Edaravone Attenuates Brain Edema and Neurologic Deficits in a Rat Model of Acute Intracerebral Hemorrhage

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Background and Purpose—Our previous studies have demonstrated that oxidative DNA injury occurs in the brain after intracerebral hemorrhage (ICH). We therefore examined whether edaravone, a free-radical scavenger, could reduce ICH-induced brain injury.

Methods—These experiments used pentobarbital-anesthetized, male Sprague-Dawley rats that received an infusion of either 100 μL autologous whole blood (ICH), FeCl2, or thrombin into the right basal ganglia. The rats were humanely killed 24 hours later. There were 4 sets of experiments. In the first, the dose-dependent effects of edaravone on ICH-induced brain injury were examined by measuring brain edema and neurologic deficits. In the second set, apurinic/apyrimidinic abasic sites and 8-hydroxyl-2′-deoxyguanosine, which are hallmarks of DNA oxidation, were investigated after treatment for ICH. In the third, the effect of delayed treatment with edaravone on ICH-induced injury was determined, whereas the fourth examined the effects of edaravone on iron- and thrombin-induced brain injury.

Results—Systemic administration of edaravone immediately or 2 hours after ICH reduced brain water content 24 hours after ICH compared with vehicle (P<0.05). Edaravone treatment immediately or 2 hours after ICH also ameliorated neurologic deficits (P<0.05). Edaravone also attenuated ICH-induced changes in apurinic/apyrimidinonic abasic sites and 8-hydroxyl-2′-deoxyguanosine and reduced iron- and thrombin-induced brain injury.

Conclusions—Edaravone attenuates ICH-induced brain edema, neurologic deficits, and oxidative injury. It also reduces iron- and thrombin-induced brain injury. These results suggest that edaravone is a potential therapeutic agent for ICH. (Stroke. 2008;39:463-469.)

Key Words: behavior □ cerebral hemorrhage □ edema □ oxidative stress □ rat

Intracerebral hemorrhage (ICH) is an often-fatal stroke subtype. It accounts for 8% to 15% of all strokes in Western populations and 20% to 30% in Asian populations.1 It frequently produces severe neurologic deficits due to secondary brain edema.

Our previous studies have demonstrated that toxic factors, including iron and thrombin released from a blood clot, may account for the formation of perihematomal edema in ICH.2,3 Iron, a hemoglobin degradation product, is associated with free-radical formation in the brain after ICH.4 However, we have also found that intracerebral thrombin can cause protein oxidation.5 There is some limited evidence that free radicals may be a target for reducing ICH-induced brain injury.6,7 The purpose of the current study was to examine whether systemic edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a free-radical scavenger (particularly of hydroxyl radicals), could reduce iron-, thrombin-, and ICH-induced brain edema and behavioral deficits in a rat model. Edavarone (Mitsubishi Pharma Corp) has been used in patients with acute cerebral infarction in Japan. The effect of edavarone on iron-, thrombin-, and ICH-induced oxidative damage was also examined by determining the changes in apurinic/apyrimidinic abasic sites (AP sites) and 8-hydroxyl-2′-deoxyguanosine (8-OHdG), markers of such damage.8

Materials and Methods

Animal Preparation and Intracerebral Infusion

Animal protocols were approved by the Animal Committee of Kagawa University. A total of 105 male Sprague-Dawley rats (CLEA, Tokyo, Japan), each weighing 300 to 400 g, were used for all experiments. Rats were allowed free access to food and water. The animals were anesthetized with pentobarbital (40 mg/kg IP), and the right femoral artery was catheterized to monitor arterial blood pressure and to sample blood for intracerebral infusion. Blood pH, PaO2, PaCO2, hematocrit, and glucose levels were monitored. Rectal temperature was maintained at 37.5°C with a feedback-controlled heating pad (CMA, Stockholm, Sweden). The rats were positioned in...
a stereotaxic frame (Narishige Instruments, Tokyo, Japan), and a cranial burr hole (1 mm) was drilled near the right coronal suture 3.5 mm lateral to the midline. A 27-gauge needle was inserted stereotaxically into the right basal ganglia (coordinates: 0 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma). Autologous whole blood (100 μL, with no anticoagulants), 10 mmol/L FeCl3 (50 μL), or 5U thrombin (50 μL) was infused at a rate of 10 μL/min with the use of a microinfusion pump (Terumo, Tokyo, Japan). The needle was removed, and the skin incision was closed with sutures after infusion.

### Experimental Groups

This study was performed in 4 parts. In the first 3 parts, all rats received an intracaudate injection of 100 μL autologous whole blood (ICH) or a needle insertion only. Part 1 investigated the dose-dependent effects of edaravone on brain edema and neurologic deficits 24 hours after ICH. Thirty rats were treated with either edaravone (0.5, 1, 3, 6, or 10 mg/kg SC, n = 5 per group) or subcutaneous vehicle (n = 5) immediately after ICH. Part 2 evaluated the effect of edaravone on ICH-induced oxidative DNA damage. Twelve rats were treated with either edaravone (6 mg/kg SC) or subcutaneous vehicle immediately after ICH. The number of AP sites and formation of the DNA modification 8-OHdG was also measured quantitatively 24 hours after ICH (n = 3 per group). Part 3 examined the therapeutic time window for edaravone on ICH-induced injury. Thirty rats were treated with either edaravone (6 mg/kg SC) or subcutaneous vehicle. Animals were divided into the following 6 groups according to the time of treatment after ICH: (1) edaravone treatment immediately after ICH; (2) vehicle treatment immediately after ICH; (3) edaravone treatment 2 hours after ICH; (4) vehicle treatment 2 hours after ICH; (5) edaravone treatment 6 hours after ICH; and (6) vehicle treatment 6 hours after ICH (n = 5 per group).

Part 4 investigated the effects of edaravone on iron- or thrombin-induced brain edema, behavioral deficits, and DNA damage (number of AP sites). In this part, rats received an intracaudate injection of 10 mmol/L FeCl3 (30 μL) or 5U thrombin (50 μL). The rats were humanely killed 24 hours later.

### Brain Water Content

Animals were reanesthetized (50 mg/kg pentobarbital IP) and decapitated 1 day after ICH for brain water content determination. The brains were removed, and a coronal brain slice (~3 mm thick) 4 mm from the frontal pole was then cut with a blade. The brain slice was divided into 2 hemispheres along the midline, and each hemisphere was dissected into the cortex and basal ganglia. The cerebellum also served as a control. A total of 5 samples from each brain were obtained: the ipsilateral and contralateral cortex, the ipsilateral and contralateral basal ganglia, and the cerebellum. Brain samples were immediately weighed on an electronic analytical balance (R160P, Sartorius, Goettingen, Germany) to obtain the wet weight. Brain samples were then dried at 100°C for 24 hours to obtain the dry weight. The formula for calculation was as follows: (wt weight – dry weight)/wet weight × 100%.

### Behavioral Tests (Corner Turn Test and Forelimb Placing Test)

Forelimb placing was scored with the vibrissae-elicited forelimb placing test. Animals were held by their bodies to allow their forelimbs to hang freely. Independent testing of each forelimb was induced by brushing the respective vibrissae on the corner of a tabletop once per trial for 10 trials. A score of 1 was given each time the rat placed its forelimb onto the edge of the table in response to the vibrissae stimulation. Percent successful placing responses were determined for the impaired and nonimpaired forelimb.

Corner turn and forelimb placing tests were used in this study. For the corner turn test, the rat was allowed to proceed into a corner, the angle of which was 30°. To exit the corner, the animal could turn either to the left or right, and this was recorded. This was repeated 10 to 15 times, and the percentage of right turns was calculated. Both forelimb placing and corner turn tests were performed and scored by an investigator blinded to the treatment conditions.

### Detection of AP Sites and 8-OHdG in DNA

DNA extraction was performed with a DNA isolation kit produced by Dojindo Molecular Technologies (Kumamoto, Japan). In this method, purified DNA (ratio of OD260/OD280 > 1.8) was isolated from brain tissues by the guanidine method with RNase A and proteinase K. This method avoids the use of phenol and the procedure of heating that may induce background. The brain tissue sampling was the same as for the brain edema measurements.

Aldehyde reactive probe (ARP) labeling and quantification of AP sites were performed with an AP sites assay kit (Dojindo Molecular Technologies). Purified DNA was dissolved at a concentration of 100 μg/mL in TE buffer, and 10 μL of the DNA solution was incubated with 10 μL of 5 mmol/L ARP solution at 37°C for 1 hour. The ARP-labeled DNA was quantified in a 96-well microplate, similar to what is used in an ELISA study. The ARP-labeled DNA and the DNA binding solution were added to each well and incubated in the dark at 37°C overnight. Each well was washed 5 times with phosphate-buffered saline/Tween-20. Horseradish peroxidase–streptavidin solution was added and incubated at 37°C for 1 hour. Afterward, the wells were washed 5 times with phosphate-buffered saline/Tween, sensitive substrate solution was added, and the preparation was incubated at 37°C for 1 hour. Then the wells were subjected to optical density measurement at 630 nm. ARP assays were done in triplicate and the means were calculated. The data, expressed as the number of AP sites per 100 000 nucleotides, were calculated on the basis of a linear calibration curve generated for each experiment with ARP-DNA standard solutions.

The levels of 8-OHdG in the samples were determined with an ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan). The kit can measure extremely low levels of 8-OHdG, and the specificity of the monoclonal antibody has been established. The wells were subjected to optical density measurement at 450 nm. 8-OHdG ELISA was performed in triplicate and the means were calculated. The data, expressed as picograms of 8-OHdG per microgram of DNA, were calculated on the basis of a linear calibration curve generated for each experiment with 8-OHdG standard solutions.

### Statistical Analysis

All data in this study are presented as mean±SD. Data were analyzed with Student’s t test or ANOVA, followed by Scheffe’s post hoc test. The correlation between brain water content and forelimb placing score was studied with Pearson’s linear-regression method. Significance levels were set at P<0.05.

### Results

All physiologic variables were measured immediately before and 1 hour after intracerebral infusions (the Table). Mean arterial blood pressure, pH, Po2, Pco2, hematocrit, and blood...
glucose were within the normal range (80 to 120 mm Hg, 80 to 120 mm Hg, 35 to 45 mm Hg, 38% to 43%, and 80 to 120 mg/dL, respectively).

Edaravone had a dose-dependent neuroprotective effect on ICH-induced injury in the rat (Figure 1). Immediate treatment after ICH with a single dose of edaravone at 3, 6, or 10 mg/kg significantly decreased brain edema in the ipsilateral cortex and basal ganglia ($P<0.05$, Figure 1A). In the basal ganglia, edaravone at a dose of 1 mg/kg also reduced water content ($P<0.05$). Edaravone (3, 6, or 10 mg/kg) also attenuated corner turn and forelimb placing deficits after ICH (Figure 1B). Brain water content in the ipsilateral basal ganglia and forelimb placing score showed a good correlation ($r=-0.726$, Figure 1C). Based on these results, which showed a maximal effect on ICH-induced injury at 6 and 10 mg/kg, a dose of 6 mg/kg was used in the other studies.

We first examined whether edaravone (6 mg/kg) reduced free radical–mediated damage after ICH. The DNA oxidation markers, AP sites and 8-OHdG, were increased in the ipsilateral basal ganglia compared with the contralateral basal ganglia 24 hours after ICH ($P<0.05$, Figure 3). Edaravone treatment significantly reduced both DNA oxidation markers ($P<0.05$, Figure 2).

Systemic administration of edaravone (6 mg/kg) immediately after ICH reduced brain water content in the ipsilateral cortex and basal ganglia 24 hours after ICH ($P<0.05$, Figure 3A). Edaravone treatment delayed for 2 hours after ICH also attenuated brain edema in the ipsilateral cortex and ipsilateral basal ganglia 24 hours after ICH ($P<0.05$, Figure 3B). However, edaravone treatment starting 6 hours after ICH failed to reduce brain edema (Figure 3C), neurologic deficits, and AP sites (data not shown) at 24 hours.

Edaravone (6 mg/kg) treatment immediately after intracerebral infusion of FeCl$_2$ ameliorated brain edema formation by guest on April 19, 2017 http://stroke.ahajournals.org/ Downloaded from
and neurologic deficits (P<0.05, Figure 4, A and B). Edaravone treatment also significantly reduced the number of AP sites (P<0.05, Figure 4C). Edaravone (6 mg/kg) also had neuroprotective effects on brain edema and neurologic deficits (P<0.05, Figure 4, A and B) after intracerebral infusion of thrombin when given immediately after thrombin. Edaravone treatment also significantly reduced AP sites (P<0.05, Figure 5C).

Discussion
The present study indicates that systemic edaravone treatment can reduce ICH-induced brain edema and neurologic deficits. Edaravone reduced both iron- and thrombin-induced injury, suggesting that it ameliorates the effects of both of these components of ICH-induced injury. Edaravone reduced the number of AP sites and 8-OHdG levels in the brain after ICH, indicating that it does not to reduce oxidative damage. Edaravone was effective in reducing injury when given...
Effect of Edaravone on ICH-Induced Brain Injury

Edaravone is a free-radical scavenger, particularly of hydroxyl radicals. It has been shown to be neuroprotective in cerebral ischemia, and it has been approved for treatment of cerebral infarction in Japan since 2001. The current study has shown that edaravone also protects against acute ICH-induced brain injury in a rat model. Thus, it reduced perihematoma brain edema, behavioral deficits, and oxidative DNA damage. The fact that it could reduce brain edema when administered 2 hours after ICH but not after 6 hours suggests that there could be a therapeutic window for this compound. These results also boost any potential use of edaravone in stroke, in that they suggest that it is protective against hemorrhagic as well as ischemic stroke.

There has been some other evidence that free-radical scavengers can protect against ICH-induced injury. Peeling et al. found some improvement in neurologic deficits when dimethylurea or α-phenyl-N-tert-butylnitrone was given 2 hours after ICH in a rat (collagenase) model, but there was no effect on edema. Peeling et al. also found that NXY-059, a free radical–trapping agent, improved neurologic deficits and reduced inflammation in the rat collagenase ICH model. The current study expands those results to a different agent that is in clinical use for stroke. It also demonstrates that edaravone can reduce acute edema formation and reduce DNA oxidative damage. There are several mechanisms of edema reduction by edaravone. Some reports showed that edaravone might block blood-brain barrier and endothelial damage. The fact that edaravone could also reduce ICH-induced behavioral deficits is very important, as a reduction in neurologic deficits is a goal of any stroke therapy. The tests performed in this study have been used extensively in this rat model of ICH and have been shown to be sensitive to this injury and to potential therapies.

Oxidative Damage After ICH

An increase in the number of AP sites is a marker of oxidative DNA damage. The present study demonstrated an increased number of AP sites in the ipsilateral basal ganglia 24 hours after ICH. Normally, AP endonuclease, a DNA repair enzyme, repairs AP sites in DNA. AP endonuclease is constitutively expressed in the noninjured brain but can decrease due to oxidative DNA damage. We previously found that AP endonuclease protein levels are reduced after ICH, and this, along with oxidative damage to DNA, may contribute to the increased number of AP sites.

DNA is vulnerable to oxidative stress. Deoxyguanosine residues in DNA are hydroxylated by various agents that produce free radicals, resulting in 8-OHdG accumulation. Marked increases in 8-OHdG have been observed after cerebral ischemia and kainic acid–induced seizure, and it results in apoptotic cell death. Detection of 8-OHdG by immunohistochemistry enables detection of cell type. We found an increase in 8-OHdG immunoreactivity in perihematoma neurons 3 days after ICH. In the present study, we found a marked increase in 8-OHdG only 1 day after ICH.

Exposure of proteins to reactive oxygen compounds can also lead to the modification and interconversion of amino acid side chains. Proteins oxidized by insults and then derivatized by 2,4-dinitrophenylhydrazine can be separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with the specific antibody to the 2,4-
in the brain can reach very high levels after red blood cell

cerebrospinal fluid. There is thus good evidence of oxidative injury early after
ICH and that it could be a therapeutic target. The current study has shown that edaravone can reduce that injury. Edaravone reduced both the number of AP sites and 8-OHdG expression after ICH. It also reduced brain edema, suggesting involvement of oxidative stress in edema formation after ICH. For cerebral ischemia, there are numerous reports that free radicals can cause cytotoxic and vasogenic brain edema and that free-radical scavengers can ameliorate such edema. For example, mice deficient in CuZn superoxide dismutase have increased brain edema and greater blood-brain barrier disruption after middle cerebral artery occlusion compared with wild-type controls. In the absence of other known mechanisms by which edaravone may reduce edema, we think it likely that reduced oxidative stress is the cause.

We have previously examined the time course of DNA oxidative damage and brain edema in this rat ICH model. The number of AP sites is elevated after 1 day, peaks at 3 days, and falls by 7 days. Similarly, there was marked edema after 1 day, a peak in edema at 3 days, and a reduction by 7 days. This, too, suggests that there may be a relation between DNA oxidative damage and brain edema. However, as noted earlier, oxidative stress causes damage to many different molecules (DNA, proteins, lipids, etc). Although edaravone reduces oxidative damage to DNA after ICH, it presumably also reduces damage to other molecules. Thus, although the results indicate a role for oxidative stress in edema formation, it is not possible to be definitive on whether oxidative damage to DNA is involved.

Effect of Edaravone on Sources of ICH-Induced Oxidative Stress
We hypothesized that toxic components, including iron and thrombin released from the hematoma, are associated with the induction of oxidative stress after ICH. Although iron is essential for normal brain function, iron overload can cause brain injury. Iron can react with lipid hydroperoxides to produce free radicals, and this contributes to neuronal damage during ischemia/reperfusion. After ICH, iron concentrations in the brain can reach very high levels after red blood cell lysis. Usually, most red blood cells start to lyse several days after ICH, although at this stage an increase in the iron-binding protein ferritin may serve to limit any iron-induced injury. However, red blood cell lysis can occur very early. For example, cerebrospinal fluid hemoglobin levels reach their peak by the second day after injection of blood into the cerebrospinal fluid. Magnetic resonance imaging indicates that in the early hours after ICH, hematoma oxyhemoglobin is converted to deoxyhemoglobin. Deoxyhemoglobin is spontaneously and nonenzymatically oxidized to methemoglobin as ferrous iron is converted to its ferric form. As long as the iron is in its ferrous state, the heme and globin moieties of hemoglobin cannot readily separate from each other. Thus, iron release as the clot begins to resolve may be primarily in the ferric form. Using Perl’s staining for ferric iron, we found perihematoma cells already positive for iron from the first day after ICH. In vitro, exposure to FeSO₄ results in lipid peroxidation in neurons and an increase in apoptotic cell death. A previous study showed that intracerebral infusion of iron causes brain edema and suggested that iron may play an important role in edema formation after ICH. Edaravone was effective when given immediately or 2 hours after ICH in the present study. There are a number of potential reasons for this mechanism. One possible reason is the Fenton reaction, whereby Fe²⁺ + H₂O₂ is converted to Fe³⁺ + OH· + OH⁻. Ferrous iron also catalyzes free-radical production and lipid peroxidation by other reactions. The present study has shown that edaravone attenuates iron-induced brain damage in a rat model of intracerebral FeCl₃ infusion. Another possibility is that edaravone may affect thrombin-induced brain damage. Previous studies have demonstrated that thrombin, a potentially toxic component released from the hematoma, might account for perihematoma edema formation. Direct intracerebral infusion of thrombin induced dinitrophenylhydrazine expression, indicating that it does cause oxidative injury. In the present study, edaravone attenuated thrombin-induced brain damage in a model of intracerebral thrombin infusion.

Time Course of Protection
The current study showed that edaravone reduced ICH-induced brain injury when given at the time of ICH or 2 hours later, but not at 6 hours. As discussed earlier, markers of oxidative stress in this model of ICH are generally elevated 1 day after ICH, peak at day 3, and fall by 7 days. This raises the question as to why edaravone should show protection 2 hours after ICH but not at 6 hours. We found a similar phenomenon with an iron chelator, defereroxamine, which is protective at 6 hours after ICH but not at 24 hours, although much iron release after ICH occurs after day 1. A possible explanation for this finding is how protective factors (e.g., endogenous free-radical scavengers, iron transporters, and iron chelators) change in the brain after ICH. Because those changes are delayed, there may be a period early after ICH when the brain is not well protected against free radicals and iron, and it is during this period that exogenous protectants are most effective. It is possible that edaravone is protective only early after ICH because it has some neuroprotective action other than free-radical scavenging. However, such a mechanism has not been described.

Summary
Oxidative damage occurs early after ICH, and systemic edaravone treatment can reduce oxidative DNA damage. Edaravone also reduces acute ICH-induced brain edema and neurologic deficits, probably by reducing iron- and thrombin-induced injury. The fact that edaravone was effective 2 hours after ICH suggests that it could be a therapeutic agent for ICH.

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None.
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