Paraplegia due to spinal cord ischemia during surgery on the thoracic aorta is one of the disastrous complications. Paraplegia is classified into 2 types with respect to its onset; one is immediate paraplegia and the other is delayed paraplegia, in which lower limb movement was once observed after operation but deteriorated afterward. The development of delayed paraplegia is considered to be due to delayed and apoptotic neuronal death after transient ischemia.

There are 2 major pathways for apoptosis induction. One is the extrinsic pathway, which is activated by plasma membrane death receptor ligation, and another is the intrinsic pathway in which release of cytochrome C from mitochondria and activation of caspase 9 are implicated. In delayed paraplegia due to spinal cord ischemia in rabbits was applied with the use of a balloon catheter. In this model, the spinal motor neuron shows selectively delayed neuronal death, whereas other spinal neurons such as interneurons survive. Immunohistochemical analysis and Western blotting for ubiquitin system molecules, ubiquitin, deubiquitylating enzyme (ubiquitin carboxy-terminal hydrolase 1), and ubiquitin-ligase parkin were examined.

Results—In cytoplasm, ubiquitin and ubiquitin carboxy-terminal hydrolase 1 were strongly induced both in interneuron and motor neuron at the early stage of reperfusion, but the sustained expression was observed only in motor neuron. Parkin was induced strongly at 3 hours after the reperfusion, but the immunoreactivity returned to the sham control level at 6 hours in both neurons. In the nuclei, ubiquitin, ubiquitin carboxy-terminal hydrolase 1, and parkin were strongly induced in interneuron, whereas no upregulation of these proteins was observed in motor neuron.

Conclusions—These results indicate that the vulnerability of motor neuron of the spinal cord might be partially attributed to the different response in ubiquitin-mediated stress response after transient ischemia. (Stroke. 2008;39:1883-1889.)

Key Words: ischemia • paraplegia • parkin • spinal cord ischemia • ubiquitin • UCH-L1

A recent study showed that neuronal survival is affected by disturbance of the ubiquitin proteolytic pathway in nonlethal stress. In the development of neurodegenerative disease, it is speculated that dysfunction of the ubiquitin proteasomal pathway plays an important role. However, the profile of expression of ubiquitin and its related molecule has never been investigated in the spinal cord after transient ischemia. Ubiquitin, a small 76-residue protein, is an essential factor for nonlysosomal protein degradation and clear unfolded and damaged protein. Ubiquitin expression level is regulated by the balance of ubiquitinating enzymes: ubiquitin-activating, ubiquitin-conjugating, ubiquitin-ligase enzymes, and deubiquitinating enzymes. Among ubiquitin-ligase enzymes, parkin is the most famous protein and its mutation is responsible for autosomal-recessive juvenile Parkinson disease. The down-regulation of parkin protein in transient focal cerebral ischemia has also been demonstrated. Upregulation of the expression of parkin protects cells from injury induced by ER stress. Therefore, parkin might possess the possibility to participate in the development of delayed neuronal death in...
the spinal cord after transient ischemia. Among deubiquitinating enzymes, ubiquitin carboxy-terminal hydrolase 1 (UCH-L1) is selectively expressed in neuronal cells and constitutes approximately 5% of the brain’s total soluble protein, which demonstrates a possibility that it plays a major role in neuronal cell function.6 In ischemic model, the possible relationship of UCH-L1 and apoptotic tolerance is demonstrated. Harada et al reported that the gracile axonal dystrophy (gad) mouse with an exon deletion for UCH-L1 showed apoptotic tolerance in ischemic retinal injury compared with the wild-type mouse.8

Therefore, we investigated the expression of ubiquitin-related molecule, ubiquitin, parkin, and UCH-L1 in motor neuron and interneuron of the spinal cord and discussed the role of ubiquitin and its related molecule on the development of delayed neuronal death after transient ischemia in the spinal cord.

Materials and Methods

Animal Models

White rabbits weighing 2 to 3 kg were used in this study and were divided into 2 groups: a 15-minute ischemia group (Group I) and a sham control group (Group S). All rabbits had free access to food and water before and after the procedures, and they were treated in accordance with the Declaration of Helsinki and the Guidelines for the Care and Use of Laboratory Animals. Experimental and animal care protocols were permitted by the Animal Care Committee of the Osaka University School of Medicine.

Anesthesia was induced by intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane (in oxygen) inhalation. A 5-French 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.7 The catheter was immediately removed without injection or balloon inflation in the sham group. In Group I, spinal cord ischemia was achieved by inflating the balloon to obstruct blood flow to the spinal cord. Our previous experiments confirmed that 15 minutes of transient spinal cord ischemia was sufficient for selective and delayed motor neuron death.8 Immediately after death, the spinal cord was quickly removed with the plunger of a 1-mL syringe. All samples were frozen in powdered dry ice and stored after death, the spinal cord was quickly removed with the plunger of a 1-mL syringe. Samples were then incubated with avidin–biotin–horseradish peroxidase complex (PK-6102; Vector Laboratories). Sections were colored with DAB/H₂O₂ solution, and the cytoplasm was counterstained with hematoxylin and eosin. A set of sections were stained in a similar way without the primary antibodies to ascertain specific binding of the antibodies to the proteins.

Western Blotting Analysis

To investigate changes of ubiquitin, parkin, and UCH-L1 expression, we performed Western blot analyses. Tissue samples (spinal cord at the L2 or L3 levels) were homogenized in 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 10 000 g for 10 minutes at 4°C (n=4 for each group). The supernatants were used as protein samples. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in a 10% polyacrylamide gel under reducing conditions. In brief, protein samples were boiled at 100°C in 2.5% sodium dodecyl sulfate and 5% β-mercaptoethanol, and lysates equivalent to 20 μg of protein from each sample were run on the gel for 90 minutes at 20 mA together with a size marker (MagicMark XP Western Standard; Invitrogen). The electrophoresis running buffer contained 25 mmol/L Tris base, 250 mmol/L glycine, and 0.1% sodium dodecyl sulfate. Proteins on the gel were then transferred to a polyvinylidene fluoride membrane (LC2002; Invitrogen) with a transfer buffer (NuPAGE Transfer buffer, NP0006; Invitrogen) and 10% methanol.

The primary antibodies used were the same as those used for immunocytochemistry mentioned previously. After washing in phosphate-buffered saline, membranes were incubated with horseradish peroxidase–conjugate anti-ubiquitin IgG (SC-2020; Santa Cruz Biotechnology Inc) and horseradish peroxidase–conjugate anti-parkin IgG (RPN2124, ECL Plus Western blotting Reagent Pack; Amersham Bioscience) at 1:10 000 dilution in phosphate-buffered saline for 90 minutes, respectively. The blots were developed using an ECL Plus detection method (RPN2132; Amersham Bioscience). To ascertain specific binding of the antibody for the protein, another membrane was stained in a similar way without primary antibody. The images of Western blot studies were quantified by plotting a 2-dimensional densitogram using the image analysis program NIH Image, version 1.63 (Research Services Branch, National Institute of Mental Health, National Institutes of Health).

Fluorescence Double-Labeling Study for Ubiquitin and Parkin

To investigate subcellular localization of ubiquitin, UCH-L1, and parkin, fluorescence study and double-labeling study (ubiquitin/parkin, UCH-L1/parkin) were performed. Spinal cord sections were prepared as previously described. A nonspecific blocking procedure was performed with 10% horse serum before application of primary antibodies. Then the sections were incubated with ubiquitin goat polyclonal antibodies (1:100) simultaneously with parkin mouse polyclonal antibodies (1:100) or with UCH-L1 goat polyclonal antibodies (1:100) simultaneously with parkin mouse polyclonal antibodies (1:100). These primary antibodies were incubated overnight at 4°C and detected by using donkey anti-goat IgG linked with fluorescein isothiocyanate 1:50 (SC-2024; Santa Cruz Biotechnology, Inc) and donkey anti-mouse IgG linked with TexasRed 1:50 (SC-2781; Santa Cruz Biotechnology, Inc). The slides were mounted in aqueous mounting media with DABCO and observed with fluorescein microscopy.
Statistical Analysis

The Mann-Whitney U test was used to compare the neurological scores and cell numbers. Quantitative analyses of the optical density of Western blots were analyzed by analysis of variance. Probability value less than 0.05 was considered statistically significant. Parametric data are present as mean±SD.

Results

Histological Study

In sham-operated control animals, the spinal cord was intact with many large motor neurons (18.6±2.0) in the ventral horn (Figure 1A). However, 7 days after blood flow restoration in Group I, approximately 60% of the motor neurons in the spinal cord were damaged (6.2±1.3; Figure 1C). The number of intact motor neurons in sham was significantly larger than in 7 days after reperfusion (P<0.0001). In contrast, the number of interneuron in intermediate gray matter was well preserved and was not changed significantly between the sham control and Group I (27.6±2.4 versus 25.2±0.83, respectively; Figure 1B, D).

Immunohistochemical Study

Ubiquitin is slightly expressed in cytoplasm of both motor neuron and interneuron, whereas no immunoreactivity was observed in nuclei (Figure 2A–B). After 3 hours ischemia, ubiquitin was upregulated in cytoplasm of both neurons and in nuclei in interneuron (Figure 2C–D). After 6 hours, the immunoreactivity in cytoplasm was preserved in motor neuron, whereas it returned to almost the same level as the sham group in interneuron (Figure 2E–F). At 1 day, the immunoreactivity in cytoplasm was somewhat decreased but upregulated compared with the sham group (Figure 2G). In nuclei of interneuron, ubiquitin is upregulated strongly at 3 hours (Figure 2D) and somewhat decreased at 6 hours (Figure 2F) but is well preserved until 1 day (Figure 2H). On the contrary, nuclei of motor neuron expressed no immunoreactivity of ubiquitin until 1 day (Figure 2A, C, E, G).

Parkin is slightly expressed in cytoplasm of both neurons, whereas no immunoreactivity was observed in nuclei of either neuron (Figure 3A–B). After 3 hours ischemia, parkin was upregulated in cytoplasm of both neurons and in nuclei in interneuron, whereas no immunoreactivity was observed in nuclei in motor neuron (Figure 3C–D). After 6 hours, the immunoreactivity in cytoplasm of both neurons returned to almost the same level as the sham group (Figure 3E–F), whereas it was preserved in nuclei of interneuron until 1 day (Figure 3G).

The pattern of UCH-L1 expression is similar to ubiquitin expression. UCH-L1 is slightly expressed in cytoplasm in both neurons, whereas no immunoreactivity was observed in nuclei in either nuclei in either neuron (Figure 4A–B). After 3 hours ischemia, UCH-L1 was upregulated in cytoplasm of both neurons and in nuclei in interneuron (Figure 4C–D). After 6 hours, the immunoreactivity in cytoplasm was preserved in motor neuron, whereas it returned to almost the same level as the sham group in interneuron (Figure 4E–F). At 1 day, the immunoreactivity in cytoplasm was somewhat decreased but upregulated compared with the sham group (Figure 4G–H). In nuclei, UCH-L1 is upregulated strongly at 3 hours (Figure 4D) and somewhat decreased at 6 hours (Figure 4F) but is well preserved until 1 day (Figure 4H), whereas no immunoreactivity was observed in nuclei of motor neuron.
Western Blot Analysis
Representative results of Western blot analysis of ubiquitin, parkin, and UCH-L1 are shown in Figure 5. With antibodies against ubiquitin, a slight band was detected in sham control samples, but that was strongly enhanced at 3 hours and peaked at 6 hours after blood flow restoration, but it became scarcely detectable 1 day after reperfusion (Figure 5A). With antibodies against parkin, band with a molecular weight of 55 kDa was scarcely detectable in sham control samples and was strongly enhanced at 3 hours and peaked at 6 hours after blood flow restoration; after blood flow restoration revealed a single band, which became scarcely detectable 1 day after reperfusion (Figure 5A). With antibodies against UCH-L1, a slight band with a molecular weight of 25 kDa was detected in sham control samples, but that was strongly enhanced and peaked at 3 hours and was preserved until 6 hours after blood flow restoration, but it became scarcely detectable 1 day after reperfusion (Figure 5A). The membrane without the primary antibodies revealed no band (data not shown). With quantitative analysis, we found that ischemia affected expression profiles of ubiquitin, parkin, and UCH-L1 (versus sham control *P<0.001, **P<0.0001; Figure 5B–D, respectively).

Fluorescence Double-Labeling Study
At 3 hours after ischemia, ubiquitin (Figure 6A) was detected by fluorescein isothiocyanate (green) and parkin (Figure 6C) was detected by Texas red (red); they were preferentially expressed in cytoplasm of motor neuron. The merged image was shown in Figure 6E to be double-positive. In interneuron, ubiquitin (Figure 6B) and parkin (Figure 6D) were expressed mainly in nuclei. The merged image was shown in Figure 6F to be double-positive. UCH-L1 was detected by fluorescein isothiocyanate (green). In motor neuron, UCH-L1 (Figure 6G) and parkin (Figure 6I) were expressed in cytoplasm and they were well colocalized (Figure 6K). In interneuron, they were expressed chiefly in nuclei (Figure 6H, J, L).

Discussion
We demonstrated a profile of the expression of ubiquitin, UCH-L1, and parkin after transient spinal cord ischemia in rabbits. Transient ischemia induced selective delayed motor neuronal death and affected the profile of expression of ubiquitin, parkin, and UCH-L1. In cytoplasm, ubiquitin and UCH-L1 were upregulated after transient ischemia both in motor and interneuron; however, the prolonged expression was observed in motor neuron. Parkin was transiently in-
duced both in motor and interneuron at 3 hours of reperfusion but returned to the sham level in both neurons. On the contrary, in nuclei, the profile of the expression of these molecules was different from that in cytoplasm. The nuclei of motor neuron showed no immunostaining of ubiquitin, UCH-L1, or parkin, whereas that of interneuron showed strong immunostaining after transient ischemia.

Ubiquitin is among the most phylogenetically conserved proteins and its primary function is to clear abnormal, foreign protein and unfolded proteins by targeting them for degradation by the 26S proteasome. Ubiquitin is thought to be one of the stress response proteins and enhanced immunoreactivity is observed under oxidative stress. UCH-L1 is a member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate the ubiquitin monomer and serves to recycle ubiquitin. Besides the role of ubiquitin recycling, UCH-L1 is reported to bind and stabilize monoubiquitin in neuron. The present study revealed that the pattern of expression of ubiquitin and UCH-L1 was almost similar in cytoplasm of motor neuron and in nuclei of interneuron. The profile of the expression and the role of UCH-L1 under oxidative condition in neuron has never been clarified. Overexpression of UCH-L1 has been demonstrated to increase the level of ubiquitin in both cultured cells and mice. Therefore, the upregulation of ubiquitin after transient ischemia in spinal cord might be partially regulated by UCH-L1. Parkin is one of the most famous ubiquitin-ligases whose mutation is responsible for autosomal-recessive juvenile Parkinson disease. After transient ischemia in the spinal cord, parkin was upregulated in cytoplasm both in motor and interneuron and in nuclei of interneuron. It has also been shown that the upregulation of parkin protects cells from injury induced by ER stress. In the spinal cord after transient ischemia, we have previously demonstrated the induction of ER stress in motor neuron. Therefore, upregulation of parkin might be one of the stress responses modulating the ischemic insult including ER stress in the spinal cord after transient ischemia. However, it has been demonstrated the downregulation of parkin protein in transient focal cerebral ischemia. Although the exact reason of such discrepancy is unclear, it might be attributed to the difference in the experimental model, including severity of ischemic insult and applied types of neuron in the experiment.

Besides upregulation of ubiquitin, UCH-L1, and parkin after transient ischemia in the spinal cord, the remarkable finding in this study was the difference in the manner of expression of these molecules between not only motor neuron that is destined to die and interneuron that survive, but also in nuclei and in cytoplasm of each neuron. Risuleo et al also reported that different immunoreactivity of ubiquitin between nuclei and dendrites of hippocampal neurons after transient ischemia. Considering the underlying mechanism, we should bear in mind ER stress response in spinal cord after transient ischemia. In the development of neurodegenerative disease, stress response and dysfunction of ER is speculated to play a role crosslinked with that of the ubiquitin proteasomal pathway. In addition, we have also demonstrated that ER stress associated death signal Caspase 12 was selectively induced in motor neuron after transient ischemia. We have also verified the different ER stress responses between motor neuron and interneuron by immunostaining of GRP78, phosphorylated eukaryotic initiation factor 2 alpha, activating transcription factor 4, and inositol requiring ER transmembrane RNAse isoform, which suggested that there existed

![Figure 5. Western blotting for ubiquitin, parkin, and UCH-L1.](image-url)

Quantitative analysis showed that transient ischemia affects expression profiles of ubiquitin (B), parkin (C), and UCH-L1 (D). *P < 0.0001 compared with the sham group.
more unfolded protein in ER of motor neuron than that of interneuron in the same model as in the present study. The unfolded protein that could not be coped within ER is eliminated in cytoplasm through retrograde transport, then degraded by proteasome, which is termed ER-associated degradation. Therefore, it could be easily speculated that much more unfolded protein was accumulated in cytoplasm in motor neuron than in interneuron. The accumulation of unfolded protein might upregulate the ubiquitin. The difference of ubiquitin immunoreactivity in cytoplasm might be partially explained by the difference of ER stress response and the amount of accumulated unfolded protein in cytoplasm between motor neuron and interneuron. Recently, it has also been reported that the gad mouse with an exon depletion for UCH-L1 prevented the upregulation of ubiquitin and gained apoptotic tolerance in ischemic retinal injury compared with the wild-type mouse, suggesting excessive ubiquitin induction after ischemic injury rather lead to neural apoptosis. This phenomenon is also observed in the present study and the difference of ubiquitin and UCH-L1 immunoreactivity in cytoplasm might partially explain the selective vulnerability of motor neuron.

In the nuclei, damaged proteins increase after transient ischemia and it is important to eliminate those proteins to survive ischemic insult. For elimination of damaged protein, ubiquitin and its related protein upregulation should be essential. The presence of the ubiquitin-specific/ATP-dependent 26S proteasome in nuclei has been demonstrated, and this fact suggested a role of ubiquitin in nuclear protein degradation. In addition, ubiquitin in nuclei could play a role in the repair of DNA acting on the damaged chromatin and the upregulation of ubiquitin may mediate the stress response to ischemic injury by influencing the expression of other stress proteins such as heat shock proteins. Therefore, upregulation of ubiquitin in nuclei in interneuron might play an important role in cell survival. On the contrary, the lack of ubiquitin upregulation in nuclei in motor neuron could be considered to be great disadvantage for motor neuron. Then it is natural that a question emerges about which factors determine subcellular localization of ubiquitin and its related molecules. Several speculated mechanism could be considered; one is "ubiquitin trap" mechanism. Ubiquitin is synthesized in cytoplasm and transduced to the nucleus. As stated previously, the unfolded protein is considered to be more accumulated in cytoplasm of motor neuron than of interneuron. Therefore, ubiquitin is "trapped" in cytoplasm to cope with the accumulated unfolded protein and is prevented from translocation into nuclei. The second speculated mechanism is relevant to the transport system of ubiquitin and its related molecule into nuclei. Macromolecular traffic between the cytoplasm and nuclear compartments of eukaryotic cells passes through nuclear pore complexes. Although small molecules diffuse freely through these pores, translocation of nucleic acids and proteins is often performed by soluble transport receptors (karyopherins) that can associate with the Ran GTPase. A recent study revealed that a murine ubiquitin-conjugating enzyme is imported into nuclei by the nuclear transport receptor importin-11. Therefore, it might be possible that the cytoplasm–nuclear transport of other ubiquitin and its associated molecules be mediated by nuclear transport receptor. The difference of the nuclear transport system, including the nuclear transport receptor, might exist and be responsible for the difference in immunoreactivity of ubiquitin and in related molecules in nuclei between neurons.

This is the first report that investigated the profile of induction of ubiquitin, UCH-L1, and parkin after transient spinal cord ischemia. Transient ischemia for 15 minutes affected the profile of expression of ubiquitin, parkin, and UCH-L1 and the different immunoreactivity of these molecules was observed not only between motor and interneuron, but also between cytoplasm and nuclei. We previously documented that transient ischemia for 10 minutes, which does not cause damage to motor neuron or interneuron, did not upregulate stress response protein such as heat shock protein 70 between both neurons. Furthermore, 20 minutes ischemia, for which both neurons were severely damaged, necrotized both neuron and stress response for cell protection like upregulation of cell-protective protein was surpassed by critical and drastic change. Therefore, the different way of upregulation of these ubiquitin system molecules under sublethal ischemic insult might partially explain selective vulnerability of...
motor neuron in the spinal cord after transient ischemia. However, further investigation is necessary to clarify the underlying molecular mechanism that could fully explain the vulnerability of motor neuron against ischemic insult.

Disclosures
None.

References
Ubiquitin-Mediated Stress Response in the Spinal Cord After Transient Ischemia
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