Autophagy-mediated stress response in motor neurons after hypothermic spinal cord ischemia in rabbits

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Objective: **The development of spinal cord injury is believed to be related to the vulnerability of spinal motor neurons to ischemia. However, the mechanisms underlying this vulnerability have not been fully investigated. Previously, we reported that spinal motor neurons are lost likely due to autophagy and that local hypothermia prevents such spinal motor neuron death. Therefore, we investigated the role of autophagy in normothermic and hypothermic spinal cord ischemia using an immunohistochemical analysis of Beclin 1 (BCLN1; B-cell leukemia 2 protein [Bcl-2] interacting protein), Bcl-2, and γ-aminobutyric acid type-A receptor-associated protein (GABARAP), which are considered autophagy-related proteins.**
Methods: **We used rabbit normothermic and hypothermic transient spinal cord ischemia models using a balloon catheter. Neurologic function was assessed according to the Johnson score, and the spinal cord was removed at 8 hours and 1, 2, and 7 days after reperfusion, and morphologic changes were examined using hematoxylin and eosin staining. A Western blot analysis and histochemical study of BCLN1, Bcl-2, and GABARAP, and double-labeled fluorescent immunocytochemical studies were performed.**
Results: **There were significant differences in the physiologic function between the normothermic model and hypothermic model after the procedure ($P < .05$). In the normothermic model, most of the motor neurons were selectively lost at 7 days of reperfusion ($P < .001$ compared with the sham group), and they were preserved in the hypothermic model ($P = .574$ compared with the sham group). The Western blot analysis revealed that the sustained expression of the autophagy markers, BCLN1 and GABARAP, was observed ($P < .001$ compared with the sham group) and was associated with neuronal cell death in normothermic ischemic conditions. In hypothermic ischemic conditions, the autophagy inhibitory protein Bcl-2 was powerfully induced ($P < .001$ compared with the sham group) and was associated with blunted expression of BCLN1 and GABARAP and neuronal cell survival. The double-label fluorescent immunocytochemical study revealed that immunoreactivitiy for BCLN1, Bcl-2, and GABARAP was induced in the same motor neurons.**
Conclusions: **These data suggest that the prolonged induction of autophagy might be a potential factor responsible for delayed motor neuron death, and the induction of the autophagy inhibitory protein Bcl-2 using hypothermia might limit autophagy and protect against delayed motor neuron death. (J Vasc Surg 2015;62:1312-9.)**

Clinical Relevance: **Patients undergoing thoracic aneurysm repair who awake with no neurologic deficit immediately after surgery can sometimes eventually develop paraplegia. In the present study, we demonstrated that after spinal cord ischemia in rabbits, the sustained expression of autophagy markers, Beclin 1 and γ-aminobutyric acid type-A receptor-associated protein, was observed in normothemic conditions, and the autophagy inhibitory protein, B-cell leukemia 2 protein, was powerfully induced and was associated with blunted expression of Beclin 1 and γ-aminobutyric acid type-A receptor-associated protein and neuronal cell survival in hypothermic conditions. The results of the present study indicate that prolonged induction of autophagy might be a potential factor responsible for delayed motor neuron death in this model.**

Spinal cord injury after successful surgery of the thoracic aorta is a disastrous complication. The reported incidence of paraplegia in such case ranges from 2.9% to 23% among patients who undergo surgery of the thoracic aorta.[1] The reasons for spinal cord dysfunction are thought to be the induction of ischemic damage during cross-clamping. However, patients who undergo thoracic aortic aneurysm repair who awaken with no neurologic deficits immediately after surgery might sometimes develop delayed-onset paraplegia.[2] The exact mechanism underlying this delayed vulnerability is not fully understood.

A recent study showed that neuronal survival is affected by the disturbance of the ubiquitin-proteasome pathway or the autophagy-lysosome pathway in conditions of nonlethal stress.[3] We reported that delayed and selective motor neuron death is associated with the activation of autophagic signals.[4-6] In addition, we previously reported the establishment of a topical cooling model of spinal cord ischemia that inhibits delayed and selective motor neuron death.[7] As a common feature of humans and rabbits, the

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motor neurons are vulnerable to ischemia. In the rabbit anatomy, the blood flow for the spinal cord is supplied from the lumbar artery. Therefore, production of the spinal cord ischemia model is possible using a simple technique that does not cause other organ injury.

Autophagy is an intracellular bulk degeneration process in which cytosolic, long-lived proteins and organelles are degenerated and recycled.[8] In autophagy, cytoplasmic materials and dysfunctional organelles are sequestered by autophagosomes, and subsequently delivered to the lysosome where they are degraded by lysosomal proteases. Autophagy occurs at the basal levels, although it can be further induced by stressors, including nutrient depletion, ischemia, and reperfusion.[9,10] Autophagy plays a dual role in cell survival, promoting survival by generating free amino acids and fatty acids that can be reused to maintain mitochondrial adenosine triphosphate production and protein synthesis, and inducing cell death in some circumstances.[11-13]

Beclin 1 (BCLN1) is part of the class III phosphoinositide 3-kinase complex that participates in autophagosome formation, mediating the localization of other autophagy proteins to the preautophagosomal membrane.[14,15] B-cell leukemia 2 protein (Bcl-2) is a key regulator of apoptosis and autophagy. Antiapoptotic Bcl-2 family proteins can bind the autophagy essential protein BCLN1 and inhibit BCLN1-dependent autophagy.[16-18] However, the function of BCLN1 and Bcl-2 in spinal cord ischemia-induced neurodegeneration is not completely understood. γ-Aminobutyric acid type-A receptor-associated protein (GABARAP) is a mammalian Atg8-related protein that localizes to autophagosomal membranes after post-translational modifications and has been shown to be an autophagosomal marker in mammals.[19] Using the rabbit spinal cord ischemia model, we previously reported the overexpression of GABARAP after transient ischemia.[6]

Therefore, in the present study, we hypothesized that motor neuron cells, which eventually die from acute spinal cord ischemia in this model, demonstrate the previous induction of BCLN1, Bcl-2, and GABARAP. In addition, differences in their expression level in moderate hypothermia conditions were assessed.

METHODS

Animal models. The animals were treated in accordance with the declaration of Helsinki and the guiding principles for the care and use of animals during the experiment. The animal care committee of the Kyushu University School of Medicine approved the experimental and animal care protocols.

Forty-five Japanese domesticated white rabbits weighing 2 to 3 kg were used in this study and divided into three groups: a normothermic ischemia group (group N), a hypothermic ischemia group (group H) and a sham control group. Anesthesia was induced via the intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane inhalation with 100% oxygen. A 4-French pediatric catheter (CI-300; Harmac Medical Products, Inc, Buffalo, NY) was inserted through the femoral artery and advanced 15 cm forward into the abdominal aorta. Then, a balloon was inflated and 15 minutes of transient ischemia was performed. The balloon was deflated after 15 minutes of ischemia and the catheter was immediately removed. Preliminary investigations conducted via laparotomy confirmed that the distal end of the balloon of the catheter was positioned approximately 0.5 to 1.5 cm immediately distal to the left renal artery.[4-7] The aortic pressure was continuously monitored at the proximal and distal positions of the balloon during the experiment. When the balloon of the catheter was inflated in the abdominal aorta, the systemic blood pressure of the rabbits did not change. In addition, the arterial pressure of the distal end of the catheter decreased to near zero, and no pulsation was recorded. The arterial blood pressure of this portion decreased for 15 minutes and returned to the normal level after deflation of the balloon. The body temperature was maintained at 37°C with a heating pad and monitored using a rectal thermistor during procedure and subsequent ischemia. Group H was treated using the same method with a cooling pad.[7] The cooling pad was attached to the lumbar region (L1-L5) of the naked skin. We confirmed the localized cooling effect according to the temperature of the rectum (34.85 ± 0.21°C vs 31.35 ± 0.30°C; $P < .001$). The animals were killed using deep anesthesia with sodium pentobarbital (100 mg/kg administered intravenously) at 8 hours and 1, 2, and 7 days after reperfusion (n = 5 in each group at each time point). In the sham control group, the animals were killed 7 days (n = 5) after reperfusion after the insertion of the catheter without inflating the balloon. Using the plunger of a 1-mL syringe, the spinal cords were quickly removed immediately after death. The tissue samples used for the Western blot analysis and immunohistochemical studies were frozen and stored at −80°C. The samples used for histology were fixed via immersion in 4% paraformaldehyde in 0.1 M phosphate buffer then stored at 4°C for 1 week; they were then cut transversely at approximately the L2 or L3 level and embedded in paraffin.

Neurological assessment. Neurologic function was evaluated before the rabbits were killed at 7 days after reperfusion. The rabbits were classified according to a five-point scale devised by Johnson et al[20] as follows: 0, hind-limb paralysis; 1, severe paralysis; 2, functional movement, no hop; 3, ataxia, disconjugate hop; 4, minimal ataxia; and 5, normal function. Two individuals without knowledge of the treatment graded the neurologic function independently.

Histological study. To determine the pathological changes in the spinal cord after ischemia, we performed hematoxylin and eosin staining of a set of sections examined using light microscopy and counted the number of intact large motor neuron cells in the ventral gray matter region in five sections per animal. An observer who was unaware of the animal groups and neurological outcomes examined each slide (magnification ×100). The neurons were considered "dead" if the cytoplasm was diffusely eosinophilic and