Acute Host-Mediated Endothelial Injury After Adenoviral Gene Transfer in Normal Rabbit Arteries

Impact on Transgene Expression and Endothelial Function

Keith M. Channon, HuSheng Qian, Scot A. Youngblood, Ercument Olmez, Geetha A. Shetty, Valentina Neplioueva, Michael A. Blazing, Samuel E. George

Abstract—Acute injury after adenoviral vascular gene transfer remains incompletely characterized. Here, we describe the early response (≤days) in 52 New Zealand White rabbits undergoing gene transfer (β-galactosidase or empty vector) or sham procedures to both carotid arteries. After gene transfer, arteries were either left in vivo for 1 hour to 3 days (in vivo arteries) or were excised immediately after gene transfer and cultured (ex vivo arteries). Within 1 hour, in vivo arteries receiving infectious titers of ≥4×10⁹ plaque-forming units (pfu)/mL showed endothelial activation, with an acute inflammatory infiltrate developing by 6 hours. Ex vivo arteries showed endothelial activation but no inflammatory infiltrate. There were also significant differences in transgene expression between in vivo and ex vivo arteries. Ex vivo arteries showed titer-dependent increases in β-galactosidase expression through 2×10¹⁰ pfu/mL, whereas in in vivo arteries, titers above 4×10⁹ pfu/mL merely increased acute inflammatory response, without increasing transgene expression. In vivo arteries showed significant time- and titer-dependent impairment in endothelium-dependent relaxation, with no effect on contraction or nitroprusside-induced relaxation. Interestingly, however, if rabbits were made neutropenic with vinblastine, their arteries maintained full endothelium-dependent relaxation, even after very high titer vascular infection (up to 1×10¹¹ pfu/mL). These findings show that recombinant adenovirus triggers an early inflammatory response, and it is the inflammatory response that in turn causes functional endothelial injury. This occurs at much lower titers than previously appreciated (though the precise threshold will undoubtedly vary between laboratories). However, titers below the inflammatory threshold produce excellent transgene expression without inflammation or vascular injury. (Circ Res. 1998;82:1253-1262.)

Key Words: adenovirus ■ gene transfer ■ endothelium ■ inflammation ■ neutrophil

Recombinant adenovirus shows promise as a tool for vascular research and therapy,¹⁻³ but significant limitations are clearly apparent. Most prominent are the long-term problems⁴⁻⁵: in vivo transgene expression declines from initially high levels to virtually nothing within 2 weeks,⁶ and an adenovirus-induced chronic inflammatory response produces significant endothelial injury and neointimal proliferation that confound data interpretation and counter any potential therapeutic effect.⁷ These untoward effects are largely attributable to a virus-induced host immune response.

Acute vascular effects of adenoviral gene transfer—those occurring within 3 days of gene transfer—have also been described but are less well characterized: (1) in normal rat arteries, complete endothelial denudation after high-titer (2×10¹¹ pfu/mL) gene transfer but no apparent injury at lower titers (≥10¹¹ pfu/mL),⁸ (2) in balloon-injured rat arteries, an acute medial inflammatory infiltrate and smooth muscle cell loss in arteries receiving 10¹¹ pfu/mL⁹ and (3) in normal rabbit carotid arteries, a medial inflammatory infiltrate, an impaired response to contractile agonists, and complete loss of endothelium-dependent vasodilation after moderately high-titer (4×10¹⁰ pfu/mL) gene transfer.¹⁰

These observations have led to 2 general hypotheses about adenovirus-induced acute injury. One is that the adenovirus itself is directly toxic to the vessel (“direct viral toxicity”),⁸,¹¹ a view supported by the cytotoxic effects of high-multiplicity recombinant adenovirus infections in cell culture and the known in vitro cytotoxicity of some adenoviral proteins, such as the penton base.¹²,¹³ The second is that the observed injury is a host-mediated effect, largely secondary to acute inflammatory cell infiltrate, that develops after high-titer gene transfer.¹⁴ A better understanding of adenovirus-induced acute effects and the cellular mechanisms through which they are produced may be particularly important for vascular gene transfer, since these effects may have an untoward impact on transgene expression⁶ and the subsequent development of neointimal proliferation.⁷

Accordingly, we undertook the present study to define more thoroughly the acute vascular effects of adenovirus and to answer the following general questions about adenoviral
gene transfer in normal rabbit arteries: (1) What is the extent of acute vascular inflammation, and what is its time course and titer dependence? (2) What is the impact of acute vascular inflammation on transgene expression? (3) To what extent does adenoviral gene transfer functionally impair the vessel, and if functional impairment is present, what is its time course and titer dependence? (4) What is the relative contribution of "direct viral toxicity" and host-mediated inflammatory effects to the observed functional deficits?

The present study shows that a titer-dependent acute inflammatory response that significantly limits transgene expression and endothelial vasomotor function develops within hours of vascular gene transfer. Moreover, our data suggest that the host response, rather than direct viral toxic effects, is largely responsible for the observed endothelial injury.

Materials and Methods

Animals

Male New Zealand White rabbits (2 to 2.5 kg) were maintained on a normal diet. Anesthesia was induced using ketamine (Ketastat, 60 mg/kg, Bristol Laboratories) and xylazine (Anased, 6 mg/kg, Lloyd Laboratories) subcutaneously. All animal care and procedures were approved by Duke University Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 80-23, revised 1985).

Preparation of Adenovirus Vector

We generated a recombinant adenovirus, Ad.β-Gal, derived from the in340 mutant strain of Ad5, containing a nuclear-targeted β-Gal transgene and expression cassette in the E1 region, as previously described.15 Virus was purified from infected 293 cells by lysis in virus storage buffer (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 5 mmol/L KCl, and 1 mmol/L MgCl2), followed by ultracentrifugation of the lysate on a 1.3 to 1.4 g/mL cesium chloride step gradient at 100 000g for 2.5 hours at 4°C. Virus was harvested, and residual cellular RNA was removed by incubation with RNase A (100 μg/mL, Sigma Chemical Co) for 30 minutes at room temperature. Pooled virus was ultracentrifuged overnight on a second cesium chloride gradient at 4°C. Pure virus was harvested and desalted by serial gel filtration on Sepharose CL-6B spin columns (Pharmacia) in virus storage buffer. Gel-filtered virus was diluted 1:1 with 80% normal rabbit serum/20% glycerol (virus storage medium) and immediately frozen in aliquots. Viral concentration was initially estimated spectrophotometrically by absorbance at 260 nm, and infectious titer of all viral stocks was determined by at least 3 independent plaque assays on 293 cells using standard techniques (1-hour infection at 37°C, without rocking).16

Screening of Preparations to Exclude Wild-Type Adenovirus Contamination

Wild-type contamination was shown to be <1 in 107 by PCR analysis, using primers designed to amplify a 560-bp product corresponding to Ad5 base pairs 690 to 1250 (sense primer, 5′-ACGAACGTATGATTAGACGC-3′ [Ad5 base pairs 690 to 711]; antisense primer, 5′-AGGCTCATGTTACAGACAGG-3′ [Ad5 base pairs 1250 to 1229]). As a positive control, serial 10-fold dilutions of Ad5 adenovirus DNA, ranging from 1 ng to 1 fg (106 to 1 copy of the in340 adenovirus genome) were used as template in the PCR. A single copy of Ad5 DNA produced a clearly visible 560-bp band. No recombinant preparations produced a positive PCR result, unless >104 copies of the recombinant adenovirus DNA were present in the PCR. In addition, no recombinant preparation produced cell death on cultured human vascular smooth muscle cells, despite high multiplicity of infection levels (≥100 pfu/cell) and observation periods of up to 10 days. In contrast, Ad5 preparations rapidly induced cell death in human vascular smooth muscle cells (within 3 days at a multiplicity of infection of 1).

Carotid Artery Gene Transfer

Rabbits underwent gene transfer to both carotid arteries in an identical manner. High-titer viral stock, maintained on dry ice until immediately before use, was thawed and diluted with DMEM/virus storage medium to ensure equal composition of virus solutions at different viral titers. Mock infections were carried out using DMEM/virus storage medium alone. At surgery, the right and left carotid arteries were carefully exposed through a midline neck incision. Heparin (700 IU IV) was administered, and gene transfer was performed by isolating the exposed vessel segment between microvascular clamps. A 24-gauge polytetrafluoroethylene (Teflon) cannula was inserted through a proximal arteriotomy, and the lumen of the vessel was cleared of blood by gentle flushing with DMEM. Approximately 200 μL of virus solution was instilled into the vessel lumen and incubated for 20 minutes. The arteriotomy was then repaired using 10/0 nylon sutures.

Induction of Neutropenia With Vinblastine

To induce neutropenia, animals received intravenous vinblastine according to previous protocols.17,18 Two doses of 1.5 mg vinblastine were given: 3 days before and, again, 1 day before surgery and gene transfer. A peripheral blood sample was taken immediately before each dose, on the day of surgery, and at the harvest of the vessels to ensure that profound neutropenia had been induced by the time of surgery and had been maintained throughout the experimental period. Peripheral blood counts were determined by Coulter counter, and absolute counts of neutrophils and lymphocytes were also determined by manual counting of blood smears. During vinblastine treatment, animals received a single daily dose of enrofloxacin (Baytril, 15 mg, Miles Inc) subcutaneously.

Vessel Harvesting and Analysis

Vessels were harvested at time points from 1 hour to 72 hours after surgery. Animals were anesthetized and heparinized (700 IU IV), and the carotid arteries were dissected free. Animals were killed with an intravenous overdose of pentobarbital sodium, and vessels were excised and washed in PBS. At the same time, ex vivo vessel rings were removed from the incubator and processed in parallel. Segments from all vessels were immediately frozen at −80°C for protein extraction or were rapidly processed for frozen sections and immunohistochemistry.

β-Gal and total protein concentration was quantified in frozen vessel segments using an ELISA kit (5 Prime-3 Prime) and a Bradford protein assay. β-Gal protein quantity was determined as nanograms β-Gal per milligram of vessel protein.

Immunohistochemistry and Image Analysis

Vessel segments were briefly equilibrated in 30% sucrose in PBS at 4°C, embedded in optimal cutting temperature compound (Miles Scientific), frozen in liquid nitrogen, and sectioned (6 μm) onto
silane-coated glass microscope slides. Vessel sections were thawed, dried, and then stained for β-Gal by incubation at room temperature in X-Gal solution for 4 hours (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L MgCl2 containing 0.5 mg/mL X-Gal). Immunohistochemistry to identify CD18 leukocytes, T lymphocytes, or macrophages was performed using primary antibodies directed against rabbit CD18 (Serotec), rabbit CD43 (T11/135, Serotec), or rabbit RAM 11 (Dako), respectively. VCAM-1 and ICAM-1 were identified using monoclonal antibodies raised against rabbit VCAM-1 and ICAM-1β (a generous gift of Dr M. Cybulsky, University of Toronto). Immunostaining of smooth muscle cells (HHF 35, Dako) and endothelial cells (anti- von Willebrand factor, Atlantic Antibodies) was also performed. Briefly, frozen sections were fixed for 10 minutes in cold acetone and then equilibrated in PBS. Blocking solution (1.5% horse serum in PBS) was applied for 1 hour at room temperature. Antibodies were diluted in blocking solution at concentrations determined in preliminary experiments and were applied to tissue sections for 1 hour. This was followed by sequential incubation with biotinylated anti-mouse IgG (except for von Willebrand factor staining) and ABC reagent (Vectorstain ABC kit, Vector Laboratories, Inc). Immune complexes were localized using the chromogenic alkaline phosphate substrate Vector Red (Vector Laboratories, Inc). The sections were lightly counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific). In all experiments an adjacent section was incubated with an irrelevant murine IgG monoclonal antibody to serve as a negative control. Staining intensity was quantified using an image analysis system (Olympus IX70 inverted microscope, Optronics DEI-750 image capturing hardware, PowerTowerPro 180 CPU). Images were captured using Adobe Premiere and quantified using NIH Image 1.61 software.

Endothelial inflammatory cell infiltration was assessed by counting the total number of CD18+ or CD43+ leukocytes on the luminal surface of arterial cross sections. Counting was performed at ×200 magnification, in a blinded fashion, using a pointing device and a manual counter. The mean number of luminal inflammatory cells was calculated from counting multiple vessel cross sections (range, 4 to 20; median, 8 sections/vessel).

Vasomotor Studies

Freshly harvested vessels were cleaned of fat and connective tissues, cut into helical strips, and mounted in 30-mL organ baths containing Krebs-Henseleit buffer (mmol/L: NaCl 120, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4, NaHCO3, and glucose 5.5 mmol/L; pH 7.4) maintained at 37°C and oxygenated with 95% O2/5% CO2. Vessels were cut into helical strips, and mounted in 30-mL organ baths containing Krebs-Henseleit buffer (mmol/L: NaCl 120, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4, NaHCO3, and glucose 5.5 mmol/L; pH 7.4) maintained at 37°C and oxygenated with 95% O2/5% CO2. Vessels were equilibrated for 60 minutes, with changes of bathing fluid every 15 minutes. Isometric tension studies were performed using a Grass model 7D polygraph. Optimal resting tension was determined in baseline studies, and the response to vasoactive drugs was then determined. Cumulative dose-response curves to PE (10^-4 to 10^-8 mol/L) were established. The vessels were then submaximally precontracted with PE (typically 3×10^-8 mol/L), and endothelial function was evaluated by vascular relaxation to ACh (10^-5 to 10^-7 mol/L). NO mediation of ACh responses was confirmed by blocking ACh-induced relaxation by N-methyl-l-arginine (1 mmol/L), a specific and competitive inhibitor of NO synthase. After they were washed, the vessels were again precontracted, and endothelium-independent relaxation responses to SNP (10^-4 to 10^-8 mol/L) were determined. Contractile responses were measured from the polygraph chart and expressed as a percentage of the maximal contraction or, for relaxations, as a percentage of the precontracted tension. Statistical significance of the differences between responses was assessed by ANOVA.

Results

Host Factors Limit Adenovirus-Mediated Transgene Expression In Vivo

We measured β-Gal transgene expression as a function of infecting titer and compared expression between (1) “in vivo arteries,” ie, carotid arteries left in situ after gene transfer and culture ex vivo for 3 days. A total of 19 carotid artery pairs were studied. They were infected with titers ranging from 4×10^8 to 5×10^11 pfu/mL (Figure 1).

The in vivo arteries showed a striking plateau in transgene expression. β-Gal protein was detectable at the lowest titer evaluated and rose to near-maximal levels at a titer of 1.6×10^9 pfu/mL. However, β-Gal levels showed no significant increase from 2×10^8 through 1×10^11 pfu/mL, and a further increase in titer (to 5×10^11 pfu/mL) significantly reduced β-Gal expression. In ex vivo arteries, transgene expression was higher than in the paired contralateral in vivo arteries at all titers studied. In addition, expression in ex vivo arteries increased steadily as infecting titers were increased from 1.6×10^8 to 2×10^10 pfu/mL. Thus, the gap between ex vivo and in vivo expression became progressively larger in this titer range (reaching a 6-fold difference at 2×10^10 pfu/mL). This suggested that some host factor(s) may be limiting transgene expression in vivo.

Adenovirus-Mediated Gene Transfer Provokes Time- and Titer-Dependent Endothelial Activation

We examined arteries for evidence of endothelial activation and acute inflammation after from 1 to 72 hours after infection with recombinant adenovirus. Cryosections were evaluated by immunohistochemistry to identify VCAM-1, ICAM-1, CD18+, and CD43+ leukocytes (T lymphocytes). CD18+ leukocytes were identified as PMNs, as they had multilobed nuclei and did not stain with RAM 11, which would identify CD18+ monocytes.

Figure 1. Adenovirus-mediated transgene expression in carotid arteries in vivo is greatly reduced compared with matched ex vivo vessels. Both rabbit carotid arteries were infected in an identical manner with increasing titers of a replication-deficient adenovirus encoding β-Gal. One artery was then left in vivo; the contralateral artery was excised and incubated ex vivo. β-Gal expression was determined 72 hours later by ELISA. Bars show the mean of determinations from arteries from at least 2 range, (2 to 6) animals for each titer studied. Difference between in vivo and ex vivo expression was statistically significant (P<0.05 at 1.6×10^9 pfu/mL; P<0.01 at all titers above 1.6×10^9 pfu/mL).
ICAM-1 was detectable at 6 hours after adenoviral infection in both the adventitia and endothelium (Figure 2). ICAM-1 immunostaining increased through 24 hours and remained at high levels at 72 hours. VCAM-1 was detectable as early as 1 hour and, like ICAM-1, increased through 24 hours and remained at high levels at 72 hours. However, VCAM-1 staining was confined to the endothelium. Mock-infected arteries showed no endothelial ICAM-1 or VCAM-1 expression (Figure 2, bottom panels), nor did arteries infected with low-titer adenovirus (1.6×10^9 pfu/mL, not shown).

Thus, high-titer recombinant adenoviral gene transfer induces endothelial activation very soon after infection.

The degree of endothelial activation was also titer dependent, as judged by immunohistochemistry combined with image analysis (Figure 3). When the infectious titer was held below 4×10^9 pfu/mL, neither ICAM-1 nor VCAM-1 showed significantly increased expression above that observed in sham-infected arteries. However, as titers met or exceeded that threshold, there was a steady increase in endothelial expression of both adhesion molecules. We observed a sigmoidal relationship between the log of infectious titer used and relative staining intensity, with the steepest increase in staining intensity occurring between 4×10^9 and 2×10^10 pfu/mL.

We next compared the degree of inflammation in mock-infected vessels and in low-titer (1.6×10^9 pfu/mL) and moderate-titer (2×10^10 pfu/mL) adenovirus-infected arteries. Arteries were harvested 72 hours after gene transfer, then cryosectioned, and (1) stained with X-Gal to demonstrate β-Gal–expressing cells, followed by (2) immunostaining for CD18 (Figure 4, all panels). We observed the following: (1) Mock-infected arteries showed no β-Gal–positive nuclei

![Figure 2. Time dependence of ICAM-1 and VCAM-1 expression. Rabbit carotid arteries were infected in vivo with a replication-deficient adenovirus encoding β-Gal (1×10^11 pfu/mL) or underwent mock infection and were harvested at time points 0, 1, 6, 12, or 24 hours later. Immunohistochemistry on vessel cryosections was used to identify ICAM-1 or VCAM-1 expression. Representative serial sections are shown for each time point.](image-url)
whereas arteries infected with moderate-titer adenovirus showed approximately equal amounts of transduced endothelial cells ($\approx 80\%$ endothelial cell nuclei positive for $\beta$-Gal). (2) In mock-infected arteries (Figure 4A) and low-titer–infected arteries (Figure 4C), endothelial inflammatory cells were observed only occasionally. (3) In contrast, vessels infected with moderate-titer adenovirus (Figure 4B) showed extensive endothelial infiltration by PMNs. Thus, low-titer– and moderate-titer–infected vessels differed primarily in the degree of endothelial inflammatory cell infiltrate and were not distinguishable in terms of transgene expression.

To quantify further the extent of inflammation, we counted endothelium-adherent CD18$^+$ and CD43$^+$ (pan T-lymphocyte) cells in cryosections (Figure 5). As was observed for endothelial activation, the degree of inflammatory infiltrate showed both time and titer dependence. A significant inflammatory infiltrate developed as early as 6 hours after high-titer adenovirus infection ($1\times10^{11}$ pfu/mL) and progressed to near-maximal levels by 12 hours after gene transfer (Figure 5, left). Next, we examined the effect of viral titer on inflammation at 72 hours after infection. At or below $1.6\times10^9$ pfu/mL, the number of endothelial inflammatory cells was not increased relative to mock-infected vessels (Figure 5, right). However, at $\geq 4\times10^9$ pfu/mL, the inflammatory infiltrate progressively increased. Numbers of PMNs continued to increase with titers up to $5\times10^{11}$ pfu/mL, whereas CD43$^+$ T lymphocytes were less numerous than PMNs and did not show progressive increase with viral titer.

To determine whether $\beta$-Gal transgene expression was contributing to the observed inflammation, we counted endothelium-adherent CD18$^+$ and CD43$^+$ (pan T-lymphocyte) cells in cryosections (Figure 5). As was observed for endothelial activation, the degree of inflammatory infiltrate showed both time and titer dependence. A significant inflammatory infiltrate developed as early as 6 hours after high-titer adenovirus infection ($1\times10^{11}$ pfu/mL) and progressed to near-maximal levels by 12 hours after gene transfer (Figure 5, left). Next, we examined the effect of viral titer on inflammation at 72 hours after infection. At or below $1.6\times10^9$ pfu/mL, the number of endothelial inflammatory cells was not increased relative to mock-infected vessels (Figure 5, right). However, at $\geq 4\times10^9$ pfu/mL, the inflammatory infiltrate progressively increased. Numbers of PMNs continued to increase with titers up to $5\times10^{11}$ pfu/mL, whereas CD43$^+$ T lymphocytes were less numerous than PMNs and did not show progressive increase with viral titer.

Adenovirus-Mediated Vascular Gene Transfer Results in Functional Endothelial Injury

To assess the functional impact of adenovirus-mediated gene transfer, we excised rabbit carotid arteries 3 days after gene transfer and evaluated their response to vasoactive agonists. Regardless of the adenovirus titer received, all vessels showed essentially identical contraction in response to PE (Figure 6A). Similarly, all vessels relaxed in response to treatment with the NO donor SNP (Figure 6C). These observations show that adenoviral gene transfer did not harm the contraction and relaxation responses of vascular smooth muscle.
Arteries stripped of endothelium showed minimal relaxation in response to ACh. Relaxations by balloon denudation and then allowed to recover for 3 days observed functional injury. Arteries stripped of endothelium progressively impaired relaxation responses to ACh (maximal degree of vasomotor impairment as did high-dose Ad.-Gal, showing that -Gal expression itself did not cause the observed functional injury. Arteries stripped of endothelium by balloon denudation and then allowed to recover for 3 days showed minimal relaxation in response to ACh. Relaxations to ACh were inhibited by N-methyl-L-arginine, thus confirming their dependence on NO production (data not shown).

We also evaluated the time course of vasomotor function at 6, 12, 24, and 72 hours after infection with 1 \times 10^{11} pfu/mL. ACh-induced relaxation was significantly impaired as early as 6 hours after infection, with further injury progressing through 72 hours (Figure 7). There was no significant impact on either PE-induced contraction or SNP-induced relaxation at any time after gene transfer (data not shown). Thus, adenovirus induces time- and titer-dependent endothelial dysfunction but does not impair vascular smooth muscle function.

**Neutrophil Inflammation Mediates Functional Endothelial Injury**

We hypothesized that inflammatory cell infiltration may play a role in the pathogenesis of adenovirus-related endothelial injury. To investigate this possibility, we induced neutropenia in rabbits undergoing carotid artery gene transfer. Four rabbits received vinblastine (1.5 mg IV) on days 3 and 1 before surgery. All animals survived until the designated time of harvest without evidence of infection. At surgery, all
Vinblastine-treated rabbits were profoundly neutropenic (15-fold reduction compared with pretreatment) and remained so at the time of harvest (Table 2). Peripheral blood lymphocyte and platelet counts were modestly reduced at surgery (2-fold and 2.5-fold, respectively), with further reductions noted by the time of harvest.

Vinblastine pretreatment had no apparent effect on either VCAM-1 or ICAM-1 expression after gene transfer; arteries from vinblastine-treated animals still mounted a vigorous adhesion molecule response after adenoviral gene transfer (Figure 8A and 8B; compare with Figure 2 at 24 hours). However, vinblastine pretreatment strikingly reduced endothelial inflammatory cell infiltration (Figure 8C and 8D; compare with Figure 4B). Endothelium-adherent PMNs were reduced by 25-fold (Figure 9A; P < 0.01), whereas endothelium-adherent T-lymphocytes were reduced by 2-fold (P = NS) 3 days after adenoviral gene transfer. Thus, vinblastine treatment did not affect endothelial cell activation and only modestly reduced T-lymphocyte infiltration but virtually abrogated endothelial PMN infiltration in infected arteries.

We next sought to determine whether the marked reduction in endothelial PMNs favorably affected endothelial vasoconstrictor function after adenoviral gene transfer. We examined vasmotor function in arteries from 4 groups of rabbits: (1) no vinblastine, mock-infected; (2) no vinblastine, high-titer gene transfer (1 × 10^11 pfu/mL); (3) vinblastine-treated, mock-infected; and (4) vinblastine-treated, high titer gene transfer. Vinblastine treatment did not alter sensitivity to PE and modestly reduced sensitivity to SNP, reflecting a slight reduction in

### Table 2. Induction of Neutropenia With Vinblastine

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Surgery</th>
<th>Harvest</th>
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<td>Hemoglobin, g/dL</td>
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<td>12.23±1.29</td>
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<td>Platelets, cells/mm³</td>
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<td>164±56*</td>
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<tr>
<td>Total leukocytes, cells/mm³</td>
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<td>2000±880*</td>
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<tr>
<td>Neutrophils, cells/mm³</td>
<td>2853±1670</td>
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<td>Lymphocytes, cells/mm³</td>
<td>4000±190</td>
<td>1796±664*</td>
</tr>
</tbody>
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*P < 0.05 vs pretreatment counts.

Shown are the peripheral blood hemoglobin concentrations, platelet counts, and counts for total leukocytes, neutrophils, and lymphocytes before vinblastine pretreatment, on the day of surgery, and at the time of vessel harvest. Values are mean±SD (n=4 animals).

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Vinblastine-induced neutropenia abolishes endothelial inflammatory cell infiltration. Rabbits were rendered neutropenic by vinblastine pretreatment, and carotid arteries were infected with Adβ-Gal (1×10^11 pfu/mL). Vessels were harvested 72 hours later, and serial tissue sections were immunostained for the adhesion molecules VCAM-1 (A) or ICAM-1 (B) or for CD18⁺ or CD43⁺ inflammatory cells (C and D, respectively). Counterstaining with X-Gal revealed blue nuclei in transduced endothelial cells expressing β-Gal (outlined arrows).
**Figure 9.** Cellular inflammation mediates functional endothelial injury after adenoviral vascular gene transfer. Rabbits were rendered neutropenic using vinblastine and then underwent vascular gene transfer or mock infection. A, The numbers of CD18+ leukocytes (PMNs) and CD43+ leukocytes (T cells) were assessed by immunohistochemistry on vessel cryosections from vinblastine-treated and untreated animals. Bars represent SD. **P<0.01 for PMN counts on vessels from treated vs untreated rabbits. Bars represent SD.**

Discussion

The present study shows that adenoviral gene transfer acutely induces endothelial activation, inflammatory cell infiltration, and endothelial dysfunction. All of these effects are directly related to infectious titer, and all become apparent within 6 hours of gene transfer. Although superficially discouraging, these findings are tempered considerably by the existence of a therapeutic window—a range of infectious titers that induce near-maximal transgene expression but little or no acute vascular injury. In our hands, this window lies roughly between 1 and $4 \times 10^9$ pfu/mL. Previously published therapeutic window estimates, based on expression data and morphological observations (ie, abrupt loss of transgene expression and endothelial denudation after very high-titer gene transfer), were substantially higher; our downward revision is made possible by more detailed immunohistochemistry and studies of vasomotor function. These approaches appear to be substantially more sensitive than morphology alone as indicators of acute vascular injury.

The precise boundaries of the therapeutic window will be affected by a number of variables. For example, our boundaries apply to rabbit carotids exposed to virus for 15 minutes, but they may be quite different, for example, for other species, shorter or longer exposures, and different organ systems. Moreover, the titers themselves may vary considerably between laboratories. Thus, therapeutic window boundaries must be determined on a case-by-case basis.

We show that adenovirus-induced inflammation is well developed as early as 6 hours after vascular gene transfer. This onset is too soon to attribute to 2 key adenovirus life cycle events: adenoviral viral DNA replication and late antigen expression.\(^1\) This has important implications for vascular gene transfer with newer adenoviral vectors, which are designed to reduce or eliminate viral replication and antigen expression.\(^2\) Our data suggest that acute vascular injury may be primarily determined by events surrounding the infection itself. If so, second-generation vectors will continue to induce titerto-dependent acute inflammation, and strict attention to the therapeutic window will continue to be important.

Reduced ACh-induced vascular relaxation reflects loss of endothelial NO production,\(^3\) and that, in turn, may facilitate the long-term chronic inflammation and vascular injury that is known to occur in this model.\(^2\) NO and related molecules reduce the expression of adhesion molecules and proinflammatory cytokines,\(^25\) inhibit platelet aggregation and adhesion,\(^27\) and directly inhibit vascular smooth muscle cell proliferation\(^29\) and migration.\(^30\) Thus, the detrimental effects of early endothelial injury may extend beyond acute loss of ACh-induced relaxation and may contribute to the long-term vascular injury induced by recombinant adenovirus.

The observed vasomotor impairment has a number of striking parallels to the inflammatory cell infiltrate in terms of temporal development, dependence on titer, and spatial distribution (ie, both inflammation and vasomotor impairment affect the endothelium and spare the vascular media). These tight parallels suggest the possibility of a causal relationship. Our results with cytopenic animals further support this hypothesis: vinblastine-induced cytopenia (1) virtually eliminated endothelial cellular infiltration and (2) prevented the loss of ACh-induced vascular relaxation that otherwise occurs after exposure to high-titer adenovirus. Moreover, it is noteworthy that vinblastine treatment had no discernible effect on endothelial ICAM-1 or VCAM-1 expression. This result shows that vasomotor impairment requires additional events beyond those necessary for endothelial activation. Again, this would implicate cellular infiltration as playing a causal role.

Our observations regarding vasomotor function contrast sharply with those of Lafont et al.\(^10\) They described complete loss of endothelium-dependent relaxation and substantial loss of PE-induced contraction in rabbit ear and femoral arteries 3
days after adenoviral gene transfer at $4 \times 10^{10}$ pfu/mL. In contrast, our carotid artery data suggest that adenoviral gene transfer does not affect vascular smooth muscle function at any titer and does not harm endothelial function if one uses titers within the therapeutic window. Finally, our data show that vasomotor dysfunction induced by high-titer adenovirus can be prevented by prior treatment with vinblastine. Vinblastine treatment induced neutropenia, lymphopenia, and thrombocytopenia. Any of these cytopenias may play an important role in reducing endothelial injury after gene transfer. The fact that PMNs were the most affected by vinblastine treatment, both in terms of reduced peripheral counts and reduced endothelial infiltration, suggests that neutrophils have the most significant role. Certainly, their ability to release proinflammatory mediators (eg, superoxide, tumor necrosis factor-α, and interleukin-1β [3]) and to generate tissue injury lends support for this view. However, a more targeted experimental approach is necessary to establish definitively whether PMNs are the agents of functional endothelial injury in this model.

Important questions remain to be answered about the pathogenesis of acute effects. What aspects of recombinant adenovirus infection trigger endothelial activation? Does the initial infection represent the sole stimulus for subsequent inflammatory events, or do viral DNA replication or protein expression contribute to the progression of injury observed from 12 to 72 hours? We show that neutrophils may mediate endothelial injury, but through what mechanisms are the neutrophils recruited and activated? Vascular selectins, C5a, interleukin-8, and platelet-activating factor all mediate acute inflammation in other models, and recent studies show that wild-type and recombinant adenoviruses rapidly upregulate interleukin-8 expression in cultured cells. Any of these mediators may be involved in adenovirus-induced acute inflammation in vivo. Clarification of these mechanisms may have important implications for vascular gene therapy.

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References


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