

Detection of Expression of E3 and Luciferase
We used 3 methods to assess expression of E3 genes by AdE3Luc: in vitro assays performed on transduced 293 cells and CHO cells (American Type Culture Collection) and in vivo assays performed on carotid arteries. E3 transcripts in AdE3Luc-transduced cells were detected by Northern analysis.16 We also detected E3 transcripts with reverse-transcriptase–mediated polymerase chain reaction (RT-PCR). Expression of the E3-encoded gp19K protein was detected by indirect immunofluorescence.8 Luciferase activity was detected with a luminometer.15

We investigated the relationship between inflammation and loss of transgene expression by measuring inflammation and luciferase expression in the same arteries. To permit this, each transduced artery was divided into 4 equal segments. Extracts of the first and fourth segments were assayed for luciferase expression. The remaining 2 segments were sectioned and stained to measure inflammation.

Vascular Inflammation and Neointima Formation
The second and third segments of each transduced artery were further divided into halves, and the 4 segments were embedded in O.C.T. medium. Sections cut from these blocks were stained with antibodies to detect T cells and measure expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). Staining intensities and neointima formation were scored by 2 blinded observers according to previously described semiquantitative scales.12,13 This technique again yielded highly reproducible scores with interobserver correlations >0.93 for all 3 stains. Neointima formation was also graded by 2 observers according to a semiquantitative scale.12 Interobserver correlation for intimal area scores was 0.87. For all end points, the mean score of the 4 sections from each artery was calculated as a single overall score for the artery.

Detection of Adenoviral Genomes After In Vivo Gene Transfer
Adenoviral genomes were detected in lysates of AdE3Luc-transduced arteries harvested 3 and 14 days after gene transfer by both Southern blotting and real-time quantitative PCR, as described.8

Blood Counts and Detection of Antibodies to Adenovirus
Complete blood counts were performed by a commercial laboratory (IDEXX Laboratories). Neutralizing anti-adenoviral antibodies were detected by incubating sera with virions and then testing virion infectivity on 293 cells.17

Statistics
Data are expressed as mean±SD if normally distributed and as median (interquartile range) if not. Luciferase expression levels were compared by t test if groups were normally distributed with equal variances and by Mann-Whitney rank-sum test if not. Scores for staining intensity and intimal size were compared by Mann-Whitney rank-sum test. The relationships between the observers’ scores and between these scores and luciferase expression were assessed by Spearman rank-order correlation.

Results
Expression of E3 Transcription Units
We infected CHO cells with AdE3Luc or AdRSVLuc and stained the cells for expression of the E3-encoded protein gp19K. Gp19K expression was detected by immunohistochemistry in cells transduced with AdE3Luc but not in cells transduced with AdRSVLuc (please see Fig. I, which can be accessed online at http://atvb.ahajournals.org). We assessed the expression of E3 transcripts by Northern analysis of transduced 293 cells. Cells transduced with AdE3Luc expressed several transcripts that hybridized to the E3 probe. The size of these transcripts was similar to that of E3-
hybridizing transcripts in 293 cells infected with wild-type adenovirus 2 (Figure 2A). E3 mRNA was also detected by RT-PCR in 293 cells infected with AdE3Luc but not in untransduced cells (Figure 2B).

To confirm expression of E3 genes after in vivo gene transfer, we performed RT-PCR for E3 sequences using mRNA from arteries transduced 3 days earlier with AdE3Luc or AdRSVLuc. E3 mRNA was detected only in arteries transduced with AdE3Luc (n=2 arteries per vector; Figure 2C). Inclusion of DNase treatment in all RT-PCR protocols and dependence of successful amplification on the presence of reverse transcriptase (Figure 2B and 2C) excluded vector DNA as a template.

**Transgene Expression In Vitro and In Vivo**

In 293 cells transduced with $2 \times 10^3$ viral particles/cell, AdRSVLuc and AdE3Luc expressed equivalent amounts of luciferase: $5.8 \pm 2.3 \times 10^7$ versus $6.9 \pm 1.0 \times 10^7$ RLU/μL of cell extract (n=3; $P=0.4$).

In vivo, 3 days after infusion, both vectors expressed luciferase at equivalent levels: $3.8 \times 10^7$ (7.3 \times 10^6 to 1.0 \times 10^8) RLU/mg for AdRSVLuc (n=6) versus $1.7 \times 10^7$ (2.7 \times 10^6 to 8.1 \times 10^7) RLU/mg for AdE3Luc (n=8; $P=0.4$; Figure 3). By 14 days after gene transfer, luciferase expression had fallen significantly in both AdRSVLuc (n=8) and AdE3Luc-transduced arteries (n=14; $P<0.005$ for 3- versus 14-day expression levels for both vectors) and did not differ between the 2 groups ($P=0.7$).

**Loss of Adenoviral Genomes After In Vivo Gene Transfer**

We previously showed that genomes of 2 first-generation vectors and 1 second-generation vector were rapidly lost from the artery wall after in vivo gene transfer. Nevertheless, because of a concern that the inability of the E3 region to prolong expression might be due to shutdown of transcription of E3 from the CMV promoter despite persistence of AdE3Luc genomes, we assayed arterial extracts for vector genomes 3 and 14 days after infusion of AdE3Luc (n=4 for each). AdE3Luc genomes were abundant at 3 days after gene transfer ($\approx 9$ copies per arterial wall cell) but were nearly undetectable at 14 days ($\approx 0.04$ copies per arterial wall cell).

**Vascular Inflammation and Neointima Formation**

T-cell infiltration and ICAM-1 and VCAM-1 expression were significantly and substantially decreased in arteries transduced with AdE3Luc (Figures 4 and 5). Indeed, T-cell and VCAM-1 staining were reduced to nearly undetectable levels (scores of $\leq 1$ in most of the AdE3Luc arteries reflect $\leq 1$ T cell and $\leq 1$ small area of VCAM-1 positivity per section). Neointima formation was also significantly less in AdE3Luc-transduced arteries. Sections of representative arteries (Figure 4) illustrate the dramatic difference in group medians.

Despite the decreased inflammation in the AdE3Luc arteries analyzed as a group, some of the 14 arteries had high levels of inflammation (Figure 5). This variability in...
Hematological Values 2 Weeks After Gene Transfer

<table>
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<tr>
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<th>AdRSVLuc (n=4)</th>
<th>AdE3Luc (n=5)</th>
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</thead>
<tbody>
<tr>
<td>White blood cell count, ×10⁹/µL</td>
<td>5.3±2.7</td>
<td>4.6±2.2</td>
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<tr>
<td>Hemoglobin, g/dL</td>
<td>12±0.8</td>
<td>11±3.0</td>
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<tr>
<td>Neutrophils, n/µL</td>
<td>890±910</td>
<td>510±280</td>
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<tr>
<td>Lymphocytes, n/µL</td>
<td>4100±1500</td>
<td>3800±1800</td>
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*P<0.4 for all comparisons with AdRSVLuc.

Discussion

We investigated whether incorporation of an E3 expression cassette in a first-generation adenoviral vector would prolong transgene expression and decrease local inflammation after adenoviral gene transfer to the artery wall. Our major findings were that (1) expression of E3 genes significantly reduced local inflammation and neointima formation; (2) reductions in inflammation and neointima growth were achieved without any apparent systemic effects on the immune system; (3) expression of E3 genes did not prolong transgene expression; and (4) in individual arteries, loss of transgene expression and intensity of inflammation were not correlated.

The limitations associated with inflammation and rapid loss of transgene expression after adenoviral vector infusion have prompted several groups to develop improved, “second-generation” adenoviral vectors. Second-generation vectors fit into 2 classes: “loss-of-function” vectors, which express fewer potentially immunogenic viral proteins,3,7 and “gain-of-function” vectors, which express proteins that decrease inflammation and permit transduced cells to evade immune-mediated cytolysis.10,18 Design of these vectors is based on the hypothesis that local inflammation and loss of transgene expression are linked: adenovirus-induced inflammation attracts cytotoxic T cells that recognize and eliminate transduced cells expressing adenoviral antigens.19

Second-generation vectors have prolonged transgene expression and decreased inflammation in animal models of liver and lung gene transfer5,8–10,18; however, these vectors have not previously been of value for vascular gene transfer. Indeed, we previously reported that none of 3 second-generation vectors (2 loss-of-function and 1 gain-of-function vector) decreased inflammation or prolonged gene expression after arterial gene transfer.8 Data in this previous study also cast doubt on whether vascular inflammation and loss of transgene were linked. Two interventions that nearly eliminated inflammation (lowering the adenoviral dose and systemic immunosuppression) had either no effect or only a marginal effect on duration of transgene expression.8

The present study extends our previous work significantly. Most importantly, expression of E3 genes resulted in decreased inflammation and neointima formation. An improved performance of AdE3Luc was noted compared with an equivalent dose of the first-generation vector AdRSVLuc, which is identical to AdE3Luc except for the E3 cassette. Moreover, the same level of luciferase transgene expression was obtained with both vectors (Figure 3). Thus, our results show that adenovector performance in a vascular gene transfer model can be improved without lowering the vector dose (thereby compromising transgene expression) and without systemic immunosuppression.2,8 Avoidance of...
adenovirus-induced inflammation and neointima formation is critical, because these processes contribute to the pathogenesis of vascular disease.\textsuperscript{20} Prolongation of expression, although desirable in many settings, is not required for all vascular applications of adenoviral gene transfer.\textsuperscript{21,22}

Inclusion of the E3 region did not prolong transgene expression or alter the systemic immune response to adenovirus. In this respect, our results differ from those of Ilan et al.,\textsuperscript{10} who reported that an E3-containing vector decreased inflammation, abrogated the systemic immune response to adenovirus, and dramatically prolonged transgene expression after hepatic gene transfer in mice. We are uncertain why our results differ. We considered whether shutdown of transcription from the CMV promoter or promoter interference might be present in our system; however, we found no evidence for either of these processes. Potential factors that could contribute to differences in results between Ilan et al and the present study include variability in species, nature of transgene, and target organ. Notably, however, our present results are consistent with our previous findings in immune-deficient mice and immunosuppressed rabbits,\textsuperscript{23} which suggested that local inflammation and loss of transgene expression from the artery wall are not linked. The present study extends this observation by establishing that in individual arteries, these 2 processes are not correlated (please see Fig. II, which can be accessed online at http://atvb.ahajournals.org). The mechanisms responsible for loss of vector DNA and transgene expression from the artery wall remain under investigation.

We can speculate on the precise mechanisms through which the E3 gene products abrogate inflammation and neointima growth. The E3 region encodes 7 identified proteins that function primarily by downregulating expression of MHC class I and counteracting the cytotoxic and proinflammatory actions of tumor necrosis factor-α (TNF-α).\textsuperscript{9} The importance of TNF-α in the host response to adenovirus is supported by several lines of evidence: (1) a large number of adenoviral proteins show activity against TNF-α, and the sequences of these proteins are highly conserved among adenoviral strains\textsuperscript{9}; (2) targeted deletion of the TNF-α gene or administration of soluble TNF-α inhibitors decreases inflammation and prolongs transgene expression after adenoviral gene transfer to liver and lung\textsuperscript{24–26}; (3) adenoviral mutants lacking specific E3 proteins known to inhibit TNF-α provoke increased inflammation\textsuperscript{27}; and (4) TNF-α upregulates cell surface adhesion molecule expression,\textsuperscript{28} which could account for the upregulation of ICAM-1 and VCAM-1 as well as the leukocyte infiltration seen in this model (Figures 4 and 5). It therefore seems likely that the efficacy of E3 expression in the artery wall is due to local antagonism of TNF-α. Because our vector expresses the entire E3 region, however, our data do not exclude effects of E3 that are independent of TNF-α.\textsuperscript{29–31} The possibility that gp19K contributes to the anti-inflammatory effect of E3 cannot be excluded without a direct comparison between a vector expressing E3 without gp19K and a vector expressing gp19K alone. Such an experiment is a substantial challenge, requiring disruption of gp19K expression from the complex E3 transcriptional cassette\textsuperscript{9} without alteration of expression of the other E3 proteins.

The ability of AdE3Luc to suppress neointima formation (Figures 4 and 5) is reminiscent of reports of other viral proteins that can inhibit neointima formation\textsuperscript{32,33} and suggests that the E3 region might also be used in gene therapy strategies to prevent neointima growth. In support of a direct role for E3 in controlling neointima growth, 2 of the E3 proteins downregulate cell-surface growth factor receptors, including those for epidermal growth factor and insulin-like growth factor-1.\textsuperscript{24,34} Insulin-like growth factor-1, in particular, has been implicated in proliferative arterial disease.\textsuperscript{35}

In summary, reintroduction of the E3 region in an adenoviral vector significantly and substantially reduced adenovirus-induced arterial inflammation and neointima formation. Inclusion of E3 genes improves the suitability of adenoviral vectors for vascular gene therapy.

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References


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