Gene Transfer of Extracellular Superoxide Dismutase Failed to Prevent Cerebral Vasospasm After Experimental Subarachnoid Hemorrhage

Mitsuo Yamaguchi, MD; Changman Zhou, MD, PhD; Donald D. Heistad, MD; Yoshimasa Watanabe, MD; John H. Zhang, MD, PhD

Background and Purpose—We examined the therapeutic effect of human extracellular superoxide dismutase (ECSOD) gene transfer in the prevention of delayed cerebral vasospasm after experimental subarachnoid hemorrhage (SAH) because it was reported ECSOD relieved early-stage vasospasm.

Methods—Twenty mongrel dogs were divided randomly into 4 groups to serve as control, SAH, SAH/AdECSOD, and SAH/AdBglII groups, respectively. An established canine double-hemorrhage model of SAH was used by injecting autologous arterial blood into the cisterna magna on day 0 and day 2. Angiography was performed at day 0 and day 7. Clinical behavior, cerebrospinal fluid (CSF) ECSOD activity, CSF leukocyte count, morphology, and human ECSOD expression (RT-PCR) in the basilar arteries were evaluated.

Results—Severe vasospasm was obtained in SAH, SAH/AdECSOD, and SAH/AdBglII gene–transferred dogs, and the residual diameters of the basilar artery were 41±1%, 39±4%, and 49±4%, respectively. Increased CSF activity of ECSOD was obtained in SAH/AdECSOD (162±23 U/mL) when compared with SAH (26±2) and SAH/AdBglII (25±3) dogs. RT-PCR confirmed successful gene transfer in the basilar arteries from SAH/AdECSOD dogs. Increased leukocyte counts were observed in the CSF and in the subarachnoid space, especially in SAH/AdECSOD and SAH/AdBglII dogs.

Conclusions—Gene transfer of human ECSOD failed to prevent delayed cerebral vasospasm. (Stroke. 2004;35:2512-2517.)

Key Words: cerebral vasospasm • gene therapy • subarachnoid hemorrhage

Gene therapy has been conducted in animal modes to prevent or reverse cerebral vasospasm.1,2 Recombinant endothelial NO synthase gene expression in the basilar arteries protects vasomotor function and prevents vasospasm in a canine subarachnoid hemorrhage (SAH) model.3 Overexpression of hemeoxygenase-1, the principal enzyme involved in the metabolism of hemoglobin, reduces contractions of cerebral arteries by hemoglobin and decreases vasospasm after experimental SAH in rats.4 Delayed treatment with adenovirus encoding the prepro-calcitonin gene-related peptide (CGRP) 2 days after initial blood injection reduces cerebral vasospasm in a double-hemorrhage model in dogs.5

It has been suspected that superoxide anion might be involved in vasospasm6-7 because overexpression of superoxide dismutase (SOD) attenuated cerebral vasospasm in mice.8 Among the 3 SOD isozymes (copper zinc SOD [CuZnSOD], manganese SOD [MnSOD], and extracellular SOD [ECSOD]), gene transfer of ECSOD has been shown to reduce early stage of cerebral vasospasm in a rabbit model.9 Although ECSOD has been tested in mouse8 and rat8 models of cerebral vasospasm, the results are not conclusive because of the nature of transient and mild to moderate vasoconstriction in rodents that does not resemble the delayed and persisted vasospasm seen in humans or large animals. We tested gene transfer of ECSOD in an established double-hemorrhage canine model that offers a severe and prolonged vasospasm with a similar time course as in patients.5

Materials and Methods

All experiments were performed according to the rules of animal experimentation and the Guide for the Care and Use of Laboratory Animals of Louisiana State University.

Adenoviral Vectors

Two recombinant adenoviruses were used for gene transfer: (1) replication-deficient human adenoviruses containing human ECSOD (hECSOD) cDNA (adenovirus ECSOD [AdECSOD]) with cytomegalovirus promoter; and (2) adenovirus with no transgene (AdBglII), which was used as a control. Adenoviral vectors and ECSOD genes were purchased from University of Iowa. Purified viruses were stored in PBS with 3% sucrose and kept at −80°C until use. A total
AdBglII was blinded to the first author who did injection and assigned randomly to each group, and the injection of AdECSOD or AdBglII was adjusted to the dosage used in a previous study that showed effects of gene transfer of ECSOD in a rabbit SAH model. All dogs are adjusted to the dosage used in a previous study that showed effects of gene transfer of ECSOD in a rabbit SAH model.9 All dogs are assigned randomly to each group, and the injection of AdECSOD or AdBglII was blinded to the first author who did injection and evaluation of angiographic and clinical results.

Animal Model of Cerebral Vasospasm

Twenty dogs of either sex weighing 15 to 20 kg were randomly assigned to 4 groups as: (1) normal controls for histological studies (without SAH; n = 2); (2) SAH + vehicle (PBS containing 3% sucrose without SAH; n = 6); (3) SAH + AdECSOD (n = 6); and (4) SAH + AdBglII (n = 6). Double-hemorrhage canine model was adapted from Varsos et al6 as described previously.5,11 Dogs were anesthetized with acepromazine (0.1 to 0.5 mg/kg), atropine (0.05 mg/kg), and xylazine (1.1 mg/kg), followed by tracheal intubation, and maintained by 1% isoflurane plus O2 (6 L/min) with mechanical ventilation. A sterile catheter was inserted into a vertebral artery via a femoral artery under fluoroscopic control. The body temperature of dogs was maintained at 37°C with a heating blanket. The mean arterial blood pressure, end tidal CO2, and saturation of O2 were monitored by using a V60046 monitor (Surgi Vet). VISIPAQUE (7 mL) was used as an internal control with specific primers for canine GAPDH. CSF samples were collected from the cisterna magna of anesthetized dogs after SAH, including AdECSOD- and AdBglII-transferred dogs, developed severe vasospasm as shown by representative angiograms on day 7 (Figure 1A). Mean values of the residual diameter of the basilar artery on day 7, as a

Clinical Behavior

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Scale</th>
<th>Score</th>
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<tbody>
<tr>
<td>Appetite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finished meal</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Left meal unfinished</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Scarcely ate</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active, barking, or standing</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Lying down, will stand and walk</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>with some stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Almost always lying down</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Deficits</td>
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<td></td>
</tr>
<tr>
<td>No deficits</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Unstable walk because of ataxia</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>or paraplegia</td>
<td></td>
<td>2</td>
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</table>

of 1.3 mL of AdECSOD (1.0×1010 pfu/mL) or AdBglII (1.0×1010 pfu/mL) was administrated into cisterna magna at 30 minutes after the first blood injection. The dosage of AdECSOD or AdBglII was adjusted to the dosage used in a previous study that showed effects of gene transfer of ECSOD in a rabbit SAH model.9 All dogs are assigned randomly to each group, and the injection of AdECSOD or AdBglII was blinded to the first author who did injection and evaluation of angiographic and clinical results.

Arterial Diameter Measurements

The basilar artery on angiogram was divided into 3 segments, and the diameter of the midpoint of each segment was measured by a computer-based image analyzer (NIH Image version 1.62) as described.9,11 To eliminate magnification differences on the angiograms, a radio-detectable scale was placed on the dog’s chin during the angiography run. Relative to the size of this scale as a standard, all arterial diametric values were adjusted.

Clinical Assessment

Three behavioral examinations (Table) were performed daily after SAH to record appetite, activity, and neurological deficits.11

Morphological Assessment

After euthanasia with Beuthanasia-D, dogs were perfused via both common carotid arteries with 200 mL 0.1 mol/L PBS and then 500 mL 4% paraformaldehyde in 0.1 mol/L PBS, pH 7.4, as described.11 The brain from each dog was removed and postfixed with 4% paraformaldehyde in 0.1 mol/L PBS, pH 7.4, at 4°C. Pons with the basilar artery was cut into sections 4-μm thick using a cryostat (Leica CM3050 S). For hematoxylin/eosin staining, sections were stained in hematoxylin for 2 minutes, eosin for 1 minute, then dehydrated, and mounted by permount. For immunohistochemistry staining, sections were incubated in 3% H2O2, which was diluted in PBS to prevent reaction with endogenous peroxidases. Sections were incubated in rabbit polyclonal anti-CDC8 (1:200) and goat polyclonal antibody anti-CF4 (Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C. Sections were then incubated with goat anti-rabbit IgG and anti-goat IgG as a secondary antibody (1:200) for 30 minutes, respectively, placed in avidin-peroxidase complex solution containing avidin-peroxidase conjugate for 30 minutes, and then mounted, air-dried, dehydrated, and cover-slipped.

Leukocyte Count in Cerebrospinal Fluid

We collected cerebrospinal fluid (CSF) before the first and second autologous blood injection and after angiogram on day 7 in dogs except dogs for ECSOD assay. Leukocyte in CSF was counted with Fuchs-Rosenthal Chamber (Hauser Scientific Co).

Reverse Transcriptase–Polymerase Chain Reaction

Basilar arteries were dissected from dogs (n = 3 in each group), and RNA was isolated and purified with RNeasy mini kit (Quagen) according to instructions of the manufacturer. To eliminate any genomic DNA from the samples, DNase I treatment (Sigma) was included in the RNA isolation procedure. Total RNA (0.5 μg) from basilar arteries was reverse transcribed in total volume of 20 μL using the iScript cDNA Synthesis Kit (Bio-Rad). Reactions were incubated for 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. Reaction lacking reverse transcriptase was also performed to generate controls for assessment of genomic DNA contamination. Thereafter, RNA message for hECSOD was amplified by RT-PCR using a TaKaRa Ex Taq (Takara) with primers specific for hECSOD (forward 5’-CTACTGTGTTCCTGCCTGCTC-3’; reverse 5’-TGGCCAGATCTGGTACCCCT-3’). Total mRNA of GAPDH was used as an internal control with specific primers for canine GAPDH (forward 5’-CTGAAACGGAAGCCTACTGG-3’; reverse 5’-TCTTGATGTCGTGATATTTGGCAG-3’). PCR products were detected after 2% agarose gel electrophoresis by ethidium bromide staining.

ECSOD Activity Assay in CSF

CSF samples were collected from the cisterna magna of anesthetized dogs to analyze tissue-binding ECSOD (n = 2 in each group). Briefly, after the final angiogram for evaluating residual diameter of the basilar artery, 20 U/kg of heparin was injected into the cisterna magna as described previously12 to release the tissue-binding ECSOD. CSF samples were collected 60 minutes after injection of heparin. CSF samples were centrifuged at 1000g for 6 minutes to remove cell components and stored at −20°C until use. To remove the contamination of other SOD isoforms CuZnSOD and MnSOD, the affinity chromatography with concanavaline A was performed as described previously.9,12 Thereafter, ECSOD activity in each sample was measured by nitroblue tetrazorium reduction method.9,12

Statistical Analysis

Results were expressed as mean ± SEM. The residual diameter, clinical score, ECSOD activity assay, and leukocyte count in CSF were analyzed by 1-way ANOVA followed by the Bonferroni–Dunn post hoc test if significant variance was found. A P value of P < 0.05 was considered statistically significant.

Results

Arterial Diameter on Angiography

All dogs after SAH, including AdECSOD- and AdBglII-transferred dogs, developed severe vasospasm as shown by representative angiograms on day 7 (Figure 1A). Mean values of the residual diameter of the basilar artery on day 7, as a
percentage of that on day 0, were 41±1, 39±4, and 49±4, for SAH, SAH+AdECSOD, and SAH+AdBglII, respectively. No statistical difference was noted among groups (P>0.05; ANOVA; Figure 1B).

**Clinical Assessment**

Behavior scores on appetite, activity, and neurological deficit are shown in Figure 2. The appetite score in SAH+AdECSOD group was worse than SAH or SAH+AdBglII groups on day 1 (P<0.05; Figure 2A). No statistical differences were found among groups at other time points in appetite score. In addition, no statistical differences were found among groups in activity or neurological deficit scores (P>0.05; ANOVA; Figure 2B and 2C).

**Morphological Study**

**H&E Staining**

No vasospasm was noted in control dogs (Figure 3A1). Morphological vasospasm was observed in basilar arteries in all SAH, including gene-transferred dogs (Figure 3B1 through 3D1), characterized by corrugation of the internal elastic lamina and contraction of smooth muscle cells. Spastic basilar arteries were surrounded by blood clots (Figure 3B1 through 3D1). Inserts showed high magnification of cell infiltrations at the adventitial layer of the basilar artery.

**Immunohistochemistry**

Limited staining of CD4 or CD8 was visible in the control group (Figure 3A2 and 3A3). In SAH dogs, moderate staining of CD4 or CD8 was observed especially at the adventitial layer (Figure 3B2 and 3B3). In dogs with AdECSOD and AdBglII transfer, strong staining of CD4 or CD8 was observed not only at the adventitial layer but also in other areas in the subarachnoid space (Figure 3C2, 3D2, 3C3, and 3D3).

**Leukocyte Count in CSF**

Leukocyte count in CSF is shown in Figure 3E. There were significant differences between SAH group from AdECSOD or AdBglII group on day 2 and day 7 (P<0.05 versus SAH).

**Reverse Transcriptase–Polymerase Chain Reaction**

RT-PCR analysis of RNA from the basilar arteries of AdECSOD dogs showed positive message of human ECSOD,
bands of 131 bp (Figure 4), indicating that human ECSOD gene was successfully transferred to the basilar artery. Although the expressions of GAPDH for dog were seen in all groups (Figure 4), no expressions of human ECSOD were seen either in SAH or in SAH/H11001 AdBglII groups (Figure 4A).

ECSOD Activity Assay in CSF
ECSOD activity was detected in the bloody CSF obtained on day 7 from AdECSOD dogs when compared with SAH or SAH+AdBglII groups (Figure 4B; P<0.05).

Discussion
This study is a follow-up on a previous report that gene transfer of ECSOD attenuated early-stage cerebral vasospasm (at 48 hours) in a single blood injection rabbit model. Inconsistent with the previous study, human ECSOD gene was transferred successfully into the basilar arteries with adenoviral vector, and transfer of ECSOD genes increased markedly the activity of ECSOD in CSF at day 7 after SAH. We confirmed ECSOD gene in the basilar artery by RT-PCR.

We did not perform Western blot to confirm protein production as reported previously because we could not obtain specific antibody for ECSOD. However, gene transferring of human ECSOD failed to prevent cerebral vasospasm. Angiographic and morphological studies revealed severe vasospasm in basilar arteries. In addition, clinical behavior scores were not improved by ECSOD gene transfer.

There are several factors that might contribute to these inconsistencies and discrepancies. The first factor is the degree of cerebral vasospasm in animal models. A mild to moderate vasospasm occurs mostly in rodents and rabbits. For example, a 25% reduction of the diameter of the basilar artery was observed after SAH in rabbit. Pharmacological or biological treatments including gene transfer may be able to prevent this mild to moderate vasoconstriction. Conversely, double-hemorrhage canine model produces severe vasospasm, up to 60% reduction of the diameter. Most medical treatment failed to prevent or reserve severe vasospasm. The second factor is the duration of cerebral vasospasm. In rodents and rabbits, a transient vasoconstriction occurs up to
gene transfer that cell infiltration in the basilar artery and cell count in CSF were observed in AdECSOD and AdBglII dogs, our data are not extensive to support the previous studies indicating normal body immunological response to viral intrusion is associated with the production of cytokines, which is postulated as an important factor for cerebral vasospasm. Another possibility for the inability of ECSOD in the protection of oxidative stress is the fast reaction between superoxide anion and NO that produces peroxynitrate, which contributes to cerebral vasospasm. A recent publication indicates that elevation of superoxide anion was markedly reduced by increased NO production in cerebral arteries. A detailed relationship among superoxide anion, NO, and SOD in cerebral arteries was discussed previously. Finally, ECSOD may be simply less effective than other powerful vasodilators such as CGRP in the prevention of delayed and severe cerebral vasospasm.

This study reconfirmed the use of large animal models for testing and evaluation of experimental therapies. We believe this caution is important in that the same level of gene transfer using the same vector may not produce similar effects in large animal models of cerebral vasospasm, which resembles the human vasospasm time course with features of delayed and persisted severe vasoconstriction. Because of the delayed onset feature of cerebral vasospasm, the therapeutic window for gene therapy exists for future therapeutic intervention.

Acknowledgments

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48 hours after a single blood injection. This early stage of cerebral vasospasm behaves differently from delayed vasospasm at day 7 after blood injection. Because the signaling pathways for early vasoconstriction and delayed vasospasm are different, pharmacological agents that can prevent or reverse early-stage vasospasm may not prevent or reserve delayed vasospasm. A third factor may relate to the duration of expression of introduced genes. It was suggested that ECSOD gene transfer by adenoviral vector is transient, and the peak of ECSOD expression may be in the early phase of vasospasm but not at day 7. Therefore, a second injection of AdECSOD on day 4 to 5 might be considered to keep a high level of ECSOD at day 7 to 8 in this canine model of cerebral vasospasm. Further experiments to determine a time course of ECSOD activity are needed to resolve this matter. The fourth factor is that adenoviral vector infects most parts of the brain, including cerebrum, cerebellum, and brain stem, after cisternal injection. Therefore, ECSOD activity measured in the present study may not represent the level of ECSOD activity in the basilar artery but contains those produced by other structures of the central nervous system. In addition, ECSOD expressed in the basilar artery is the tissue-binding type ECSOD. Therefore, although an overall production of ECSOD was increased in the CSF, the limited amount of tissue-binding ECSOD in the basilar artery might not be sufficient to attenuate severe and delayed vasospasm in this double-hemorrhage canine model at day 7.

Another common factor or side effect for viral vector-mediated gene therapy is the inflammatory response. Although we have confirmed the inflammatory responses after

Figure 4. A, Expression of human ECSOD mRNA in canine basilar arteries is shown by RT-PCR using specific primers for human ECSOD. The bands of 131 bp of human ECSOD were recognized only in basilar arteries treated with AdECSOD. Specific primers for canine GAPDH were used as control. B, ECSOD activity in CSF on day 7 is shown (n=2 for each group). ECSOD activity was 26±2 (U/mL) in SAH dogs, 162±23 in AdECSOD dogs, and 25±1 in AdBglII dogs, respectively.


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