Gene Transfer of Extracellular Superoxide Dismutase Increases Superoxide Dismutase Activity in Cerebrospinal Fluid

Hiroshi Nakane, MD; Yi Chu, PhD; Frank M. Faraci, PhD; Larry W. Oberley, PhD; Donald D. Heistad, MD

Background and Purpose—Copper-zinc superoxide dismutase (CuZnSOD) is expressed intracellularly, while extracellular SOD (EC-SOD) is released from cells. The purpose of this study was to determine whether gene transfer of CuZnSOD increases SOD activity predominantly in tissues, and gene transfer of EC-SOD increases SOD activity in cerebrospinal fluid (CSF). We also determined whether heparin or dextran sulfate releases EC-SOD into CSF.

Methods—We injected recombinant adenoviruses expressing EC-SOD (AdEC-SOD), CuZnSOD (AdCuZnSOD), or β-galactosidase (Adβ-gal) into the cisterna magna of rabbits.

Results—Total SOD activity in CSF was 39±11 U/mL (mean±SE) before virus injection. Three days later, total SOD activity in CSF increased to 148±22 U/mL after AdEC-SOD and 92±10 U/mL after AdCuZnSOD (P<0.05 versus AdEC-SOD), with no change after Adβ-gal (49±5 U/mL). EC-SOD protein was detected in CSF after AdEC-SOD but not AdCuZnSOD or Adβ-gal. Injection of heparin or dextran sulfate into the cisterna magna increased total SOD activity 27-fold and 32-fold over basal values, respectively, in CSF of rabbits that received AdEC-SOD. In contrast to effects in CSF, total SOD activity in basilar artery and meninges was significantly higher after AdCuZnSOD and tended to be higher after AdEC-SOD than after Adβ-gal.

Conclusions—We have developed a method for intracranial gene transfer of CuZnSOD and EC-SOD. After gene transfer, CuZnSOD was expressed mainly in tissues, and EC-SOD was released into the CSF, especially after injection of heparin or dextran sulfate. Gene transfer of different isoforms of SOD may be useful in studies of cerebral vascular physiology and pathophysiology. (Stroke. 2001;32:184-189.)

Key Words: adenovirus ■ basilar artery ■ gene transfer ■ heparin ■ superoxide dismutase

Superoxide may contribute to vascular dysfunction in several pathological states in the central nervous system, including ischemia-reperfusion injury,1–3 vasospasm after subarachnoid hemorrhage,4,5 and meningitis.6 Although there are several potent antioxidant systems, superoxide dismutase (SOD) appears to play a key role in protection against oxygen radicals. There are 3 known isoforms of SOD: copper-zinc SOD (CuZnSOD), manganese SOD (MnSOD), and extracellular SOD (EC-SOD). CuZnSOD is an isoform of SOD that exists intracellularly and dismutates superoxide within cells.7,8 MnSOD is located in mitochondria and plays a critical role as an antioxidant.8,9 EC-SOD is the only isoform of SOD that is released from cells into the extracellular space.10 EC-SOD is the primary extracellular antioxidant enzyme and is highly expressed in blood vessels,11,12 uterus,13 and airways.12 We speculated that, after intracranial gene transfer of CuZnSOD, CuZnSOD would remain within cells lining the subarachnoid space and SOD activity would increase within the cells. In contrast, we anticipated that after intracranial gene transfer of EC-SOD, EC-SOD would be produced within the cells and released into extracellular space. Thus, SOD activity might increase in both cells and cerebrospinal fluid (CSF).

Previous studies have reported that EC-SOD is released into plasma from liver after injection of heparin or dextran sulfate intravenously.14 The mechanism of this release is thought to be displacement of EC-SOD by heparin or dextran sulfate from heparan sulfate proteoglycan on cell surfaces.15 We speculated that heparin or dextran sulfate might release EC-SOD into CSF after transduction of EC-SOD to cells in the subarachnoid space. Our goals were (1) to determine whether gene transfer of CuZnSOD and EC-SOD by injection into the cisterna magna increases SOD activity in cells lining the subarachnoid space and in CSF, (2) to determine whether the increase in SOD activity in CSF is greater after gene transfer of EC-SOD than CuZnSOD, and (3) to determine whether heparin or dextran sulfate enhances release of EC-SOD into the CSF.

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Materials and Methods

Adenoviral Vectors

Three different recombinant adenoviruses were used for gene transfer: (1) AdCMVβ-gal carried the reporter gene of β-galactosidase and was used as a control virus; (2) AdCMVCuZnSOD, a gift from Dr John Engelhardt (University of Iowa), contained cDNA for human CuZnSOD; and (3) AdCMVEC-SOD carried cDNA for human extracellular SOD. The adenoviral vectors were produced by the University of Iowa Gene Transfer Vector Core with the method described previously.14 Phosphate-buffered saline (PBS) containing 3% sucrose, which is the solvent for viruses, was used as virus-free control.

In Vivo Gene Transfer to Rabbits

All animal procedures were approved by the Animal Care and Use Review Committee at the University of Iowa. Adult male New Zealand White rabbits (weight, 2.5 to 3.4 kg; n = 34) were anesthetized with intramuscular ketamine 50 mg/kg and xylazine 10 mg/kg. A 25-gauge needle was aseptically inserted into the cisterna magna as previously described.17 Three hundred microliters of CSF was withdrawn for measurement of total SOD activity, and 300 μL of viral suspension (artificial CSF with Adβ-gal, AdCuZnSOD, or AdEC-SOD in 3% sucrose) was injected slowly. The dose of adenovirus was 3 × 10^9 plaque-forming units per injectate.

We observed the distribution of β-gal expression in the brain of rabbits using X-gal staining after injection of AdCMVβ-gal into the cisterna magna. We found that the ventral surface of the brain was extensively stained in rabbits (data not shown), as we had shown previously in rats,17 mice,18 and rabbits.19 One day after injection of virus suspension, rabbits were anesthetized with intramuscular ketamine 50 mg/kg and xylazine 10 mg/kg, and CSF was collected. Three days after injection, rabbits were killed by an overdose of pentobarbital. After collection of CSF, the basilar artery and meninges (dura) of the brain stem were obtained. Each CSF sample was centrifuged at 3000 rpm for 6 minutes to remove cell components. Supernatant was stored at −20°C until used. Basilar artery and meninges were frozen in liquid nitrogen and stored at −80°C until used.

In some rabbits, heparin (20 U/kg) or dextran sulfate (5000 molecular weight, 8 μg/kg) was injected into the cisterna magna 3 days after gene transfer of EC-SOD or CuZnSOD, and CSF samples were collected 15, 60, and 120 minutes after injection of heparin or dextran sulfate. We estimated the dose of heparin and dextran sulfate from a previous study, in which 2000 U/kg of heparin and 800 mg/kg of dextran sulfate were injected intravenously.14 Total blood volume is approximately 100× blood volume of the basilar artery, and was then separated on an SDS/10% polyacrylamide gel with 5% stacking gel in SDS/Tris/glycine running buffer.22 We used a commercial CuZnSOD standard (Sigma) and made an EC-SOD standard using gene transfer with AdEC-SOD to C6 glioma cells. The protein was electrophoretically transferred to a nitrocellulose membrane, which was then blocked with 5% (wt/vol) nonfat milk in PBS containing 0.1% Tween-20 for 1 hour at room temperature. The membrane was then incubated with primary antibody, goat anti-human EC-SOD (1:5000), or sheep anti-human CuZnSOD (1:500) in PBS containing 0.1% Tween-20 for 1 hour. After it was washed, the blot was incubated with horseradish peroxidase–conjugated rabbit anti-goat IgG (1:10 000) or rat anti-sheep IgG (1:10 000) for 1 hour at room temperature. After it was washed, the blot was incubated with Super Signal ULTRA chemiluminescent substrate (Pierce Laboratories) and exposed to x-ray film.

Data Analysis

Results are expressed as mean ± SE. Total SOD activity data in CSF, basilar arteries, and meninges were analyzed with 1-way ANOVA with post hoc Bonferroni correction.

SOD Assay

Total SOD activities of tissue and CSF were determined by the method of Spitz and Osherley.20 Measurement of enzyme activity was based on inhibition of O_2^−–mediated reduction of NBT to formazan, the photosabsorbant product. Solutions containing 1 U/mL catalase, 5.6 × 10^−1 mol/L NBT, 0.1 mmol/L xanthine, 1.0 mmol/L diethyl- enetriaminepentaacetic acid, 0.13 mg/mL bovine serum albumin, and 50 μmol/L bathocuproine disulfonic acid in 0.05 mol/L phosphate buffer (pH 7.8) were incubated with varying amounts of supernatant (0 to 100 μg of protein in tissue samples, 0 to 100 μL in CSF samples). Superoxide radicals were generated by adding xanthine oxidase (10^−3 to 10^−5 U/mL) to the cuvettes, and measurements were recorded at 560 nm, 37°C for 5 minutes, with the use of a spectrophotometer. Total SOD activity of the supernatant was measured as the effectiveness of the supernatant to inhibit dismutation of O_2^− generated by the xanthine–xanthine oxidase reaction. Total SOD activity were expressed in units per milligram of protein measured, as described by Lowry et al.21 for tissue samples or units per milliliter for CSF samples.

Western Blotting for CuZnSOD and EC-SOD in CSF

To determine the isoforms of SOD in CSF, Western blotting was used. First, CSF samples were concentrated 10-fold by cryoporation. CSF that contained 50 μg of protein was denatured with SDS loading buffer at 95°C for 5 minutes and then separated on an SDS/10% polyacrylamide gel with 5% stacking gel in SDS/Tris/glycine running buffer.22 We used a commercial CuZnSOD standard (Sigma) and made an EC-SOD standard using gene transfer with AdEC-SOD to C6 glioma cells. The protein was electrophoretically transferred to a nitrocellulose membrane, which was then blocked with 5% (wt/vol) nonfat milk in PBS containing 0.1% Tween-20 for 1 hour at room temperature. The membrane was then incubated with primary antibody, goat anti-human EC-SOD (1:5000), or sheep anti-human CuZnSOD (1:500) in PBS containing 0.1% Tween-20 for 1 hour. After it was washed, the blot was incubated with horseradish peroxidase–conjugated rabbit anti-goat IgG (1:10 000) or rat anti-sheep IgG (1:10 000) for 1 hour at room temperature. After it was washed, the blot was incubated with Super Signal ULTRA chemiluminescent substrate (Pierce Laboratories) and exposed to x-ray film.

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Immunochemistry for EC-SOD

Unfixed frozen tissues were cut into 6-μm sections on a cryostat at −20°C. Sections were incubated with primary antibody (goat anti-human EC-SOD, 1:500) overnight. After they were washed with PBS, sections were incubated for 30 minutes with the secondary antibody (mouse anti-goat IgG, 1:1000), rinsed again with PBS, and developed with Vectastain ABC Kit alkaline phosphatase (Vector Laboratories Inc).

Total SOD Activity

Tissue Preparation

Transduced basilar arteries and meninges were homogenized with a tissue homogenizer (5-second bursts for 3 minutes) in 3 volumes of 0.05 mol/L phosphate buffer, pH 7.8, and sonicated with an ultrasonic processor (10-second bursts for 1 minute). All procedures were performed on ice. The homogenate was centrifuged at 3000 rpm for 10 minutes, and the supernatant was used for determination of protein content and total SOD activity.

SOD in Tissue

Extensive expression of EC-SOD was observed in the adventitia of basilar artery from rabbits that were injected with AdEC-SOD. No EC-SOD staining was seen in basilar artery from rabbit that received Adβ-gal (Figure 1). Endogenous EC-SOD of rabbit was not stained because the goat anti-human EC-SOD antibody did not detect rabbit EC-SOD.

After injection of AdCuZnSOD into the cisterna magna, total SOD activity of basilar arteries was 2.5 times higher than that after injection of Adβ-gal. Total SOD activity of basilar artery tended to increase after gene transfer of AdEC-SOD but was not significantly higher than that after Adβ-gal (Figure 2).

Results were similar in the meninges. SOD activity in meninges was significantly higher 3 days after gene transfer of CuZnSOD than after EC-SOD or β-gal (Figure 2).
SOD in CSF
There was no significant difference among the 3 groups in basal levels of total SOD activity in CSF. Three days after gene transfer of AdEC-SOD, total SOD activity was 3 times higher than basal level and was significantly higher than after injection of Adβ-gal or AdCuZnSOD (Figure 3). Total SOD activity tended to increase after injection of AdCuZnSOD compared with Adβ-gal, but this was not statistically significant.

Western blotting of EC-SOD showed a band in CSF at the same position as the EC-SOD standard after injection of AdEC-SOD (Figure 4). This band was not detected in CSF samples from rabbits that were treated with AdCuZnSOD or Adβ-gal. In contrast, CuZnSOD protein was undetectable in any samples of CSF. This result indicates that there is no detectable CuZnSOD protein in CSF even after gene transfer of CuZnSOD.

Effects of Heparin and Dextran Sulfate
After transduction with AdEC-SOD, injection of heparin into the cisterna magna of rabbits increased total SOD activity by 6.7-fold, which was 27-fold the basal value for total SOD activity (Figure 5, left panel). The elevation was sustained for at least 2 hours after injection of heparin.

After transduction with AdEC-SOD, injection of dextran sulfate increased total SOD activity by 7.4-fold, which was a 33-fold increase above basal value in total SOD activity (Figure 5, right panel). Injection of heparin or dextran sulfate in rabbits that received AdCuZnSOD did not significantly increase total SOD activity in CSF.

Figure 1. Immunocytochemistry for EC-SOD in basilar artery from rabbits after injection of AdEC-SOD (B, magnification ×128; D, ×400) or Adβ-gal (A, magnification ×160; C, ×400). Adv indicates adventitia. There was extensive blue staining for EC-SOD in adventitia of basilar artery after AdEC-SOD but not Adβ-gal. No staining was observed in the absence of primary antibody. The sections were counterstained with nuclear fast red.

Figure 2. Total SOD activity in basilar arteries and meninges 3 days after gene transfer of EC-SOD, CuZnSOD, or β-gal. SOD activity in basilar arteries from rabbits that received AdCuZnSOD was significantly higher than after injection of Adβ-gal. SOD activity in meninges from rabbits that received AdCuZnSOD was significantly higher than after injection of Adβ-gal and AdEC-SOD. *P<0.01 vs Adβ-gal. †P<0.01 vs AdEC-SOD. Values are mean±SE; n=5 for Adβ-gal, n=5 for AdEC-SOD, and n=6 for AdCuZnSOD.
MnSOD, it has been assumed that this enzyme does not play a major role in dismutation of superoxide in extracellular space. However, recent studies indicate that in some tissues, such as blood vessels, uterus, and airways, EC-SOD is the predominant isoform of SOD. Thus, effects of EC-SOD under physiological and pathological conditions are of interest. Recently, several studies using EC-SOD transgenic mice have reported that EC-SOD has neuroprotective effects and anti-inflammatory effects.

EC-SOD has a heparin binding site and can bind to heparan sulfate proteoglycans on the cell surface. On the basis of affinity for heparin, EC-SOD is separated into type A (without affinity), type B (with weak affinity), and type C (with high affinity) for heparin. There are large interspecies differences in total plasma EC-SOD activity and affinity for heparin. EC-SOD of rats is mainly present in the extracellular fluid because rats have no type C EC-SOD. On the other hand, as predicted from the affinity for heparin, EC-SOD of humans (type C) binds to the cell surface. In rabbits, type C is the predominant isoform of EC-SOD.

In this study we transduced human EC-SOD to the brain of rabbits. Thus, human EC-SOD was overexpressed in cells and released into the extracellular space and then presumably bound to extracellular matrix of tissues. Immunocytochemistry for EC-SOD demonstrated that human EC-SOD was expressed in the adventitia of basal artery and meninges after gene transfer. Interestingly, and as anticipated, total SOD activity in CSF also increased after injection of AdEC-SOD. On the basis of the Western blotting, the increase of SOD activity was due to gene transfer by immunocytochemistry and Western blotting. EC-SOD is the only isoform of SOD that is released into the extracellular space.

Discussion

In this study we demonstrated that, first, overexpression of CuZnSOD and EC-SOD can be achieved in the subarachnoid space by in vivo gene transfer. Second, the distributions of CuZnSOD and EC-SOD are different after gene transfer. CuZnSOD is expressed mainly in cells after gene transfer and, in contrast, EC-SOD is released into the CSF. Third, injection of heparin or dextran sulfate into the cisterna magna greatly enhanced the release of EC-SOD into the CSF in rabbits that received AdEC-SOD.

Immunocytochemistry of EC-SOD showed extensive staining in the adventitia of basilar artery from rabbits that received AdEC-SOD. Furthermore, total SOD activity in basilar artery and meninges from rabbits that received AdCuZnSOD increased significantly compared with β-gal transduced–rabbits. These results indicate that CuZnSOD and EC-SOD can be transduced in the basilar artery and meninges by injection of adenoviral vectors into the cisterna magna. In this study we used anti-human EC-SOD antibody, which detects human but not rabbit EC-SOD. Thus, we could detect the EC-SOD that is expressed by gene transfer by immunocytochemistry and Western blotting.

EC-SOD is the only isoform of SOD that is released into the extracellular space. Because the amount of EC-SOD in most organs is relatively small compared with CuZnSOD and CuZnSOD protein was not detected in any CSF samples, even after gene transfer of CuZnSOD. Lane 4 and 5 were EC-SOD standard and CuZnSOD standard, respectively.

Figure 4. Western blotting of EC-SOD protein and CuZnSOD protein in CSF from rabbits 3 days after gene transfer of β-gal (lane 1), CuZnSOD (lane 2), or EC-SOD (lane 3). EC-SOD protein was detected in CSF only from rabbits that received AdEC-SOD. CuZnSOD protein was not detected in any CSF samples, even after gene transfer of CuZnSOD. Lane 4 and 5 were EC-SOD standard and CuZnSOD standard, respectively.

Figure 5. Total SOD activity in CSF from rabbits that received AdCuZnSOD or AdEC-SOD 3 days before these measurements. Left, After injection of heparin (20 U/kg) into the cisterna magna, total SOD activity increased in CSF of rabbits that received AdEC-SOD but not AdCuZnSOD. *P<0.01 vs AdCuZnSOD. Values are mean±SE; n=5 for AdEC-SOD, n=5 for AdCuZnSOD. Right, After injection of dextran sulfate (5000 molecular weight, 8 μg/kg) into the cisterna magna, total SOD activity increased in CSF of rabbits that received AdEC-SOD but not AdCuZnSOD. *P<0.01 vs AdCuZnSOD. Values are mean±SE; n=4 for AdEC-SOD, and n=3 for AdCuZnSOD.
proteoglycans. Thus, some amount of type C EC-SOD may be released into the CSF by heparin.

Injection of heparin or dextran sulfate into the cisterna magna produced a large increase of total SOD activity in CSF in rabbits after gene transfer of EC-SOD but not CuZn-SOD. Because total SOD activity is measured in CSF, the values represent both endogenous and exogenous SOD. Thus, the minimal increase in SOD activity shown in Figure 5, after heparin or dextran sulfate in rabbits that received AdCuZn-SOD, indicates that neither exogenous nor endogenous isoforms increased substantially. We speculate that heparin and dextran sulfate bind to the heparin binding site of EC-SOD and displace EC-SOD into CSF from cells lining the subarachnoid space. This result is consistent with a previous report that showed an increase in SOD activity in plasma after gene transfer of EC-SOD and injection of heparin or dextran sulfate intravenously.14

It is likely that, after injection of AdEC-SOD into the cisterna magna, some EC-SOD was released into the CSF, but the majority of the EC-SOD was bound to the extracel-

lular matrix of tissues. Heparin and dextran sulfate then presumably displaced EC-SOD, which was bound to cells, from extracellular matrix into extracellular fluid. In a previous report, a 3-fold increase in CuZn-SOD in neurons of transgenic mice had a protective effect against glutamate neurotoxicity.27 In other reports, a 3- to 5-fold increase in EC-SOD in the brain or lung of transgenic mice had a protective effect against cerebral ischemia24 or hyperoxic

neurotoxicity.25 Thus, we anticipate that, although SOD activity in tissues, with only a small increase in CSF. This finding is consistent with the concept that CuZnSOD is produced within cells and, after gene transfer, largely remains intracellular. However, total SOD activity in CSF tended to increase after gene transfer of CuZnSOD. One possible mecha-
nism for the increase in SOD activity in CSF is that some cells, which were infected with AdCuZnSOD, may have undergone cell death, and endogenous rabbit SOD was released into the CSF. Because no human CuZnSOD protein was detected in CSF by Western blotting after gene transfer of CuZnSOD, it is unlikely that the elevation of SOD activity was produced by human SOD that was transduced to the rabbits.

As discussed above, it is most likely that the higher activity of SOD in CSF after gene transfer of EC-SOD than CuZnSOD is due to release of EC-SOD, but not CuZnSOD, from the extracellular compartment into the CSF. It is possible, however, that both SODs are released into CSF, and more rapid clearance of the smaller protein (CuZnSOD) contributes to low concen-
trations in CSF.

CuZnSOD may play an important role in protection against neuronal injury1–3 and vasospasm after subarachnoid hemorrhage.5 CuZnSOD improves vasomotor function28–30 by dismutation of superoxide anion within cells. Several reports have suggested that increases in intracellular CuZnSOD, using liposomal-encapsulated SOD31–33 or polyethylene glycol–conjugated SOD,29,30 are effective in protection of cells. Using gene transfer techniques, CuZn-

SOD can be overexpressed within cells. Thus, gene transfer of CuZnSOD also appears to be a promising approach to protect vessels from pathological conditions.

Efficiency of gene transfer varies among species and may be influenced by serotype of adenoviral vectors that is used for different species. Applications of this approach to differ-
ent species, including humans, will require optimization of the vector, including perhaps the serotype of the adenovirus.

Studies using transgenic mice suggest that CuZnSOD and EC-SOD are key enzymes in protection against neuronal injury or vasomotor dysfunction.1,15,24 Furthermore, EC-SOD may be involved in regulation of vasomotor function.14 Because there are substantial amounts of EC-SOD in blood vessels,11,12 We suggest that this intracranial in vivo gene transfer approach may be useful in clarifying the role of EC-SOD and CuZnSOD in cerebral vascular biology.

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Although transgenic or knockout mice of these SOD isoforms have been generated and used in stroke studies,\(^3\)\(^-\)\(^6\) it is not clear whether these enzymes can be expressed and upregulated by means other than the transgenic technology. In this nicely written and carefully done study, Nakane and his colleagues have injected an adenovirus-mediated overexpression of endothelial nitric oxide synthase. Am J Physiol. 1997;273: H265–H270.

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