Vascular Endothelium Has a Local Anti-Adenovirus Vector System and Glucocorticoid Optimizes Its Gene Transduction

Takahisa Murata, Masatoshi Hori, Sheng Lee, Akio Nakamura, Kazuhiro Kohama, Hideaki Karaki, Hiroshi Ozaki

Objective—Although adenovirus is a powerful tool for vascular research and therapy, endothelial impairment after infection has been reported. We investigated the mechanisms of this impairment and the effect of dexamethasone (DEX) on gene transfer into the vascular endothelial cells.

Methods and Results—β-Galactosidase gene encoding adenovirus vector (β-gal-Ad) (7.5×10⁶ plaque-forming units/mL) transduced β-gal into the rabbit organ–cultured pulmonary endothelium, followed by an apoptosis and an impairment of endothelium-dependent relaxation (EDR). Endothelial cell infected by β-gal-Ad expressed proinflammatory genes mRNAs and suppressed endothelial nitric oxide synthase (eNOS) mRNA. Treatment with DEX dramatically increased β-gal protein expression in the endothelium, attenuated β-gal-Ad–induced apoptosis, and prevented the impairment of EDR. DEX also suppressed the mRNAs expressions of proinflammatory genes and recovered eNOS mRNA expression in organ-cultured vascular endothelium. In addition, we confirmed the DEX’s beneficial effects in an endothelial cell line (in vitro) and rat femoral artery (in vivo) experiments.

Conclusion—These results suggest that adenovirus vector induces host-immune responses and apoptosis in vascular endothelial cells. DEX is found to be a useful and potent tool to prevent the Ad-induced impairments of the endothelium and to optimize gene expression efficiency by adenovirus vector at the protein translation level in both in vitro and in vivo experiments. (Arterioscler Thromb Vasc Biol. 2005;25:1796-1803.)

Key Words: adenovirus ■ apoptosis ■ endothelial cells ■ gene therapy ■ inflammation

Vascular endothelium plays important roles in local regulation of vascular tone and smooth muscle cell proliferation via the release of vasoactive products such as nitric oxide (NO). Impairment of endothelium has long been considered to be a pathogenic mechanism of various vascular diseases, such as arteriosclerosis.¹

A number of methods for delivering recombinant genetic material to vascular endothelial cells have been investigated in vitro and in vivo.²⁻⁴ Because they allow an effective transient gene expression in proliferating and nonproliferating cells, first-generation (E1/E3-deleted) adenovirus (Ad) vectors have become the most popular choice for use in arterial gene therapy and basic vascular experiments. However, in use of the vascular wall, high-titer E1/E3-deleted Ad vector infection has a number of disadvantages, such as the production of inflammatory reactions, resulting in transient recombinant protein expression, significant endothelial injury, and neointimal proliferation.⁵⁻⁷ These disadvantages seriously hamper its clinical application, and researchers have been struggling to alter the vector genome to reduce its toxicity.⁸⁻⁹

Several studies have demonstrated that these disadvantages are largely attributable to Ad-induced systemic cellular and humoral immune responses.⁷,¹⁰ However, the mechanism of direct adenoviral toxicity to endothelial cells remains to be clarified in detail. Further investigation and modification of the direct toxicity to vascular endothelial cells, along with optimization of gene expression efficiency, will be needed to advance the efficiency of arterial gene therapy.

In this study, we hypothesized that the vascular endothelium may exert a specific antiviral immuno-activity that results in endothelial impairment and low efficiency of transgene expression. To examine this possibility, we focused on the direct effects of Ad vector on the vascular endothelium using organ-cultured pulmonary arteries. In addition, we investigated the effects of 2 respective immunosuppression regimens, dexamethasone (DEX) and cyclosporine A (CSA), on gene transfer into the vascular endothelium to evaluate the usefulness of the Ad vector in gene therapy for vascular diseases. The organ culture method makes it possible to dissociate the influence of systemic cellular and humoral immune responses so that the direct effect of the Ad vector on...
the vascular endothelium can be investigated, and to easily examine
the tissue for both morphological and functional changes. In this
study, based on results from the organ culture procedure, we applied
the technique to in vitro and in vivo gene transfer into endothelium.

Materials and Methods
Adenovirus Construction and Purification
We made β-galactosidase gene-encoding Ad vector (β-gal-Ad) as
previously described. Briefly, a nuclear-targeted bacterial β-gal
transgene fragment was inserted into a cosmid vector pAXCawt with
CAG promoter (Takara Shuzo, Japan) derived from adenovirus
subtype 5 with the E1 and E3 regions deleted at the Swal site.
Recombinant adenoviruses containing the insert were constructed by
the COS/TPC (terminal protein complex) method using an adenovi-
rus expression vector kit (Takara Shuzo, Japan). The concentration
of β-gal-Ad in the stock was 3 × 10^10 plaque forming units (PFU)/
ml, as determined by plaque titration on 293 cells. An Ad vector
(5.3 × 10^10 PFU/ml) (Takara Shuzo, Japan) without a transgene
was used as a control vector.

Organ Culture Procedure and Gene Transfer
Animal care and treatment were conducted in conformity with the
institutional guidelines of the University of Tokyo. Male Japanese
White rabbits (2 to 2.5 kg) were euthanized. The organ culture
procedure was performed as described previously. In brief, the
main branches of the intrapulmonary arteries were isolated. Each
artery was cut into rings with ~1.3 to 1.4 mm diameter and 1.3 to
1.4 mm width. Each arterial ring was then placed in 100 μL of
DMEM and exposed to the respective adenoviral vectors in 1.5
cm^2) HUVECs at passage 31 to 34 showing cobblestone-like
morphology in a well plate as previously reported. Subconfluent
(1.8 × 10^6 cells/cm^2) HUVECs at passage 31 to 34 showing cobblestone-like
morphology were used for the experiments. Cells were cultured in
1% fetal bovine serum DMEM in the presence or absence of 0.3 to
1 μmol/L DEX (Sigma) or 1 to 10 μmol/L CSA (Wako, Japan) for 2
hours (infection period). After infection, the media containing the
viruses were removed. The arterial rings were rinsed 3 times with
fresh DMEM and incubated in 100 μL DMEM for a period between
6 hours and 21 days in the presence or absence of these compounds
(expression period) to induce the transfection β-gal protein. The
incubators were maintained at 37°C under an atmosphere of 95% air
and 5% CO2.

Cell Culture Procedure and Gene Transfer
The human umbilical vein endothelial cell (HUVEC) line was
purchased from the American Type Culture Collection and cultured
in 1 mL DMEM containing 10% fetal bovine serum using a 20-mm
well plate as previously reported. Subconfluent (1.8 × 10^6 cells/
cm^2) HUVECs at passage 31 to 34 showing cobblestone-like
morphology were used for the experiments. Cells were cultured in
1% fetal bovine serum DMEM in the presence or absence of 0.3 to
3 μmol/L DEX 24 hours before the 3 × 10^6 to 3 × 10^7 PFU/mL Ad
infection and during the infection period (2 hours) and the expression
period (48 hours).

In Vivo Gene Transfer
Six hours before the surgery, 0.1 mg/kg DEX-containing physio-
logical salt solution or DEX-free physiological salt solution was injected
into Sprague-Dawley rabbits (280 to 310 grams) subcutaneously. Rats
were anesthetized by pentobarbital (40 mg/kg), and the 1-cm
segments of the right (for mock infection) and left (for Ad infection)
femoral arteries were surgically exposed. For each artery, 20 μL of
3 × 10^7 to 3 × 10^9 PFU/mL Ad containing DMEM or Ad-free DMEM
was infected for 30 minutes. After the infection, 0.1 mg/kg DEX was
infected subcutaneously every 24 hours. Two days after the infection,
the femoral arteries were used for each experiment.

Vasomotor Studies
After the expression period, vascular muscle tension was recorded by
a method described elsewhere. In brief, muscle tension was
recorded isometrically with a force-displacement transducer under a
resting tension of 10 mN. Data are shown as the percent relaxation
of the steady-state preconstriction.

Historical Methods: β-Gal Staining
After the expression period, arterial rings or HUVECs were rinsed 3
times with phosphate-buffered saline and fixed with 4% formalde-
hyde. Subsequently, arterial rings or HUVECs were stained for β-gal
by incubation at room temperature in X-gal solution, and then the
ring was opened. Images were captured using a light microscope, and
the number of β-gal–positive endothelial cells was counted.

Cross-Section Hematoxylin and Eosin Staining
and Terminal Deoxynucleotidyl Transferase-Mediated
DUTP Nick End-Labeling Staining
After the β-gal staining, arterial rings were embedded in paraffin.
The 4-μm-thick sections were stained with hematoxylin and eosin.
For the detection of apoptosis, the transfection-mediated dUTP nick
end-labeling (TUNEL) method was applied to the sections using an
In Situ Apoposis Detection Kit-POD (Roche Diagnostics, Japan).
For visualization, the sections were treated with 0.05% DAB solution
with hematoxylin and eosin counterstaining.

Whole-Mount Immunostaining
and TUNEL Staining
Whole-mount immunostaining was performed as previously de-
scribed. After the fixation, arteries were probed with anti-CD31
monoclonal antibody (1:10 dilution) (Dako, Denmark). In the other
experiment for the detection of apoptosis, fixed arteries were
subjected to the TUNEL method using fluorescein isothiocyanate
(FITC)-labeled dUTP. The images were captured using a confocal
laser scanning microscope LSM510 imaging system (Carl Zeiss,
Germany).

Semi-Quantitative Reverse-Transcription
Polymerase Chain Reaction
After the incubation, the endothelial cells were scraped, and total
RNA was extracted from the endothelial cells. Reverse-transcription
polymerase chain reaction (RT-PCR) was performed as described
previously. In some target genes, we performed partial sequence
cloning (accession numbers in DDBJ; GAPDH; AB128158, endo-
theilial nitric oxide synthase [eNOS]; AB128159, IL-1β; AB128152,
tumor necrosis factor [TNF]-α; AB128153, vascular cell adhesion
molecule [VCAM]-1; AB128156, intercellular adhesion molecule-1
[ICAM]-1; AB128157, interferon [IFN]-α; AB128154). The oligo-
nucleotide primers were designed, as follows: GAPDH: sense,
TCCCTCAAGATGTTCAGCA; antisense, AGATCCACAGCGA-
TACATT; eNOS: sense, ATAGAATTCACCAGCTTTGG-
GAATGCGGAT; antisense, ATAGAATTCGGCGATCTCCT-
GTTGTTCTGGACTCCTT; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG; antisense, TCTCATGATTTG-
CTGCCTGCC; IL-1β: sense, CGTCTCCGTG-
TGATGAAAG; antisense, CAGGAGAGCAGGATGATC;
TNF-α: sense, ATGGTCCACCTGATCAGGATAGG; antisense,
GGAGACTGAGAATGGCTTGG;
The Effects of DEX and CSA on Transgene Expression in Organ-Cultured Pulmonary Arterial Endothelium

In the pulmonary artery organ infected with 7.5 × 10^4 PFU/mL β-gal-Ad for 2 hours, β-gal–positive cells were observed in the endothelium (Figure 1A). As expected, the predominant staining was intranuclear, and a nuclear dominant blue staining was not seen in null-Ad–infected arteries (n = 4, data not shown). We first sought to assess the effect of virus-titer on Ad gene transfer in the pulmonary endothelium. In the pulmonary arteries infected with β-gal-Ad at various concentrations (1.5 × 10^4 to 1.5 × 10^6 PFU/mL) for 2 hours, β-gal–positive endothelial cells increased in a titer-dependent manner (1.5 × 10^{10} PFU/mL; 108.2 ± 2.1 cells/mm^2, 7.5 × 10^{10} PFU/mL; 199.5 ± 4.3 cells/mm^2, 1.5 × 10^{10} PFU/mL; 470.2 ± 3.1 cells/mm^2; n = 10 each).

We next examined the effect of treatment with the immunosuppressive agents DEX and CSA on β-gal-Ad–mediated gene expression into endothelium (Figure 1). DEX (1 to 10 μg/mL) and CSA (1 to 10 μg/mL) treatments during both the infection and the expression period optimized the β-gal expression efficiency (the maximum increase in efficiency was 3.5-fold and 2.8-fold by 3 μg/mL DEX and 3 μg/mL CSA, respectively; n = 10 each).

In an attempt to further enhance the DEX-induced increase in gene transfer into the endothelium, we investigated the effects of different treatment regimens. Treatment with 3 μg/mL DEX during the β-gal-Ad infection period alone (before DEX treatment; Figure 1C) did not improve β-gal-Ad–mediated gene expression in comparison with pulmonary endothelium infected with 7.5 × 10^4 PFU/mL β-gal-Ad under a DEX-free condition (n = 8; Figure 1C). In response to 3 μg/mL DEX treatment only during the expression period after the infection (after DEX treatment; Figure 1C), optimization of gene expression efficiency was observed similar to that in the DEX-treated endothelium during both the infection and the expression periods (before and after DEX treatment; n = 8 each).

We next conducted an experiment to assess the optimal length of DEX treatment for stable β-gal expression. We infected pulmonary arteries with 7.5 × 10^6 PFU/mL β-gal-Ad for 2 hours in the presence of DEX (during a 2-hour infection and a 48-hour expression period), washed out the medium, and incubated the infected arteries in the DEX-free DMEM for 2 or 3 weeks. In these arteries, β-gal expression was maintained at a high level at both 2 and 3 weeks after infection (2 weeks: 564.3 ± 15.8 μg-gal–positive cells/mm^2; 3 weeks: 540.3 ± 19.2 μg-gal–positive cells/mm^2; n = 8 each).

Endothelial Cell Death and Denudation

Figure 2A shows a paraffin section of the vascular tissues stained first with β-gal and then hematoxylin and eosin, and Figure 2B shows a whole-mount of CD31 (endothelium marker)–immunostained tissues. β-gal–positive cells have blue-stained nuclei. In the nontreated pulmonary artery, no β-gal–positive cells were observed, and the endothelial cells attached tightly to the tunica interna (n = 5) (Figure 2A) and

**Statistical Analysis**

The results of the experiments are expressed as the means ± SEM. Statistical evaluation of the data were performed by analysis of variance (ANOVA), followed by the Tukey post-test for comparison between groups using Prism 3.0. *P* < 0.05 was regarded as statistically significant.

**Results**

The Effects of DEX and CSA on Transgene Expression in Organ-Cultured Pulmonary Arterial Endothelium

In the pulmonary artery organ infected with 7.5 × 10^4 PFU/mL β-gal-Ad for 2 hours, β-gal–positive cells were observed in the endothelium (Figure 1A). As expected, the predominant staining was intranuclear, and a nuclear dominant blue staining was not seen in null-Ad–infected arteries (n = 4, data not shown). We first sought to assess the effect of virus-titer on Ad gene transfer in the pulmonary endothelium. In the pulmonary arteries infected with β-gal-Ad at various concentrations (1.5 × 10^4 to 1.5 × 10^6 PFU/mL) for 2 hours, β-gal–positive endothelial cells increased in a titer-dependent manner (1.5 × 10^{10} PFU/mL; 108.2 ± 2.1 cells/mm^2, 7.5 × 10^{10} PFU/mL; 199.5 ± 4.3 cells/mm^2, 1.5 × 10^{10} PFU/mL; 470.2 ± 3.1 cells/mm^2; n = 10 each).

We next examined the effect of treatment with the immunosuppressive agents DEX and CSA on β-gal-Ad–mediated gene expression into endothelium (Figure 1). DEX (1 to 10 μg/mL) and CSA (1 to 10 μg/mL) treatments during both the infection and the expression period optimized the β-gal expression efficiency (the maximum increase in efficiency was 3.5-fold and 2.8-fold by 3 μg/mL DEX and 3 μg/mL CSA, respectively; n = 10 each).

In an attempt to further enhance the DEX-induced increase in gene transfer into the endothelium, we investigated the effects of different treatment regimens. Treatment with 3 μg/mL DEX during the β-gal-Ad infection period alone (before DEX treatment; Figure 1C) did not improve β-gal-Ad–mediated gene expression in comparison with pulmonary endothelium infected with 7.5 × 10^4 PFU/mL β-gal-Ad under a DEX-free condition (n = 8; Figure 1C). In response to 3 μg/mL DEX treatment only during the expression period after the infection (after DEX treatment; Figure 1C), optimization of gene expression efficiency was observed similar to that in the DEX-treated endothelium during both the infection and the expression periods (before and after DEX treatment; n = 8 each).

We next conducted an experiment to assess the optimal length of DEX treatment for stable β-gal expression. We infected pulmonary arteries with 7.5 × 10^6 PFU/mL β-gal-Ad for 2 hours in the presence of DEX (during a 2-hour infection and a 48-hour expression period), washed out the medium, and incubated the infected arteries in the DEX-free DMEM for 2 or 3 weeks. In these arteries, β-gal expression was maintained at a high level at both 2 and 3 weeks after infection (2 weeks: 564.3 ± 15.8 μg-gal–positive cells/mm^2; 3 weeks: 540.3 ± 19.2 μg-gal–positive cells/mm^2; n = 8 each).
were tightly blocked like cobblestones (n=5) (Figure 2B). In the arteries infected with $7.5 \times 10^8$ PFU/mL β-gal-Ad, in contrast, a few β-gal–positive cells were observed in the endothelium; the excoriation of endothelial cells from the tunica interna was observed in some regions; and apoptosis-like concentrated and rounding endothelial nuclei were often observed (n=5). The $7.5 \times 10^8$ PFU/mL null-Ad–infected pulmonary artery had no β-gal–positive endothelial cells (n=5), but in these arteries, similar morphological endothelial impairments were observed (n=5). In the arteries infected with $7.5 \times 10^8$ PFU/mL β-gal-Ad in the presence of 3 μmol/L DEX during the infection and expression periods, many β-gal–positive cells were observed in the endothelium (Figure 2A). No conspicuous changes, such as excoriation or apoptosis-like changes, were observed (n=5 each). We confirmed by β-gal and CD31 double-staining that the morphology of β-gal–expressing cells was completely intact (n=4; data not shown). In the arteries infected with $7.5 \times 10^8$ PFU/mL β-gal-Ad and treated with 3 μmol/L CSA during the infection and expression periods, in contrast, many β-gal–positive cells were observed in the endothelium (Figure 2A), and excoriation and apoptosis-like changes were often observed (n=5 each).

We also performed a TUNEL assay in paraffin-sectioned and whole-mounted tissues to label apoptosis-induced DNA strand breaks (Figure 3A and 3B, respectively), and counted TUNEL-positive endothelial cells by whole-mount TUNEL assay (Figure 3C). In the nontreated arteries, there were no TUNEL-positive endothelial cells (n=5). In the arteries infected with $7.5 \times 10^8$ PFU/mL β-gal-Ad, many TUNEL-positive endothelial cells were visible (n=5 each). Treatment with 3 μmol/L DEX during both the infection and expression periods...
periods significantly inhibited β-gal-Ad–induced endothelial apoptosis (n=5); 3 μmol/L CSA treatment also inhibited β-gal-Ad–induced endothelial apoptosis significantly, but its inhibitory effect was not pronounced as that of 3 μmol/L DEX (n=5).

To assess the optimal regimen length of DEX treatment for the inhibition of β-gal-Ad (7.5×10^6 PFU/mL)-induced apoptosis, we also investigated the effects of different treatment regimens. Treatment with 3 μmol/L DEX during the β-gal-Ad infection period alone (before DEX treatment) did not inhibit β-gal-Ad–induced endothelial apoptosis (n=5) (Figure 3D). Treatment with 3 μmol/L DEX during the expression period only (after DEX treatment) significantly inhibited Ad-induced apoptosis, but this inhibitory effect was smaller than that by treatment during both the infection and expression periods (before and after treatment) (n=5).

**Impairment of Endothelial-Dependent NO Production**

In the nontreated pulmonary arteries with endothelium, substance P (0.1 to 30 nmol/L) caused vasorelaxation of the muscle contraction elicited by 1 μmol/L prostaglandin F_2α in a concentration-dependent manner (n=5) (Figure 4A). The substance P (100 nmol/L)-induced endothelium-dependent relaxation was abolished by treatment with N^6^-monomethyl-L-arginine, a NOS inhibitor (200 μmol/L, 30 minutes before the addition of prostaglandin F_2α) (n=4 each; data not shown). Taken together, these results indicate that the substance P-induced vasorelaxation of the cultured arteries may be attributable mainly to NO production. In the β-gal-Ad–infected pulmonary arteries, the substance P-induced endothelium-dependent relaxation was almost completely abolished (Figure 4A) (n=5). The 3 μmol/L DEX treatment, but not the 3 μmol/L CSA treatment (during both the infection and expression periods), significantly improved the β-gal-Ad–induced endothelium-dependent relaxation impairment (n=5 each). In all of the arteries, the impairments in smooth muscle contractility and susceptibility to NO were not observed (n=5 each; data not shown).

**Endothelial Inflammation and Antivirus Reaction**

In the pulmonary endothelial cells, the elevation of proinflammatory cytokines, TNF-α, IL-1β, and interferon-α gene expressions were observed from 6 hours after infection, reached a peak at 12 hours after infection, as measured by semiquantitative RT-PCR analysis (n=4; data not shown). Figure 4B shows typical traces of semiquantitative RT-PCR analysis for GAPDH, eNOS, IL-1β, TNF-α, interferon-α, VCAM-1, ICAM-1, and LacZ (β-gal) in the pulmonary endothelial cells at 12 hours after infection with β-gal-Ad (7.5×10^6 PFU/mL) in the presence or absence of 3 μmol/L DEX. In addition, we performed real-time RT-PCR analysis of these molecules for quantification (Figure 4C). The eNOS mRNA expression slightly decreased, whereas proinflammatory cytokine and the related gene expressions were increased in β-gal-Ad–transfected endothelial cells (n=4 each). LacZ (β-gal) mRNA expression was increased by β-gal-Ad transfection. DEX treatment suppressed the elevation of the cytokines and those related genes expressions completely.

**The Effect of DEX on Transgene Expression in the Endothelial Cell Line**

We next examined the effect of DEX on Ad-mediated gene transfer into HUVECs (Figure 5). In the HUVECs infected with β-gal-Ad at various concentrations (3×10^5 to 3×10^7 PFU/mL) for 2 hours under the nontreated condition, a few β-gal–positive endothelial cells were observed. However, even in the low-titer β-gal-Ad–infected cells, morphological changes in HUVECs, such as apoptotic rounding, blebbing, and denudation from the plate (Figure 5A), were observed, and the number of the excoriated cells increased in a titer-dependent manner (Figure 5B).

We next examined the effect of treatment with DEX on β-gal-Ad–mediated gene expression in HUVECs. The transforming efficiency was increased ≈2.7-fold by 1 μmol/L DEX in the 3×10^6 PFU/mL β-gal-Ad–infected HUVECs (Figure 5B; n=16). The higher (3 μmol/L) and lower (0.3 μmol/L) concentration of DEX slightly decreased the efficiency of gene
expression (n=12 each; data not shown). In addition, β-gal–positive HUVECs treated with 1 μmol/L DEX maintained their morphological characteristics (Figure 5A).

The Effect of DEX on Transgene Expression in Rat Femoral Artery

Finally, we examined the effects of DEX on in vivo transgene expression using rat femoral artery and found that 0.1 mg/kg DEX treatment increased the number of β-gal–positive endothelial cells significantly in a titer-dependent manner (3×10⁸ to 3×10¹⁰ PFU/mL) (Figure 6B). However, in the endothelium of femoral artery treated with Ad-free DMEM, no β-gal–positive stained or impaired endothelial cells were observed (data not shown). In the 0.1-mg/kg DEX-treated rat, the submaximum transfection efficiency of β-gal was obtained by 3×10⁹ PFU/mL β-gal-Ad infection (Figure 6B). Morphological study revealed that in the arteries infected with 3×10⁹ PFU/mL β-gal-Ad, most endothelial cells were excoriated at 2 days after infection (Figure 6A) (n=4). The degree of severity of these impairments was dependent on β-gal-Ad titer (data not shown). In the 3×10⁹ PFU/mL β-gal-Ad–infected femoral arteries of 0.1 mg/kg DEX-treated rats, 325.4±16.1 cells/mm² β-gal–positive stained cells were detected in the endothelium (Figure 6B) (n=4). Additionally, no excoriation of endothelial cells from the tunica interna was observed in the DEX-treated arteries.

Discussion

In the present study, we found that β-gal-Ad infection into the endothelium induced impairment of endothelium-dependent relaxation and morphological abnormality with proinflammatory cytokines and related genes mRNA expressions. Because the organ culture method to vascular tissues is a reliable means of distinguishing between the influence of systemic cellular and/or humoral immune response and the direct effects of Ad infection into the endothelial cells, we concluded, based on the present results, that endothelial cells have an endothelium-specific antivirus system.

Another important finding of this study is that the immunosuppressive agents DEX and CSA can increase foreign gene transfection efficiency via improvement of the antiviral responses in endothelial cells. DEX, but not CSA, can prevent apoptosis of endothelial cells. These beneficial effects of DEX were confirmed in both in vitro and in vivo experiments. Taken together, these results suggest that DEX treatment during both the virus infection period and the protein expression period has good potential for use in the design of preclinical and clinical gene therapy experiments for vascular diseases.

Tripathy et al reported that host immune responses are directed predominantly against foreign transgene-encoded proteins.² In this study, however, infection with an empty Ad vector yielded results similar to those by the bacterial β-gal-Ad in morphological studies (Figure 2), demonstrating that our results are not unique to the β-gal transgene, but rather occurred as a result of the direct effects of Ad infection itself.

Cytokines such as TNF-α, IL-1β, and interferon-α play prominent roles in the host defense against viruses. Our results demonstrated that 7.5×10⁹ PFU/mL β-gal-Ad infection immediately induced these cytokines in endothelial cells. TNF-α and IL-1β are multifunction cytokines with many
proinflammatory properties.\textsuperscript{15} TNF-α is particularly important in the induction of apoptosis and in the upregulation of adhesion molecules such as ICAM-1 and VCAM-1, which recruit leukocytes to the site of inflammation.\textsuperscript{16} As mentioned, in an in vivo study, the Ad-induced T cell infiltration was reported to play an important role as a hindrance factor in vascular impairments. In this study, we did not examine the Ad-induced T cell infiltration, but it is expected that in the in vivo treatments, the inhibition of ICAM-1 and VCAM-1 expression observed in DEX-treated pulmonary arteries might lead to the attenuation of Ad-induced T cell infiltration and secondary impairment.

Interferon-α/interferon-β are secreted by virus-infected cells and inhibit virus replication through cessation of the protein translation of infectious viral RNA.\textsuperscript{17,18} In the present study, endothelial cells released interferon-α at 6 to 12 hours after infection. In fact, 7.5×10\textsuperscript{8} PFU/mL β-gal-Ad without DEX did not result in a large expression of β-gal protein even though a similar amount of LacZ mRNA was induced by β-gal-Ad with or without DEX, suggesting that interferon-α released from endothelial cells may protect against Ad transfection at the protein translation level.

Ad vector (subgroup C adenovirus) infection is mediated via 2 cell surface receptors. The fiber protein of Ad first binds to the cell surface of coxsackievirus and adenovirus receptor.\textsuperscript{19} Virus internalization is then mediated through cell surface αvβ3 or αvβ5 integrins. We observed that post-DEX treatment (in which DEX was absent during Ad-gal-Ad infection) resulted in transfer of β-gal protein into the endothelium at a level similar to that by DEX treatment during both the infection and the expression period. Taken together, these findings suggested that DEX optimizes the Ad transfection efficiency by increasing viral cell adhesion and entry, but by protecting against viral β-gal RNA translation. These results are also consistent with the mechanism of interferon action.

DEX prevented Ad-induced apoptosis and maintained endothelial function. Previous reports suggest TNF-α upregulates FAS receptor and resulted in inducing apoptosis in lymphocytes.\textsuperscript{20} In the present study, TNF-α was induced in endothelial cells by infection with Ad, indicating that similar apoptosis signaling with lymphocytes may be induced in endothelial cells. The actions of glucocorticoids are mediated by alteration of the expression of specific target genes.\textsuperscript{21,22} Suppression of some genes and/or cell survival signal activations may contribute to the inhibition of Ad-induced endothelial cell death by DEX treatment. We found a slight inhibitory effect of CSA against Ad-induced endothelial apoptosis, but CSA could not prevent the impairment of endothelial function significantly. A possible explanation is that most apoptosis negative endothelial cells treated with CSA may be just before or in the middle stage of the apoptosis cascade. Further experiments are needed to clarify this point. Additionally, we observed that post-DEX treatment is not sufficient in terms of Ad-induced apoptosis inhibition, even though post-DEX treatment can increase the Ad transfection efficiency.

NO and related molecules reduce the expression of adhesion molecules and proinflammatory cytokines\textsuperscript{23,24} and directly inhibit vascular smooth muscle cell proliferation\textsuperscript{25} and migration.\textsuperscript{26} Thus, the protective effects of DEX against Ad-induced endothelial injury may contribute to long-term protection from vascular injury induced by recombinant Ad.

Considering the differences between in vivo and in vitro experimental models, including various experimental conditions, we cannot make an accurate evaluation of the efficiency of Ad infections in the present study relative to that of those in other studies. However, our results indicate that high transfection efficiency can be achieved by quite low concentrations of Ad (in vitro isolated cells: 3×10\textsuperscript{8} PFU/mL organ culture; 7.5×10\textsuperscript{6} PFU/mL; in vivo: 3×10\textsuperscript{8} PFU/mL) in the presence of DEX. Because the host response to Ad vector can vary depending on the dose of Ad,\textsuperscript{27} treatment with a relatively small amount of Ad may also help protect against the liver inflammation observed in in vivo Ad treatment.

Ad was rarely transduced into smooth muscle cells both in organ culture experiments and in vivo experiments. One probable explanation is that smooth muscle cells do not express enough coxsackievirus and adenovirus receptor as reported.\textsuperscript{27}

Previous studies in endothelial cells in vitro have shown that gene expression peaks at ∼7 days after infection and persists for at least 14 days, and the same is true in endothelial cells in vivo.\textsuperscript{5–7} Our results using the organ culture procedure indicated that transient DEX treatment during and after infection could achieve enhanced and prolonged gene expression. Considering the side effects induced by long-term steroid treatments, these results have several potentially important implications for the design of preclinical and clinical gene therapy experiments.

In conclusion, we have shown that the vascular endothelium exhibits unique antivirus reactions, including apoptotic cell death and cytokine production in an autocrine and/or paracrine manner, and provided a simple method by which to markedly improve adenoviral delivery to endothelial cells using the immunosuppressive agent DEX. The present results have several potentially important implications for the design of preclinical and clinical gene-therapy experiments for vascular disease.

Acknowledgments

This work was partly supported by a grant-in-aid for Scientific Research from the Ministry of Education of Japan, by research fellowships of the Japan Society for the Promotion of Science for Young Scientists, and by the Program for the Promotion of Basic Research Activities for Innovative Biosciences.

References

Antiviral Activity of Vascular Endothelium


Vascular Endothelium Has a Local Anti-Adenovirus Vector System and Glucocorticoid Optimizes Its Gene Transduction

Takahisa Murata, Masatoshi Hori, Sheng Lee, Akio Nakamura, Kazuhiro Kohama, Hideaki Karaki and Hiroshi Ozaki

*Arterioscler Thromb Vasc Biol.* 2005;25:1796-1803; originally published online June 16, 2005; doi: 10.1161/01.ATV.0000174130.75958.b7

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2005 American Heart Association, Inc. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/25/9/1796

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/