Protective Vasomotor Effects of In Vivo Recombinant Endothelial Nitric Oxide Synthase Gene Expression in a Canine Model of Cerebral Vasospasm

Vini G. Khurana, MD, PhD; Leslie A. Smith; Timothy A. Baker, BS; Daihiko Eguchi, MD, PhD; Timothy O’Brien, MD; Zvonimir S. Katusic, MD, PhD

Background and Purpose—Post-subarachnoid hemorrhage (SAH) cerebral vasospasm is a potentially devastating condition whose pathogenesis involves impaired nitric oxide (NO) bioavailability. We aimed to determine whether recombinant endothelial NO synthase (eNOS) gene expression may protect vasomotor function and prevent vasospasm in a canine experimental SAH model.

Methods—Recombinant adenoviral vectors (5×10⁹ plaque-forming units/animal) encoding genes for eNOS (AdeNOS) and β-galactosidase (AdLacZ) or vehicle were injected into the cerebrospinal fluid (CSF) of dogs on day −1 (ie, 24 hours before the first intra-CSF injection of blood on day 0). Cerebral angiography was performed at day 0 (baseline) and day 7 (immediately before death), and tissues were harvested for additional studies.

Results—Western analysis and immunohistochemistry detected recombinant eNOS exclusively in cerebral arteries isolated from AdeNOS-transduced dogs, and in this group of animals CSF NO concentrations were significantly elevated by day 2. Analysis of day 7 versus day 0 cerebral angiograms for each group revealed significant spasm at the basilar artery midpoint in AdLacZ-transduced and nontransduced dogs but not in AdeNOS-transduced dogs. Isometric force recording of basilar arteries isolated from AdeNOS-transduced dogs showed significantly augmented relaxations to bradykinin and reduced contractions to endothelin-1.

Conclusions—Our results suggest that expression of recombinant eNOS in the adventitia of cerebral arteries may contribute toward protection against post-SAH vasospasm. (Stroke. 2002;33:782-789.)

Key Words: basilar artery ■ gene therapy ■ nitric oxide ■ subarachnoid hemorrhage ■ vasospasm, intracranial ■ dogs

The expression and function of recombinant genes in cerebral arteries after adenovirus-mediated gene transfer have been extensively characterized, although the ability of recombinant endothelial nitric oxide synthase (eNOS) gene expression to ameliorate a disease involving intracranial arteries has not been demonstrated. The present study, representing a continuation of efforts to translate cerebrovascular gene transfer technology closer to the clinical arena, was performed to characterize this ability, particularly in view of the important role played by NO in normal vasomotor function and the pathogenesis of cardiovascular diseases, including post-subarachnoid hemorrhage (SAH) cerebral vasospasm. We hypothesized that intrathecal delivery of an adenoviral vector encoding recombinant eNOS gene could be used in a canine experimental SAH model to augment cerebrovascular eNOS expression and protect against vasospasm. Our study lends support to the possibility of gene therapy for post-SAH vasospasm and underscores important benefits and limitations of this therapeutic approach.

Subjects and Methods

Animals

Male mongrel dogs weighing 20 to 22 kg were used for experiments. General anesthesia was induced with 10 mg/kg IV methohexital (Brevital; Jones Pharma) and maintained with 2% inhaled isoflurane (Fort Dodge Animal Health). All procedures were approved by the Institutional Biosafety and Animal Care and Use Committees. Biological parameters measured in animals included core temperature, blood pressure, heart rate, hemoglobin, arterial pH and pCO₂, and cerebrospinal fluid (CSF) total nucleated cell count (sum of automated lymphocyte plus polymorphonuclear lymphocyte cell counts) with differential into percentage lymphocytes versus polymorphonuclear lymphocytes.

Adenoviral Vectors and Gene Transfer

Replication-incompetent recombinant adenoviral vectors encoding bovine eNOS (AdeNOS) and Escherichia coli β-galactosidase (AdLacZ; a vector-control in this study) were generated as described elsewhere. “Control” dogs or arteries refers to those not exposed to vector (ie, nontransduced) but instead exposed to the appropriate “vehicle” (140 mmol/L NaCl, 10 mmol/L Tris buffer, 2 mmol/L MgCl₂, 10% glycerin; pH 8.0). For each dog, vector (5×10⁹ plaque-forming units) was injected into the cerebrospinal fluid (CSF) of the dogs (AdeNOS and AdLacZ; 5×10⁹ plaque-forming units/animal).

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plaque-forming units (PFU/dog) or vehicle was delivered directly into the CSF using a spinal needle inserted aseptically into the cisterna magna immediately inferior to the midline-occiput (ie, intrathecal route; intracisternal injection). One half milliliter of CSF was aspirated and mixed with 50 μL vector or vehicle before its intracisternal injection. After injection, animals were maintained in a 30° head-down prone position for 20 minutes before transfer to post-anesthesia recovery. Intrathecal injection of vector or vehicle was carried out on day 1; ie, 24 hours before the first intrathecal injection of venous blood (day 0). The transduction titer of 5×10^8 PFU/dog was chosen based on previous canine in vivo gene transfer studies.3,13

**Experimental SAH**

Induction of experimental double SAH followed by cerebral vasospasm was performed as described elsewhere.10,14 On day 0, with the anesthetized animal in the prone position as above, 4 mL CSF was aspirated through aseptic cisterna magna puncture. Four milliliters of autologous venous blood was then introduced into the CSF through intracisternal injection performed for 2 minutes. Two days later (day 2), this procedure was repeated with a further 4 mL autologous blood.

**Cerebral Angiography**

Basilar artery angiography was performed on day 0 (baseline; before first injection of blood) and day 7 (immediately before death) as described elsewhere.10 A transmemoral 7-F catheter was advanced to the proximal vertebral artery, after which iodine radiocontrast was injected into the posterior cerebral circulation. The basilar artery was imaged radiographically and its diameter measured as described below. One-centimeter diameter circular radio-opaque washers laid flatly on the angiogram table next to the head of the animal were used as external references for radiograph magnification.

**Histochemistry and Immunohistochemistry**

Histochemical staining for recombinant β-galactosidase was performed using X-Gal (Life Technologies) in whole brains obtained from dogs on day 7. For immunohistochemical staining of recombinant eNOS, basilar arteries were harvested on day 7 except in the case of 1 AdeNOS-transduced dog, which died on day 2 and whose basilar artery was immediately harvested post-mortem. Anti-eNOS mouse monoclonal IgG1 antibody (1:500 dilution; BD-Transduction Laboratories) with cross-reactivity against bovine eNOS was used for recombinant eNOS detection.3,4,7

**NO Measurement**

NO levels were measured indirectly from CSF NO metabolites (nitrate, nitrite) by automated chemiluminescence assay using an NO analyzer (Ionics-Sievers).13

**Western Analysis**

Middle cerebral arteries from all SAH dogs after day 7 death were studied in parallel. Total protein was determined using the DC Protein Assay Kit (Bio-Rad). Size-fractionated proteins were hybridized with anti-eNOS mouse monoclonal IgG antibody (1:500 dilution; BD-Transduction) and bands visualized with ECL-Detection System (Amersham). Positive controls were bovine aortic endothelial cells lysates; negative controls were middle cerebral artery homogenates from nontransduced dogs.

**Isometric Force Recording**

Vascular reactivity was studied by isometric force recording.3,4,7,14 Vasomotor responses were compared between basilar arteries from transduced and nontransduced dogs. Uridine triphosphate (UTP), diethylamine-NONOate (DEANONOate), endothelin-1 (ET-1), and papaverine (PPV) were obtained from Sigma.

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**Data Analysis**

Data were analyzed from 14 dogs exposed to experimental double SAH (“SAH dogs”: 4 nontransduced, 5 AdenOS transduced, 5 AdeNOS transduced) that survived to day 7 and had macroscopically normal brains. Data from a separate group of 9 dogs not injected with vehicle, vector, or blood (ie, “non-SAH” dogs) were analyzed exclusively for CSF NO concentrations. Excluded from analysis were data from an additional 2 SAH dogs based on early mortality (day 2) in 1 and intracranial pus (day 7) in the other. Results are expressed as mean±SEM; herein, n indicates the number of animals from which tissues were harvested, while probability values of P<0.05 are considered significant.

**Results**

**Biological Parameters**

Transduced animals demonstrated an early-onset, sustained febrile response (Table). Total nucleated cell counts in AdeNOS-transduced dogs were approximately 10-fold greater than in AdenOS-transduced dogs on day 0, which in turn were approximately 6-fold greater than in control dogs (both P<0.001). By day 2, compared with day 0 neutrophil and lymphocyte counts, relative CSF neutrophilia and lymphocytopenia were observed in control dogs, whereas relative CSF neutropenia and lymphocytosis were observed in transduced dogs (all P<0.05; Table).

**Morphology**

Using X-Gal histochemistry at day 7, diffuse expression of recombinant eNOS was detected immunohistochemically in basilar arteries obtained on day 2 from an AdeNOS-transduced dog (Figure 1). Basilar arteries from other AdeNOS-transduced dogs euthanized on day 7 stained positively for recombinant eNOS (n=5), although less strongly than those observed on day 2. No positive staining was observed in basilar arteries from nontransduced animals or in basilar arteries treated without primary antibody or with an isotype-matched non–anti-eNOS primary antibody (n=5).

**Biochemistry**

Biochemical evidence for expression of recombinant eNOS exclusively in AdeNOS-transduced dogs was found after Western analysis of cerebral arteries obtained at day 7 (Figure 2A). Further evidence for recombinant eNOS expression in
Adenovirus-transduced dogs was found by comparing CSF NO metabolite measurements in SAH and non-SAH dog groups (Figure 2B). By day 2, CSF NO levels were significantly elevated in AdeNOS-transduced dogs compared with other groups (all \( P < 0.05 \); Figure 2B).

**Radiology**

Angiographic vasospasm in AdLacZ-transduced dogs (Figure 3A) appeared similar to that in nontransduced dogs. Angiographic evidence for protection against vasospasm was found exclusively in AdeNOS-transduced dogs (Figure 3B), however, significantly attenuated spasm in these animals occurred only at the basilar artery midpoint (Figure 3C). From basilar artery midpoint diameters measured from angiograms in SAH dogs, significant spasm was observed in day 7 compared with day 0 angiograms in both nontransduced (32 ± 7%; \( P = 0.025 \);
4 measurements from n=4 dogs) and AdLacZ-transduced (25±6%; P=0.026; 5 measurements from n=5) groups, but not in the AdeNOS group (18±7%; P=0.088; 5 measurements from n=5; Figure 3C). When all basilar artery diameter measurements were considered (ie, proximal, midpoint, distal), differences between day 0 versus day 7 diameters within each group were significant (all P<0.01; nontransduced: 31±3% spasm, 12 measurements from n=4; AdLacZ-transduced: 28±4% spasm, 15 measurements from n=5; AdeNOS-transduced: 20±3% spasm, 15 measurements from n=5). However, there was a small but significant (P=0.047) decrease in the amount of day 7 spasm seen in the aggregate of basilar artery diameters in AdeNOS-transduced dogs (20±3% spasm) compared with nontransduced dogs (31±3% spasm). Differences in the amount of day 7 spasm seen in the aggregate of basilar artery diameters between AdLacZ- and nontransduced groups, between AdLacZ- and AdeNOS-transduced groups, and between nontransduced and transduced groups were not significant (all P>0.05).

**Vasomotor Function**

Bradykinin-induced relaxations were significantly enhanced and greatest in AdeNOS-transduced basilar arteries (Figures 4A and 4B). Maximal relaxations to DEANONOate (10⁻⁵ mol/L) were not different between control (97±1%; n=4) and AdLacZ-transduced (98±1%; n=5) and AdeNOS-transduced (99±0%; n=5) groups (all P>0.05). However, relaxations to DEANONOate between 10⁻⁸ and 10⁻⁶ mol/L were significantly augmented in AdeNOS-transduced arteries (log EC₅₀=8.1±0.1, n=5, versus 7.9±0.1, n=5 and 7.5±0.1, n=4, for AdeNOS versus AdLacZ and control arteries, respectively; both P<0.05).

Maximal contractions to ET-1 (10⁻₈ mol/L) were not different between control (124±8%; n=4) and AdLacZ-transduced (133±8%; n=5) and AdeNOS-transduced (142±8%; n=5) arteries (all P>0.05; Figure 4C). However, contractions to ET-1 between 10⁻¹¹ and 11⁻⁹ mol/L were significantly reduced in AdeNOS-transduced arteries (log EC₅₀=8.9±0.1, n=5, versus 9.2±0.1, n=5 and 9.2±0.1, n=4, for AdeNOS versus AdLacZ and control arteries, respectively; both P<0.05; Figure 4C).

**Discussion**

Although we were unable to achieve optimal angiographic reversal of vasospasm using intracisternal AdeNOS gene transfer, we did achieve the following statistically significant findings in AdeNOS-transduced animals: (1) no significant spasm at the basilar artery midpoint; (2) less whole basilar artery vasospasm; (3) augmented relaxations to bradykinin; and (4) attenuated contractions to ET-1. Our report is the first to indicate that recombinant gene transfer can confer some degree of angiographic and functional vasomotor protection against vasospasm in a large-animal model closely mimicking human cerebrovascular disease.

**Biological Parameters**

Consistent with the effects of recombinant adenoviral vector delivery in the central nervous system, a febrile response with relative CSF neutropenia and lymphocytosis occurred in...
transduced dogs. Nontransduced dogs, however, developed neutrophilia and lymphocytopenia, findings consistent with inflammation secondary to the presence of blood rather than adenovirus. We cannot explain why the nucleated cell count in AdeNOS-transduced dogs was approximately 10-fold higher than in AdLacZ-transduced dogs at day 0, given that the same titer of vector was delivered in both groups and that use of replication-incompetent adenoviral preparations was confirmed by reverse-transcriptase polymerase chain reaction. The possibility that the observation was related to a different degree of acute immunologic response mounted against each recombinant protein (derived from different sources, namely, \textit{E} coli in AdLacZ and bovine in AdeNOS) cannot be excluded.\textsuperscript{16}

**Morphology**

We found that intracisternal AdLacZ delivery resulted in diffuse expression of recombinant protein despite mechanical head-tilt aimed at facilitating vector targeting to the basilar artery region.\textsuperscript{2} This was most likely because of the effect of normal CSF circulation on diffusion of vector suspended in a low-viscosity, nonthrombogenic vehicle. Further, consistent with perivascular transduction after intracisternal adenovirus delivery,\textsuperscript{2,4,7} recombinant eNOS expression occurred in basilar artery adventitia. Notably, strong adventitial expression of recombinant eNOS was detected at day 2; the lower level of expression detected at day 7 is consistent with the transient nature of recombinant gene expression observed using adenoviral vectors.\textsuperscript{5,15}

**Biochemistry**

Evidence supporting recombinant eNOS expression in AdeNOS-transduced dogs was found by Western analysis and CSF NO assay, findings consistent with reported biochemical effects of intracisternal AdeNOS delivery in dogs.\textsuperscript{4,13} In our study, CSF NO levels in AdeNOS-transduced dogs were significantly elevated from day 2 onwards. Paradoxically, we found that recombinant eNOS expression was more pronounced immunohistochemically on day 2, but CSF NO levels in AdeNOS-transduced animals were higher on day 7. Ng et al\textsuperscript{17} suggest that in humans after SAH, 2 temporal modes of NO metabolite measurements are observed: a monophasic pattern with peak NO metabolites at day 2 versus a biphasic pattern with peaks at days 1 and 3. We recognize the apparent discrepancy between our immunohistochemical and biochemical data. One possible explanation is that recombinant eNOS expression may be occurring in noncerebroarterial cells found elsewhere in the subarachnoid space, such as in leptomeningeal or venous fibroblasts; we have no information regarding the dynamics of expression in such tissue and its temporal correlation with CSF NO levels.
Inclusion of a non-SAH group of dogs aided in interpreting the effects of experimental SAH on NO levels. In all SAH groups, NO levels were increased compared with the non-SAH group, a finding that may reflect an underlying inflammatory response triggered by the presence of blood in the subarachnoid space. Although we have no information regarding the presence of inducible NOS (iNOS) in our animals, this would seem plausible given the association between adenoviral vectors, inflammation, and iNOS induction in vascular smooth muscle cells and infiltrating leukocytes. Further, SAH itself is associated with inflammation and increased CSF NO levels, findings supported by our data. We believe that the larger elevation of CSF NO levels observed in AdeNOS-transduced dogs is a result of expression and activity of recombinant eNOS exclusively in these animals as indicated by our immunohistochemical and Western analysis data. However, despite finding no consistent association between CSF nucleated cell counts and CSF NO levels, we recognize that the increase in CSF leukocytes noted in AdeNOS-transduced animals may be associated with a long-lasting inflammatory reaction in the subarachnoid space that may, in turn, be associated with increased NO levels from upregulated leukocyte iNOS.

Radiology
Cerebral artery diameter reduction in experimental models of vasospasm may be up to 50% to 60% by day 7, depending on the species; in dogs the average reduction is 30% to 50%. In our nontransduced dogs, the reduction was 32%, ie, on the lower side of published canine experimental data. This may be a result of the fact that our model is somewhat different from previous canine SAH models: in our current model, a manipulation of the animal occurred first on day 1 when we injected vehicle or vector intracisternally. It is conceivable that this may somehow contribute to an overall reduction of spasm, given that in our previous reports involving nontransduced dogs (similar protocol using 4 or 5 mL of blood per injection except no day -1 manipulation) we induced vasospasm associated with approximately 55% diameter reduction.

We found that day 7 angiographic spasm was significantly attenuated in AdeNOS-transduced dogs, however, only at the basilar artery midpoint. The reason for protection occurring only at the arterial midpoint in this group is unknown but may relate to focal differences in hemodynamics or vascular anatomy or increased vector transduction at this location. It does not seem to relate to focal absence of clot, which typically encased the entire artery. When the aggregate of basilar artery diameters was calculated for each group and compared, significant spasm was found in all 3 SAH dog groups, however, there was significantly less spasm in AdeNOS-transduced compared with nontransduced animals. Although our angiographic data do not show marked prevention of vasospasm in AdeNOS-transduced animals, limited but still significant protection from spasm in this group remains encouraging. This limitation may in future be overcome by improved vector transduction efficiency and specificity and expression of more therapeutic recombinant transgenes from knowledge of the precise molecular pathogenesis of vasospasm. Furthermore, pertaining to reasons for the absence of protection from angiographic spasm in the study by Stoodley et al, those investigators used a lower titer of AdeNOS (1.2 x 10^9 PFU/dog) compared with that used in the present work (5 x 10^9 PFU/dog) and delivered vector simultaneously with the first blood injection; in our study vector was delivered 24 hours before the first blood injection. In fact, Toyoda et al delivered adenoviral vector up to 5 days before blood injection in their rabbit SAH model. It is possible that any beneficial vasoprotective effect to be gained at the lower titer used by Stoodley et al was reduced even further by the concomitant presence of blood, which may have impaired transduction partly by rapidly forming a mechanical barrier around the brain stem and basilar artery (V.G. Khurana, MD, PhD, Z.S. Katusic, MD, PhD, unpublished data, 2001).

Vasomotor Function
Endothelium-dependent relaxations were considerably augmented in arteries harvested from AdeNOS-transduced dogs. This finding, consistent with previous canine studies, is attributable to coupling generated between bradykinin receptors present in adventitial fibroblasts and biosynthesis of recombinant eNOS in these cells. To our knowledge, vasomotor responses to this peptide have not been reported at day 7 in any SAH model. Evidence for augmented relaxations to the endothelium-independent NO donor, DEANONOate, was also found in AdeNOS-transduced dogs. This finding may be explained by additional relaxing effects conferred by the presence of basally active eNOS exclusively in this group of animals. The possibility of some NO contributed by concomitant iNOS induction cannot be excluded, although this is unlikely to have occurred exclusively in AdeNOS-transduced animals. Interestingly, we observed small but significant augmentations of relaxations to bradykinin and DEANONOate in arteries harvested from AdLacZ-transduced animals compared with arteries from control animals. This may be partly a result of effects on signal transduction pathways caused by β-galactosidase or of effects of increased NO biosynthesis in these arteries from iNOS induction, the occurrence of which is supported by our biochemical data. However, we do not have direct evidence for these possibilities at present.

Contractions to ET-1, a putative vasospasm mediator, were significantly attenuated in arteries from AdeNOS-transduced dogs. A similar finding was reported in an ex vivo basilar artery gene transfer study by Onoue et al who postulated that because activation of fibroblast ET\(_{\alpha}\) receptors causes increased intracytosolic calcium levels, ET-1 may stimulate recombinant eNOS (a calcium-activated enzyme) through an ET\(_{\alpha}\) receptor-mediated pathway, thereby facilitating NO production by adventitial fibroblasts and paradoxically protecting against contractions. It is therefore apparent that functional vasoprotection conferred by recombinant eNOS expression in AdeNOS-transduced arteries is mediated through both augmentation of relaxation and attenuation of contraction. Finally, we recognize that enhanced relaxation
responses to bradykinin were greater in absolute magnitude than reduced contractile responses to ET-1 in AdeNOS-transduced animals; this discrepancy may be partly related to differences in the number, spatial distribution, and activation of bradykinin versus ET-1 receptors.

Benefits and Limitations of Gene Therapy Model

Pertaining to the present vasospasm model, the following benefits deserve mention. First, canine and simian vasospasm models most closely mimic human vasospasm from the perspectives of both time-course (peak incidence at day 7) and magnitude (30% to 50%). Second, gene transfer approaches involving intracisternal delivery have the advantage of diffuse transgene expression, particularly beneficial in vasospasm, which likely affects both large and small arteries across the cerebrum10 and not simply the arteries closest to a ruptured aneurysm. Third, the transient nature of recombinant protein expression by adenoviral vectors9 may be advantageous in vasospasm whose natural history is transient, measured in days to a few weeks.

Certain limitations of the present model also deserve mention. First, our vector, an early-generation adenovirus,3 is immunogenic, and relatively high titers (>10^9 PFU/dog) are required to produce functional benefits. At higher titers (eg, 10^10 PFU/dog), such vectors may be fatal, as observed in a pilot study that inadvertently resulted in the deaths of 3 of the 4 dogs (V.G. Khurana, MD, PhD, Z.S. Katusic, MD, PhD, unpublished data, 2000). Second, although diffuse cerebrovascular expression of therapeutic transgenes may be needed in post-SAH vasospasm, undesirable collateral neuronal transduction may occur after intracisternal vector delivery. This underscores the need to develop accurate and efficient delivery systems and specifically targetable vectors before this technology can be used clinically. Third, regarding the clinical value of eNOS gene transfer, we acknowledge that NO bioavailability may be one of several factors contributing to the vascular dysfunction seen in vasospasm. However, our data are encouraging inasmuch as they suggest that eNOS overexpression is associated with vasoprotection in the setting of post-SAH vasospasm. Fourth, we recognize that delivery of vector 24 hours in advance of the first blood injection, the equivalent of therapeutic intervention 24 hours before aneurysmal rupture, does not accurately reflect the clinical situation in aneurysmal SAH where treatment follows the ictus. In our study, vector was delivered relatively “early” to test the hypothesis that AdeNOS can be used to favorably modify the natural history of canine post-SAH vasospasm. Administration of vector in the absence of blood may be akin to vector delivery into the CSF or directly onto at-risk vessels nearest the ruptured aneurysm after the amount of subarachnoid blood has been reduced by operative clot removal and subarachnoid space CSF irrigation during aneurysm surgery. Last, there remains a need to distinguish between the role of iNOS versus recombinant eNOS in this experimental paradigm because it is conceivable that increased expression of iNOS in the central nervous system can contribute to increased CSF NO metabolites, decreased vasospasm, and altered responses to NO-related vasoactive substances. Despite the aforementioned limitations, we believe that systematic evolution of gene transfer technology holds great promise for the successful treatment of diseases across the entire clinical spectrum.


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