Autophagy-mediated stress response in motor neuron after transient ischemia in rabbits

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Objective: **Spinal cord injury is considered to be related to a vulnerability of spinal motor neurons to ischemia. However, the mechanisms underlying this vulnerability are not fully understood. We investigated the role of autophagy, which is an intracellular bulk degradation process, at motor neuron as a potential mechanism of neuronal death by immunohistochemical analysis for microtubule-associated protein light chain3 (LC3) and γ-aminobutyric-acid type-A-receptor-associated protein (GABARAP) which are considered as markers of autophagy.**

Methods: **We used a rabbit spinal cord ischemia model with the use of a balloon catheter. The spinal cord was removed at 8 hours, 1, 2, or 7 days after 15 minutes of transient ischemia, and histologic changes were examined with hematoxylin-eosin staining. Western blot analysis for LC3 and GABARAP, temporal profiles of LC3 and GABARAP immunoreactivity, and double-label fluorescence immunocytochemical studies were performed.**

Results: **In the ischemia group, about 85% of motor neurons were preserved until 2 days after reperfusion, but were selectively lost at 7 days ($P < .001$ compared with sham group). Western blot analysis demonstrated slight immunoreactivity for LC3 and GABARAP in the sham-operated spinal cords. In contrast, the ischemia group LC3 and GABARAP immunoreactivity became apparent at 8 hours after reperfusion. With quantitative analysis we found that ischemia affected expression profiles of LC3-II and GABARAP. At 8 hours after reperfusion, co-labeling of LC3 and GABARAP were observed in the same motor neurons that eventually died.**

Conclusion: **These data suggest that autophagy was induced in motor neurons by transient spinal cord ischemia in rabbits. (J Vasc Surg 2009;50:381-7.)**

Clinical Relevance: **Patients undergoing thoracic aneurysm repair who awake with no neurologic deficit immediately after operation can sometimes eventually develop paraplegia. However, the exact mechanism of this delayed vulnerability is not fully understood. In the present study, we demonstrated that following spinal cord ischemia in rabbits, expression of both LC3 and GABARAP were increased in the same motor neurons which eventually died, suggesting strong induction of autophagy in those cells. Thus, the results of the present study indicate that autophagy may be a potential mechanism of neuronal cell death in this model.**

The mechanism of acute spinal cord dysfunction is believed to be due to its ischemic damage occurring during cross-clamping. 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.[1] However, patients undergoing thoracic aneurysm repair who awake with no neurologic deficit immediately after operation can sometimes eventually develop paraplegia.[2] The exact mechanism of this delayed vulnerability is not fully understood.

A recent study showed that neuronal survival is affected by disturbance of the ubiquitin-proteasome pathway or the autophagy-lysosome pathway in non lethal stress.[3,4] In the development of neurodegenerative disease, it is speculated that dysfunction of the ubiquitin proteasomal pathway or the autophagy lysosomal pathway is also considered to play an important role.[5,6] An upregulated expression of ubiquitin, UCH-L1, and parkin was demonstrated after transient spinal cord ischemia in rabbits.[7] However, the profile of expression of autophagy-related proteins and its related molecule has never been investigated in the spinal cord after transient ischemia.

Autophagy is an intracellular bulk degradation process whereby cytosolic, long-lived proteins and organelles are degraded and recycled.[8] In autophagy, cytoplasmic materials and dysfunctional organelles are sequestered by an autophagosome, and subsequently delivered to the lysosome where they are degraded by lysosomal proteases. During the formation of mammalian autophagosomes, two ubiquitylation-like modifications are required, autophagy-related genes (Atg) 12-conjugation and autophagy-related genes (Atg) 8-modification. Microtubule-associated protein light chain3 (LC3) and γ-aminobutyric-acid type-A-receptor-associated protein (GABARAP) are mammalian Atg8-related proteins which localize to autophagosomal membranes after post-translational modifications, and LC3-II, a lipidated form of LC3, and GABARAP have been shown to be autophagosomal markers in mammals.[9,10] Autophagy occurs at basal levels, but can be further induced by stressors, including nutrient depletion, ischemia,

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Competition of interest: none.

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and reperfusion.[11-13] Autophagy has a dual role in cell survival as it can promote cell survival by generating free amino acids and fatty acids, which can be reused to maintain mitochondrial adenosine triphosphate production and protein synthesis, but can also promote cell death in some circumstances.[14,15] Recent studies have also reported that altered autophagy has been observed in various neurodegenerative disease, including amyotrophic lateral sclerosis (ALS), Huntington's disease, Parkinson's disease, and Alzheimer's disease,[16-19] but it remains unclear whether autophagy plays a beneficial or detrimental role in the motor neurons after transient spinal cord ischemia.

Thus in the present study, we hypothesized that motor neuron cells eventually dying in this model of acute spinal cord ischemia demonstrate prior induction of microtubule-associated protein LC3 and GABARAP.

MATERIALS AND METHODS

Animal models. Twenty-five Japanese domesticated white rabbits weighing 2.5-3 kg were used in this study, and were divided into two groups: a 15-minute ischemia group, and a sham control group. All rabbits were allowed free access to food and water before and after the procedure, and were treated in accordance with the Declaration of Helsinki and the "Guide for the Care and Use of Laboratory Animals". The experimental and animal care protocols were approved by the Animal Care Committee of the Kyushu University School of Medicine.

Anesthesia was induced with intramuscular administration of ketamine at a dose of 50 mg/kg, and maintained with 2% halothane in oxygen inhalation. A 5F pediatric balloon-tipped catheter (model 405; Braun, Melsungen, Germany) was inserted through the right femoral artery and was advanced 15 cm forward into the abdominal aorta. Preliminary experiments had already confirmed that the balloon in the distal end of the catheter was positioned 0.5-1.5 cm just distal to the left renal artery.[20] In the sham group, the catheter was immediately removed without injection or balloon inflation, while in the ischemia group, 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.[21]

During the experiment, aortic pressures were continuously monitored both at the proximal and distal positions of the balloon. When the balloon of the catheter was inflated in the abdominal aorta, systemic blood pressure of the rabbits did not change. The arterial pressure distal to the inflated balloon fell to near zero and no pulsation was recorded. Upon deflation of the balloon, systemic blood pressure of this portion decreased for 15 minutes and then returned to the normal level (data not shown). Body temperature was monitored with a rectal thermostat, and was maintained at 37°C with a heating pad during surgery and subsequent ischemia. Animals were allowed to recover at ambient temperature, and were sacrificed with deep anesthesia of sodium pentobarbital (100 mg/kg intravenously) at 8 hours, and 1, 2, and 7 days after reperfusion (n = 5 at each time point). In the sham control, animals were sacrificed at 8 hours (n = 3) or 7 days (n = 5) of reperfusion after insertion of the catheter into the abdominal aorta without inflating the balloon. Spinal cords were quickly removed immediately after sacrificing, using the plunger of a 1-ml syringe. The tissue samples for Western blot analysis and immunohistochemical studies were frozen in powdered dry ice and stored at −80°C. The samples for histology were fixed by immersing in 4% paraformaldehyde in 0.1 M phosphate buffer, and then stored at 4°C for 1 week; they were then cut transversely at about the L2 or L3 level, and finally embedded in paraffin.

Neurologic assessment. Neurologic function was assessed before the rabbits were sacrificed at 7 days after reperfusion. The rabbits were classified according to a five-point scale devised by Johnson et al[22] as follows: 0: Hind-limb paralysis; 1: Severe paraparesis; 2: Functional movement, no hop; 3: Ataxia, disconjugate hop; 4: Minimal ataxia; and 5: Normal function. Two individuals without knowledge of the treatment graded neurologic function independently.

Histologic study. Sections taken 7 days after reperfusion were stained with hematoxylin and eosin and examined by light microscopy (n = 5 for each group). The number of intact large motor neuron cells in the ventral gray matter region was counted by an observer who was unaware of animal group or neurologic outcome while each slide was examined. With hematoxylin and eosin staining, the cells were considered dead if the cytoplasm was diffusely eosinophilic, and viable if the cells demonstrated basophilic stippling (that is, contained Nissl substance).

Western blot analysis. To investigate changes of LC3 and GABARAP expression, we performed Western blot analyses. The tissue samples (spinal cord at the L2 or L3 levels) 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 12,000g for 15 minutes at 4°C. The supernatants were used as the protein samples. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in a 10% polyacrylamide gel under non reducing conditions. In brief, protein samples were boiled at 100°C in 2.5% SDS 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 (Precision Plus Protein Standards; Bio-Rad Laboratories, Hercules, Calif). The electrophoresis running buffer contained 25 mmol/L Tris base, 250 mmol/L glycine, and 0.1 % SDS. The proteins on the gel were then transferred to a polyvinylidene fluoride membrane (IPVH20200; Millipore, Bedford, Mass) with a transfer buffer consisting of 25 mmol/L Trisbase, 20 mmol/L glycine, and 10% methanol.

After the transfer, the membranes were placed in 4% powdered milk in phosphate-buffered saline (PBS) to block nonspecific binding. Then they were incubated with primary antibodies at 1:1000 dilution for 20 hours at 4°C. The primary antibodies used were as follows: mouse mono-

Table. Neurological score at 2 days and 7 days after procedure

Animal number	*Sham control 7 days*	*15-minute ischemia*	
		2 days	*7 days*
1	5	3	2
2	5	2	0
3	5	2	0
4	5	0	0
5	5	0	0
Mean ± SD	5 ± 0*	1.4 ± 1.34	0.4 ± 0.89

SD, Standard deviation.
Compared to the 15-minute ischemia group at 7 days after procedure.
*$P = .0079$.

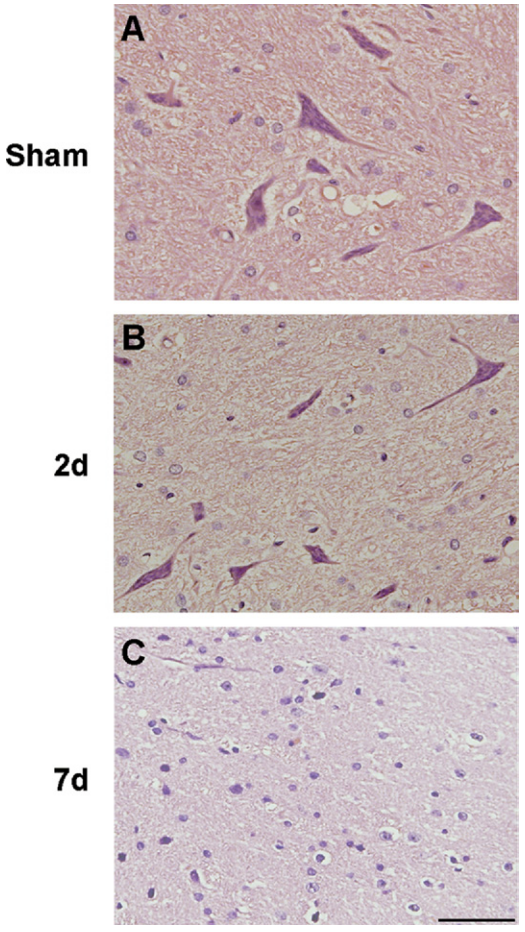

Fig 1. Histological findings in the spinal cord after 15 minutes of ischemia stained with hematoxylin-eosin (HE). The spinal cord of the sham group **(A)** and the ischemia group at 2 days (2d) after ischemia **(B)** showed no histologic changes. At 7 days (7d) after ischemia **(C)**, there was selective loss of motor neurons in the ischemia group, without apparent gliosis or cellular infiltration. **Bar = 50 μm**.

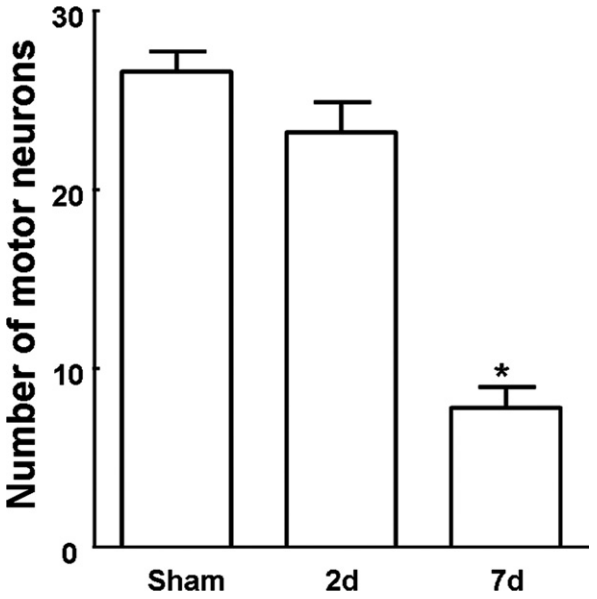

Fig 2. More than 70% of the motor neurons in the spinal cord were damaged at 7 days (7d) after reperfusion. *2d,* 2 days. *$P <$ .001 compared with sham group.

clonal anti-LC3 antibody (M152-3; Medical and Biological Laboratories Co, Ltd, Nagoya, Japan) and goat polyclonal anti-GABARAP antibody (SC-9190; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). After washing in PBS, the membranes were incubated with horseradish peroxidase-conjungate anti-mouse IgG (#7076; Cell Signaling Technology, Danvers, Mass) and horseradish peroxidase-conjungate anti-goat IgG (PI-9500; Santa Cruz Biotechnology, Inc) at 1:1000 dilution in PBS for 90 minutes, respectively. The blots were developed using ECL Plus detection method (RPN2132; Amersham Bioscience, Buckinghamshire, England). To ascertain specific binding of the antibody for the protein, another membrane was stained in a similar way without the primary antibody. The images of Western blot studies were quantified by plotting a two-dimensional densitogram using the image analysis program NIH Image, version 1.63 (Research Services Branch, NIMH, National Institute of Health, Bethesda, Md).

LC3 and GABARAP immunohistochemistry. We also performed an immunohistochemical study to investigate changes in the expression of LC3 and GABARAP at 8 hours, 1 day, and 2 days after reperfusion in the ischemia group, and at 8 hours after reperfusion in the sham group (n = 5 for each group). Spinal cord sections were rinsed in 0.1M PBS for 20 minutes, and blocked in 2% normal horse serum for 2 hours at room temperature. Then they were incubated with primary antibodies in 10% normal horse serum or 10% normal rabbit serum and 0.3% Triton-X 100 for 20 hours at 4°C, respectively. The primary antibodies used were the same as those used for Western blot analysis noted above, and each dilution was as follows: antibody against LC3 at 1:25 and that against GABARAP at 1:200.

After quenching endogenous peroxidase activity by exposing slides to 0.3% H2O2 and 10% methanol for 20 minutes, the slides were washed in PBS and incubated for 3 hours with biotinylated anti-mouse IgG (PK-6102; Vector

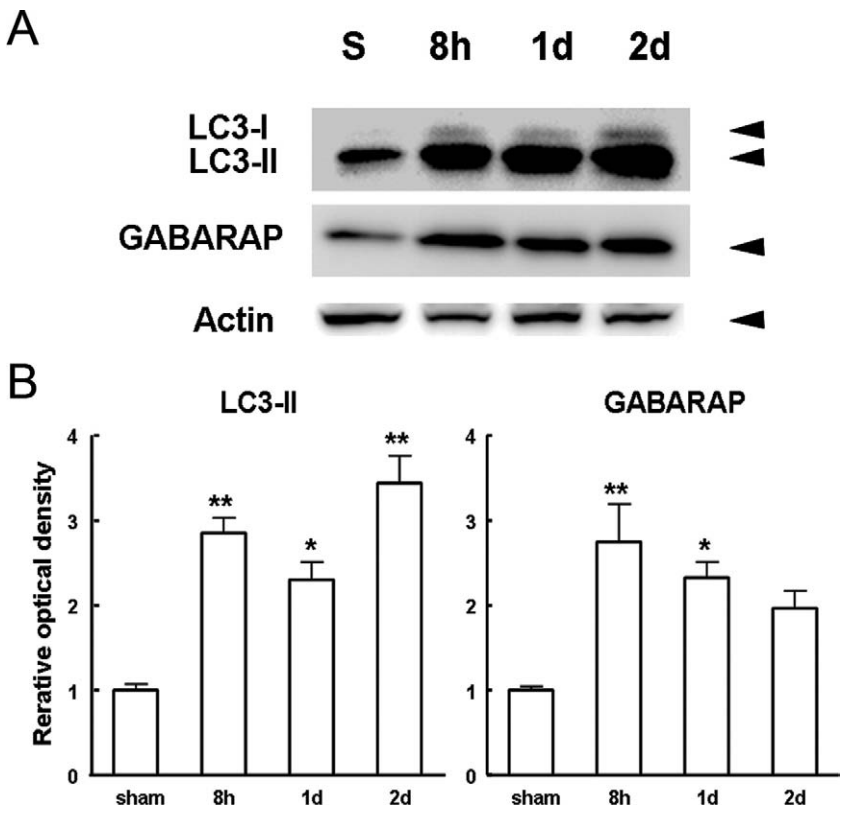

Fig 3. Representative Western blot for LC3 and GABARAP. **A,** LC3-I is cytosolic form of LC3 and LC3-II is a lipidated form of LC3. Immunoreactivity was only weakly (LC3 and GABARAP) detected in the sham group. In the ischemia group, strong bands for LC3 and GABARAP were observed at the expected sizes (16kD and 14kD, respectively) at 8 hours (8h) after reperfusion, and LC3 expression was preserved until 2 days (2d) after reperfusion. There was no change in actin expression in either group. **B,** Quantitative analysis showed that 15 minutes ischemia significantly increased LC3 and GABARAP expression at 8 hours after reperfusion. *S,* sham; *1d,* 1 day. $*P < .01$. $**P < .001$ compared with sham group.

Laboratories, Burlingame, Calif) and biotinylated anti-goat IgG (PK-6105; Vector Laboratories) at 1:200 dilution in PBS containing 0.018% normal horse and rabbit serum. Subsequently they were incubated with avidin-biotin-horseradish peroxidase complex (PK-6102; Vector Laboratories). The slices were colored with DAB/H2O2 solution, and the cytoplasm was counterstained with hematoxylin. To ascertain specific binding of antibody for the protein, a set of sections were stained in a similar way without the primary antibody.

Fluorescence double-labeling study of LC3 and GABARAP. Spinal cord sections were prepared as described above. A nonspecific blocking procedure was performed using 10% horse serum before application of primary antibodies. Then the sections were incubated with LC3 mouse monoclonal antibodies (1:50) simultaneously with GABARAP goat polyclonal antibodies (1:50). These primary antibodies were incubated overnight at 4°C and were detected by using goat anti-mouse lgG linked with green-fluorescent Alexa Fluor 488 (1:500; A11001; Invitrogen, Carlsbad, Calif) and donkey anti-goat lgG linked with orange-fluorescent Alexa Fluor 555 (1:500; A-21432; Invitrogen). The slides were mounted in aqueous mounting media with 1,4-diazabicyclo[2.2.2]octane and observed with fluorescent microscopy.

Statistical analysis. The Mann-Whitney *U* test was used to compare the neurologic scores and cell numbers. Quantitative analyses of the optical density of Western blots were analyzed by analysis of variance. A *P* value less than .05 was considered statistically significant. Parametric data are present as mean ± standard deviation.

RESULTS

Neurologic outcome. The results are summarized in the Table. In the sham group (n = 5), all rabbits were normal (grade 5). In the ischemia group, at 2 days after reperfusion (n = 5), one rabbit (20%) had ataxia (grade 3), two rabbits (40%) did not hop (grade 2), and two rabbits (40%) had hind-limb paralysis (grade 0), while at 7 days after reperfusion (n = 5), one rabbit (20%) did not hop (grade 2), and four rabbits (80%) had hind-limb paralysis

(grade 0). Fifteen minutes of ischemia did affect neuronal function.

Histologic study. In the sham group, the spinal cord was intact with many large motor neurons (26.6 ± 2.4 cells) in the ventral horn (Fig 1, *A*). However, in the ischemia group at 7 days after reperfusion, approximately 70% of the motor neurons in the spinal cord were damaged (7.8 ± 2.6 cells), although most motor neuron cells had remained intact after 2 days of reperfusion (23.2 ± 3.8 cells; Fig 1, *B* and *C*). The number of intact motor neurons in the sham group was significantly larger than that in the ischemia group at 7 days after reperfusion ($P = .0079$; Fig 2). Small motor neurons and intermediate neurons survived the ischemia. Dorsal horn neurons were also intact after 15-minute ischemia (data not shown). The 15-minute ischemia at 7 days after the procedure affected the number of motor neuronal loss cells in contrast to sham control. Thus, selective loss of motor neurons was confirmed, in accordance with our previous reports.[23]

Western blot analysis. Representative results of Western blot analysis are shown in Fig 3. For LC3-II, a slight band with a molecular weight of 16kDa was detectable in the sham group samples, which was then strongly enhanced at 8 hours, and was preserved until 2 days after reperfusion in the ischemia group. For GABARAP, a slight band with a molecular weight of 14kDa was detectable in the sham group samples, which was then strongly enhanced and peaked at 8 hours, but which returned to almost the same level as the sham group at 2 days after reperfusion in the ischemia group. The membranes without the primary antibodies revealed no band (data not shown). With quantitative analysis, we found that ischemia affected expression profiles of LC3-II and GABARAP (vs sham control $*P < .001$, $**P < .0001$; Fig 3, *B*, respectively).

Immunohistochemical study. Immunoreactive LC3 and GABARAP of sections from spinals cords are shown in Fig 4. The spinal cords of sham-operated animals slightly showed LC3 (Fig 4, *A*) and GABARAP (Fig 4, *E*) immunoreactivities in the cytoplasm of motor neurons. After 8 hours ischemia, LC3 expression was upregulated in the cytoplasm of motor neurons (Fig 4, *B*). After 2 days, the immunoreactivity in the cytoplasm was preserved in motor neurons (Fig 4, *C* and *D*). After 8 hours ischemia, GABARAP expression was also upregulated in the cytoplasm of motor neurons (Fig 4, *F*). After 1 day, the immunoreactivity in the cytoplasm was somewhat decreased but upregulated compared with the sham group (Fig 4, *G*). At 2 days, the immunoreactivity in the cytoplasm of motor neurons returned to almost the same level as the sham group (Fig 4, *H*).

Fluorescence double-labeling study. The results of LC3 and GABARAP double-staining immunohistochemistry are shown in Fig 5. At 8 hours after ischemia, LC3 (Fig 5, *A*) was detected by green-fluorescent Alexa Fluor 488 (*green*) and GABARAP (Fig 5, *B*) was detected by orange-fluorescent Alexa Fluor 555 (*red*); they were preferentially expressed in the cytoplasm of motor neurons. The merged image was shown in Fig 5, *C* to be double-positive. In motor neuron, LC3 (*green*; Fig 5, *A*) and GABARAP (*red*; Fig 5, *B*) were expressed in the cytoplasm and they were well colocalized (Fig 5, *C*).

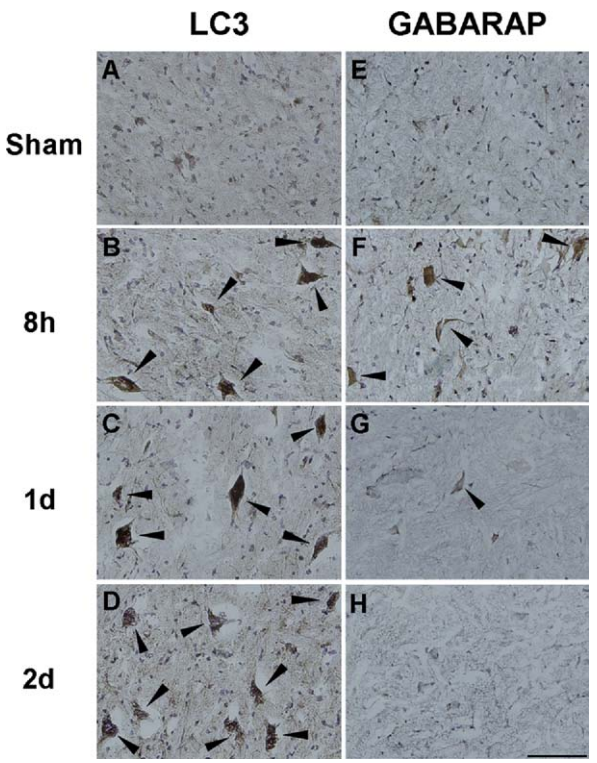

Fig 4. Immunostaining against LC3 and GABARAP in motor neurons in a sham group spinal cord **(A, E)** and in an ischemia group spinal cord at 8 hours (8h) **(B, F)**, 1 day (1d) **(C, G)** and 2 day (2d) **(D, H)** after reperfusion. *Arrows* show motor neurons that express immunoreactive LC3 **(B, C, and D)** and GABARAP **(F, G)**, respectively. **Bar = 100 μm**.

DISCUSSION

We demonstrated a profile of the expression of LC3 and GABARAP after transient spinal cord ischemia in rabbits. Transient ischemia induced selective delayed motor neuronal death and affected the profile of expression of LC3 and GABARAP. LC3 and GABARAP were induced strongly in motor neurons after transient ischemia. The prolonged expression of LC3 was observed until 2 days after reperfusion, whereas expression of GABARAP gradually decreased until 2 days after reperfusion.

In every eukaryotic cell, there are two main systems for the degradation of cytoplasmic protein; the ubiquitin-proteasome system and autophagy.[4,7,24] In contrast to the ubiquitin-proteasome system, which accounts for most of the selective intercellular protein degradation, autophagy is less selective.[25] A recent study indicated the existence of autophagy in cerebral ischemia.[11,26] However, the existence and the role of autophagy in the motor neurons after transient spinal cord ischemia have been unclear. We therefore investigated whether autophagy plays a beneficial or

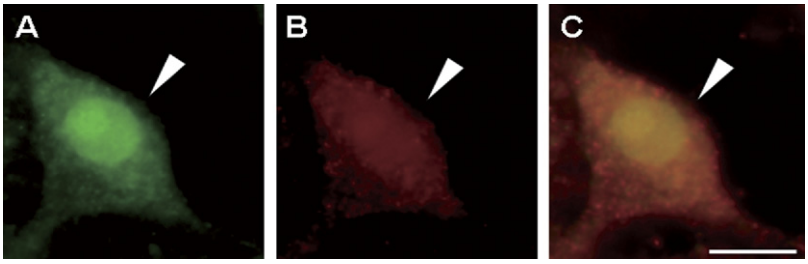

Fig 5. Co-localization of LC3 and GABARAP in motor neurons at 8 hours after ischemia. LC3 was detected by green-fluorescent Alexa Fluor 488 (*green*) **(A)**, and GABARAP by orange-fluorescent Alexa Fluor 555 (*red*) **(B)**. The merged image is shown in **(C)** with double positive (*arrow*) as yellow color. **Bar = 20 μm**.

detrimental role in the motor neurons after transient spinal cord ischemia.

Recently, 27 autophagy-related (ATG) genes were identified whose products appear to be related to the autophagy process.[27] It was found that the molecular basis of autophagy may well be highly conversed from yeast to humans.[8] For example, microtubule-associated protein 1 LC3 and γ-aminobutyric-acid type-A ($GABA_A$)-receptor-associated protein (GABARAP) play a critical role in the formation of autophagosomes.[10] LC3, a mammalian homologue of yeast Atg8, is a microtubule-associated protein that is lapidated upon activation of autophagy. The C-terminal fragment of LC3 is cleaved immediately following synthesis to yield a cytosolic form called LC3-I. A subpopulation of LC3-I is further converted to an autophagosome-associating form, LC3-II. LC3-II associates with the preautophagosomal and autophagosomal membrane and thus is considered a marker of autophagy. It was previously reported, for instance, that the amount of LC3-II was correlated with the existent of autophagosome formation.[10] Consequently, we measured the extent of autophagy in motor neurons after transient ischemia in the spinal cord by determination of LC3-II expression. In the present study, LC3-II was upregulated in motor neurons after transient ischemia in the spinal cord. Although in the present study the expression of LC3-II was slightly induced at sham group, it was reasonable because autophagy is an intracellular bulk degradation process and occurs at basal levels. A recent study demonstrated that the LC3-II protein levels were upregulated in the rat brain following ischemia induced by middle cerebral artery occlusion.[28] Overexpression of LC3-II has also been demonstrated to increase the activity of autophagy.[10,13] As such, the upregulation of LC3-II in the present study might be considered to increase activity of autophagy in motor neuron after transient ischemia in the spinal cord.

GABARAP, the other mammalian homologue of yeast Atg8, was identified as a $GABA_A$ receptor-associated protein of molecular weight 16kDa. The precise function and localization of GABARAP is unclear. However, a recent study demonstrated that GABARAP confers the capacity for binding to autophagosomes as well as LC3.[10] In the present study, we demonstrated that GABARAP was upregulated from 8 hours to 1 day after reperfusion in motor neurons after transient ischemia in the spinal cord. This result suggests that GABARAP can be localized on autophagosomal membranes under transient ischemia in the spinal cord.

Our previous study demonstrated that overexpression of the apoptosis-inducing protein, such as Akt and caspase3, was demonstrated in the motor neuron cells of the spinal cord at 8 hours after reperfusion, and the peak of Akt and caspase3 induction preceded the DNA fragmentation in the spinal cord after the ischemic insult.[23] The expression of Akt and caspase3 precede the appearance of neuronal damage, and may therefore be implicated in the activation of apoptosis in our model. A more recent study also reported that autophagy was involved in mechanisms of cell death after focal cerebral ischemia.[28] Thus, in the present study, LC3 and GABARAP were upregulated in motor neuron at an early phase after transient ischemia in the spinal cord. The upregulation of LC3 and GABARAP in the motor neurons after transient spinal cord ischemia may represent enhanced autophagy as a mechanism to recycle injured cells and reduce damage, or a process leading to cell death. Therefore, one can speculate that whether autophagy plays a beneficial or detrimental role in the motor neurons after transient spinal cord ischemia is considered to be dependent on the character of the stress and degree of the expression of LC3 and GABARAP.

A recent study demonstrated that overexpression of LC3 was observed in motor neurons of the spinal cord prior to the onset of ALS in transgenic mice with an Cu/Zn superoxide dismutase mutation.[16] In this study, the increase in the immunoreactivity of LC3 was demonstrated selectively in ventral motor neuron cells in the spinal cord after 8 hours of reperfusion. This finding suggests that the oxidative injury may activate LC3 induction as a compensatory mechanism. Thus, our results suggest that the mechanism of motor neuron death in the spinal cord after ischemia may have similar features with that of ALS.

This study also demonstrates that immunoreactivities for both LC3 and GABARAP were induced in the same motor neurons which eventually died. These results suggest that autophagy was induced in motor neurons, and contributed to neuronal death by transient spinal cord ischemia in rabbits.

AUTHOR CONTRIBUTIONS

Conception and design: HB, MS
Analysis and interpretation: HB, MS
Data collection: HB
Writing the article: HB
Critical revision of the article: MS, KA, RT
Final approval of the article: RT
Statistical analysis: HB, MS
Obtained funding: Not applicable
Overall responsibility: HB

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