MCI-186 prevents spinal cord damage and affects enzyme levels of nitric oxide synthase and Cu/Zn superoxide dismutase after transient ischemia in rabbits

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Objective: The mechanism of spinal cord injury is believed to be related to the vulnerability of spinal motor neuron cells against ischemia. We tested whether MCI-186, which is useful for treating ischemic damage in the brain, can protect against ischemic spinal cord damage.

Methods: After induction of ischemia, MCI-186 or vehicle was injected intravenously. Cell damage was analyzed by observing the function of the lower limbs and by counting the number of motor neurons. To investigate the mechanism by which MCI-186 prevents ischemic spinal cord damage, we observed the immunoreactivity of Cu/Zn superoxide dismutase, neuronal nitric oxide synthase, and endothelial nitric oxide synthase.

Results: MCI-186 eased the functional deficits and increased the number of motor neurons after ischemia. The induction of neuronal nitric oxide synthase was significantly reduced by the treatment with MCI-186. Furthermore, the increase in the induction of endothelial nitric oxide synthase and Cu/Zn superoxide dismutase was more pronounced.

Conclusion: These results indicate that MCI-186 may protect motor neurons from ischemic injury by reducing neuronal nitric oxide synthase and increasing endothelial nitric oxide synthase. MCI-186 may be a strong candidate for use as a therapeutic agent in the treatment of ischemic spinal cord injury.

Spinal cord injury after successful operation on the thoracic aorta is a disastrous and unpredictable complication in human beings. In an attempt to prevent this complication, various methods of spinal cord protection have been suggested, including temporary shunts, partial bypass, and systemic or regional hypothermia. Regardless of the surgical technique or method of spinal cord protection used, no method has been developed that totally prevents the development of paraplegia. The reported prevalence of paraplegia ranges from 2.3% to 23% in operations on the thoracic aorta. The mechanism of spinal cord injury during operations on the thoracic aorta is believed to be related primarily to direct tissue ischemia. Ischemia can occur when there is permanent exclusion of the essential intercostal arterial blood supply to the spinal cord or temporary interruption of the spinal cord blood flow. Spinal motor neurons are believed to be more vulnerable to ischemia than dorsal horn neurons, but the reason for this difference is not fully understood. To investigate the exact mechanism of the vulnerability of spinal motor neurons to ischemia, we created a reproducible model of spinal cord ischemia in which we statistically analyzed cell damage.
Our previous report, in which we examined rabbit spinal cords immunohistochemically after 15 minutes of transient ischemia, demonstrated that neuronal nitric oxide (NO) synthase (nNOS), endothelial NO synthase (eNOS), and Cu/Zn superoxide dismutase (SOD) are induced to increased levels in motor neurons.10 In rabbits, the death of motor neurons after 15 minutes of ischemia is caused by apoptosis. The mechanism of cytotoxicity may be associated with NOS.10 NO is a free gas that is synthesized from arginine by NOS, and highly induced NOS results in the production of too much NO. NO reacts with superoxide and produces the powerful oxidant peroxynitrite (ONOO−). Recently, it has been suggested that NO and ONOO− may cause oxidative damage. Cu/Zn SOD acts as a radical scavenger, so it has a neuroprotective effect. However, Cu/Zn SOD reacts with peroxynitrite to form nitrotyrosine, and too much accumulation of nitrotyrosine may be more harmful to motor neurons.11,12 The induction of too much nNOS causes neurons to die. However, eNOS protects against transient ischemic damage even if the mechanism is not known.13 Nevertheless, nNOS, eNOS, and Cu/Zn SOD might be useful markers for investigating the mechanism of neuronal death after spinal cord ischemia.11

MCI-186 inhibits a peroxidative mechanism in vitro14 and markedly protects against ischemic and postischemic brain edema in rats, perhaps by scavenging hydroxy and peroxyl radicals.15 However, the possibility that MCI-186 is effective in protecting motor neurons of the spinal cord from ischemic injury has not yet been examined. In this study, we investigated the protective effect of MCI-186 after transient ischemia and showed that MCI-186 reduces the distribution of nNOS, increases the distribution of eNOS and Cu/Zn SOD, and protects spinal cord motor neurons from ischemic injury.

Materials and Methods

Animal Models

Thirty-three male domesticated white rabbits (Kumagai Company, Sendai, Japan), weighing 2 to 3 kg, were divided into 3 groups: sham control group, transient ischemia and treatment with vehicle group (group I), and transient ischemia and treatment with MCI-186 group (group M). All rabbits were allowed free access to food and water before and after the procedure, and they were treated in accordance with the Declaration of Helsinki and the Guidelines for the Care and Use of Laboratory Animals. The experimental and animal care protocols were approved by the Animal Care Committee of the Tohoku University School of Medicine.

Anesthesia was induced with intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane inhalation in oxygen. A 5F pediatric balloon-tipped catheter (model 405; Braun, Melsungen, Germany) was inserted through the right femoral artery and advanced 15 cm forward into the abdominal aorta. Preliminary experiments had already confirmed that the balloon in the distal end of the catheter should be positioned 0.5 to 1.5 cm distal to the left renal artery.16 The catheter was immediately removed without injection or balloon inflation in the sham control animals. In groups I and M, spinal cord ischemia was achieved by the inflation of the balloon to obstruct blood flow to the spinal cord as previously described.16 Our previous experiments confirmed that 15 minutes of transient spinal cord ischemia is sufficient to result in selective motor neuron death.5,9 Saline (vehicle, 1 mL/kg rabbit body weight) or MCI-186 (3 mg/mL saline/kg rabbit body weight) was administered intravenously after 30 minutes of reperfusion. Immediately after the animals died, the spinal cord was quickly removed with the plunger of a 1-mL syringe.17 All samples were frozen in powdered dry ice and stored at −80°C until use. Then the spinal cords were cut transversely at approximately the L2 or L3 level and mounted on glass slides.

Neurologic Assessment

Neurologic function was assessed before the animals were killed 8 hours, 1 day, 2 days, or 7 days (n = 3 at 8 hours, 1, or 2 days; n = 5 at sham, 7 days) after reperfusion. Animals were classified by a 5-point scale devised by Johnson and colleagues as follows: 0 = hind-limb paralysis; 1 = severe paraparesis; 2 = functional movement, no hop; 3 = ataxia, uncoordinated hop; 4 = minimal ataxia; and 5 = normal function.18 Two individuals without knowledge of the treatment independently graded neurologic function. The Mann-Whitney U test was used to compare the neurologic scores and cell numbers.

Histologic Study

The sections taken 7 days after reperfusion in both groups were stained with hematoxylin-eosin and examined by light microscopy. An observer who was unaware of the animal group or neurologic outcome examined each slide. With hematoxylin-eosin staining, the cells were considered dead if the cytoplasm was diffusely eosinophilic and viable if the cells showed basophilic stripping (contained Nissel substance).19 The Mann-Whitney U test was used to compare the neurologic scores and cell numbers.

Immunohistochemistry

A mouse monoclonal anti-Cu/Zn SOD antibody (sc8637, 1:200 dilution; Santa Cruz Biotechnology Associates Inc, Santa Cruz, Calif), a mouse monoclonal anti-nNOS antibody (sc5302, 1:200 dilution; Santa Cruz Biotechnology Associates Inc), and a mouse monoclonal anti-eNOS antibody (SA-258, 1:200 dilution; BIOMOL Research Laboratories, Plymouth Meeting, Pa) were used for immunohistochemical studies. For the immunohistochemical analysis, the sections were fixed for 10 minutes in ice-cold acetone, air dried, and rinsed in 0.01 mol/L phosphate buffer containing 0.15 mol/L NaCl (pH 7.4). After being blocked with 10% normal horse serum for 2 hours, the slides were washed and incubated overnight at 4°C with a mouse monoclonal antibody. Endogenous peroxidase was blocked for 20 minutes with 0.3% H2O2 and 10% methanol. Then, sections were washed and incubated for 3 hours with biontylated anti-mouse immunoglobulin G (1:200 dilution; PK6102, Vector Laboratories, Burlingame, Calif) in the buffer, followed by incubation for 30 minutes with an avidin-biotin-peroxidase complex (PK6102). Staining was developed with 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/mL in 50 mmol/L Tris-HCl buffer, pH 7.4) in the presence of H2O2. Staining was categorized in 4 grades in the following manner: no
staining (−), slight staining (+), moderate staining (2+), or dense staining (3+). The results are expressed as the mean ± SD of the mean. The Mann-Whitney U test was used to compare the neurologic scores and cell numbers.

**Results**

**Neurologic Assessment**
The neurologic results are summarized in Table 1. All of the rabbits survived until they were killed. All sham-operated controls (n = 5) demonstrated normal neurologic function (score of 5). Both group I (n = 14) and group M (n = 14) were divided into their individual reperfusion times (8 hours, 1 day, 2 days, and 7 days). All rabbits in group I demonstrated severe paraparesis by 7 days to a score of less than 3 points (n = 5). All group M rabbits demonstrated almost normal function after the procedure. There was a significant difference in physiologic function between group I and group M animals 7 days after the procedure as assessed by the Mann-Whitney U test (P = .02).

**Histologic Study**
The numbers of intact motor neurons are summarized in Table 2. In sham-operated control animals, the spinal cord was intact with many large motor neurons in the anterior horn (AH) (Figure 1). However, 7 days after blood flow restoration in group I, more than 70% of the motor neurons in the spinal cord were damaged. Seven days after reperfusion in group M, there was less evidence of damaged motor neurons. The number of intact motor neurons in group I was significantly smaller than in group M (P = .009) (Table 2).

**TABLE 1. Neurologic functional scores on the seventh day after the procedure**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sham</th>
<th>Group I</th>
<th>Group M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
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<td>5</td>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5 ± 2.4</td>
<td>4.2 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

P = .02 compared with the scores during group I: Mann-Whitney test.

**TABLE 2. Numbers of large motor neurons in the ventral gray matter on the seventh day after the procedure**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sham</th>
<th>Group I</th>
<th>Group M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
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<tr>
<td>5</td>
<td>21</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>21.2 ± 1.5</td>
<td>16.2 ± 1.9</td>
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</table>

P = .009 compared with the sham control group: Mann-Whitney test. P = .009 compared with group I: Mann-Whitney test.

**Immunohistochemical Study**
Table 3 is a summary of the immunohistochemical analysis in each group. The motor neurons in the AHs of sham-operated rabbits were slightly positive for Cu/Zn SOD. Whereas the Cu/Zn SOD immunoreactivity was induced in the motor neurons of group M with a peak at 2 days, it was only slightly induced at 2 days in group I (Figure 2). Other neurons such as dorsal neurons and glial cells did not show any significant inductions (data not shown). The motor neurons in the AHs of sham controls were slightly positive for eNOS. The eNOS immunoreactivity was strongly induced in the motor neurons of group M with a peak at 2 days, it was only slightly induced at 2 days in group I (Figure 3). The antibody for nNOS only slightly labeled the motor neurons in the AHs of the sham controls. The nNOS immunoreactivity was strongly induced in the motor neurons of group I at 8 hours and 1 day, whereas it was only slightly induced in group M (Figure 4, Table 3).

**Figure 1. Histologic findings of the spinal cord 7 days after 15 minutes of ischemia; stained with hematoxylin-eosin. The spinal cord of sham-control (A) was intact. In the spinal cord of group I (B), more than 70% of motor neurons were lost; motor neurons indicate eosinophilic changes. Seven days after reperfusion in group M (C), there was less evidence of damaged motor neurons, and no glial changes were found. Motor neurons (black arrows). Bar = 400 μm.**
TABLE 3. Immunoreactivity for Cu/Zn SOD, nNOS, and eNOS in the spinal motor neuron

<table>
<thead>
<tr>
<th></th>
<th>Cu/Zn SOD</th>
<th>nNOS</th>
<th>eNOS</th>
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<tr>
<td>Sham</td>
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<td>Group I</td>
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<tr>
<td>7 d</td>
<td>$+++$</td>
<td>$+++$</td>
<td>$2+2+2+$</td>
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</tbody>
</table>

*Group I, Transient ischemia group; group M, transient ischemia and treatment with MCI-186 group; --, negative; $\pm$, subtle positive reaction; +, positive reaction; $2+$ strongly enhanced reaction.*

*SOD, Superoxide dismutase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase.*

Figure 2. Immunohistochemical staining for Cu/Zn superoxide dismutase (SOD) in spinal cord at sham (A), 8 hours (B), 1 day (C), and 2 days (D) after ischemia in group I and at 8 hours (E), 1 day (F), and 2 days (G) after ischemia in group M. The Cu/Zn SOD immunoreactivity in group I was only slightly induced at 2 days. The density of Cu/Zn SOD immunoreactivity in group M was stronger than that in group I at 2 days. Motor neurons staining strongly with Cu/Zn SOD (black arrows). Bar = 400 μm.

Figure 3. Immunohistochemical staining for endothelial nitric oxide synthase (eNOS) in spinal cord at sham (A), 8 hours (B), 1 day (C), and 2 days (D) after ischemia in group I and at 8 hours (E), 1 day (F), and 2 days (G) after ischemia in group M. The eNOS immunoreactivity in group I was only slightly induced at 2 days. The density of eNOS immunoreactivity in group M was stronger than that in group I at 1 and 2 days. Motor neurons staining strongly with eNOS (black arrows). Bar = 400 μm.
Discussion

MCI-186 is known to inhibit a peroxidative mechanism in vitro, and peroxidative mechanisms have been implicated in ischemic and postischemic cell damage. There are 2 pathways for generating peroxidative reactions in the damaged brain, as there are in any tissue: auto-oxidation (nonenzymatic peroxidation) and enzyme-processing peroxidation. Cell damage induced by nonenzymatic peroxidation within an in vitro system is suspected to be attributable to iron, SOD, hydrogen peroxide, and lipoxygenase products from arachidonic acid (enzyme-processing peroxidation).

It is widely appreciated that, in a middle cerebral artery occlusion model, a marked increase in water content at the ischemic area may be the result of an increase in water supply resulting from reperfusion and may correspond to the potential for tissue swelling. Furthermore, marked generation of superoxide derived from arachidonate metabolism may also worsen the cell damage and result in the aggravation of brain edema during reperfusion. The introduction of arachidonate or a potent initiator of free radicals leads to brain edema in vivo. Recent reports showed that MCI-186 markedly attenuates ischemic and postischemic brain in rats. We therefore tested whether MCI-186 has a preventive effect in the AH of the spinal cord after transient ischemia. Treatment with MCI-186 resulted in preservation of paraplegia and demonstrated a protective effect on motor neurons.

Immunoreactivity for Cu/Zn SOD is slightly positive in the motor neurons of the AH under normal conditions and is induced in rabbit spinal cords after 15 minutes of transient ischemia, with a peak at 24 hours after reperfusion. Both nNOS and eNOS are slightly present in the motor neurons in the AH. In this study, nNOS was induced by transient ischemic insult with a peak at 8 hours of reperfusion, as was eNOS with a peak at 24 to 48 hours. MCI-186 increased the expression of eNOS but decreased that of nNOS. Although both nNOS and eNOS introduced NO, each had a different effect on motor neurons. Our findings demonstrate that overexpression of nNOS results in neuronal death, whereas increased introduction of eNOS has a neuroprotective effect. Thus, it is suggested that moderate production of NO by eNOS has an apparent neuroprotective effect, and that overproduction of NO by nNOS induces neurotoxicity. Therefore, MCI-186 inhibits ischemic damage to motor neurons by both reducing the neurotoxic factor nNOS and increasing the neuroprotective factor eNOS. MCI-186 may also have the effect of increasing the expression of Cu/Zn SOD. Cu/Zn SOD may work as a radical scavenger to protect neurons from ischemic damage.

Many reports of experiments in vitro and in vivo have suggested that MCI-186 reacts with hydroxy and peroxyl radicals by scavenging, whereas there has been little evidence that MCI-186 inhibits the activity of NO in ischemic tissue. However, we hypothesize that MCI-186 also reduces the degree of cell damage in ischemic spinal cords by scavenging the free radicals that superoxide, NO, and ONOO⁻ are believed to easily become. A previous report showed that MCI-186 reduces the activity of endothelium-derived NO. Our results indicate that MCI-186 may react with NO and ONOO⁻ directly or may scavenge the free radicals induced by them.

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease characterized by relatively selective degeneration of motor neurons in the spinal cord, brain stem, and motor cortex. Approximately 10% of ALS is familial, and the remainder is sporadic. Recently, mutations in the Cu/Zn SOD gene were identified in a number of patients with...
familial ALS and are believed to be a result of the cytotoxicity of the mutant Cu/Zn SOD protein. The cytotoxicity of nitrotyrosine is currently suspected in the mechanism of motor neuron death in ALS. Recent studies showed that nitrotyrosine increased in transgenic mice with familial ALS mutations. Both in the brain and spinal cord, once ischemic change occurs, the amount of NO is highly increased because nNOS is highly induced. NO reacts with superoxide to produce the powerful oxidant ONOO⁻, whereas Cu/Zn SOD reacts with peroxynitrite to form nitrotyrosine. Furthermore, in the present study, we demonstrated that MCI-186 reduces nNOS but induces eNOS and normal Cu/Zn SOD. Therefore, we suspect that the amount of nitrotyrosine with MCI-186 induction may be reduced in the spinal cord after transient ischemia, and our results indicate that the mechanism of motor neuron death in the spinal cord after ischemia may have a feature in common with familial ALS.

Conclusion
This report shows that MCI-186 protects the motor neurons of the spinal cord from ischemic injury. MCI-186 reduces the induction of nNOS and increases that of both eNOS and Cu/Zn SOD. MCI-186 should be a strong candidate for use as a therapeutic agent in the treatment of ischemic spinal cord injury in the near future.

References