Gene Transfer of Extracellular Superoxide Dismutase Reduces Cerebral Vasospasm After Subarachnoid Hemorrhage

Yoshimasa Watanabe, MD; Yi Chu, PhD; Jon J. Andresen, BA; Hiroshi Nakane, MD; Frank M. Faraci, PhD; Donald D. Heistad, MD

Background and Purpose—Superoxide may play an important role in cerebral vasospasm after subarachnoid hemorrhage (SAH). Our first goal was to determine the effect of gene transfer of extracellular superoxide dismutase (ECSOD) on vasospasm after experimental SAH. Our second goal was to determine whether tissue binding of ECSOD via the heparin-binding domain (HBD) is important for the effect of the enzyme. Thus, we examined effects of gene transfer of ECSOD with the HBD deleted (ECSODΔHBD).

Methods—Adenovirus expressing human ECSOD (AdECSOD), ECSODΔHBD (AdECSODΔHBD), or no transgene (AdBglII) was injected into the cisterna magna of anesthetized rabbits 30 minutes after induction of experimental SAH. Cerebral angiography, an assay for ECSOD activity in cerebrospinal fluid (CSF), and Western blotting for human ECSOD in the basilar artery were performed.

Results—Baseline diameter of the basilar artery averaged 0.77±0.01 mm (mean±SEM) and was similar in all treatment groups. Decreases in diameter of the basilar artery 2 days after SAH were smaller after AdECSOD (11±3%; n=10) than after AdBglII (25±4%; n=7; P<0.05). ECSOD activity was not detected in CSF before SAH and gene transfer. Of 8 rabbits treated with AdECSOD, in which ECSOD activity in CSF was measured after SAH, 5 animals had detectable ECSOD activity in CSF (46±13 U/mL). In these 5 rabbits, the diameter decreased by only 6±3%, and ECSOD protein was detected in the basilar artery. After AdECSODΔHBD (n=4), despite high levels of ECSOD activity in CSF (91±19 U/mL), vessel diameter decreased by 20±2%, and no ECSODΔHBD protein was detected in the basilar artery.

Conclusions—Gene transfer of ECSOD reduces cerebral vasospasm after experimental SAH. Tissue binding of the enzyme is essential for cerebral vascular effects of ECSOD. (Stroke. 2003;34:●●●●●●●)

Key Words: gene transfer ■ subarachnoid hemorrhage ■ superoxide dismutase ■ vasospasm, intracranial ■ rabbits

Delayed cerebral vasospasm after subarachnoid hemorrhage (SAH) may be caused by multiple and complex mechanisms, including vascular dysfunction caused by oxidative stress.1,2 Several studies indicate a pathophysiological role of superoxide (O$_2^-$) in cerebral vasospasm. After experimental SAH in dogs, production of O$_2^-$ is increased in the subarachnoid space.3 Administration of superoxide dismutase (SOD) into cerebrospinal fluid (CSF) reduces cerebral vasospasm in rabbits,4 although this is not consistent in different animal models and treatment protocols.5,6 In studies using transgenic mice that overexpress copper/zinc SOD (Cu/ZnSOD) or extracellular SOD (ECSOD), diameter of the middle cerebral artery after experimental SAH is larger in transgenic mice than in nontransgenic mice.7,8 Some of these studies imply that production of O$_2^-$ in the extracellular space after SAH is important in the pathophysiology of vasospasm. Thus, we speculated that local overexpression of ECSOD, the only isoform of SOD that is secreted into the extracellular space,9 in CSF or cerebral arteries may be a useful approach to reduce vasospasm. The first goal of this study was to determine effects of gene transfer of ECSOD on cerebral vasospasm after experimental SAH. ECSOD binds to tissue via its heparin-binding domain (HBD), which provides affinity of the enzyme for heparan-sulfate proteoglycans on cell surfaces and in extracellular matrices.10 ECSOD is released into blood by intravenous injection of heparin.11 After gene transfer into the cisterna magna of normal rabbits, ECSOD is overexpressed in cerebral arteries and CSF, with the majority being bound to tissue and released into CSF by intracisternal heparin.12 Distribution of overexpressed ECSOD may be a critical determinant of efficacy of gene transfer because there are at least 2 different possible sources of extracellular O$_2^-$, oxyhemoglobin in CSF13 and NAD(P)H oxidase in cerebral arteries,14 that may...
contribute to the pathophysiology of vasospasm after SAH. Recently, we have constructed a human ECSOD with the HBD deleted (ECSOD\textsubscript{HBD}).\textsuperscript{15} This variant of ECSOD, which has normal activity but does not have affinity for heparin or heparan-sulfate proteoglycans,\textsuperscript{10} is readily released into blood after systemic gene transfer in rats. We speculated that ECSOD\textsubscript{HBD} would be released freely into CSF after gene transfer into the cisterna magna and that loss of tissue binding might change the efficacy of the enzyme for reduction of vasospasm. Thus, the second goal of this study was to compare effects of gene transfer of ECSOD and ECSOD\textsubscript{HBD} after SAH.

Materials and Methods

Adenoviral Vectors
We constructed replication-deficient adenoviruses containing the gene for human ECSOD (AdECSOD) or human ECSOD\textsubscript{HBD} (AdECSOD\textsubscript{HBD}) with a cytomegalovirus promoter, using standard methods.\textsuperscript{16} An adenovirus with no transgene (AdBglII) was used as a control virus.

Gene Transfer in Rabbits Without SAH
All animal procedures were approved by the Animal Care and Use Review Committee of the University of Iowa.

Changes in total SOD activity in CSF were compared after gene transfer of ECSOD and ECSOD\textsubscript{HBD} in rabbits without SAH. A method for gene transfer into the cisterna magna of the rabbit was described elsewhere.\textsuperscript{17} In brief, a recombinant adenovirus in phosphate-buffered saline (PBS) with 3% sucrose was diluted to 0.3 mL of viral suspension containing 1 \times 10^{10} particles with artificial CSF (composition in mmol/L: 132 NaCl, 2.95 KCl, 1.71 CaCl\textsubscript{2}, 0.65 MgCl\textsubscript{2}, 24.6 NaHCO\textsubscript{3}, and 3.69 d-glucose). The titers of adenovirus was regarded to be optimal on the basis of previous experiments.\textsuperscript{12} Adult male New Zealand White rabbits weighing 2.6 to 3.7 kg were anesthetized with intramuscular xylazine 10 mg/kg and ketamine 50 mg/kg, and a 25-gauge needle was aseptically inserted into the cisterna magna. After withdrawal of 0.5 mL of CSF, the viral suspension was slowly injected into the cisterna magna. The head was tilted nose-down by 30 degrees for 30 minutes after injection of virus.

One, 2, and 3 days after gene transfer, CSF samples were collected from the cisterna magna of anesthetized rabbits. Three days after gene transfer, a CSF sample was collected again 60 minutes after injection of heparin (20 U/kg) into the cisterna magna as described previously.\textsuperscript{12} CSF samples were centrifuged at 1000 \times g for 6 minutes to remove cell components and stored at −20°C until used. Total SOD activity in each CSF sample was measured by the modified nitroblue tetrazolium reduction method\textsuperscript{18} as described previously.\textsuperscript{12}

Simulated SAH and Gene Transfer
Experimental SAH was produced in rabbits as described previously.\textsuperscript{19} In brief, a 25-gauge needle was aseptically inserted into the cisterna magna of an anesthetized rabbit, and 0.8 mL/kg of fresh nonheparinized autologous blood taken from the ear artery was injected after withdrawal of 0.8 mL of CSF. The head was tilted nose-down by 30 degrees for 30 minutes after injection of blood.

About 40 minutes after injection of blood, the viral suspension was slowly injected into the cisterna magna through a 25-gauge needle. Then the head was tilted nose-down by 30 degrees for 30 minutes.

Vertebral Angiography
Digital subtraction angiography was performed immediately before (day 0) and 2 days after (day 2) experimental SAH as previously described.\textsuperscript{18} In brief, anesthetized rabbits were placed in a supine position and mechanically ventilated. An angiogram was obtained by injection of 0.8 mL of nonionic contrast medium through a microcatheter (Renegade 130/20/1TIP; Boston Scientific) introduced into the left vertebral artery from an exposed femoral artery. Arterial pressure at the thoracic aorta was recorded directly through the catheter, and arterial blood gases were measured during angiography. Angiograms were converted to image files on a computer. Diameter of the basilar artery was measured with the NIH Image program by an observer blinded to the experimental protocol.

ECSOD Activity in CSF After SAH
CSF samples were collected from rabbits on day 2 after SAH, centrifuged, and stored at −20°C until used. To eliminate contamination of SOD activity from erythrocytes in the subarachnoid space, ECSOD and ECSOD\textsubscript{HBD} were separated from other 2 isoforms, Cu/ZnSOD and manganese SOD (MnSOD), with affinity column chromatography on concanavalin A.\textsuperscript{20} Each sample (0.3 mL) was applied to a 2-mL disposable column containing 0.5 mL of concanavalin A sepharose (Sigma). Cu/ZnSOD and MnSOD lack affinity for concanavalin A and were collected in the effluent from 1.5 mL of PBS added on the column. After the column was washed with 10 mL of PBS, ECSOD was eluted in 5 fractions of 0.3-mL effluent by addition of 0.5 mol/L o-methyl-d-mannoside (Sigma) dissolved in PBS. SOD activity in each fraction containing ECSOD was determined by the nitroblue tetrazolium method and summed for each original sample. We estimated the recovery rate of SOD activity after the chromatography procedure as 60% for both ECSOD and ECSOD\textsubscript{HBD}, using control enzymes made by gene transfer to A549 cells with AdECSOD or AdECSOD\textsubscript{HBD}, respectively. Values of ECSOD activity in this study were corrected for this recovery rate. An activity of <10 U/mL is undetectable by the SOD activity assay used in this study, and therefore the lower detectable limit of ECSOD activity was calculated as 17 U/mL. In some animals, ECSOD activity also was measured in CSF samples withdrawn before induction of experimental SAH.

Western Blotting for ECSOD in Basilar Artery
In preliminary experiments, activity of ECSOD in the basilar artery was not measurable because the tissue sample was too small. Thus, overexpression of human ECSOD in the basilar artery was confirmed by Western blotting. Rabbits were euthanatized by an overdose of pentobarbital after angiography and after CSF samples were obtained on day 2. The basilar artery was removed, frozen in liquid nitrogen, and stored at −80°C until use. The frozen sample was ground into a coarse powder state, 100 \mu L of 0.05-mmol/L phosphate buffer (pH 7.8) was added, and protein extract was collected as a supernatant after centrifugation at 4°C and 15 000 \times g for 15 minutes. Concentration of protein was determined by Lowry assay (Bio-Rad). Ten micrograms of protein was electrophoresed in a 10% native polyacrylamide gel and immunoblotted with a rabbit anti-ECSOD antibody (provided by Dr James Crapo of National Jewish Medical and Research Center, Denver, Colo). The blot was incubated with chemiluminescent substrate (Femto Maximum Sensitivity; Pierce) and exposed to x-ray film.

Data Analysis
Values are expressed as mean±SEM. Paired or unpaired t test was used for comparison of 2 values. Time-dependent changes in total SOD activity in CSF were analyzed by repeated-measures ANOVA and contrast analysis. One-way ANOVA followed by Scheffé's F test was used for multiple comparisons. Mann-Whitney U test or Kruskal-Wallis test followed by Scheffé's F test was used for comparison of ECSOD activity in CSF. P<0.05 was considered statistically significant.

Results

Gene Transfer in Rabbits Without SAH
All rabbits without SAH tolerated an injection of AdECSOD (n=3) or AdECSOD\textsubscript{HBD} (n=3) into the cisterna magna. In both groups total SOD activity in CSF increased during the 3 days after gene transfer (P<0.05), and the values at 2 and 3
days after gene transfer tended to be higher in AdECSODΔHBD-injected rabbits than in AdECSOD-injected animals (Figure 1A). Three days after gene transfer, total SOD activity in CSF was greatly increased by intracisternal injection of heparin (Figure 1B). In contrast, total SOD activity in CSF was not changed by heparin after gene transfer of ECSODΔHBD.

Experimental SAH
Experimental SAH was induced in 36 rabbits (14 for AdBglII, 14 for AdECSOD, and 8 for AdECSODΔHBD group). Of these rabbits, 21 animals (7 for AdBglII, 10 for AdECSOD, and 4 for AdECSODΔHBD group) had subarachnoid clot covering the ventral surface of the brain stem. The other animals, which died (n=1) or had severe neurological deficits (n=5) immediately after the experimental procedures on day 0 or had only a small amount of blood in the subarachnoid space on postmortem examination (n=9), were excluded from further analyses.

Mean arterial blood pressure and arterial blood Pco2 during angiography were not significantly different between day 0 and 2 in each group. There were small changes in arterial P02 and pH in some groups (Table).

Effect of Gene Transfer of ECSOD on Vasospasm
Baseline diameter of the basilar artery was similar in the AdBglII and AdECSOD groups. In both groups, the diameter was decreased on day 2 after SAH (Figure 2A). The decrease in the diameter was smaller in the AdECSOD group than in the AdBglII group (Figure 2B).

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<th>Arterial Pressure and Blood Gases During Angiography in Rabbits Treated With AdBglII, AdECSOD, or AdECSODΔHBD After Subarachnoid Hemorrhage</th>
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Values are mean±SEM.
*P<0.05 vs day 0.
ECSOD Activity in CSF After Gene Transfer of ECSOD

ECSOD activity was not detected in CSF obtained before experimental SAH and gene transfer (n=7). On day 2, a CSF sample was measured for ECSOD activity from 5 rabbits of the AdBglII group and 8 animals of the AdECSOD group. In rabbits treated with AdBglII, ECSOD activity was not detected in CSF except for 1 animal that had 18 U/mL of activity in CSF, while in AdECSOD-treated rabbits it was detected in 5 animals (46±6 U/mL) except for 3 with no detectable activity. ECSOD activity was significantly higher in rabbits treated with AdECSOD than in animals treated with AdBglII (P<0.05).

To determine whether attenuation of the decrease in diameter depends on ECSOD activity in CSF after gene transfer of ECSOD, data from the rabbits described above were plotted against ECSOD activity (Figure 3A). Of 8 rabbits treated with AdECSOD, the percent decrease in the diameter was smaller in 5 animals that had detectable ECSOD activity in CSF than in animals treated with AdBglII (P<0.05).

Effect of Gene Transfer of ECSODΔHBD

In all rabbits treated with AdECSODΔHBD, ECSOD activity in CSF after SAH was detectable (n=4). Baseline diameter of the basilar artery (0.80±0.03 mm; n=4) was similar to the AdBglII and AdECSOD groups. In contrast to rabbits treated with AdECSOD, there was no reduction in the severity of vasoconstriction, despite a large increase in ECSOD activity in CSF, in AdECSODΔHBD-treated animals (Figure 3B). The decrease in diameter in the AdECSODΔHBD group was comparable to that in the AdECSOD[act(−)] group or in the AdBglII group (Figure 4B).

Overexpression of Human ECSOD Protein in Basilar Artery

Binding of ECSOD to the basilar artery after treatment with AdECSOD, but not AdECSODΔHBD, was confirmed by Western blotting with the use of anti-human ECSOD antibody (Figure 5). After treatment with AdECSOD, a small amount of human ECSOD protein was detected in the basilar artery of rabbits with no detectable ECSOD activity in CSF, while a large amount was detected in rabbits with elevated activity in CSF. No human ECSOD protein was detected in the basilar artery of rabbits treated with AdBglII or AdECSODΔHBD.

Discussion

This study indicates that gene transfer of ECSOD reduces cerebral vasospasm after experimental SAH. The decrease in arterial diameter was greatly reduced in AdECSOD-treated rabbits with increased ECSOD activity in CSF. In contrast,
gene transfer of ECSODΔHBD failed to reduce vasospasm, despite a large increase in ECSOD activity in CSF. After gene transfer, a large amount of ECSOD, but no ECSODΔHBD, was bound to the basilar artery. These results suggest that tissue-bound ECSOD is essential for reduction of vasospasm after SAH.

Role of Superoxide in Vasospasm

Attenuation of cerebral vasospasm by exogenous SOD,4 in ECSOD transgenic mice,8 and by gene transfer of ECSOD (this study) suggests that $O_2^-$ produced in the extracellular space plays an important role in the pathophysiology of vasospasm. In preliminary experiments, we could not demonstrate an increase in $O_2^-$ level in the basilar artery after SAH either by lucigenin-enhanced chemiluminescence or by laser confocal microscopy using dihydroethidium (data not shown). We had anticipated that the lucigenin-enhanced signal would be increased by extracellular $O_2^-$, whereas intracellular $O_2^-$ detected by dihydroethidium would not be increased. However, an increase in extracellular $O_2^-$ production after experimental SAH has been demonstrated previously in situ, in the subarachnoid space and in adventitia of the basilar artery.3

Extracellular $O_2^-$ may contribute to cerebral vasospasm after SAH through several mechanisms. $O_2^-$ inactivates nitric oxide (NO) to impair NO-mediated vasorelaxation.21,22 Reaction of $O_2^-$ with NO produces the highly reactive oxidant peroxynitrite, which can alter protein function by nitration of tyrosine residues,23 damage cell membranes by lipid peroxidation,24 and break DNA.25 There is evidence for possible involvement of these mechanisms in development of cerebral vasospasm.8,26,27

Increased production of $O_2^-$ may result in elevation of levels in cerebral arteries of hydrogen peroxide ($H_2O_2$), which induces constriction in isolated basilar arteries.28 Hydrogen peroxide can generate highly reactive hydroxyl radical in the presence of ferrous ion that may be increased after SAH,7 and studies using scavengers of hydroxyl radical indicate a role for this radical in vasospasm.29 In vascular tissue, $H_2O_2$ can also activate key signaling molecules such as protein kinase C, mitogen-activated protein kinases, and nuclear factor-kB,30 which may be implicated in vasospasm after SAH.31–33 Gene transfer of ECSOD could enhance these $H_2O_2$-mediated mechanisms by facilitating dismutation of $O_2^-$ to $H_2O_2$. However, 1 mol or more of $H_2O_2$ can be formed per mole of $O_2^-$ through oxidation of biomolecules such as catecholamines, whereas only 0.5 mol of $H_2O_2$ is produced by dismutation of 1 mol of $O_2^-$. Thus, when production of $O_2^-$ is enhanced in the subarachnoid space, efficient dismutation of $O_2^-$ by ECSOD may decrease levels of $H_2O_2$ by preventing stoichiometrically greater formation of $H_2O_2$ through the oxidizing action of $O_2^-$.34

Importance of Local Overexpression of ECSOD in Basilar Artery

Overexpression of ECSOD is achieved mainly in adventitia of cerebral arteries and meninges after in vivo gene transfer.12 Thus, we speculate that vasospasm was attenuated by dismutation of extracellular superoxide in adventitia of the basilar artery, where ECSOD accumulates after gene transfer. Reduction of tyrosine nitration in adventitia of the middle cerebral artery after SAH in ECSOD transgenic mice provides support for this possibility.8 The half-life of $O_2^-$ at physiological pH is short so that $O_2^-$ does not diffuse far from its production site. Thus, it is possible that $O_2^-$, which contributes to vasospasm after SAH, may be produced within adventitia of cerebral arteries. There are at least 2 possible sources of extracellular $O_2^-$ that may contribute to the pathophysiology of vasospasm after SAH. First, oxyhemoglobin, which is released from erythrocytes in the subarach-
noid blood surrounding cerebral arteries, can release O$_2^-$ through autoxidation.\textsuperscript{11} Second, NAD(P)H oxidase in cerebral artery may be activated after SAH and contribute to development of vasospasm.\textsuperscript{14} NAD(P)H oxidase activity may be attributable to inflammatory cells infiltrating into cerebral arteries and perivascular tissue after SAH.\textsuperscript{55} Adventitial fibroblasts also have components of NAD(P)H oxidase and produce extracellular O$_2^-$ in aorta,\textsuperscript{22} although it is not known whether a similar mechanism is present in intracranial arteries.

We suggest that tissue binding of ECSOD, from local overexpression of the enzyme in the basilar artery after gene transfer, is essential for the effect of the enzyme on vasospasm after SAH. For reduction of vasospasm by administration of exogenous SOD,\textsuperscript{4} repetitive intracisternal injections of an extremely large amount of Cu/ZnSOD is required to maintain adequate concentration of the enzyme in cerebral arteries, presumably because it does not bind to tissue.

**ECSOD Activity in CSF After Gene Transfer**

Absolute values of SOD activity in CSF were lower in rabbits after SAH than in rabbits without SAH, mainly because we measured ECSOD activity after SAH and total SOD activity in rabbits without SAH. It is known, however, that SOD activity in CSF is decreased after SAH.\textsuperscript{26} Nevertheless, overexpression of ECSOD achieved in this study was sufficient for pronounced reduction of vasospasm. Increased ECSOD activity in CSF after gene transfer is associated with a large amount of ECSOD bound to the basilar artery (Figure 5). Only a small amount of ECSOD was overexpressed in 3 rabbits treated with AdECSOD, perhaps because we failed to successfully inject the virus into CSF through the cisterna magna.

**Clinical Implications**

In the rabbit model of SAH, vasospasm usually peaks approximately 2 days after injection of blood and resolves within 10 days.\textsuperscript{4,36} After injection of AdECSOD into CSF of rabbits, the time course of overexpression of ECSOD coincides with the onset of vasospasm (Figure 1). A single injection of AdECSOD provides continuous production and accumulation of ECSOD in CSF and cerebral arteries for several days and was effective in reducing vasospasm. Thus, adenovirus-mediated gene transfer of ECSOD might potentially be useful as an antioxidant therapy for prevention of cerebral vasospasm after SAH. Previously, we reported that gene transfer of CGRP after induction of experimental SAH reduced vasospasm.\textsuperscript{19} Although CGRP may be more effective than ECSOD, gene therapy with ECSOD also is attractive because it may eliminate one of the possible causes of vasospasm after SAH.

In a large clinical trial, pegorgttein (polyethylene glycol–conjugated Cu/ZnSOD) failed to improve neurological deficits after head injury in patients.\textsuperscript{37} There are many possible explanations for the finding, including the dose of SOD, timing and duration of treatment, and severity and type of injury. We would like to speculate about another possibility. Perhaps extracellular binding to the outer cell membrane is necessary for a protective effect of SOD. If extracellular O$_2^-$ contributes to secondary damage after head injury, our data imply that ECSOD might be beneficial only if it has the HBD, and Cu/ZnSOD, which does not have the HBD, would not be protective.

**Limitations of the Study**

We recognize that injection of blood into CSF of rabbits is not equivalent to SAH in patients. Rabbits develop relatively mild vasospasm (20% to 30% reduction in diameter in most studies),\textsuperscript{4,19,26,33} which usually does not produce neurological deficits, with a short interval after onset of SAH, compared with patients. Further studies using canine or primate models of vasospasm, with comparable severity and temporal profile to patients, are needed to address this limitation.\textsuperscript{36}

Because inflammation may contribute to vasospasm after SAH,\textsuperscript{35} inflammatory response to adenoviral vectors in CSF\textsuperscript{17} may have complicated the results of this study. To address this concern, we injected AdBglIII as a control virus and compared effects of AdECSOD and AdBglIII. In addition, previous experiments indicate that vasoconstriction after SAH is similar after SAH alone and SAH with a control virus.\textsuperscript{19} Inflammatory response to viral vectors will be one of the major obstacles to effective gene therapy for vasospasm after SAH. Further progress in gene transfer vectors is needed to overcome this problem.\textsuperscript{38}

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**References**


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