Apoptosis of Motor Neurons With Induction of Caspases in the Spinal Cord After Ischemia

T. Hayashi, MD; M. Sakurai, MD; K. Abe, MD, PhD; M. Sadahiro, MD, PhD; K. Tabayashi, MD, PhD; Y. Itoyama, MD, PhD

Background and Purpose—Some neuronal subpopulations are especially vulnerable to ischemic injury. In the spinal cord, large motor neurons are vulnerable to ischemia and are selectively lost after transient ischemia. However, the mechanisms of the neuronal loss have been uncertain. We hypothesized that spinal motor neurons might be lost by apoptosis and investigated a possible mechanism of neuronal death by detection of double-strand breaks in genomic DNA and immunohistochemical analysis for caspases, ie, interleukin-1β converting enzyme (ICE), Nedd-2, and CPP32.

Methods—We used a rabbit spinal cord ischemia model created with a balloon catheter. The spinal cord was removed at 8 hours, 1, 2, or 7 days after 15 minutes of transient ischemia, and histological changes were studied with hematoxylin-eosin staining. To detect double-strand breaks in DNA, a staining with terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick end labeling (TUNEL) was performed. Furthermore, expression of ICE, Nedd-2, and CPP32 was investigated by Western blotting and immunohistochemical analysis.

Results—Motor neurons were selectively lost at 7 days after transient ischemia. TUNEL study demonstrated that no cells were positively labeled until 1 day after ischemia, but nuclei of some motor neurons were positively labeled at 2 days. Western blot analysis revealed no immunoreactivity for ICE and slight immunoreactivities for Nedd-2 and CPP32 in the sham-operated spinal cords. However, immunoreactivity became apparent at 8 hours after transient ischemia, decreased at 1 day, and returned to baseline level at 2 days. Immunohistochemical analysis demonstrated that motor neurons were responsible for induction of those caspases.

Conclusions—Double-strand breaks in genomic DNA and induction of three caspases were demonstrated. These results indicate that motor neuron death in the spinal cord after transient ischemia is profoundly associated with activation of apoptotic processes. (Stroke. 1998;29:1007-1013.)

Key Words: apoptosis ■ ischemia ■ motor neuron ■ rabbit ■ spinal cord

Most neurons in the central nervous system are destined to die in response to severe ischemia, even if blood flow would be restored later. However, after relatively mild or short ischemia, certain neuronal subpopulations die although others do not. This phenomenon is known as “selective vulnerability.” Previous studies demonstrated that hippocampal CA 1 pyramidal cells, Purkinje cells in the cerebellum, and neurons in the third to fifth layers of cerebral cortex are selectively vulnerable to ischemia in the brain. As for the spinal cord, it is known that motor neurons are peculiarly vulnerable to ischemia. We previously demonstrated that motor neurons in the spinal cord selectively degenerated after experimental transient ischemia, which was compatible with the delayed deterioration of neurological function after spinal cord ischemia.

Although it is still controversial, some studies have revealed that delayed neuronal death of hippocampal CA 1 pyramidal cells after transient forebrain ischemia has some features of apoptosis, such as positive staining with TUNEL. and internucleosomal DNA fragmentation. In the rat brain with MCA occlusion, neurons that are going to die also have some attributes of apoptosis. Furthermore, apoptotic neuronal death in these models is demonstrated to be associated with induction of caspases. In the rat brain with MCA occlusion, expression of Nedd-2 (caspase-2) and CPP32 (caspase-3) was induced, whereas only Nedd-2 was induced in the gerbil brain after transient ischemia. ICE (caspase-1) was not induced in neuronal cells in these models but was expressed in microglia that were activated in the brain tissue after ischemia. In the spinal cord, however, the mechanisms of cellular death of motor neurons after transient ischemia are yet to be resolved.

In the present study, we demonstrated selective motor neuronal death in the spinal cord after transient ischemia and found that these dying motor neurons showed double-strand breaks in genomic DNA. Furthermore, we performed West-
ern blot study and immunohistochemical analysis to investigate how the expression of caspases such as ICE, Nedd-2, and CPP32 changes after ischemia. The results showed all of these caspases were induced with a slight difference in chronological profile. These findings are essential for the elucidation of the mechanisms of motor neuron death in spinal cord ischemia and might provide clues for the possible treatment of spinal cord infarction in the future.

Materials and Methods

Animal Model

During the experiment, animals were treated in accordance with the declaration of Helsinki and the guiding principles in the care and use of animals. Experimental and animal care protocols were approved by the animal care committee of Tohoku University School of Medicine.

Twenty-two Japanese White rabbits weighing 2 to 3 kg each (15 weeks old) were used. Anesthesia was initially induced with intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane inhalation. Spinal cord ischemia was conducted as described in our previous report. Briefly, a 5F pediatric Swan-Ganz catheter (model 405, Braun) was percutaneously inserted through the femoral artery and placed 1.0 cm caudal to the left renal artery. Body temperature was monitored with a rectal thermistor and maintained at 37°C with a heating pad during the operation. Spinal cord ischemia was performed by inflation of the balloon at that point, and after a 15-minute ischemic period, the catheter was removed. The animals were then allowed free access to water and food at ambient temperature.

The animals were divided into two experimental groups: group A, for investigation with histological, TUNEL, and immunohistochemical studies (n=14); and group B, for Western blot study (n=8).

Group A

At 8 hours, 1, 2, or 7 days after blood flow restoration, the animals were killed by use of deep anesthesia with diethyl ether (n=3 at each time point). Two sham-operated control were killed just after insertion of the catheter into the abdominal aorta without inflation of the balloon. After the animals were killed, the spinal cords were quickly removed with the use of the plunger of a 1-mm syringe and frozen in powdered dry ice. Then, 10-μm-thick sections at the L2 to L3 level were cut on a cryostat at −20°C, collected on glass slides coated with polylysine, and stored at −80°C until use.

Group B

For Western blot analysis, the animals were killed at 8 hours or 1 or 2 days after blood flow restoration (n=2 at each time point), and samples were obtained as noted above for group A and kept at −80°C until use. Sham-control samples (n=2) were also obtained.

Histological Study

To observe the pathological changes of the spinal cord after ischemia, we performed HE staining with a set of sections and examined them by light microscopy.

In Situ Detection of DNA Fragmentation

For detection of double-strand breaks in genomic DNA, which is one of the features seen in apoptosis, we performed TUNEL study in accordance with our previous report with use of a kit (TACS in situ apoptosis detection kit model 4810–30K, Trevigen). Briefly, 10-μm thick tissue sections were fixed, digested, and then incubated with terminal deoxynucleotidyl transferase and biotinylated dNTP for 1 hour at 37°C. After they were washed with PBS, the sections were incubated with streptavidin-biotin–horseradish peroxidase complex and developed using dianinobenzidine. Sections were counterstained with methyl green.

For quantitative measurement of the number of cells that underwent apoptosis, we counted motor neurons that were positive or negative in TUNEL. The results were expressed as mean±SD.

Western Blot Analysis

To investigate changes of expression of caspases, ie, ICE, Nedd-2, and CPP32, we performed Western blot analysis. Tissue samples were homogenized in a lysis buffer (0.1 mol/L NaCl, 0.01 mol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, and 1 μg/mL aprotinin), and then the homogenates were centrifuged at 7000g for 15 minutes at 4°C. Supernatants were used as protein samples. Assays to determine the protein concentration of the supernatants were subsequently performed by comparison with a known concentration of bovine serum albumin with use of a kit (BCA protein assay reagent kit No. 23225, Pierce). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to our previous report. In brief, protein samples were boiled at 100°C in 2.5% SDS and 5% β-mercaptoethanol, and lysates equivalent to 6 μg of protein were electrophoresed on polyacrylamide gels with a continuous gradient from 10% to 15% (Phast Gel 10 to 15, Pharmacia LKB) using the Phast system (Pharmacia LKB). The proteins were transferred to a polyvinylidene fluoride membrane (Micron Separations Inc) with a transfer buffer consisting of 25 mmol/L Tris-HCl (pH 7.5), 192 mmol/L glycine, and 20% methanol.

After the transfer, the membrane was placed in 1% powdered milk in PBS to block nonspecific binding. Then it was incubated with primary antibodies at 1:200 dilution for 20 hours at 4°C. The primary antibodies used were as follows: goat polyclonal antibody against ICE p20 (E307T), goat polyclonal antibody against Nedd-2 p19 (L046T), and goat polyclonal antibody against CPP32 p20 (H047T; all from Santa Cruz Biotechnology). After it was washed in PBS, the membrane was incubated with biotinylated anti-goat IgG (7023508R, Zymed Laboratories) at 1:200 dilution in PBS for 90 minutes. It was washed in PBS and incubated with avidin-biotin–horseradish peroxidase complex (PK-6102, Vector Laboratories) for 60 minutes. The membrane was then developed with dianinobenzidine used as a color substrate. The reaction was stopped by washing it in distilled water. To ascertain specific binding of the antibody for the protein, another membrane was stained in a similar way without the first antibody.

Immunohistochemical Analysis for Caspases

We performed immunohistochemical studies to investigate which types of cells expressed immunoreactive ICE, Nedd-2, and CPP32. Frozen sections of 10-μm thickness were fixed in acetone, rinsed in PBS, and blocked with 10% normal rabbit serum for 2 hours. They were then incubated with primary antibodies in 10% normal rabbit serum and 0.3% Triton-X 100 for 20 hours at 4°C. The primary antibodies used were the same as those used for Western blot analysis noted above, and each dilution was as follows: antibody against ICE p20 at 1:400, that against Nedd-2 p19 at 1:100, and that against CPP32 p20 at 1:400. After quenching endogenous peroxidase activity by exposing the slides to 0.3% H2O2 and 10% methanol for 20 minutes, we washed the slides in PBS and incubated them for 3 hours with biotinylated anti-goat IgG (7023508R, Zymed Laboratories) at 1:200 dilution in PBS containing 0.018% normal rabbit serum. Subsequently, the slides were incubated with avidin-biotin–horseradish peroxidase complex (PK-6102, Vector Laboratories) for 30 minutes and developed with dianinobenzidine used as a color substrate. The reaction was stopped by washing the slides in distilled water. Hematoxylin was used for nuclear staining. To ascertain specific binding of the antibodies for the proteins, a set of sections was stained in a similar way without the first antibody. Staining was
categorized into four grades in the following manner: no staining (−), slightly stained (+), moderately stained (1+), or densely stained (2+).

Results

Histological Study

Representative photographs of sections stained with HE are shown in Figure 1. In sham-operated animals, the spinal cord was intact, with many large motor neurons in the anterior horn (Figure 1a). The spinal cord at 1 day (not shown) and 2 days after blood flow restoration (Figure 1b) also exhibited no pathological changes. However, in the spinal cord at 7 days after blood flow restoration (Figure 1c), approximately 70% of motor neurons were lost without involvement of other neurons or glial cells. Apoptotic bodies were verified (Figure 1c, black arrowhead), and some neurons exhibited cell shrinkage (Figure 1c, white arrowhead). On the other hand, there were no neurons with eosinophilic structureless cytoplasm indicative of necrosis. No apparent gliosis or cellular infiltration was observed. Thus, selective loss of motor neurons was confirmed, in accordance with our previous report.5

In Situ Detection of DNA Fragmentation

Cells with double-strand breaks in DNA are detected by TUNEL staining in brown, because 3'-hydroxyl groups are labeled with biotinylated dUTP.18 In the present study, the spinal cord with sham operation showed no stained cells. Also, in the spinal cord of animals that underwent 15 minutes of ischemia, no cells were positively stained with TUNEL until 1 day after blood flow restoration (Figure 2a). At 2 days after reperfusion, however, nuclei of some motor neurons were detected in brown with a granular pattern (Figure 2b), indicating cells that were undergoing double-strand DNA breaks mainly associated with apoptosis.

For quantitative measurement, the number of motor neurons positive or negative in TUNEL was recorded in each specimen in a blinded fashion (Table 1). At 2 days, approximately 50% of motor neurons were positive in TUNEL, but the others were negative (Figure 2c). The decrease in total number of motor neurons was larger than the number of TUNEL-positive cells at 2 days.

Western Blot Analysis

Representative results of Western blot analysis are shown in Figure 3. With antibody against ICE p20, no band was
detectable in samples of sham control, but samples at 8 hours after blood flow restoration revealed one band (Figure 3a). The MW of this band was 20 kDa and corresponded to the p20 subunit of ICE. This band became less distinct at 1 day and scarcely detectable at 2 days. Western blot analysis for Nedd-2 showed a barely detectable band in samples of sham control (Figure 3b). The MW was 19 kDa and corresponded to the p19 subunit of Nedd-2. At 8 hours after reperfusion, this band became more evident, but it became less dense at 1 day and much less dense at 2 days. With antibody against CPP32 p20, a feeble band of 20-kDa MW was revealed in sham-control samples, which corresponded to the p20 subunit of CPP32 (Figure 3c). This band became intense at 8 hours and 1 day but returned to the baseline level at 2 days after

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TABLE 1. Number of Large Motor Neurons That Are Positive or Negative in TUNEL

<table>
<thead>
<tr>
<th></th>
<th>TUNEL Negative</th>
<th>TUNEL Positive</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>18.0±1.4</td>
<td>0</td>
<td>18.0±1.4</td>
</tr>
<tr>
<td>8 h</td>
<td>18.7±2.5</td>
<td>0</td>
<td>18.7±2.5</td>
</tr>
<tr>
<td>1 d</td>
<td>16.7±3.8</td>
<td>0</td>
<td>16.7±3.8</td>
</tr>
<tr>
<td>2 d</td>
<td>9.3±3.2</td>
<td>9.0±2.0</td>
<td>18.3±2.1</td>
</tr>
<tr>
<td>7 d</td>
<td>4.5±0.7</td>
<td>0.5±0.7</td>
<td>5.0±1.4</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD. n=2 in control group and n=3 in other groups.
reperfusion. The membrane without the primary antibody revealed no bands (not shown).

**Immunohistochemical Analysis for Caspases**

In the sham-operated spinal cords, no neuronal or glial cells produced immunoreactive ICE (Figure 4a). At 8 hours after blood flow restoration, most motor neurons in the anterior horn strongly expressed immunoreactive ICE (Figure 4b), whereas no other cellular components expressed it (Figure 4c). At 1 day after reperfusion, however, the expression markedly decreased (Figure 4d), and it returned to the baseline level at 2 days (Figure 4e). As for Nedd-2 expression, very slight immunoreactivity was noticed in spinal motor neurons of the sham-operated animals (Figure 5a). At 8 hours after reperfusion, immunoreactivity became very conspicuous in almost all motor neurons (Figure 5b), whereas there were no immunoreactivities in other cellular components (Figure 5c). Immunoreactivity in motor neurons returned to baseline level at 1 day (Figure 5d) and 2 days (Figure 5e) after blood flow restoration. Immunoreactivity for CPP32 was detected to a slight degree in the spinal motor neurons of sham-operated animals (Figure 6a), whereas no cells other than motor neurons expressed it. Expression was markedly increased at 8 hours after reperfusion in almost all motor neurons (Figure 6b) but not in other cells (Figure 6c). Immunoreactivity in motor neurons, although substantially decreased, could also be noted at 1 day after reperfusion (Figure 6d). At 2 days after reperfusion (Figure 6e), however, immunoreactivity returned almost to the baseline level. Sections without the first antibody showed no staining (not shown). Results of immunohistochemical analysis for ICE, Nedd-2, and CPP32 are summarized in Table 2.
TABLE 2. Immunoreactivity for ICE, Nedd-2, and CPP32 in Spinal Motor Neurons After 15 Minutes of Ischemia

<table>
<thead>
<tr>
<th></th>
<th>ICE</th>
<th>Nedd-2</th>
<th>CPP32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>8 h</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
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<tr>
<td>1 d</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 d</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

The symbols —, ±, +, and 2+ represent no, slight, moderate, and dense immunoreactivity, respectively. n=3 in each time point.

Discussion

It is known that clinical motor function deteriorates in a delayed manner after spinal cord ischemia.6 We previously reported that ≈70% of motor neurons were selectively lost after experimental transient spinal cord ischemia and that the motor neuronal loss could be the reason for the delayed neurological dysfunction.5 Because there are no differences in spinal blood flow between ventral and intermediate gray matter in this experimental model of spinal cord ischemia,19 the result of our previous study should be interpreted to mean that motor neurons are selectively vulnerable to ischemia. However, the exact mechanism of the selective vulnerability of motor neurons in the spinal cord after ischemia has been unclear. If the mechanisms of this selective vulnerability were disclosed, a treatment for spinal cord infarct might be developed. Therefore, it is important to elucidate the mechanisms of motor neuron death in the spinal cord after ischemia.

Similar to the case of motor neurons in the spinal cord, some neuronal subpopulations in the brain, such as hippocampal CA 1 pyramidal cells, Purkinje cells in the cerebellum, and neurons in the third to fifth layers of cerebral cortex, are known to be selectively vulnerable to ischemia.1-3 Although the mechanisms of delayed neuronal death in hippocampal CA 1 pyramidal cells is not fully elucidated, there is evidence to indicate that their death includes some apoptotic features; many neurons were positively stained with TUNEL,7,8 and formation of DNA ladders was detected with gel electrophoresis.9 It is still debated whether hippocampal CA 1 pyramidal cells undergo apoptosis, because cytoplasmic eosinophilia, which is one of the features of necrosis, precedes changes of nuclei in these cells.7 However, many previous reports9,20,21 lead us to consider that activation of apoptotic machinery actually takes place in such a model. For example, upregulation of Nedd-2 mRNA in the hippocampus at 3 to 6 hours after transient ischemia has been reported.14 Because induction of caspases is an attribute of apoptosis,22-24 we could consider that apoptotic machinery is involved in the delayed neuronal death of hippocampal CA 1 pyramidal cells.

In the present study, we demonstrated that ≈70% of motor neurons in the spinal cord were selectively lost at 7 days after transient ischemia, which was in accordance with our previous report. On the other hand, TUNEL study revealed that ≈50% of motor neurons had breaks in genomic DNA at 2 days (Table 1). This discrepancy in percentages is not in conflict because apoptosis is a relatively short-lived process, and some neurons might not yet be in the final apoptotic process at 2 days; the number of cells that undergo apoptosis is likely to be underestimated.25 Nuclei of neurons undergoing apoptosis show positivity in TUNEL with a granular pattern, although those in necrosis show slight staining with a homogenous pattern.26 The fact that nuclei at 2 days after ischemia in the present study were densely stained with a granular pattern (Figure 2b) suggests that the motor neurons were undergoing apoptosis. In addition, neuronal cells showed some morphological features of apoptosis with HE staining (Figure 1c).

Expression of ICE, Nedd-2, and CPP32, were induced in these cells at the protein level. Expression of Nedd-2 and CPP32 to a slight degree in sham controls is compatible with previous reports.13,14,24 Because induction of these caspases is known to induce apoptosis in vitro22-24 and in vivo,27,28 all of these proteins might be involved in neuronal death in this model. The time course of their induction, with a peak at 8 hours, is compatible with their involvement in delayed neuronal death because the preparatory event does not coincide with but precedes oligonucleosomal DNA cleavage in apoptosis.29 The results of the present study differ in part from previous ischemic models. In hippocampal CA 1 pyramidal cells in the gerbil brain, only Nedd-2 mRNA was increased at 4 to 16 hours after transient ischemia, with no change in ICE and CPP32 mRNA levels.34 Furthermore, in the rat brain with MCA occlusion, mRNA levels of Nedd-2 and CPP32 were increased, whereas that of ICE showed no change.13 Although the reason for the difference in the pattern of caspase inductions is unclear, this discrepancy is not surprising. Different types of cells would undergo apoptosis through different pathways.30 Previous studies demonstrated that ICE is involved in apoptosis triggered by Fas,30 whereas Nedd-2 is not; on the other hand, Nedd-2 is involved in developmental neuronal death in the embryo.23 Although ICE was not induced in the brain with MCA occlusion,13 its selective inhibitor decreased the infarct volume.28 Therefore, it is suspected that many factors, including ICE, are involved in apoptotic death of neuronal cells after ischemia. In the model in the present study, caspases were induced in almost all motor neurons, although ≈70% of them were lost by 7 days. Thus, expression of caspases itself might not be sufficient for the execution of apoptosis in these cells. The role of apoptosis repressor genes, such as bcl-2, might also play a role in neuronal loss in this model.
Spinal cord infarction is a relatively rare disease compared with cerebral infarction. However, 1200 consecutive necropsies revealed that 52 cases (4%) had hypoxic myelopathy. Furthermore, it has been reported that ~40% of patients who undergo operation of the thoracic aorta develop paraplegia. Thus, ischemic spinal cord injury is an important cause of physical disabilities today. In spite of this, treatment remains only supportive, and the neurological prognosis is generally poor. The present study demonstrated that motor neurons were lost mainly by an apoptotic mechanism. In animal models of traumatic spinal cord injury, neuronal cells and oligodendrocytes in the white matter underwent apoptosis, and treatment with cycloheximide was effective for reduction of this injury. In the future, administration of agents that inhibit apoptosis could be an effective means of therapy for spinal cord infarction.

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References
Neuronal apoptosis, a process known as programmed cell death during early CNS development, has been proposed to be involved in the death of postmitotic neurons after acute ischemic and traumatic brain injuries as well as in chronic neurodegenerative diseases. Recent studies have extended this cell death process to neurons and glia of the spinal cord after traumatic injuries.\textsuperscript{1,2} It is known that in the spinal cord, the large motor neurons are selectively degenerated after experimental transient ischemia,\textsuperscript{3} which is compatible with delayed neurological dysfunction.\textsuperscript{4} However, the mechanisms of such an ischemic neuronal loss had not been clearly established.

Using a well-established and reliable rabbit model of spinal cord ischemia,\textsuperscript{5} Hayashi et al have now provided evidence that spinal cord motor neurons were selectively lost at 7 days after a brief 15 minutes of transient ischemia. The apoptotic cell death process was identified in these neurons because the appearance of the early immunocytochemical expression of caspases at 8 hours followed by DNA fragmentation at 2 days support this possibility.

This study is novel because it supports the contention that spinal cord motor neurons are vulnerable to the apoptotic cell death process even after a brief transient spinal cord ischemia. This study also provides an impetus for future therapeutic strategies in the clinical setting so that the motor neuronal loss and the resulting devastating paraplegia can be ameliorated.

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\textbf{References}
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