Expression and Function of Recombinant Endothelial Nitric Oxide Synthase Gene in Canine Basilar Artery After Experimental Subarachnoid Hemorrhage

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**Background and Purpose**—Gene transfer with recombinant viral vectors encoding vasodilator proteins may be useful in therapy of cerebral vasospasm after subarachnoid hemorrhage (SAH). Relaxations mediated by nitric oxide are impaired in cerebral arteries affected by SAH. The present study was designed to determine the effect of SAH on the efficiency of ex vivo adenovirus-mediated gene transfer to canine basilar arteries and to examine whether expression of recombinant endothelial nitric oxide synthase (eNOS) gene may have functional effects on vasomotor reactivity of spastic arteries affected by SAH.

**Methods**—Replication-deficient recombinant adenovirus vectors encoding bovine eNOS (AdCMVeNOS) and *Escherichia coli* β-galactosidase (AdCMVβ-Gal) genes were used for ex vivo gene transfer. Rings of basilar arteries obtained from control dogs and dogs exposed to SAH were incubated with the vectors in minimum essential medium. Twenty-four hours after gene transfer, expression and function of the recombinant genes were evaluated by (1) histochemical or immunohistochemical staining, (2) β-galactosidase protein measurement, and (3) isometric tension recording.

**Results**—Transduction with AdCMVβ-Gal and AdCMVeNOS resulted in the expression of recombinant β-galactosidase and eNOS proteins mostly in the vascular adventitia. The expression of β-galactosidase protein was 2-fold higher in SAH arteries than in normal arteries. Endothelium-dependent relaxations caused by bradykinin and substance P were suppressed in SAH arteries. The relaxations to bradykinin were significantly augmented in both normal and SAH arteries after AdCMVeNOS transduction but not after AdCMVβ-Gal transduction. The relaxations to substance P were augmented by AdCMVeNOS transduction only in normal arteries. Bradykinin and substance P caused relaxations even in endothelium-denuded arteries, when the vessels were transduced with AdCMVeNOS. These endothelium-independent (adventitia-dependent) relaxations to bradykinin observed after AdCMVeNOS transduction were similar between normal and SAH arteries, whereas those to substance P were significantly reduced in SAH arteries compared with normal arteries.

**Conclusions**—These results suggest that expression of recombinant proteins after adenovirus-mediated gene transfer may be enhanced in cerebral arteries affected by SAH and that successful eNOS gene transfer to spastic arteries can at least partly restore the impaired nitric oxide–mediated relaxations through local (adventitial) production of nitric oxide. (Stroke. 1998;29:1959-1966.)

**Key Words:** cerebral vasospasm ■ gene therapy ■ genetic vectors ■ nitric oxide synthase ■ subarachnoid hemorrhage

**D**elayed cerebral vasospasm is a major cause of morbidity and mortality in patients with subarachnoid hemorrhage (SAH).1,2 The exact mechanism underlying pathogenesis of vasospasm is not completely understood; however, existing evidence suggests that impaired function of the l-arginine–nitric oxide (NO) pathway in the vascular endothelium and perivascular nerves may contribute to the reduced NO-mediated vasodilatation and the development of arterial spasm after SAH.2-4 Consistent with this concept are previous findings demonstrating that experimental vasospasm could be reversed by intracarotid infusion of NO,5 intravenous admin-
arteries and that expression of eNOS gene in the adventitia can modulate vascular tone. However, it has not been determined whether recombinant eNOS protein can be functionally expressed in the diseased cerebral arteries affected by SAH. Therefore, the present study was designed to compare the efficiency of ex vivo adenovirus-mediated gene transfer between basilar arteries obtained from control dogs and dogs exposed to SAH and to examine whether transduced eNOS gene may have functional effects on vasomotor reactivity of spastic cerebral arteries.

Materials and Methods

Construction, Propagation, and Purification of Adenoviral Vectors

The adenoviral vector encoding an eNOS gene (AdCMV eNOS), driven by the cytomegalovirus immediate early promoter, was generated through homologous recombination. These vectors, based on serotype 5 wild adenovirus, have been made replication deficient by a deletion of the early region 1, which was replaced by a cDNA sequence encoding bovine aortic endothelial cell eNOS (kindly provided by Dr David G. Harrison, Emory University, Atlanta, Ga). The generation, propagation, purification, and evaluation of the adenoviral vector containing eNOS gene were described in detail previously. The recombinant adenoviral vector encoding β-galactosidase gene driven by cytomegalovirus promoter (AdCMV β-Gal), used in all experiments as control, was a kind gift of Dr James M. Wilson (University of Pennsylvania, Philadelphia).

Experimental Model of SAH

Mongrel dogs of either sex weighing 12 to 17 kg were used for experiments. Induction of SAH followed by cerebral vasospasm was conducted as described in our previous study. Under general anesthesia with 15 mg/kg IV sodium thiopental, the cisterna magna was aseptically punctured with a spinal needle (No. 22), and 5 mL anesthetic arterial blood was injected through the spinal needle over 2 minutes. After anesthesia with 15 mg/kg IV sodium thiopental, the cisterna magna was repeated in the same manner. Seven days after the first injection (on day 7), the animals were killed to isolate the brain. Two days later (on day 2), the injection of venous blood was injected through the spinal needle over 2 minutes. After 15 minutes in the head-down position, the animal was allowed to recover. Two days later (on day 2), the injection of venous blood into the cisterna magna was repeated in the same manner. Seven days after the first injection (on day 7), the animals were killed to isolate basilar arteries. Because the identical procedures evoked reproducible vasospasm (diameter of basilar artery on day 7 was 57±7% of diameter before intracisternal injection of blood [n=6]), angiography was not performed in the present study. All the procedures and handling of the animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Foundation.

Gene Transfer

Rings (3 mm long) of basilar arteries were taken from control dogs and dogs exposed to SAH, anesthetized with 30 mg/kg IV sodium pentobarbital, and killed by bleeding from the carotid arteries. To remove intraluminal blood, arterial rings were gently rinsed with cold modified Krebs-Ringer bicarbonate solution (control solution; pH 7.4, 37°C) aerated with 95% O2/5% CO2. Isometric tension was recorded continuously. The rings were allowed to stabilize at a resting tension of 0.2 to 0.4 g for 1 hour. Each ring was then gradually stretched to the optimal point of its length-tension curve (∼3.0 g) as determined by the contraction to 10−3 mol/L UTP. All experiments were conducted in the presence of 10−5 mol/L indomethacin to eliminate the possible influence of endogenous cyclooxygenase. To evaluate relaxation responses, the rings were contracted with UTP (3×10−6 to 3×10−3 mol/L) before the addition of agonists. Each concentration was used 15 minutes before the concentration-response curve for each vasodilator agent was obtained.

Histochemical and Immunohistochemical Analyses of Gene Expression

For histochemical staining of β-galactosidase, the rings of basilar arteries were fixed for 30 minutes in 2% paraformaldehyde/0.2% glutaraldehyde in PBS. After they were rinsed with PBS, the rings were placed in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside reagent for 2 hours. The stained vessels were frozen in O.C.T. compound (Miles, Inc). Serial 5-μm cross sections were cut from each arterial and counterstained for nuclei. For immunohistochemical staining of recombinant eNOS, arterial rings were frozen in O.C.T. compound, and serial 5-μm cross sections were cut. After immersion fixation in acetone (4°C), the sections were incubated in 0.1% sodium azide/0.3% hydrogen peroxide and then incubated with 5% normal goat serum/PBS-Tween 20 to block the nonspecific protein binding sites. A monoclonal antibody for eNOS (5 μg/mL, 1:50 of stock; Transduction Laboratory) was applied for 60 minutes at room temperature, followed by incubations with biotinylated rabbit anti-mouse F(ab)’2 (1:200, 20 minutes) secondary antibody and peroxidase-conjugated streptavidin (1:300, 20 minutes) (Vector Laboratories, Inc). A 30-second immersion in 0.1 mol/L sodium acetate buffer (pH 5.2), eNOS immunoreactivity was visualized with 3-amino-9-ethylcarbazole and hematoxylin counterstaining.

Quantitative Analysis of β-Galactosidase Protein

Expression of β-galactosidase protein in vessels was quantified by ELISA. Twenty-four hours after gene transfer, arterial rings were homogenized in a solution containing 0.1 mol/L K3HPO4, 0.2% Triton X-100, and 10−3 mol/L phenylmethylsulfonyl fluoride, pH 7.8 at 4°C. After centrifugation at 12 000g for 10 minutes, β-galactosidase protein levels in supernatants were determined using the use of β-galactosidase ELISA kit (5 Prime 3 Prime, Inc). Total protein levels in the supernatants were measured by DC Protein Assay Kit (Bio-Rad).

Analyses of Vascular Reactivity

Twenty-four hours after gene transfer, each ring was connected to an isometric force-displacement transducer (Grass FT03; Grass Instrument Co) and suspended in an organ chamber filled with 25 mL modified Krebs-Ringer bicarbonate solution (control solution; pH 7.4, 37°C) aerated with 95% O2/5% CO2. Isometric tension was recorded continuously. The rings were allowed to stabilize at a resting tension of 0.2 to 0.4 g for 1 hour. Each ring was then gradually stretched to the optimal point of its length-tension curve (∼3.0 g) as determined by the contraction to 10−3 mol/L UTP. All experiments were conducted in the presence of 10−5 mol/L indomethacin to eliminate the possible influence of endogenous cyclooxygenase. To evaluate relaxation responses, the rings were contracted with UTP (3×10−6 to 3×10−3 mol/L) before the addition of agonists. Care was taken to match the contractions induced with UTP in control and SAH rings. Concentration-response curves were obtained in a cumulative fashion. Several rings prepared from the same artery were studied in parallel. The relaxations were expressed as a percentage of maximal relaxations induced by 3×10−4 mol/L papaverine.

Drugs

The following pharmacological agents were used: UTP, bradykinin, substance P, Nnitr-o-l-arginine methyl ester (L-NNAME), indomethacin, papaverine hydrochloride (Sigma), and diethylamine NONOate (DEA-NONOate; Cayman Chemical Co). Drugs were dissolved in distilled water so that volumes of <0.15 mL were added to the organ chambers. Concentrations of all drugs are expressed as final molar (mol/L) concentration in the control solution. For experiments with L-NNAME, L-NNAME was added 15 minutes before the concentration-response curve for each vasodilator agent was obtained.
Results

Effect of SAH on Expression of β-Galactosidase Reporter Gene

Expression of β-galactosidase reporter gene in the vessel wall was compared between normal (untreated) and SAH arteries by quantitative measurements of β-galactosidase protein. Twenty-four hours after AdCMVβ-Gal transduction, β-galactosidase protein levels were significantly higher (≈2-fold) in SAH arteries than in normal arteries (Figure 1). In nontransduced control arteries, β-galactosidase protein levels were not different between normal and SAH arteries.

Histochemical and Immunohistochemical Analyses of Gene Expression in Basilar Artery After SAH

Twenty-four hours after AdCMVβ-Gal transduction, recombinant β-galactosidase protein was expressed mainly in the adventitia of a basilar artery exposed to SAH (Figure 2A and 2B). Transgene expression of eNOS was also observed mostly in the adventitia of an AdCMVeNOS-transduced artery (Figure 2C and 2D).

Effect of SAH on Relaxations of Basilar Artery

During contractions induced by UTP, bradykinin (10^{-11} to 10^{-7} mol/L) and substance P (10^{-11} to 10^{-8} mol/L) caused concentration-dependent relaxations in basilar arteries with endothelium. These relaxations were significantly reduced in arteries obtained from SAH dogs (Figure 3A and 3B). In contrast, relaxations to DEA-NONOate, a donor of NO, were not affected by SAH (Figure 3C).

Statistical Analysis

The results are expressed as mean±SEM; n refers to the number of animals studied. Each set of concentration-response curves was statistically analyzed by repeated-measures ANOVA. Levels of β-galactosidase were compared with ANOVA with the Bonferroni/Dunn post hoc test. Statistical significance was accepted at the level of P<0.05.
Effect of Adenovirus-Mediated Gene Transfer on Relaxations of Basilar Artery

The relaxations to bradykinin were significantly augmented in AdCMVeNOS-transduced basilar arteries with endothelium obtained from normal dogs, whereas they were not altered in AdCMVβ-Gal–transduced arteries (Figure 4A). This augmentation of bradykinin-induced relaxations after eNOS gene transfer was also detected in arteries exposed to SAH (Figure 4B). The relaxations to substance P were also augmented in AdCMVeNOS-transduced arteries obtained from normal dogs (Figure 5A); however, no significant augmentation was detected in the relaxations to substance P in SAH arteries (Figure 5B). The relaxations caused by DEA-NONOate were not affected by gene transfer in both normal and SAH arteries (Figure 6A and 6B).

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The relaxations to bradykinin were significantly augmented in AdCMVeNOS-transduced basilar arteries with endothelium obtained from normal dogs, whereas they were not altered in AdCMVβ-Gal–transduced arteries (Figure 4A). This augmentation of bradykinin-induced relaxations after eNOS gene transfer was also detected in arteries exposed to SAH (Figure 4B). The relaxations to substance P were also augmented in AdCMVeNOS-transduced arteries obtained from normal dogs (Figure 5A); however, no significant augmentation was detected in the relaxations to substance P in SAH arteries (Figure 5B). The relaxations to bradykinin and substance P observed in AdCMVeNOS-transduced arteries were abolished by treatment with L-NAME (3×10⁻⁴ mol/L), an inhibitor of nitric oxide synthase (NOS) (Figures 4 and 5). The relaxations caused by DEA-NONOate were not affected by gene transfer in both normal and SAH arteries (Figure 6A and 6B).

Relaxations to Bradykinin and Substance P in Endothelium-Denuded Basilar Arteries after Adenovirus-Mediated Gene Transfer

In AdCMVβ-Gal–transduced arteries without endothelium obtained from both normal and SAH dogs, bradykinin and substance P did not induce any relaxations (Figure 7A and 7B). However, these endothelium-dependent vasodilator peptides caused relaxations in AdCMVeNOS-transduced arteries even after the removal of endothelium. The relaxations induced by bradykinin after endothelial removal were similar between arteries obtained from normal dogs and dogs exposed to SAH (Figure 7A). In contrast, the relaxations induced by substance P were significantly reduced in SAH arteries compared with normal arteries (Figure 7B).

Discussion

In the present study we demonstrated, for the first time, functional expression of recombinant eNOS gene in cerebral arteries affected by SAH. Transgene expression was mainly
localized in the adventitial layer of basilar arteries. Efficiency of ex vivo adenovirus-mediated transfer of β-galactosidase gene appeared greater in arteries exposed to SAH than in normal (untreated) arteries. Our results also suggest that expression of recombinant eNOS protein in SAH arteries could restore impaired endothelium-dependent relaxations through NO release from the adventitia.

Successful gene transfer with subsequent expression of recombinant proteins in canine basilar arteries exposed to SAH was assessed by recombinant proteins in canine basilar arteries exposed to SAH through NO release from the adventitia. Consistent with our previous findings on normal adventitia, positive staining of recombinant proteins was detected predominantly in the vascular eNOS immunohistochemistry. Positive staining of recombinant proteins in SAH arteries might affect the endogenous eNOS gene expression, consistent with our previous findings on normal canine cerebral arteries.10–12 The expression of recombinant eNOS protein in SAH arteries could restore impaired endothelium-dependent relaxations through NO release from the adventitia.

The relaxations induced by papaverine (3×10^{-4} mol/L) on the relaxations of eNOS gene–transduced arteries with inflammatory response and resultant fibrosis were detected in the tunica adventitia.16–19 In our previous gene transfer studies on normal canine cerebral arteries, electron microscopy immunogold labeling indicated that expression of recombinant eNOS protein was localized to adventitial fibroblasts.11,12 Therefore, it is possible that the enhanced transgene expression in SAH arteries might result from increased proliferation of adventitial fibroblasts. Alternatively, upregulation of adenosine receptor expression or increased activity of the cytomegalovirus promoter may also explain enhanced transgene expression after SAH.

Both bradykinin and substance P are endogenous peptides and cause endothelial NO-mediated relaxations in canine cerebral arteries.20,21 The relaxations to bradykinin, especially the relaxations to bradykinin and substance P were reduced in basilar arteries affected by SAH.

Figure 5. Relaxation responses to substance P in nontransduced (Control), AdCMVβ-Gal–transduced (β-Gal), and AdCMV(eNOS)–transduced (eNOS) basilar arteries with endothelium obtained from untreated (Normal) dogs (A) and dogs exposed to SAH (B). Effect of L-NAME (3×10^{-4} mol/L) on the relaxations of eNOS gene–transduced arteries is also presented. Relaxations were obtained during contractions induced by UTP. Data are shown as mean±SEM and are expressed as percentage of maximal relaxations induced by papaverine (3×10^{-4} mol/L); 100% = 2.7±0.4 g (n=7), 2.2±0.3 g (n=7), 2.2±0.3 g (n=7), and 2.3±0.4 g (n=5; A), and 2.3±0.3 g (n=5; B), 1.8±0.2 g (n=7), 1.7±0.3 g (n=7), and 2.5±0.4 g (n=7; B), for Control, β-Gal, eNOS, and eNOS plus L-NAME, respectively. *Significantly different from β-galactosidase reporter gene–transduced arteries; **P<0.05 by repeated-measures ANOVA.
These findings demonstrate that eNOS gene transfer selectively augmented the relaxations to the endothelium-dependent vasodilator (bradykinin) in spastic cerebral arteries as well as normal arteries. Although a previous study has reported that adenoviral vectors may cause an inflammatory response and inhibit endothelium-dependent relaxations, endothelial dysfunction and altered reactivity of smooth muscle were not observed in our experiments. The augmented relaxations to bradykinin in AdCMVeNOS-transduced arteries were abolished by L-NAME, a NOS inhibitor, reinforcing our conclusion that activation of recombinant eNOS protein is responsible for increased formation of NO.

The relaxations to bradykinin and substance P in canine cerebral arteries are dependent on the presence of endothelium, because these peptides did not cause any relaxations in nontransduced and AdCMVβ-Gal-transduced basilar arteries without endothelium. However, bradykinin and substance P induced relaxations in AdCMVeNOS-transduced arteries, even after the endothelium was removed. This is consistent with our previous results demonstrating a restoration of relaxations to bradykinin in AdCMVeNOS-transduced cerebral arteries without endothelium. The relaxations induced by bradykinin and substance P in endothelium-denuded arteries are best explained by activation of recombinant eNOS enzyme and subsequent NO production in the adventitia. In addition, the relaxations to an NO donor, DEA NONOate, were not affected by SAH, suggesting that the vasoreactivity to exogenous NO is not impaired by exposure to autologous blood followed by adenovirus-mediated gene transfer. Therefore, the relaxations to bradykinin and substance P observed in endothelium-denuded arteries are thought to reflect the activation of recombinant eNOS in normal and SAH arteries. The relaxations to bradykinin in AdCMVeNOS-transduced arteries without endothelium were similar between normal and SAH arteries, and those to substance P were reduced in SAH arteries. This may explain our results demonstrating that a significant augmentation of relaxations to substance P after eNOS gene transfer was detected only in normal arteries but not in SAH arteries with endothelium. Although in the target cells (presumably adventitial fibroblasts) precise cellular mechanisms underlying response to both peptides have not been fully understood, the selective reduction of substance P–induced relaxations in SAH arteries suggests that prolonged SAH may have a deferal effect on the signal transduction pathways, including receptor function activated by bradykinin and substance P. Significantly augmented expression of recombinant β-galactosidase protein in SAH arteries suggests that higher expression of recombinant eNOS may also be present in SAH arteries. However, we did not quantify the expression of eNOS protein in transduced arteries, and the presented results do not allow any conclusion regarding the augmentation of eNOS gene transfer efficiency or increase in expression of recombinant eNOS protein in SAH arteries.

Gene transfer with recombinant viral vectors encoding vasodilator proteins may be useful in therapy of cerebral vasospasm after SAH. The narrowing of cerebral arteries exposed to autologous blood is associated with an impaired function of the endothelial L-arginine–NO pathway, decreased eNOS messenger RNA level, and loss of perivascular neuronal NOS immunoreactivity. The present study indicates that recombinant eNOS protein can be successfully expressed after adenovirus-mediated gene transfer in the diseased cerebral arteries affected by SAH and that adventitial expression of eNOS gene can, at least in part, restore the impaired NO-mediated relaxations to endogenous vasodilators. Cerebral vasospasm becomes clinically evident within 10 days after the onset of SAH and continues for several days. Transgene expression in the vascular wall could be maintained 14 days after adenoviral transduction. Limited duration of transgene expression, a primary problem of gene therapy for chronic diseases, may be advantageous in the treatment of transient narrowing of cerebral arteries. In addition, a recent study has demonstrated that perivascular expression of recombinant β-galactosidase could be obtained by intracisternal delivery of adenoviral vectors in dogs with SAH. Thus, functional expression of recombinant eNOS gene in cerebral arteries may provide a novel approach for the prevention and/or treatment of cerebral vasospasm.

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References


Editorial Comment

Production and release of NO by endothelium play a major role in vascular biology. The source of NO in endothelium of normal blood vessels is the endothelial isoform of NOS (eNOS).1 NO is a potent vasodilator that binds to and activates soluble guanylate cyclase in vascular muscle, resulting in relaxation.2 Many studies, including studies in humans and gene-targeted mice, indicate that NO is a major mediator of endothelium-dependent relaxation and an important regulator of vascular growth.3 Several disease states, including SAH, are associated with impairment of this NO signaling pathway.4 Although responses of cerebral arteries to NO may be attenuated after SAH, some studies suggest that administration of exogenous NO can inhibit vasospasm after SAH.2

Some previous studies have used molecular approaches in an attempt to inhibit vasospasm after SAH. These approaches include the use of oligonucleotides with high affinity for nuclear factor-κB to act as “decoy DNA”5 and antisense oligonucleotides for mRNA for preproendothelin-1.6 The present study used a new approach—adenoviral-mediated gene transfer—to overexpress eNOS and thus increase pro-
duction of NO in cerebral arteries after SAH. There are several interesting new findings in the study. First, the efficacy of ex vivo gene transfer (as indicated by levels of activity of the reporter gene, β-galactosidase) is increased in the basilar artery after SAH. Expression of β-galactosidase occurred predominately in adventitia. Second, relaxation of the basilar artery in response to bradykinin (which normally causes release of NO from endothelium) was increased in arteries from control animals and animals exposed to SAH. After gene transfer of eNOS, relaxation in response to bradykinin occurred even in arteries without endothelium, suggesting that the response was mediated by cells in the adventitia. Thus, after gene transfer of eNOS, cells in the adventitia may function as an important source of NO in normal and diseased blood vessels.

Although current adenoviral vectors have limitations that prevent their therapeutic use, the present results nonetheless illustrate the potential for a gene transfer approach to alter vascular function after SAH. A key question in relation to SAH is whether the quantity of NO produced under basal conditions (ie, in the absence of bradykinin or other exogenous stimuli) after gene transfer of eNOS in vivo is sufficient to alter vascular tone and inhibit vasospasm. Interestingly, recombinant eNOS expressed in adventitia may also be activated by endothelin-1, and levels of endothelin in vessels and cerebrospinal fluid are increased after SAH. Thus, one could speculate that production of endothelin, which may contribute to development or maintenance of vasospasm under normal conditions, may be paradoxically protective after gene transfer of eNOS to blood vessels by activating eNOS in adventitia.

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