Edaravone (MCI-186) Scavenges Reactive Oxygen Species and Ameliorates Tissue Damage in the Murine Spinal Cord Injury Model

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Abstract

The present study evaluated the effect of the free radical scavenger edaravone on lesion volume and neurological dysfunction after spinal cord injury (SCI) in mice, and investigated its protective effects on superoxide generation. Female C57BL/6 mice were subjected to SCI using a pneumatic impact device and were treated with 3 mg/kg of edaravone or vehicle 30 minutes before the insult. Motor functions were quantitatively evaluated. Lesion volume was assessed by Dohrmann's two-cone method after one week. In situ detection of superoxide in the injured cord was carried out using the superoxide-sensitive dye dihydroethidium (DHE) staining technique. Pretreatment with edaravone significantly improved motor dysfunction and reduced the lesion volume to about 63% of the control (p < 0.05). Semi-quantitative measurements of red fluorescence emitted from DHE revealed that the superoxide concentration increased in the lesion periphery at 1 and 3 hours after the insult, and that pretreatment with edaravone significantly inhibited the increase of superoxide concentration in the lesion periphery at both time points (p < 0.0001). Double staining with DHE and monoclonal antibody against MAP2 showed that most cells positive for DHE were also positive for MAP2. These findings suggest that edaravone ameliorates tissue damage by scavenging reactive oxygen species, especially in the neurons, after SCI.

Key words: dihydroethidium, edaravone, free radical, secondary injury, spinal cord injury, superoxide

Introduction

Spinal cord injury (SCI) often leads to serious neurological sequelae including tetraparesis and incontinence. The generation of reactive oxygen species (ROS), such as superoxide, H2O2, and hydroxyl radicals, is believed to cause secondary tissue injury in the acute stage of SCI, through lipid peroxidation, protein oxidation, and deoxyribonucleic acid (DNA) damage. Injection of H2O2 and Fe2+ into the spinal cord through microdialysis fibers found that the generated hydroxyl radicals induced both necrosis and apoptosis of the neurons and astrocytes. Very recently, L-buthionine-(S,R)-sulfoximine, an inhibitor of endogenous glutathione, was shown to enhance neutrophil infiltration, lipid peroxidation, histological damage, apoptosis, nitrotyrosine formation, poly(adenosine diphosphate-ribose) expression, and motor dysfunction in a murine SCI model. Therefore, pharmacological intervention has been used to prevent the occurrence of secondary tissue injury in the acute stage of SCI. High dose methyl prednisolone therapy has been adopted in clinical practice for patients with SCI, with limited efficacy.

Edaravone (MCI-186; 3-methyl-1-phenyl-2-pyrazolin-5-one), a free radical scavenger, is beneficial for patients in the acute ischemic stroke. Edaravone protects the vascular endothelial cells and neurons against oxidative stress. Edaravone has neuroprotective effects in animal models of global or focal cerebral ischemia and traumatic brain injury, and markedly reduces the degree of edema after cerebral ischemia and traumatic brain injury. Edaravone significantly reduces tissue damage in animal models of spinal cord ischemia and SCI. However, the underlying mechanisms remain unclear.

The present study aimed to clarify whether edaravone...
vone reduces ROS production and ameliorates tissue damage in the murine SCI model.

**Materials and Methods**

I. Murine model of incomplete SCI

Female C57BL/6 mice, 5 to 10 weeks of age, weighing 15–20 g (CLEA Japan Inc., Tokyo) were maintained under a dark/light cycle of 12 hours, and food and water were available ad libitum. All surgical procedures, interventions, and preoperative and postoperative care followed the Hokkaido University Graduate School of Medicine Guide for the Care and Use of Laboratory Animals.

All mice were subjected to incomplete SCI using a controlled pneumatic impact device, as previously described. Anesthesia was induced by inhalation of 4.0% isoflurane in air. During surgery, the rectal temperature was maintained at 34.5 to 36.0°C with a heating pad, which was controlled with a rectal thermometer. A midline incision was made in the back from the T9 to T13 levels. The muscles at the T10 level were retracted to expose the spinous processes under the operating microscope. A T10 laminectomy was performed, leaving the dura mater intact. The animals were transferred to the pneumatic impact device and were placed in a prone position. The impact velocity was set at 2 m/sec and the depth of impact was kept constant at 0.25 mm. Following impact at the T10 level, the midline incision wound was closed, and the animal was placed on a table for acute neurological assessment.

II. Clinical assessment

The animals were divided into two groups, the vehicle-treated group (n = 10) received an intraperitoneal injection of vehicle 30 minutes before the SCI, and the edaravone-treated group (n = 10) received an intraperitoneal injection of 3 mg/kg of edaravone (kindly donated by Mitsubishi Pharma, Tokyo) at 30 minutes prior to the SCI.

Bladder expression was performed twice per day until reflex bladder emptying was established. Neurological functions were assessed in all animals by a technician unaware of the treatment group, using the behavioral function test, including motor score, toe spread, pain withdrawal, platform hang, wire mesh descent, rope walk, and hind foot bar grab test, before and at 0, 1, 4, and 7 days after SCI.

To assess the lesion volume due to SCI, the animals were sacrificed 7 days after the onset. The animals were anesthetized by inhalation of 4.0% isoflurane and were transcardially perfused with 50 ml of heparinized saline, followed by 4% paraformaldehyde. The spinal cord was carefully removed, embedded in paraffin, and cut into 7-mm thick sagittal sections. The sections were stained with Luxol fast blue. Lesion volume was measured by the two-cone method, in which the injured spinal cord is divided into 2 cones and the lesion volume is determined using the formula: Lesion volume = \( \pi D \times D(H1 + H2)/6 \), where D is the occipitofrontal diameter, H1 the rostral head from the epicenter, and H2 is the sum of the caudal head.

III. Superoxide distribution

To evaluate the effect of edaravone on the temporal and spatial profiles of superoxide during the acute stage of SCI, vehicle- (n = 6) and edaravone-treated animals (n = 6) were sacrificed at 1 and 3 hours after the onset of SCI by inhalation of 4% isoflurane. The spinal cord was removed and frozen immediately in liquid nitrogen. The spinal cord was embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Zoeterwoude, Zoeterwoude, Netherlands) and cut into 10-mm sagittal sections. The sections were incubated with dihydroethidium (DHE) (5 mmol/l; Molecular Probes, Inc., Eugene, Ore., U.S.A.) in phosphate buffered saline in a dark, humidified chamber (30 min, 37°C). DHE is oxidized by superoxide to ethidium bromide, which then binds to the DNA in the nucleus and emits red fluorescence. The red fluorescence was detected through a 580-nm long-pass filter, using a fluorescence microscope (BX51; Olympus, Tokyo), and digitally recorded with a CCD camera (model VB-6000/6010; Keyence Co., Osaka). Two regions of interest (ROIs) were selected on each section (n = 6), located on the lesion periphery at 1 and 2 mm rostral from the epicenter. The intensity of red fluorescence was measured using VH Analyzer image analysis software (VH-H1A5 ver 2.2; Keyence Co.). For semiquantitative analysis, the ratio of the red fluorescence intensities in the two ROIs was calculated.

Fluorescence immunocytochemistry was employed to assess whether the neurons were the main location of superoxide in the acute stage of SCI. Following superoxide detection using the DHE technique as described above, the sections were treated with a mouse monoclonal antibody against MAP2 (diluted 1:200; Chemicon, Temecula, Calif., U.S.A.) labeled with mouse immunoglobulin G labeling kit (Zenon Alexa Fluor 488; Molecular Probes, Inc.) as a fluorescence probe (n = 3). Green and red fluorescence emitted from the anti-neuronal nuclear antigen antibody and ethidium bromide, respectively, were detected using a fluorescence microscope and digitally recorded as above.
IV. Statistical analysis
All data are expressed as the mean ± standard deviation. Continuous data were compared by the unpaired t-test. Non-parametric data were compared by the Mann-Whitney U test. Differences of p < 0.05 were considered statistically significant.

Results

I. Clinical effect of edaravone
No animals died during the study period. Figure 1 shows the course of Kuhn’s neurological score in the vehicle- and edaravone-treated groups. SCI caused marked deterioration of hind limb function followed by gradual improvement in both groups. The motor scores of the vehicle- and edaravone-treated groups were 0.2 ± 0.6 and 0.2 ± 0.4 on day 0, respectively, with no significant difference, but improved to 3.3 ± 1.1 and 4.2 ± 1.2 on day 7, respectively, indicating that edaravone significantly improved the motor function in the hind limb on day 7 (p = 0.0146). However, there were no significant differences in toe spread, pain withdrawal, platform hang, wire mesh descent, and rope walk scores between two groups throughout the study. The hind foot bar grab score in the edaravone-treated animals was 2.3 ± 0.8, significantly higher than 1.7 ± 0.7 in the vehicle-treated animals on day 4 (p = 0.0364). However, the difference disappeared on day 7 (2.2 ± 0.6 and 2.6 ± 0.7, respectively).

Figure 2 shows representative sagittal sections of the spinal cord at 7 days after the onset of SCI in the vehicle- and edaravone-treated animals. Quantitative measurements revealed that the lesion volume was significantly smaller in the edaravone-treated group than in the vehicle-treated group (2.43 ± 0.48 and 3.82 ± 1.71 mm³, respectively; p = 0.0412).

II. Effect of edaravone on superoxide distribution
DHE staining showed that red fluorescence was markedly low at 1 and 3 hours after SCI in the epicenter of both groups (Fig. 3). Red fluorescence was much higher at the periphery of the epicenter at 1 and 3 hours after SCI in the vehicle-treated animals, but was less prominent in the edaravone-
Fig. 2 Photomicrographs showing Luxol fast blue staining of the spinal cord in representative examples of the vehicle- (A) and edaravone-treated mice (B). Original magnification ×40.

Fig. 3 Fluorescence micrographs of the spinal cord stained with the superoxide-sensitive dye dihydroethidium (red fluorescence) in representative examples of the vehicle- (A, B) and edaravone-treated mice (C, D) at 1 (A, C) and 3 hours (B, D) after the onset of spinal cord injury. Asterisks show the epicenter. The periphery rostral from the epicenter (arrows) had significantly higher red fluorescence at 1 and 3 hours after the onset of spinal cord injury in the vehicle-treated mice. Images of the vehicle- and edaravone-treated mice were acquired at the identical location at each time point. Original magnification ×40.

Fig. 4 Fluorescence micrograph of the spinal cord stained with the superoxide-sensitive dye dihydroethidium showing the red fluorescence intensity was measured in regions of interest at the periphery 1 (a) and 2 mm (b) rostral from the epicenter (asterisk) at 1 and 3 hours after the onset of spinal cord injury. The ratio of red fluorescence was given by a/b. Original magnification ×40.

The ratio of the intensities of red fluorescence at 1 and 2 mm rostral from the epicenter at 1 hour post-injury was 1.45 ± 0.20 in the vehicle-treated animals, significantly higher than 0.87 ± 0.07 in the edaravone-treated animals (p < 0.0001) (Fig. 4). The ratio of red fluorescence at 3 hours post-injury was 1.29 ± 0.18 in the vehicle-treated animals, significantly higher than 0.82 ± 0.07 in the edaravone-treated animals (p < 0.0001). Therefore, pretreatment with 3 mg/kg of edaravone significantly reduced superoxide production in the lesion periphery at 1 and 3 hours after SCI.

Combined DHE and MAP2 staining at the periphery 1 mm rostral from the epicenter of the vehicle-treated animal showed that more than 90% of cells positive for DHE staining were positive for MAP2 (Fig. 5).

Discussion

The present study found that pretreatment with 3 mg/kg of edaravone significantly reduced lesion volume and improved motor dysfunction after SCI in mice. Previously, injection of edaravone at 5 minutes, 24 hours, and 48 hours after the onset of SCI in rats significantly improved motor function and reduced the volume of white matter lesions.23

The present investigation of the mechanisms involved in the protection of the injured spinal cord used in situ DHE staining to evaluate the temporal and spatial profiles of superoxide production in the central nervous system of mice.26 Superoxide production was shown to increase in the periphery...
Fig. 5 Photomicrographs showing simultaneous staining with the superoxide-sensitive dye di-hydroethidium (red, A) and monoclonal antibody against MAP2 (green, B) in the lesion periphery at 1 hour after the onset of spinal cord injury in a representative vehicle-treated animal. Merged image (C) showing that the majority of the cells emitting red fluorescence were also positive for MAP2. Original magnification ×1000.

of the injured tissue by 1 and 3 hours after the onset of SCI in the vehicle-treated animals. Immunostaining against MAP2, a specific neuronal marker, revealed that the most of the superoxide was located in the neurons, strongly indicating that the main target of superoxide is the neurons in the acute stage of SCI. Previous evaluation of ROS production after SCI found that SCI significantly enhances superoxide production in the periphery up to 24 hours after the insult.2) Direct microdialysis measurement of the production of hydroxyl radical, one of the most destructive ROS, in the injured spinal cord found that hydroxyl radicals significantly increased at 5 minutes, 1 hour, and 3 hours, but not at 5 hours after SCI.17) The present and previous observations suggest that superoxide may be generated in the lesion periphery for a longer time after SCI than hydroxyl radical.

Edaravone significantly inhibited the increase in superoxide in the periphery of injured cord at 1 and 3 hours after the onset of SCI, which strongly suggests that edaravone significantly reduced the lesion volume by scavenging ROS production in the acute stage of SCI. Superoxide is important in the oxidative chain reaction, yielding highly reactive oxidants such as hydroxyl radical.16,27) Hydroxyl radical is generated from superoxide in the gray matter of the spinal cord through the iron-catalyzed Haber-Weiss reaction.17) Furthermore, dialysate levels of 3,4-dihydroxybenzoic acid correlate well with the intensity of DHE fluorescence.18) The present and previous observations strongly suggest that edaravone inhibits the cycle of ROS production by preventing any increase in superoxide concentration and, in turn, hydroxyl radical concentration in the lesion periphery after SCI. Evaluation of the effect of edaravone on lipid peroxide formation, downstream of the cascades of ROS production, by measuring malondialdehyde in the injured spinal cord homogenates found that edaravone significantly attenuated lipid peroxide formation by >45% in the acute stage of SCI.23) Protective effects of edaravone have also been observed in the ischemia-reperfusion model of spinal cord.28-30) Edaravone protects the motor neurons by reducing oxidative DNA damage after spinal cord ischemia in rabbits.29,30) Edaravone also significantly attenuates ROS production during reperfusion.28)

The present study strongly suggests that pretreatment with 3.0 mg/kg of edaravone, a free radical scavenger, prevents the increase of superoxide concentration in the lesion periphery at 1 and 3 hours after SCI, and ameliorates tissue damage. Edaravone also promotes improved motor function at 7 days after SCI. Edaravone may directly inhibit the increase in ROS generation especially in the neurons after SCI. Further studies are necessary to elucidate the therapeutic time window after the insult in order to establish the clinical usefulness of edaravone treatment for patients with acute SCI.

References


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Commentary

Aoyama and colleagues have demonstrated nicely through histological and fluorescence immunocytochemistry techniques that the free radical scavenger edaravone (MCI-186) reduces lesion volume and superoxide presence in the periphery of the lesion in a mouse contusion model of spinal cord injury (SCI). Currently no medical treatment has proven to be very effective in improving both motor and autonomic functional recovery following SCI. Just as in traumatic brain injury (TBI), secondary insults are responsible for the ongoing neuronal destruction following SCI. Neuroprotective medications, such as edaravone, interrupt this destructive progression, and theoretically have the potential to reduce damage and yield improved functional recovery. The search for neuroprotective agents which demonstrate efficacy in SCI is of paramount importance given the rising incidence and devastating nature of the disease.

In their study, Aoyama and colleagues administered edaravone 3 mg/kg 30 minutes prior to creating a contusion injury in female C57BL/6 mice at the T10 level. The study had two aims, one to demonstrate improved motor functional recovery and two to show through histological and immunocytochemistry techniques the effects of edaravone in SCI. Although the authors note that the mice had their bladders emptied twice daily, they did not discuss autonomic function recovery in their paper.

In the first aim, mice were tested for motor score (the authors do not state which standard they used, I presume BMS), toe spread, pain withdrawal, platform hang, wire mesh descent, rope walk, and hind foot bar grab test prior to injury and on post injury days 0, 1, 4, and 7. The authors show that on post injury day 7 there was significant difference in motor score in the treatment group $3.3 \pm 1.1$ vs. $4.2 \pm 1.2$ ($p = 0.01460$). This result is promising, however, motor score especially at low levels of function can be subjective. In more objective measures such as platform hang, wire mesh descent, rope walk, and hind foot bar grab there was no significant differences between the groups at post injury day 7. Perhaps if the study was extended to 14 days post injury these differences would become more apparent.

The goal of the second aim was demonstrated nicely. Quantitative measurements showed that lesion volume was significantly smaller in the edaravone-treated animals $2.43 \pm 0.48$ vs. $3.82 \pm 1.71$ mm$^3$ ($p = 0.0412$). In addition, when comparing intensities of red fluorescence, and hence presence of superoxide, at the periphery of the lesion at 1 and 3 hours post injury the authors found significant decreased fluorescence in the edaravone-treated animals $(1.45 \pm 0.20$ vs. $0.87 \pm 0.07$ [p < 0.0001] at 1 hour post injury and $1.29 \pm 0.18$ vs. $0.82 \pm 0.07$ [p < 0.0001] at 3 hours post injury).

In conclusion, Aoyama and colleagues have demonstrated that edaravone (MCI-186) has potential as a therapeutic agent for reducing secondary damage and improving outcome following SCI. Future directions will be to evaluate the agent administered post injury to show if the effects are still present in a more clinically applicable setting. In addition an evaluation of the agent’s effect on autonomic function recovery and an extended evaluation of motor function recovery would be interesting.

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This is an interesting article investigating the neuroprotective effects of edaravone which is used clinically in the acute ischemic stroke, in terms of neurological functions, histological findings, and superoxide production in the acute spinal cord injury mouse model. Edaravone has been reported to have neuroprotective effects in animal experiments of cerebral and spinal cord ischemia, and traumatic brain injury. However, few studies have appeared on acute spinal cord injury. The present study first showed the attenuation effects of edaravone on superoxide production in the periphery of the lesion caused by mechanical spinal cord injury in mice.

Although the experimental design of this study such as pretreatment is unsuitable for clinical validity, this paper is still of value because it suggests the ameliorating effects of edaravone, which is known as a free radical scavenger, on tissue damage and motor dysfunction after the injury by scavenging reactive oxygen species mainly in the neurons. It is an important matter for the authors to refer to the necessity of further studies to elucidate the therapeutic window from the clinical point of view.

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