Amelioration of Vasospasm After Subarachnoid Hemorrhage in Transgenic Mice Overexpressing CuZn–Superoxide Dismutase

Hideyuki Kamii, MD, PhD; Ichiro Kato, MD, PhD; Hiroyuki Kinouchi, MD, PhD; Pak H. Chan, PhD; Charles J. Epstein, MD; Atsushi Akabane, MD, PhD; Hiroshi Okamoto, MD, PhD; Takashi Yoshimoto, MD

Background and Purpose—To clarify the effect of superoxide dismutase (SOD) on vasospasm after subarachnoid hemorrhage (SAH), we investigated sequential changes in arterial diameter after SAH in transgenic mice overexpressing CuZn-SOD (SOD-1).

Methods—SOD-transgenic mice and nontransgenic littermates (35 to 40 g) were subjected to SAH produced by endovascular perforation of the left anterior cerebral artery. At 4 hours and 1, 3, 7, and 14 days after SAH, the mice were perfused with 10% formalin and consequently with a mixture of carbon black and 10% gelatin to cast all vessels. Vasospasm was evaluated by measuring the diameter of the left middle cerebral artery (MCA) with a microscope.

Results—In nontransgenic mice, the diameter of the MCA on day 3 after SAH (110.5 ± 20.5 μm [mean ± SD]; n = 16) was significantly reduced compared with that without SAH (138.5 ± 14.5 μm; n = 12) (P<0.01). Moreover, on day 3 after SAH, the diameter of the MCA in SOD-transgenic mice (127.9 ± 20.2 μm; n = 20) was significantly larger than that in nontransgenic mice (110.5 ± 20.5 μm; n = 16) (P<0.05).

Conclusions—These results suggest that SOD is effective on the amelioration of vasospasm after SAH and that oxygen free radicals, particularly superoxide, play an important role in the pathogenesis of vasospasm after SAH. (Stroke. 1999;30:867-872.)

Key Words: free radicals • mice, transgenic • subarachnoid hemorrhage • superoxide dismutase • vasospasm

Erythrocytes are essential for causing vasospasm, and oxyhemoglobin released from erythrocytes in the subarachnoid clot is believed to be a most potent trigger of vasospasm.1,2 However, the pathophysiology of cerebral vasospasm after subarachnoid hemorrhage (SAH) still remains unclear. Recently, 2 major derangements in the cerebral artery have been indicated as a cause for cerebral vasospasm after SAH. One is augmentation of contraction, which is protein kinase C (PKC) dependent, and the other is suppression of dilation, which is mediated by endothelium-derived relaxing factor/nitric oxide (NO).3 Oxygen free radicals are involved in both systems; active oxygens can activate the PKC system and lead to lipid peroxidation through activation of phospholipase A2,4 and superoxide (O2•−) is known to inactivate NO,5 resulting in the occurrence of vasospasm after SAH. Therefore, superoxide dismutase (SOD), an enzyme converting O2•− to hydrogen peroxide (H2O2), could prevent contraction of the cerebral artery after SAH. Experiments in vivo, however, have not always proven the efficacy of SOD in preventing vasospasm after SAH. Kamiyama et al6 initially showed that SOD is effective against vasospasm induced by oxyhemoglobin in cats. In addition, intracisternal injection of SOD reduced endothelial injury and prevented the occurrence of vasospasm in a rabbit SAH model.7 However, intrathecal administration of both SOD and catalase failed to protect against oxyhemoglobin-induced vasospasm in monkeys.8 The discrepancy in the effect of SOD on vasospasm after SAH may result from differences in methods of administration of SOD.

In the present study we established a new mouse SAH model and investigated sequential changes in arterial diameter after SAH in transgenic mice overexpressing CuZn-SOD (SOD-1) to clarify the effect of SOD on vasospasm after SAH. In SOD-transgenic mice, the CuZn-SOD gene (Sod1) is expressed in all nervous elements, including neurons, glia, and endothelial cells.9 Therefore, the complicating issues regarding the half-life of SOD in cerebrospinal fluid and potential side effects of exogenously supplied enzyme could be eliminated in our studies.
Materials and Methods

In making transgenic mice that carry the human CuZn-SOD gene (Sod1), for the present study, a linear 14.5-kb EcoRI-BamHI fragment of human genomic DNA was excised from the recombinant plasmid pHGSOD-SVneo and separated from the plasmid sequences before microinjection. The EcoRI-BamHI DNA fragment contains the entire SOD-1 gene, including the promoter sequences required for expression in transfected cells. In the heterozygous SOD-1 transgenic mice designated as TgHS/SF 218/3, a 3-fold increase in CuZn-SOD activity has been observed in all brain regions including the cerebral cortex, where CuZn-SOD levels were 7.9 ± 0.5 and 22.7 ± 1.41 units per milligram protein in nontransgenic and SOD-transgenic mice, respectively. Neurons, astroglia, and cerebral vessels were immunocytochemically stained for a polyclonal antibody against SOD-1, suggesting that SOD-1 immunoreactivity is expressed in all brain cells in the transgenic mice. There were no observable phenotypic differences between SOD-transgenic mice and nontransgenic normal littermates in the present study as well as in our previous studies. In SOD-transgenic mice were identified by qualitative demonstration of human CuZn-SOD in blood samples with the use of nondenaturing gel electrophoresis followed by nitroblue tetrazolium staining.

In the present study we used a new mouse model of SAH, which modified rat SAH models on the basis of endovascular arterial rupture near the bifurcation of the anterior cerebral artery (ACA) and the middle cerebral artery (MCA). Since these rat models often occluded the MCA because of mechanical injury by endovascular perforation in our pilot study, we perforated the ACA near the anterior communicating artery by an endovascular technique to prevent MCA occlusion. Briefly, anesthesia was induced in a chamber with a mixture of 2% halothane, 68% N2O, and 30% O2.

To assess the degree of vasospasm after SAH in rat and rabbit SAH models, anesthetized in a chamber with a mixture of 2% halothane, 68% N2O, and 30% O2. We were allowed to respire spontaneously, and the anesthesia was maintained with a mixture of 0.5% halothane, 69.5% N2O, and 30% O2 during the operation. The left femoral artery was cannulated for measurement of mean arterial blood pressure, PaO2, PaCO2, and pH before and after SAH. The left common carotid artery was exposed, and the external carotid artery (ECA) and its branches were isolated and coagulated. A 5.0-monofilament nylon suture, blunted at the tip, was introduced into the internal carotid artery (ICA) through the ECA stumps up to the left ACA near the anterior communicating artery, where resistance was encountered, as in a mouse ischemia model used in our previous studies. The suture was advanced 5 mm further to perforate the artery and was immediately withdrawn through the ICA into the ECA, allowing reperfusion and producing SAH. When endovascular SAH occurred, mice showed respiratory failure, from which they recovered spontaneously after several seconds. Sham-operated control mice underwent identical procedures except that the suture was withdrawn just after the resistance was felt.

At 4 hours and 1, 3, 7, and 14 days after SAH, the mice were anesthetized with an intraperitoneal injection of 20 mg/kg pentobarbital and perfused through the left ventricle with 10% formalin and consequently with a mixture of carbon black and 10% gelatin to cast all vessels. All experimental protocols were approved by the Tohoku University Animal Research Committee.

Results

In our mouse SAH model, SAH was diffusely distributed in the basal cistern of the brain, especially around the left ACA (Figure 2A). The mortality rate within 72 hours was 29% in nontransgenic and 27% in SOD-transgenic mice, which was not significantly different between nontransgenic and SOD-transgenic mice. Severe SAH occurred in some mice, all of which died within several hours (Figure 2B).

Figure 3 demonstrates representative photographs of sequential changes in the diameter of the MCA after SAH in nontransgenic mice. On day 3 (Figure 3B) after SAH, the diameter of the MCA was reduced compared with that in sham-operated control mice (Figure 3A). On day 7 (Figure 3C), it was almost recovered to the extent of that in the control mice. As shown in Figure 4, in nontransgenic mice, the diameter of the MCA on day 3 after SAH (110.5 ± 20.5 μm [mean ± SD]; n = 16) was significantly reduced compared with that in sham-operated control mice (138.5 ± 14.5 μm; n = 12) (P < 0.01, Student’s t test), whereas that on day 1 (124.6 ± 22.1 μm; n = 15) and day 7 (139.4 ± 13.1 μm; n = 14) showed no significant differences from that in the control. In SOD-transgenic mice, the MCA diameter on day 1 (133.5 ± 24.2 μm; n = 12), day 3 (127.9 ± 20.2 μm; n = 20),
and day 7 (137.3±18.9 μm; n=10) demonstrated no significant changes from that in the control (140.1±12.5 μm; n=8). On day 3 after SAH, the diameter in SOD-transgenic mice (127.9±20.2 μm; n=20) was significantly larger than that in nontransgenic mice (110.5±20.5 μm; n=16) (P<0.05).

Discussion

With the recent successful development of genetically engineered mice (transgenic or knockout) in stroke research, a mouse ischemic model has become more prevalent. However, mouse SAH models have never been reported because of the technical difficulties in making a reliable model, although a number of SAH model in rats are available for research on SAH. Recently, new rat SAH models without craniectomy have been developed on the basis of an endovascular perforation of the cerebral artery. These models more closely resemble aneurysmal rupture in clinical cases but have not demonstrated delayed vasospasm after SAH. In the present study we established a new SAH model in the mouse, which modified rat SAH models without craniectomy. Our mouse model has demonstrated delayed vasospasm after SAH by the use of the casting method, with a mixture of carbon black and gelatin. The application of this model to transgenic or knockout mice can contribute to the elucidation of molecular mechanisms underlying the pathogenesis of SAH, including delayed vasospasm.

Despite intensive research efforts, the mechanism underlying vasospasm after SAH still remains unclear. Recent reports have shown that endothelial injury and resultant impairment in endothelium-dependent relaxation play an important role in the development of vasospasm after SAH. However, the cause of the derangement in endothelium-dependent relaxation is not known. The classic endothelium-derived relaxing factor has been identified as NO, which is synthesized from the amino acid L-arginine by the Ca2+-dependent enzyme NO synthase. Hino et al. showed that endothelial NO synthase mRNA decreased in cerebral arteries 7 days after SAH, suggesting that decreased production of NO by NO synthase in endothelial cells could contribute to vasospasm after SAH. In addition, it was reported that hemoglobin binding of NO inhibited endothelium-dependent relaxation in the cerebral artery. In smooth muscle cells, soluble guanylate cyclase or guanosine monophosphate, which is necessary for the relaxation response, was reduced in the canine basilar artery after SAH. Thus, scavenging of NO or decreased response of smooth muscle to NO may also result in impaired endothelium-dependent relaxation.

It is believed that oxyhemoglobin in its conversion to methemoglobin releases superoxide (O2•−). This O2•−, in turn, rapidly reacts with NO to form peroxynitrite, which is a strong oxidant and could form a species with the reactivity of hydroxyl radical during decomposition. Thus, a high level of CuZn-SOD activity may reduce the amount of O2•−, leading to increase of NO level by prolonging the half-life of NO itself. In addition, decrease in O2•− may result in a smaller amount of peroxynitrite and hydroxyl radical, which can diminish endothelial injury and increase NO production. Therefore, in SOD-transgenic mice, a high level of CuZn-SOD activity can contribute to increase in NO level and consequently amelioration of vasospasm after SAH. Shishido et al. demonstrated that intrathecal injection of SOD prevented morphological endothelial injury and attenuated the occurrence of vasospasm in a rabbit SAH model. It is also reported that injection of SOD enhanced and prolonged the vasodilatation induced by sufficient exogenous l-arginine on the spastic basilar artery after SAH in dogs. In addition, Medele et al. demonstrated that brains of rats with angiographic vasospasm revealed nitrotyrosine, which is a peroxidation product of peroxynitrite with tyrosine contained in tissue proteins, predominantly located with a perivascular distribution and in the pia. These previous reports are consistent with our results.

On the other hand, it is suggested that the increase in SOD alone may accumulate H2O2 and subsequently increase hydroxyl radical by an iron-catalyzed Haber-Weiss reaction and Fenton reaction. Therefore, to reduce hydroxyl radical production and prevent the occurrence of vasospasm after SAH, both SOD and catalase or glutathione peroxidase might be necessary, since H2O2 is detoxinized by catalase and/or glutathione peroxidase to H2O and O2. In SOD-transgenic mice, a higher level of H2O2 is produced in the brain than in nontransgenic littermates under normal physiological condi-
tions; however, enzymatic activity of catalase is also induced to convert increased amount of H$_2$O$_2$ to H$_2$O and O$_2$. Although we did not measure the catalase activity in the present study, overproduced H$_2$O$_2$ might be catalyzed by induced catalase after SAH, resulting in diminished production of hydroxyl radical and consequently amelioration of vasospasm in SOD-transgenic mice.

It has been also emphasized that increase in PKC-dependent smooth muscle contraction plays an important role in causing vasospasm after SAH.53–56 The arterial smooth muscle has 2 contractile systems; one is the Ca$^{2+}$/calmodulin/myosin light-chain kinase system, and the other is the PKC-mediated system, which can be activated without a precipitous rise in intracellular Ca$^{2+}$ concentration. In the canine basilar artery after SAH, recent reports have shown an increase in diacylglycerol, 57 an intrinsic PKC activator, and a decrease in cGMP, 45 an inhibitor of the PKC system, as well as an increase in PKC activity. 55,58 The vascular smooth muscle has two mechanisms for vascular relaxation: the first is the cGMP-mediated relaxation system in cerebral arteries, and the other is the PKC-mediated system, and the other is the PKC-mediated system, which can be activated without a precipitous rise in intracellular Ca$^{2+}$ concentration. In the canine basilar artery after SAH, recent reports have shown an increase in diacylglycerol, 57 an intrinsic PKC activator, and a decrease in cGMP, 45 an inhibitor of the PKC system, as well as an increase in PKC activity. 55,58 The present study clearly demonstrated that preexisting high level of CuZn-SOD contributed to improvement of vasospasm after SAH, since the complicating issues regarding potential side effects of exogenously supplied enzyme and differences in the administration method of the enzyme could be eliminated in our studies. Hence, there may have been a technical failure in the administration of SOD in the previous studies, which could not demonstrate the efficacy of SOD in preventing vasospasm after SAH, although it is not known whether catalase and/or glutathione peroxidase as well as SOD may be necessary to reduce hydroxyl radical production.

In conclusion, we established a new SAH model in mice and demonstrated a significant amelioration of vasospasm after SAH in SOD-transgenic mice compared with nontransgenic littermates. A high level of CuZn-SOD activity could maintain endothelial NO production and inhibit PKC activation, resulting in amelioration of prolonged smooth muscle contraction in the cerebral artery. Oxygen free radicals, particularly superoxide, may play a pivotal role in the pathogenesis of vasospasm after SAH.

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References


Vasospasm following SAH carries a serious consequence. The molecular mechanisms of vasospasm after SAH remains to be fully delineated, but there are likely contributions by multiple factors. Among them, the generation of free radicals has been extensively studied. Experimental evidence supporting the role of free radicals is derived from the observations showing reduction of vasospasm by antioxidants or free radical scavenging enzymes such as superoxide dismutate (SOD). In the preceding article, Kamii and associates report the development of an SAH model in mice and the application of this model in transgenic mice overexpressing SOD to confirm the putative role of free radicals (superoxide in particular) in the pathogenesis of vasospasm. They found that vasospasm after SAH was reduced in mice overexpressing SOD compared with their littermates. The significance of this study is the notion that SOD has been applied extensively to prevent vasospasm in animal models of SAH with mixed results. The short half-life of SOD and its low uptake by cells probably have contributed to the lack of SOD effects. Endogenous overexpression of SOD in mice apparently circumvents these problems.

Kamii et al assessed the extent of vasospasm based on the diameter of the proximal middle cerebral artery in formalin-fixed and gelatin-cast brains. It remains to be confirmed how faithfully this measure reflects the magnitude of vasospasm as determined by angiography. However, it appears clear that overexpression of SOD has reduced the alteration of vasoreactivity after SAH. The authors have also eloquently addressed 2 relevant molecular cascades underlying SOD inhibition of vasospasm, namely, the reduction of the interaction of superoxide with nitric oxide to form peroxynitrite and the inhibition of protein kinase C activity. Further studies comparing the content of nitric oxide or its metabolites and protein kinase C activity between mice overexpressing SOD and their littermates are needed to substantiate these 2 molecular mechanism(s) that may be altered by SOD activity to affect vasospasm after SAH.

Chung Y. Hsu, MD, PhD, Guest Editor
Department of Neurology
Cerebrovascular Disease Section
Washington University School of Medicine
St Louis, Missouri
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