Gene Transfer of Calcitonin Gene–Related Peptide Prevents Vasoconstriction After Subarachnoid Hemorrhage

Kazunori Toyoda, Frank M. Faraci, Yoshimasa Watanabe, Toshihiro Ueda, Jon J. Andresen, Yi Chu, Shoichiro Otake, Donald D. Heistad

Abstract—We sought to determine whether adenovirus-mediated gene transfer in vivo of calcitonin gene–related peptide (CGRP), a potent vasodilator, ameliorates cerebral vasoconstriction after experimental subarachnoid hemorrhage (SAH). Arterial blood was injected into the cisterna magna of rabbits to mimic SAH 5 days after injection of AdRSVCGRP (8×10⁹ pfu), AdRSVβgal (control virus), or vehicle. After injection of AdRSVCGRP, there was a 400-fold increase in CGRP in cerebrospinal fluid. Contraction of the basilar artery to serotonin in vitro was greater in rabbits after SAH than after injection of artificial cerebrospinal fluid (P<0.001). Contraction to serotonin was less in rabbits with SAH after AdRSVCGRP than after AdRSVβgal or vehicle (P<0.02). Basal diameter of the basilar artery before SAH (measured with digital subtraction angiogram) was 13% greater in rabbits treated with AdRSVCGRP than in rabbits treated with vehicle or AdRSVβgal (P<0.005). In rabbits treated with vehicle or AdRSVβgal, arterial diameter after SAH was 25±3% smaller than before SAH (P<0.0005). In rabbits treated with AdRSVCGRP, arterial diameter was similar before and after SAH and was reduced by 19±3% (P<0.01) after intracisternal injection of CGRP-(8-37) (0.5 nmol/kg), a CGRP receptor antagonist. To determine whether gene transfer of CGRP after SAH may prevent cerebral vasoconstriction, we constructed a virus with a cytomegalovirus (CMV) promoter, which results in rapid expression of the transgene product. Treatment of rabbits with AdCMVCGRP after experimental SAH prevented constriction of the basilar artery 2 days after SAH. Thus, gene transfer of CGRP prevents cerebral vasoconstriction in vivo after experimental SAH. (Circ Res. 2000;87:818–824.)

Key Words: gene therapy ■ cerebral vasospasm ■ vasodilation ■ neuropeptide ■ rabbit

Cerebral vasospasm is a delayed, prolonged arterial constriction that follows subarachnoid hemorrhage (SAH), which can lead to brain ischemia and infarction.¹ Vasoconstriction occurs in 30% to 40% of patients after SAH1 and is the leading cause of mortality (28%) and morbidity (39%) in patients after SAH.² Several mechanisms may contribute to vasoconstriction after SAH, including impaired endothelium-dependent relaxation; production of endothelium-derived contracting factors, including endothelin; and impaired activity of potassium channels in cerebral blood vessels.³ Cerebral vascular muscle is depolarized after SAH, most likely as a result of inhibition of potassium channels, and this depolarization is thought to contribute to vasospasm.⁴ Although several dilator mechanisms, including NO and nitrovasodilators, are impaired after experimental SAH in most studies,⁵–⁷ dilation of large cerebral arteries is not impaired in response to synthetic (aprikalim and cromakalim) and endogenous (calcitonin gene-related peptide [CGRP]) openers of potassium channels.⁶–⁸ Thus, we speculated that openers of potassium channels in vascular muscle may prevent vasospasm after SAH.

CGRP is an extremely potent vasodilator, which hyperpolarizes arterial muscle at least in part via opening of potassium channels.⁹,¹⁰ The peptide is abundant in perivascular nerve fibers surrounding cerebral blood vessels,¹¹ and depletion of CGRP may have functional effects in the cerebral circulation after SAH.¹²,¹³ Intrathecal and intravenous injection of exogenous CGRP increases arterial diameter in animals with vasospasm after experimental SAH.¹⁴–¹⁶ Thus, it seemed likely that, if a sufficient amount of CGRP is released around cerebral arteries after SAH, vasospasm might be prevented.

Gene transfer to vascular adventitia via cerebrospinal fluid (CSF) is an appealing strategy to modulate cerebral vascular function.¹⁷,¹⁸ We made a recombinant adenovirus that encodes prepro-CGRP and reported that gene transfer via CSF in normal rabbits expresses biologically active CGRP, increases the level of cAMP (a second messenger for the vasodilator response to CGRP) in the basilar artery, and attenuates contraction of the artery in vitro.¹⁷ Exposure of canine cerebral arteries to blood in vivo does not diminish, or may even augment, efficiency of expression after gene transfer to vascular adventitia via CSF.¹⁷,¹⁸ Therefore, it seemed likely that gene transfer of CGRP might be effective in preventing vasospasm after SAH.
transfer in vivo and in vitro. Thus, we speculated that perivascular application in vivo of a virus that expresses CGRP might prevent constriction of cerebral arteries after SAH. The goal of this study was to test the hypothesis that gene transfer of CGRP in vivo ameliorates cerebral vasoconstriction after SAH.

Materials and Methods

We made a replication-deficient adenovirus encoding prepro-CGRP (AdCGRP) or β-galactosidase (Adβgal) with a Rous sarcoma virus (RSV) promoter. We also constructed an adenovirus with a cytomegalo-virus (CMV) promoter encoding prepro-CGRP (AdCMVCGRP) and used AdCMVβgal. Male New Zealand White rabbits weighing 2.4 to 3.2 kg were anesthetized intramuscularly with xylazine and ketamine. A needle was inserted into the cisterna magna, 250 μL of CSF was withdrawn, and 250 μL of viral suspension (PBS with 3% sucrose with 3×10^9 pfu/mL virus) or vehicle (PBS with 3% sucrose) was infused.

Experimental SAH was produced in rabbits 5 days after injection of virus (AdRSVCGRP or AdRSVβgal) or vehicle. The anesthetized rabbit was placed in a prone position, and a needle was inserted into the cisterna magna. After withdrawal of 1 mL of CSF, 1 mL/kg of fresh, nonheparinized autologous arterial blood was injected.

Two days after experimental SAH (day 7), rabbits were anesthetized with pentobarbital IV and exsanguinated. Basilar arteries were removed and cut into rings (3 mm in length) for recording of isometric tension to examine vascular reactivity. Contraction to KCl, serotonin, and histamine was examined. Contraction was expressed as a percentage of response to 40 nmol/L KCl. Relaxation to acetylcholine and sodium nitroprusside was examined after vasorelaxation with 1 to 3 μmol/L of histamine. Relaxation was expressed as percentage of precontraction. In separate experiments, arterial rings were examined for relaxation to synthetic CGRP 2 days after injection of arterial blood or artificial CSF (without gene transfer).

Digital subtraction angiography was performed immediately before and 2 days after experimental SAH in rabbits treated with virus. The anesthetized rabbit was placed in a supine position, and an angiocatheter was introduced through the femoral artery. The anesthetized rabbit was placed in a supine position, and an angiocatheter was introduced through the femoral artery to measure systemic blood pressure. Then the angiocatheter was introduced through the femoral artery of the rabbit and advanced into the left vertebral artery, and angiography into the thoracic aorta was performed immediately before injection of blood to mimic SAH.

Results

Effects of SAH or Gene Transfer on Release of CGRP Into CSF

Before studying rabbits after experimental SAH treated with adenoviral vectors, we performed experiments to determine effects of experimental SAH or gene transfer alone on expression of CGRP in CSF. CGRP immunoreactivity was measured in CSF from rabbits after injection of blood into CSF without gene transfer (Figure 1A). Peak concentration of CGRP 30 minutes after injection of blood was 0.17±0.10 (mean±SEM) nmol/L. Concentration of CGRP in serum from the same rabbits was 0.44±0.09 nmol/L.

CGRP concentration was measured in CSF from rabbits treated with AdRSVCGRP without experimental SAH (Figure 1B). Between 3 and 7 days after injection of the virus, concentration of CGRP exceeded 1 nmol/L, with peak expression of CGRP 5 days after injection of the virus (2.2±0.9 nmol/L). This course of expression of the transgene product in CSF was similar to the previous finding of expression in other tissues after injection of virus with the RSV promoter. On the basis of this result, we injected blood into the cisterna magna of rabbits 5 days after injection of virus in the following study.

Gene Transfer In Vivo

We injected adenovirus into CSF of rabbits, then injected blood to mimic SAH 5 days later (day 5), and performed final assays 2 days later (day 7). All rabbits tolerated injection of adenovirus without detectable neurological deficits. Immediately after injection of blood, almost all rabbits developed transient apnea. Seven of 53 rabbits (13%) died within a few minutes after injection of blood. Data from those rabbits were excluded. Rectal temperature was 39.3±0.1°C before injection of virus, 39.4±0.1°C before injection of blood (day 5), and 39.4±0.1°C on day 7. Leukocyte count in CSF increased from 3×10^6/mm^3 to 327±59/mm^3 5 days after injection of AdRSβgal (before injection of blood) and 417±76/mm^3 5 days after AdRSVCGRP. We did not measure leukocyte count on day 7, because contamination with blood affected the counts.

In rabbits treated with AdRSβgal or vehicle, baseline concentration of CGRP on day 0 was minimal (0.006±0.003

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Concentration of CGRP in CSF of rabbits. A, After experimental SAH without gene transfer (n=6). B, After gene transfer with AdRSVCGRP in normal rabbits (n=8). C, Before injection of virus or vehicle (day 0) and before (day 5) and 2 days after (day 7) experimental SAH. \*P<0.0001 vs vehicle or Adβgal (n=14 for vehicle, n=14 for Adβgal, and n=16 for AdCGRP). Scales differ in the 3 panels. Values are mean±SEM.
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With histochemistry, there was extensive staining for β-galactosidase on the ventral surface of the brain from rabbits treated with AdRSVβgal and blood injection into CSF, as we previously demonstrated in other species.2,24,26 Thus, widespread overexpression of CGRP was also expected on the surface of brain after gene transfer of CGRP with experimental SAH.

**Effect of Gene Transfer on Vasoconstriction In Vitro After Experimental SAH**

Contraction to 40 mmol/L KCl was similar in arteries exposed to blood and treated with vehicle (1.48±0.14 g), AdRSVβgal (1.40±0.11 g), and AdRSVCCGRP (1.50±0.12 g), and in arteries from rabbits that received an injection of artificial CSF (instead of blood) into the cisterna magna and treated with vehicle (control rabbits, 1.58±0.23 g).

The contractile response to serotonin (0.01 to 3 μmol/L) was greatly augmented in arteries from rabbits after experimental SAH that were treated with vehicle, compared with control rabbits without SAH (P<0.001 for the entire concentration-response curve; maximal response [Rmax] was 98±7% versus 48±11%, P<0.0005, Figure 2A). Contraction was less after AdRSVCCGRP (Rmax 70±11%) than after vehicle or AdRSVβgal (Rmax 93±8%) (P<0.02 for entire curve; P<0.05 for Rmax).

Contractile responses to histamine (0.03 to 30 μmol/L) were similar in arteries from rabbits after experimental SAH (treated with vehicle) and from control rabbits without SAH (Figure 2B). In arteries treated with AdRSVCCGRP, contraction to 3 and 10 μmol/L histamine was less than after vehicle or AdRSVβgal (P<0.05).

Relaxation to high concentrations of acetylcholine (1 to 10 μmol/L) were attenuated after SAH (in rabbits treated with vehicle) compared with control rabbits (Rmax 78±8% versus 99±1%, P<0.05, Figure 2C). After SAH, relaxation to acetylcholine did not change after AdRSVCCGRP compared with vehicle or AdRSVβgal. Relaxation to nitroprusside was similar among the 4 groups (Figure 2D).

In separate experiments, relaxation to synthetic CGRP (0.03 to 10 μmol/L) was virtually identical between the arteries from rabbits 2 days after experimental SAH and after injection of artificial CSF instead of blood (without gene transfer, Figure 2E).

Thus, vasoconstriction to serotonin, which was augmented after SAH, and vasoconstriction to histamine, which was not augmented, were both attenuated by gene transfer of CGRP. The response to synthetic CGRP was preserved after SAH.

**Effect of Gene Transfer on Vasoconstriction In Vivo After Experimental SAH**

We tested the hypothesis that gene transfer of CGRP prevents constriction of arteries in vivo. First, we determined effects on resting diameter of the basilar artery 5 days after gene transfer (Figure 3A). Injection of AdRSVβgal did not change diameter on day 5 (897±19 μm) compared with vehicle (887±13 μm). In rabbits treated with AdRSVCCGRP, diameter on day 5 (1000±25 μm) was 13% greater than in rabbits treated with vehicle or AdRSVβgal (P<0.005). Thus, gene transfer of CGRP increased resting diameter of the basilar artery.
Arterial diameter was compared on day 7 (2 days after SAH) and on day 5. In rabbits treated with vehicle, diameter was 25 ± 3% smaller on day 7 (670 ± 28 μm) than on day 5 (*P < 0.001, Figures 3A, 4A, and 4B). In rabbits treated with AdRSVβgal, diameter was 23 ± 3% smaller on day 7 (696 ± 31 μm) than on day 5 (*P < 0.001). Vertebral arteries and vessels of the circle of Willis, in addition to the basilar artery, were constricted on day 7 (Figures 4A and 4B). In rabbits treated with AdRSVCGRP, diameter was similar on days 5 and 7 (963 ± 10 μm, *P > 0.1, Figures 3A, 4D, and 4E).

After intracisternal injection of CGRP (0.1 nmol/kg) to rabbits treated with vehicle on day 7, diameter of the constricted basilar artery returned to resting values (1 ± 3%, *n = 5, Figure 4C). After intracisternal injection (0.5 nmol/kg) of a CGRP₁ receptor antagonist, CGRP-(8-37), to rabbits treated with AdRSVCGRP on day 7, the basilar artery constricted by 19 ± 3% (*n = 5, *P < 0.01, Figures 3B and 4F). These findings suggest that either gene transfer of CGRP or an intracisternal bolus injection of synthetic CGRP can prevent vasoconstriction after experimental SAH by an effect on CGRP₁ receptors.

Systemic arterial pressure and arterial blood gases during angiography were not significantly different between treatment groups or at the time of the 2 angiograms in each group (Table). Thus, these physiological factors did not account for differences in arterial diameter.

In the studies described above, we examined effects of treatment with AdRSVCGRP before SAH. In other studies, we examined effects of gene transfer of CGRP after experimental SAH. For these studies, we constructed a recombinant virus with a CMV promoter, because maximal expression is much more rapid with a CMV than an RSV promoter.²⁶ In rabbits treated with vehicle or AdCMVβgal, diameter of the basilar artery was 21 ± 2% smaller 2 days after than before SAH (Figure 5, control). Reduction in diameter after SAH was similar in rabbits treated with vehicle (−23 ± 3%, *n = 4) or AdCMVβgal (−18 ± 2%, *n = 4, *P > 0.1). In rabbits treated with AdCMVCGRP, diameter was similar before and 2 days after SAH (Figure 5). Systemic arterial pressure and arterial blood gases during angiography were not significantly different between treatment groups or at the time of the 2 angiograms in each group (data not shown).

**Discussion**

This is the first successful use of gene transfer of a vasoactive protein or peptide to prevent cerebral vasoconstriction in vivo after experimental SAH. There are 2 major new findings. First, gene transfer of CGRP in vivo prevented excessive contraction of the basilar artery to serotonin in vitro after experimental SAH. Second, gene transfer of CGRP prevented

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**Physiological Parameters During Angiography**

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<td>Day 5</td>
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<tr>
<td>Mean arterial pressure, mm Hg</td>
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<td>Arterial blood gas</td>
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<td>Po₂, mm Hg</td>
<td>66 ± 7</td>
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Values are mean ± SEM.
constriction of the basilar artery in vivo after experimental SAH both when the virus was applied before SAH and after SAH. Thus, gene transfer of CGRP is a promising approach for prevention of vasospasm after SAH.

**Gene Transfer to Cerebral Arteries**

A unique implication of the present study is for gene therapy for cerebrovascular disease. Although clinical trials for patients with coronary and peripheral vascular disease are under way, gene transfer has not received extensive study for even animal models of cerebrovascular diseases. A reason for this lag may be that intravascular gene delivery, which is feasible for coronary and peripheral arteries, has not yet been accomplished for intracranial cerebral arteries, because intravascular approaches require interruption of blood flow to the brain.

As an alternative approach, perivascular gene delivery via CSF can be used for overexpression in the cerebral arteries of vasoactive proteins and peptides, including CGRP. An advantage of perivascular gene delivery by injection into CSF versus intravascular delivery is that expression of transgene products is not limited to small regions but is distributed over an expanded area. Because multiple cerebral arteries both in anterior and posterior circulations are at risk for vasospasm after SAH, perivascular gene delivery may be more beneficial than intravascular delivery for prevention of vasospasm. In a recent study, perivascular application of liposomes with oligonucleotides with high affinity for nuclear factor-kB to act as decoy DNA also prevented cerebral vasospasm after experimental SAH.

**Vasodilators in SAH**

The early release and subsequent depletion of CGRP in and around cerebral arteries after SAH suggest that endogenous CGRP may be protective in the cerebral circulation after SAH and that depletion of CGRP may contribute to vasospasm. The findings in Figure 1A confirm a previous finding that, 30 minutes after experimental SAH, CGRP concentration in CSF reached a peak, possibly from release of endogenous CGRP after SAH. Because the peak concentration of CGRP in CSF in both the present and previous studies was lower than concentrations in serum, however, blood contamination may contribute substantially to increases in CSF levels of CGRP. Importantly, the increase in endogenous CGRP in CSF derived from SAH was far less than the enormous increase in transduced CGRP after gene transfer.

The present and previous findings in vitro indicate that relaxation of cerebral arteries to CGRP is similar with and without SAH. The findings are in contrast to the previous finding in vivo that dilatation of the basilar artery to CGRP after SAH is augmented as compared with arteries without SAH. One possible explanation for differences in findings in vivo and in vitro is that effects of SAH on arteries may be attenuated in vitro, because arteries are removed from clot remnants and products in CSF that may contain vasoconstrictor substances.

The present and previous findings indicate that endothelium-dependent vasorelaxation is impaired after SAH. Several mechanisms have been proposed to account for this vascular dysfunction after SAH, including a reduction in production or activity of NO, impairment of activation of soluble guanylate cyclase or production of cGMP, inhibitory effect of hemoglobin on NO, and adhesion and infiltration of leukocytes in cerebral arteries. Adenovirus encoding eNOS (AdeNOS) has been used extensively for gene transfer to blood vessels. Adenovirus encoding eNOS (AdeNOS) has been used extensively for gene transfer to blood vessels. The virus, however, has not yet been shown to be effective in vivo in prevention of vasospasm after SAH.

**Vasoconstrictors in SAH**

In this study, vasoconstriction in vitro to serotonin was potentiated, and vasocontraction to KCl and histamine was not altered, after experimental SAH. Results were similar to those of previous studies in vitro of the basilar artery from rabbits. Serotonin has been used commonly to test vascular reactivity after SAH, and previous studies demonstrated that contraction to serotonin was increased 2-fold or more after SAH. Denervation hypersensitivity of cerebral arteries to serotonin and norepinephrine, from a marked reduction or alteration in perivascular adrenergic nerves, may contribute to altered vascular response to serotonin.

In contrast, contraction of the rabbit basilar artery to KCl or histamine in vitro was not altered after experimental SAH. Contraction to histamine, however, was augmented in arteries after SAH when oxyhemoglobin was added to the organ bath. Thus, it is possible that, although SAH has the potential to alter vascular responses to histamine, augmented responses were not demonstrated in the present and previous studies in the organ bath because vasoconstrictor substances from clot remnants and products in CSF were not present in the bath.

After gene transfer of AdRSVCGRP, contraction of the basilar artery to serotonin and histamine in vitro was attenuated in the present study and in a previous study in normal vessels. Attenuation of contraction was mediated by CGRP receptors, because pretreatment with CGRP-(8-37), an antagonist for CGRP receptors, restored the response to normal. AdRSVCGRP did not alter responses to KCl, possibly because depolarization from KCl may prevent hyperpolarization from CGRP. Thus, the present findings of altered vascular reactivity in vitro strongly suggested that gene transfer of CGRP might prevent vasoconstriction in vivo after SAH. Because studies in the organ bath have important limitations described above, however, confirmation in vivo was essential.
Altered Vascular Tone After Gene Transfer

The present angiographic findings demonstrated several effects of gene transfer on the basilar artery. First, arterial diameter was similar after treatment with Adβgal and vehicle. Thus, although leukocytosis in CSF demonstrated a substantial inflammatory response after injection of adenovirus, the inflammation itself did not alter arterial diameter before or after exposure to blood. Responses of normal arteries to several stimuli in vitro in a previous study and in arteries after SAH in the present study also were virtually identical in arteries treated with Adβgal and vehicle.

Second, gene transfer of CGRP increased resting diameter in vivo before SAH and prevented vasoconstriction after experimental SAH by an effect on CGRP$_1$ receptors. Vaso-dilator effects of transduced CGRP were greater in constricted arteries on day 7 (44% increase in diameter compared with vehicle-treated rabbits) than in nonconstricted arteries on day 5 (13%). After SAH, because endogenous CGRP is depleted in cerebral arteries, sensitivity of the basilar artery to CGRP is augmented as compared with nonconstricted arteries on day 5 (13%). After SAH, because endogenous CGRP is depleted in cerebral arteries, sensitivity of the basilar artery to CGRP may be greater. This hypothesis is consistent with previous findings that dilatation of the basilar artery in vivo to CGRP after SAH is augmented as compared with arteries without SAH. Another possible reason for augmented dilator effects in constricted arteries may be that vessels that are submaximally constricted have greater capacity for dilation than do nonconstricted vessels.

Limitations of the Study

Vasospasm is a frequent and often fatal complication after SAH in patients. Novel strategies, including perhaps gene therapy, are needed to prevent vasospasm. An important limitation of this study, however, is that experimental SAH in rabbits does not replicate vasospasm after SAH in humans. The rabbit is commonly used, however, for experimental SAH, with intracisternal injection of autologous blood. Previous studies reported constriction of the rabbit basilar artery by 12% to 35% after injection of blood into CSF. Therefore, the present finding of 25% reduction in diameter is comparable with those in previous studies. Constriction of cerebral arteries does not produce signs of ischemia unless constriction is much greater (perhaps 50% or more), at least in patients, so it is not clear whether the present strategy will also be useful for prevention of symptomatic vasospasm.

Another difference between vasospasm in rabbits and patients is the interval after onset of SAH. Previous reports in rabbits demonstrated that both angiographic evidence of vasoconstriction and augmented vasoconstriction to serotonin in vitro are maximal 2 days after SAH. In this study, we first pretreated rabbits with virus before injection of blood to match the peak of transgene expression with the peak of vasoconstriction after SAH, as in a previous study of gene transfer for brain ischemia. In application of this method in patients, it obviously will not be possible to pretreat patients with gene transfer before SAH. Thus, we constructed a recombinant virus with a CMV promoter, for which duration of 2 days seems to be appropriate for maximal transgene expression. The finding that AdCMVCGRP can prevent cerebral vasoconstriction when it is injected after SAH suggests that gene therapy may eventually be used for prevention of vasoconstriction when the virus is injected after SAH. Patients frequently develop vasospasm 4 to 14 days after SAH. Thus, it is possible that a virus with the RSV promoter may be preferable to the CMV promoter for use in patients.

The inflammatory response to this recombinant virus, demonstrated in this study by an increase in leukocytes in CSF, would prevent use in patients. To minimize unwanted inflammatory or immune responses, coadministration of immunosuppressant drugs or immunomodulatory proteins may be useful. Newer adenoviral vectors may be less immunogenic and thus applicable to human use.

Implications

Intravenous administration of synthetic CGRP to patients after SAH altered velocity of blood flow in cerebral arteries, suggesting dilatation of spastic arteries, and appeared to improve neurological outcome. The use of intravenous CGRP, however, is limited by pronounced hypotension. Intracisternal gene transfer of CGRP is likely to be more useful than intravenous administration, because local gene transfer may avoid systemic effects of CGRP, and sustained release of CGRP is likely to be required for efficacy. Thus, we suggest that gene transfer has important potential advantages over usage of exogenous peptides for prevention of vasospasm. Although there are limitations in the current vectors and animal models, as discussed above, the present study provides evidence, or perhaps proof of principle, that gene transfer may be useful for prevention of vasospasm after SAH.

Acknowledgments

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References


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