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EFFECT OF A FREE RADICAL SCAVENGER, EDARAVONE, ON FREE RADICAL REACTIONS — RELATED SIGNAL TRANSDUCTION AND CEREBRAL VASOSPASM IN THE RABBIT SUBARACHNOID HEMORRHAGE MODEL —

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Abstract **OBJECTIVE:** It is hypothesized that free radical reactions evoked by oxyhemoglobin (oxyHb) cause cerebral vasospasm after aneurysmal subarachnoid hemorrhage (SAH), even though the detailed mechanisms have not yet been fully established. The aims of this study were thus to investigate, through the use of the double-hemorrhage rabbit model, the possibility that using a potent free radical scavenger, edaravone, will show amelioration of cerebral vasospasm, and to delineate the mechanism of signal transduction that causes cerebral vasospasm. **METHODS:** In the SAH group, SAH was simulated using the double-hemorrhage rabbit model. In the treatment group, edaravone (0.6 mg/kg) was injected into the central ear vein. Ninety-six hours after SAH, the basilar artery was excised. The degree of cerebral vasospasm was evaluated by measuring the diameter of each basilar artery, and the expression of Rho-kinase in the vascular wall was examined by Western blotting. **RESULTS:** The diameter of the basilar artery in the edaravone-treated group was 0.71 ± 0.06 mm, which was statistically significantly larger than that in the nontreated SAH group (0.50 ± 0.03 mm; $P < 0.01$). The expression of Rho-kinase in the edaravone-treated group was statistically significantly reduced in comparison to that of the nontreated SAH group ($P < 0.01$). **CONCLUSION:** Results from this study indicate that edaravone may potentially serve as an agent in the prevention of cerebral vasospasm in patients after SAH. In addition, it might be suggested that the free radical reaction mediated by oxyHb is concerned with the regulation of the Rho/Rho-kinase pathway.

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Key words: Cerebral vasospasm; edaravone; free radical scavenger; Rho-kinase; subarachnoid hemorrhage.

Objective

It is hypothesized that free radical reactions, such as lipid peroxide production in the arterial smooth muscle layer, evoked by oxyhemoglobin (oxyHb) released from a subarachnoid clot cause cerebral vasospasm after aneurysmal subarachnoid hemorrhage (SAH).[9,15,22,23,27,49] In fact, previous studies using free radical scavengers in experimental SAH models showed amelioration of cerebral vasospasm as a result of scavenging free radicals.[3,11,25,53]

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a potent free radical scavenger which is widely used in Japan to improve functional outcomes in patients suffering from acute ischemic stroke.[43,52] In addition, we found a trend toward a lower incidence of delayed ischemic neurological deficits (DINDs) and lower incidence of poor outcome caused by cerebral vasospasm in edaravone-treated patients with aneurysmal SAH.[33]

Several recent studies have revealed that intracellular signal transduction of vascular smooth muscle cells, especially the Rho/Rho-kinase pathway[8,18,38,39,49], is activated during the development of cerebral vasospasm and, as a result, a sustained contraction of arterial smooth muscle cells occurs.[18,38,49] Even though it has been speculated that free radical reactions, evoked by oxyHb released from a subarachnoid clot, induce sustained contraction through an

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activated intracellular signal transduction, detailed mechanisms of this free radical reaction-induced sustained contraction have not yet been clarified.[49)]

The aims of this study were to investigate, through the use of the double -hemorrhage rabbit model, the possibility that using edaravone in experimental SAH models will show amelioration of cerebral vasospasm and to delineate the mechanism of signal transduction that causes sustained contraction in cerebral vasospasm.

Materials and Methods

All experimental protocols were approved by the Hirosaki University Animal Research Committee. Thirty Japanese white rabbits weighing 2.5 to 3.0 kg were used. All animals were randomly assigned to three groups: group 1 (sham, n = 10), the animals were given an intravenous injection of edaravone with sham surgery; group 2 (SAH, n = 10), SAH was produced; and group 3 (SAH-edaravone, n = 10), SAH was produced and the animals were given an intravenous injection of edaravone.

Production of SAH

In groups 2 and 3, SAH was produced according to the double-hemorrhage method.[4,31,45)] The animals were anesthetized with an intravenous injection of pentobarbital (30 mg/kg) and an intramuscular injection of ketamine (20 mg/kg).

After anesthesia, under spontaneous breathing, a 23-gauge butterfly needle was percutaneously placed in the cisterna magna, and cerebrospinal fluid (1.0 to 1.5 mL) was aspirated under aseptic technique. Animals in group 1 (Sham) were injected 1.5 mL of saline solution. In groups 2 and 3 (SAH and SAH-edaravone), nonheparinized arterial blood from the femoral artery, 1.5 ml, was injected into the cisterna magna over one to two minutes. Animals were then placed in a 30° head-down, tilted position for 15 minutes to ensure that blood spread into the basal cistern. Forty-eight hours afterwards the second SAH and sham surgery were performed in the same manner as the first.

Intravenous injection of edaravone

In groups 1 and 3, edaravone (0.6mg/kg) was injected into the central ear vein over 1 to 2 minutes, twice a day for 96 hours after SAH.

Histological Evaluation

Perfusion-fixation was performed at ninety-six hours after SAH. Five animals of each group were deeply anesthetized using 100 mg/kg pentobarbital, the thorax was opened, and a cannula was immediately inserted into the ascending aorta via the left ventricle. Perfusion fixation was performed at 75 mm Hg with 400 mL heparinized physiological saline (5000 U / 500 mL), followed by 500 mL of phosphate-buffered 4% paraformaldehyde (pH 7.4). Finally, the brain was carefully removed so as not to stretch and injure the basilar artery.

The tissue was dehydrated in graded alcohol and embedded in paraffin. All 6-μm-thick sections were cut vertically, mounted on a glass slide, and stained with hematoxylin-eosin (HE) or with periodic acid-Schiff stain (PAS).

Cerebral vasospasm was evaluated using the HE-stained sections. Vessel patency was quantified by measuring the basilar artery circumference with the National Institutes of Health image program (version 1.62). To correct for vessel deformation and off-transverse sections, the internal circumferences of five different sections of each vessel, separated by 200 μm, were measured and averaged. The luminal cross-sectional area of each vessel was estimated with the use of the calculated radius (r) value obtained from the measured circumference (r = measured circumference / 2π; area of circle = πr^2). The mean diameter of the basilar arteries was calculated from the calculated radius (diameter = 2r).

Lipid peroxide production was evaluated using the PAS-stained sections[34)]. Expressions of lipid

peroxide production in vascular smooth muscle were reviewed by two blinded, independent observers (TN and MN). The intensity in the vascular smooth muscle layer was graded on a 3-point scale, from 0 (least intense) to 2 (most intense). Differences in the score graded were resolved by discussion with a third observer (HO).

Western blotting

Another five animals of each group were killed by the intravenous injection of high dose pentobarbital (300 mg/kg) on day four after SAH. The basilar arteries were immediately removed and stored at -80℃ until analysis.

Western blotting followed the standard technique. Primary antibody included goat anti-RockⅡ (sc1851; Santa Cruz Biotechnology; Santa Cruz, CA). The membrane was incubated with the appropriate Cruz Marker compatible secondary antibody. Bands were detected with a chemiluminescence detection kit (ECL plus; Amersham Bioscience). Blot bands were quantified using the densitometry method (Scion image Beta 4.02), n = 5 for each group. The value of the sham is expressed as 100%, and other groups are expressed as a percentage of the sham group.

Statistical analysis

Data are expressed as mean ± SEM. Differences between individual groups were analyzed using *post hoc* t-tests supported by JMP® (Version 5; SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Evaluation of cerebral vasospasm

In group 2 (SAH, n = 5), mean ± SEM basilar artery diameter was statistically significantly reduced four days after SAH versus group 1 (Sham, n = 5) (0.50 ± 0.03 mm versus 0.73 ± 0.05 mm, $P < 0.01$). After SAH, mean ± SEM basilar artery diameter was greater in group 3 (SAH + edaravone, n = 5) versus group 2 (0.71 ± 0.06 mm versus 050 ± 0.03 mm, $P < 0.01$). In addition, marked corrugation of the internal elastic lamina around the wall, with thickening of the vascular smooth muscle layer, was seen in group 2. In contrast, the corrugation was much less remarkable in group 3.

Lipid peroxide production in the vascular smooth muscle layer

In group 1 (Sham, n = 5), lipid peroxide production in the vascular smooth muscle layer was evaluated as a score of 0.2. In group 2 (SAH, n = 5), lipid peroxide production was detected diffusely at the vascular smooth muscle layer, and the average score was 2.0. In contrast, all sections of group 3 (SAH + edaravone, n = 5) showed lower lipid peroxide production, with an average score of 0.4. With the *Chi Squared* test, a statistically significant difference in lipid peroxide production scores was found between groups 2 and 3 ($P < 0.01$).

Expression of Rho-kinase

In group 2 (SAH, n = 5), expression of Rho-kinase was significantly increased in the vascular smooth muscle cells by Western blotting ($P < 0.01$; versus groups 1 and 3).

Discussion

The transformation of oxyHb, released from a subarachnoid clot, into methemoglobin generates activated species of oxygen such as the superoxide anion, hydrogen peroxide and singlet oxygen[10,17,32,50] . Oxygen radicals can initiate peroxidative reactions in membrane polyunsaturated fatty acid, producing lipid peroxides. And it has been speculated that lipid peroxide production in vascular smooth muscle layer causes cerebral vasospasm after SAH. It has thus been considered that scavenging free radicals in the subarachnoid space will ameliorate cerebral vasospasm. In fact, previous studies using free radical scavengers in experimental SAH models showed statistically significant amelioration of cerebral vasospasm, even though detailed mech-

anisms as to how free radicals induce sustained contraction of vasucular smooth muscle have not yet been clarified. This study also revealed that using edaravone in an experimental rabbit SAH model also statistically significantly ameliorated lipid peroxide production in the vascular smooth muscle layer and cerebral vasospasm.

Several recent studies have revealed that the Rho/Rho-kinase pathway plays an important role in vascular diseases. And Sato et al. showed that the Rho/Rho-kinase pathway is activated during the development of cerebral vasospasm after SAH. It has been considered that the Rho/Rho-kinase pathway is activated by some trimeric G-protein-coupled receptors, including lysophosphatidic acid, thrombin, and serotonin receptors, which are linked to the Rho/Rho-kinase pathway. The α subunits of G_i, G_q, G_{12} and G_{13} activate Rho by regulating GDP/GTP exchange factors for Rho. Rho-kinase, which is activated by Rho, phosphorylates the myosin-binding subunit (MBS) of myosin phosphatase and, as a result, activity of myosin phosphatase is decreased. In addition, the activated Rho-kinase phosphorylates myosin light chain (MLC) at the Ser19 residue, which is the site phosphorylated by Ca^{2+}/calmodulin-dependent MLC kinase. Thus a sustained contraction of vascular smooth muscle was induced by Rho-kinase. But, the mechanism as to how SAH activates the Rho/Rho-kinase pathway has not yet been clarified.

Wickman et al. have shown that the oxyHb-mediated sustained contraction of vascular smooth muscle is dependent on the Rho/Rho-kinase pathway and PKC, by using the selective inhibitors of Rho-kinase, Y-27632, and HA-1077, even though the mechanism by which oxyHb activates the Rho/Rho-kinase pathway has not yet been clarified. OxyHb has been shown to exhibit a number of signaling processes such as free radical reactions, elevation of intracellular Ca^{2+}, activation of tyrosine kinases and mitogen-activated protein kinases. Whether free radical reactions or other reactions mediated by oxyHb play a role in the activation of the Rho/Rho-kinase pathway has not yet been clarified. Since the Rho-kinase expression on Western blotting was statistically significantly reduced due to the amelioration of lipid peroxide production in the vascular smooth muscle layer by using edaravone, a potent free radical scavenger in the experimental rabbit SAH model, it could be speculated that the free radical reaction mediated by oxyHb is concerned with the regulation of the Rho/Rho-kinase pathway.

In conclusion, results from this study indicate that edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a potent free radical scavenger, may serve as an agent in the prevention of cerebral vasospasm in patients after SAH, since intravenous administration of edaravone after the onset of SAH statistically significantly ameliorated lipid peroxide production in the vascular smooth muscle layer and basilar artery vasospasm after experimental SAH in rabbits. In addition, results from this study also indicate, for the first time, that free radical reactions mediated by oxyHb might play an important role in the Rho/Rho-kinase pathway by expressing Rho-kinase.

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