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Adenovirus-Mediated Gene Transfer In Vivo to Cerebral Blood Vessels and Perivascular Tissue in Mice

Stuart D. Christenson, MD; Kristy D. Lake, PhD; Hiroaki Ooboshi, MD, PhD; Frank M. Faraci, PhD; Beverly L. Davidson, PhD; Donald D. Heistad, MD

Background and Purpose—Gene transfer to cerebral blood vessels has been accomplished in rats and dogs by injection of replication-deficient adenovirus into cerebrospinal fluid. In this study we examined transgene expression after injection of adenovirus into the cerebrospinal fluid of mice. Responses were observed in ICR mice and C57BL/6 mice, which are outbred and inbred strains, respectively.

Methods—We injected replication-deficient recombinant adenovirus expressing nuclear targeted β -galactosidase, driven by either the Rous sarcoma virus promoter (AdRSV- β Gal) or the cytomegalovirus promoter (AdCMV- β Gal), into the cisterna magna of anesthetized ICR and C57BL/6 strains of mice. The brains were examined from 1 to 21 days after injection by chemiluminescent enzyme activity assay or histochemical staining.

Results—After injection of AdRSV- β Gal, expression of β -galactosidase in ICR mice peaked on day 7 and returned to basal by day 14. Expression of β -galactosidase in C57BL/6 mice was maximal on days 7 to 14 and was minimal by day 21 after injection of AdRSV- β Gal. After injection of AdCMV- β Gal in C57BL/6 mice, peak expression of transgene occurred on day 1 and was greatly diminished by day 3. Transgene expression was observed primarily on the ventral surface of the brain, with preferential expression in leptomeninges and adventitia along the major cerebral arteries of that region.

Conclusions—Injection of recombinant adenovirus in the cisterna magna resulted in transgene expression in leptomeninges and perivascular tissue of cerebral blood vessels in two strains of mice. The CMV promoter elicited rapid but short-lived expression of the transgene, while the RSV promoter elicited slower, more sustained transgene expression. Expression of AdRSV transgene was prolonged in C57BL/6 mice compared with ICR mice. This approach for gene transfer may be useful to study cerebral vascular biology in genetically altered strains of mice. (*Stroke*. 1998;29:1411-1416.)

Key Words: adenovirus ■ cerebral arteries ■ gene transfer ■ promoter regions (genetics) ■ mice

Gene transfer to blood vessels offers a new tool to study vascular physiology, with potential for treatment of vascular disease. Adenoviral vectors have been used to achieve gene transfer in several types of vessels.¹⁻³ Gene transfer to blood vessels usually has been accomplished by intraluminal administration of a vector.⁴⁻⁶ This approach, however, is generally limited by the need to transiently interrupt blood flow or to use a double-balloon catheter that produces expression limited to a small segment of vessel. Alternative approaches have been developed recently that circumvent these obstacles by perivascular administration of adenovirus to the carotid arteries of monkeys⁷ and to cerebral blood vessels of rats⁸ and dogs.⁹

The major goal of this study was to achieve gene transfer to cerebral blood vessels and perivascular tissue in mice by administration of a viral vector into the cerebrospinal fluid. Gene transfer in mice is attractive because it allows studies of vascular mechanisms in genetically altered strains.

The time course of transgene expression appears to be dependent on host immune responses to either the vector¹⁰ or

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the transgene product.¹¹ We examined transgene expression in ICR mice, a common outbred strain of heterogeneous background, and in C57BL/6 mice, an inbred strain that is commonly used as a background for genetically altered strains.

The time course of transgene expression also may be dependent on the promoter used in the vector. The CMV promoter, for example, provides strong constitutive expression, and the RSV promoter generally provides slower transgene expression. We assessed the time course of expression using vectors driven by either the RSV or the CMV promoter.

Materials and Methods

Adenoviral Vector

We used two different replication-deficient recombinant adenoviruses (serotype 5) encoding the reporter gene for bacterial β -galac-

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Selected Abbreviations and Acronyms

AdCMV- β Gal	= adenovirus expressing nuclear targeted β -galactosidase driven by cytomegalovirus promoter
AdRSV- β Gal	= adenovirus expressing nuclear targeted β -galactosidase driven by Rous sarcoma virus promoter
CMV	= cytomegalovirus
PBS	= phosphate-buffered saline
RSV	= Rous sarcoma virus

tosidase driven by either the Rous sarcoma virus promoter (AdRSV- β Gal) or the immediate early cytomegalovirus promoter (AdCMV- β Gal). These vectors were constructed according to methods similar to those described previously.^{12,13} Briefly, these replication-deficient adenoviral vectors have been deleted of sequences in the E3, E1A, and E1B regions, impairing the ability of the virus to replicate. Either the RSV or CMV promoter was used to drive transcription of *lacZ*, the gene for *Escherichia coli* β -galactosidase, with a simian virus 40 polyadenylation sequence cloned downstream from this reporter gene. Recombinant viruses were grown in human embryonic kidney (293) cells that complement the E1 early viral promoters. After purification, the virus was suspended in PBS with 3% sucrose and kept at -80°C until used.

Animals and Surgical Procedure

All animal procedures were approved by the Institutional Animal Care and Use Review Committee. Male ICR mice (Harlan Sprague; weight, 16 to 26 g) ($n=36$) and C57BL/6 mice (Harlan Sprague; weight, 16 to 24 g) ($n=64$) were used. Mice were anesthetized with pentobarbital (≈ 75 mg/kg IP) or ketamine (40 mg/kg SC) and acepromazine (1.5 mg/kg SC). The head was placed in a stereotaxic apparatus with the nose approximately 30° below horizontal. With the use of aseptic techniques, the skin was incised, and muscle was cleared to expose the atlanto-occipital membrane. A 30-gauge needle and Hamilton syringe were mounted on the arm of the stereotaxic device, and the needle was inserted 1.0 mm into the cisterna magna. Twenty microliters of viral suspension ($\approx 3 \times 10^{10}$ plaque-forming units per milliliter) was infused over 20 minutes. The needle was withdrawn 10 minutes after the infusion was completed, and the nuchal muscle and skin were closed with sutures. Approximately 95% of mice survived the procedure.

Histochemical Analysis for β -Galactosidase

After 1 to 21 days, the mice were killed with pentobarbital (≈ 120 mg/kg IP) and perfused transcardially with heparinized (100 mU/mL) normal saline. The brain was removed and rinsed thoroughly with normal saline. For histochemical staining of β -galactosidase activity, the brains were placed in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS (<1 hour), then rinsed with PBS. The whole brain or coronal sections were incubated in 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-Gal) solution (2 hours at 24°C), rinsed in normal saline solution, and then postfixed with 7% buffered formalin. The fixed tissue was then processed for paraffin embedding, and microtome sections were cut from the block, placed on slides, and counterstained with hematoxylin and eosin or nuclear fast red. Brain sections were examined for positive staining of β -galactosidase (blue nuclei) by light microscopy.

Chemiluminescent Assay

To quantify β -galactosidase activity, the ventral region of the brain, corresponding to the brain stem (including basilar artery) and circle of Willis, was sectioned for chemiluminescent enzyme assay. The section of tissue was homogenized and lysed with $150 \mu\text{L}$ of lysis buffer containing 0.2% Triton X-100 and 100 mmol/L potassium phosphate, pH 7.8. The suspension was centrifuged at $10\,000g$ for 10 minutes, and the supernatant was assayed for β -galactosidase activ-



Figure 1. Expression of β -galactosidase on the ventral surface of the brain of a C57BL/6 mouse 1 day after intracisternal injection of AdCMV- β Gal. There was dark blue staining of leptomeninges near the basilar artery, circle of Willis, middle cerebral artery, and smaller arteries.

ity with the use of the Galacto-Light Plus assay kit. Light emission was measured with a Monolight 2010 luminometer and calibrated with a standard curve generated with the use of purified *E coli* β -galactosidase. Protein concentrations were determined with a BioRad DC Protein Assay, and normalized β -galactosidase activity was expressed as milliunits β -galactosidase per milligram protein.

Statistical Analysis

Data are presented as mean \pm SEM. A natural log transformation of the data was used in the analysis to normalize the variance among the treatment groups. To compare β -galactosidase activity with control value, ANOVA was performed, followed by the Dunnett test for multiple comparisons. For comparisons between groups (ICR versus C57BL/6, AdRSV- β Gal versus AdCMV- β Gal), a two-way ANOVA was performed, with treatment and day as the two factors of analysis. Treatment groups were then compared at each day with P values adjusted by Bonferroni's method. $P < 0.05$ was considered a significant difference.

Results

Histochemical Analysis of Gene Expression for β -Galactosidase

Staining for β -galactosidase was observed primarily on the ventral surface of the brain, especially along the major cerebral arteries (Figure 1). Transgene expression was also observed on the medial dorsal surface of the cerebellum and brain stem near the injection site. The dorsal cortical surface had few positively stained cells. Nuclei were well transduced

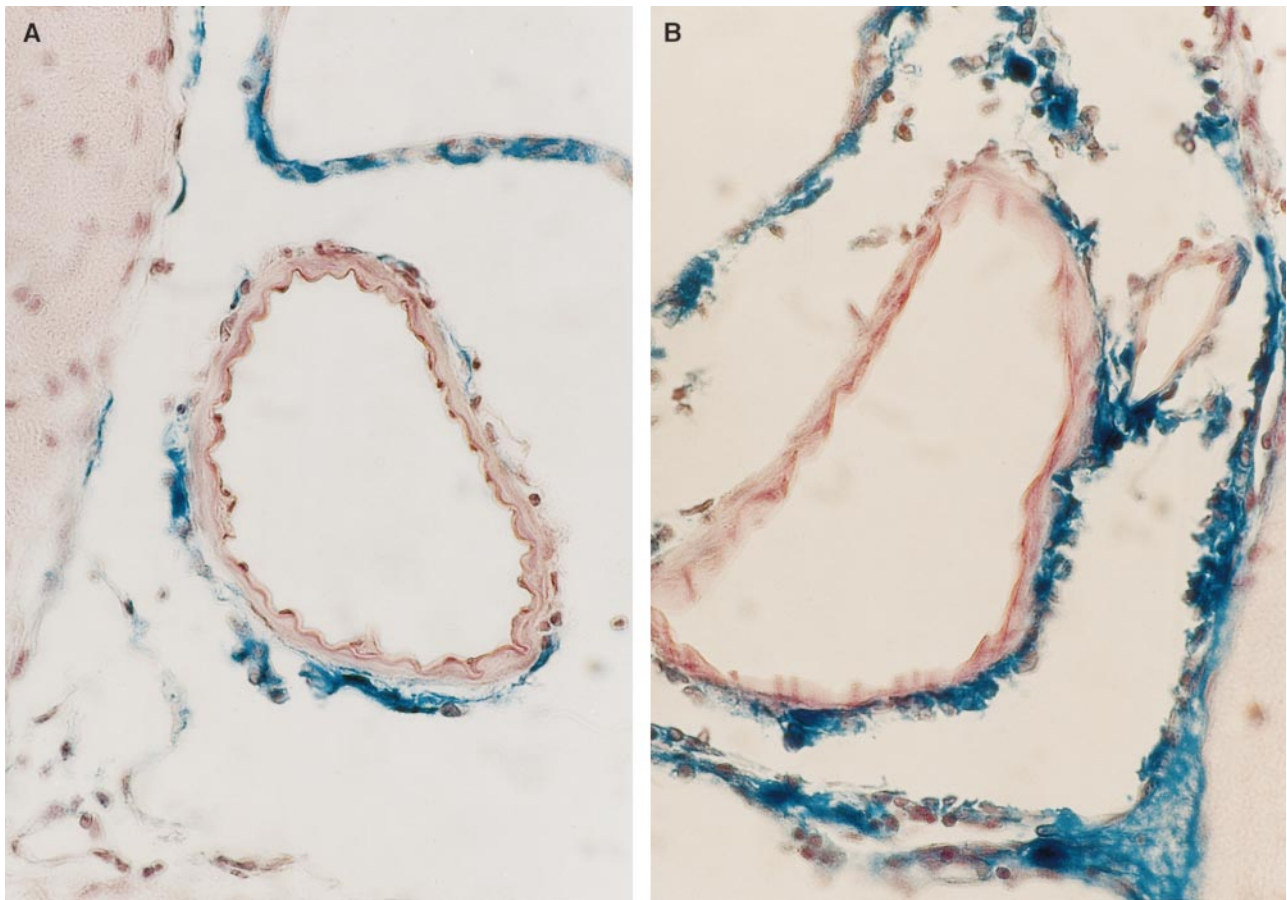


Figure 2. Expression of β -galactosidase in the brain of a C57BL/6 mouse 1 day after intracisternal injection of AdCMV- β Gal. Brain sections were stained with X-Gal and counterstained with hematoxylin and eosin. High-power views ($\times 320$) show expression of β -galactosidase in the basilar artery (A) and the communicating artery (B). There was dark blue staining of leptomeninges and adventitia. In both panels, leukocytes can be seen around transfected tissues.

in the leptomeningeal cells, especially those overlying cerebral blood vessels on the ventral surface (Figure 2A and 2B). Medial and intimal cells did not demonstrate staining, and adventitial cells were often stained.

β -Galactosidase Activity in ICR and C57BL/6 Mice

In ICR mice, peak expression of β -galactosidase was observed 7 days after injection of AdRSV- β Gal (Figure 3). Transgene expression virtually disappeared 14 days after injection of the virus. In C57BL/6 mice, after injection of

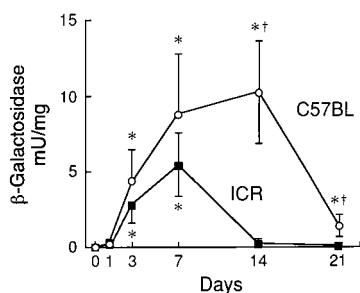


Figure 3. Expression of β -galactosidase on ventral brain stem and cerebrum after intracisternal injection of AdRSV- β Gal in ICR and C57BL/6 mice. Each value indicates mean \pm SEM of 3 to 7 mice. * $P < 0.05$ vs day 0; † $P < 0.05$ vs ICR mice.

AdRSV- β Gal, peak expression was observed at 7 to 14 days (at a time when expression had virtually disappeared in ICR mice) and diminished (but was not gone) by 21 days (Figure 3). After injection of AdCMV- β Gal in C57BL/6 mice, peak expression of transgene was observed at 1 day and was greatly diminished by 3 to 7 days (Figure 4).

Inflammatory Response

Polymorphonuclear and mononuclear leukocytes were observed in vascular adventitia and surrounding leptomeninges

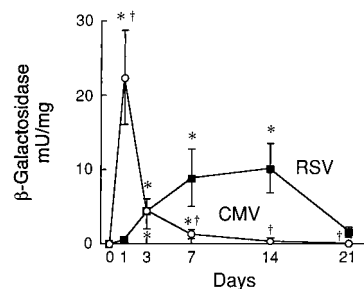


Figure 4. Expression of β -galactosidase on ventral brain stem and cerebrum after intracisternal injection of AdRSV- β Gal or AdCMV- β Gal in C57BL/6 mice. Each value indicates the mean \pm SEM of 3 to 8 mice. * $P < 0.05$ vs day 0; † $P < 0.05$ vs AdRSV- β Gal (Figure 3).

on the ventral and lateral surfaces of the brain after injection of AdCMV- β Gal (Figure 2) or AdRSV- β Gal at day 1 and day 7. Vehicle-injected mice had few or no leukocytes. No animals had head tilts, paralysis, or detectable behavioral changes for up to 21 days.

Discussion

The major finding of this study was that administration of replication-deficient adenovirus into the cisterna magna of mice produced expression of the transgene, which demonstrates that the method is feasible in mice. The gene product was expressed in a time-dependent manner on the ventral surface of the brain stem and cerebrum, especially in leptomeningeal cells along the major cerebral blood vessels and in adventitial cells. Transgene expression appeared to persist longer in C57BL/6 mice than ICR mice. Finally, we found that peak expression was greater, occurred much sooner, and diminished earlier with the use of an adenoviral vector driven by a CMV immediate early promoter than with the use of an adenoviral vector driven by an RSV promoter.

Gene Transfer to Mice

Gene transfer to blood vessels is generally accomplished by intraluminal delivery with the use of perforated,^{14,15} double-balloon,^{6,14,16} or hydrogel-coated balloon^{6,15} catheters or by direct injection^{4,5,14} into blood vessels. For gene transfer to cerebral blood vessels, this approach is limited by the apparent need to interrupt cerebral blood flow and to disrupt the blood-brain barrier.⁸ As an alternative approach, two groups have accomplished gene transfer to cerebral blood vessels in rats⁸ and dogs^{9,17} by intracisternal injection. In this study transgene expression was observed in perivascular tissue and adventitia of cerebral arteries in both strains of mice. As in previous studies,^{8,9,17} transgene expression was not observed in media, endothelium, or neurons.

Gene transfer to cerebral blood vessels in mice is an attractive possibility because genetically altered mice are available. Administration of a vector to deliver a functional transgene to one of these genetically altered strains could offer a new means of studying vascular biology.

Determinants of Transgene Expression

Promoters are an important determinant of the level and time course of expression after gene transfer.^{18–20} Promoters have different levels of constitutive activity and different response elements that induce or inhibit activity. In this study we used adenovirus vectors driven by CMV or RSV promoters. The CMV major immediate early promoter/enhancer drives constitutive expression, and response elements within the enhancer allow inducible expression through binding of active transcription factors, such as cAMP response element binding protein and nuclear factor- κ B. The CMV, but not RSV, promoter is associated with inflammatory response elements, nuclear factor- κ B, and cAMP response element binding protein.^{21–24} We observed that peak expression was reached much sooner (day 1 versus day 7 to 14) and appeared to be greater with the CMV promoter than with the RSV promoter. Thus, different intensity and duration of transgene expression

in cerebral vessels and perivascular tissue can be provided on the basis of choice of viral promoters.

Inbred animals (eg, C57BL/6 mice) are genetically more homozygous than outbred animals (eg, ICR mice). Inbred and genetically altered strains are useful for investigating specific questions that require distinct genetic alterations, and outbred strains are useful for screening.²⁵ The C57BL/6 mouse appears to be more susceptible to bacterial infection than outbred mice such as ICR.^{25,26} Peak expression of the transgene in C57BL/6 mice occurred at a time when expression had virtually disappeared in ICR mice, and the duration of expression was longer in C57BL/6 mice. Duration of expression after gene transfer is affected by the host immune response to the viral proteins¹⁰ and the transgene product.¹¹ Both cellular and humoral immune responses play a role in the response to these foreign proteins in brain^{27,28} and peripheral tissues.^{29–32} Immunocompetence of the host, therefore, can greatly affect the time course of transgene expression.^{25,26} We speculate that differences in the immune status between the two strains of mice used in this study may contribute to the difference in time course of transgene expression.

Limitations of the Method

Although this method offers promise for study of cerebral vascular function, it has important limitations. First, the duration of transgene expression was only 2 to 3 weeks when the RSV promoter was used, and longer expression may be desirable for some studies and therapeutic approaches. Second, transient inflammation, with leukocytosis produced by injection of adenovirus into the cerebrospinal fluid, may affect studies of vascular function. For studies of vascular biology, the inflammatory responses can be addressed with appropriate controls. Inflammation, nevertheless, will present a major obstacle to gene therapy in which adenoviral vectors are used.

Conclusions and Speculation

The duration of expression noted in this study is relatively short (<1 and 3 weeks for CMV and RSV promoters, respectively). This duration is too brief for some therapeutic applications for chronically diseased vessels. The duration may be useful, however, when applied to physiological studies and pathophysiological applications. For example, an adenovirus that encodes for endothelial nitric oxide synthase has been demonstrated to be functional in carotid³³ and cerebral blood vessels.⁹ Transient gene replacement may be feasible in cerebral vessels of endothelial nitric oxide synthase-deficient mice.³⁴ In relation to pathophysiology, we speculate that prevention of vasospasm may be feasible after subarachnoid hemorrhage.^{35–38}

In summary, the present study demonstrates successful use of an adenoviral vector to induce transgene expression in perivascular tissues near cerebral blood vessels. The onset and duration of transgene expression are dependent on the promoter that is used and the strain of mice. This *in vivo* technique may provide a useful tool to evaluate the role of selected enzymes or receptors in normal and genetically altered mice.

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Editorial Comment

In this article, the authors show that intracisternal injection of adenovirus containing the *lacZ* gene results in successful transfection of cerebral blood vessels and meningeal cells. There were differences in the length of gene expression depending on the viral promoter and strain of recipient mice. The authors note that this strategy may be useful as a tool to study cerebrovascular function and may even represent a method for therapeutic gene delivery.

Points to be made from this study include the following: (1) Viral vectors were introduced through direct intracisternal injection. This is a relatively easy approach with which to deliver drugs or other agents to rodents and, indeed, to humans (through a lateral cervical puncture or direct injection into the cisterna magna). (2) Cells successfully transfected included meningeal cells and cells of the vascular adventitia. Thus, this method would not be appropriate for circumstances in which transfection of

cells on the cortical surface (neurons and glia) or vascular media or endothelial cells is desired. (3) Gene expression was transient and thus not appropriate for circumstances in which ongoing gene expression is desired. (4) An inflammatory response occurs, which, as the authors note, may limit the application of this method.

In spite of such limitations, however, the transfection method described is likely to be useful for studies in which transient local gene expression in adventitial and meningeal cells is desired. Moreover, in the case of genes encoding secreted proteins, gene products expressed locally in these cells may diffuse to affect adjacent cells in the cerebral vasculature and cortical surface.

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