Changes in autophagy proteins in a rat model of spinal cord injury

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【**Abstract**】**Objective:** Autophagy is involved in several neurodegenerative diseases and recently its role in acute brain injury has won increasing interest. Spinal cord injuries (SCIs) often lead to permanent neurological deficit. Therefore, in this study, we examined the profiles of autophagy-linked proteins (MAP-LC3) after SCI to investigate whether the expression of autophagy contributes to neurological deficit after SCI.

Methods: Adult female Sprague-Dawley rats were used and randomly divided into control and SCI groups. All the rates received laminectomy at T_8-T_{10} level. Those in the SCI group received additional exposure of the dorsal surface of the spinal cord, followed by a weight-drop injury. Thereafter we investigated the expression levels of MAP-LC3, beclin-1, Cathepsin D and the beclin-1-binding protein bcl-2 by western blot analysis at 12 h, 24 h, 3 d, 7 d, 21 d and 28 d. One-way ANOVA with Tukey post hoc test was used to compare data between groups.

Results: We observed significant increase in the level of LC3 (LC3-II/LC3-I) at 3 d and 7 d after SCI when compared with the sham group. While the level of beclin-1 and ratio of beclin-1/bcl-2 was found to have increased from 12 h to 24 h after injury. Cathepsin D expression was also elevated at 7 d (P<0.01).

Conclusion: Based on the above mentioned data, we proposed that autophagy plays a role in the manifestation of cell injury following SCI.

Key words: ***Spinal cord injuries; Autophagy; LC3 protein, rat; Cathepsin D***

Chin J Traumatol 2014;17(4):193-197

Autophagy is an intracellular catabolic mechanism for the degradation of cytoplasmic constituents in the autophagosomal-lysosomal pathway.[1] It is characterized by the presence of double membrane cytoplasmic vesicles that are called autophagosomes. They sequester cytosolic components before fusing with lysosomes where the lysosomal hydrolases breakdown the sequestered organelles. Autophagy is involved not only in the balance between protein synthesis and degradation but also in the execution of cell death.[2] Autophagy contributes to the mechanism of non-apoptotic programmed cell death, which is known as autophagic cell death.[3] Increased autophagy has now been reported in some experimental models of traumatic brain injury, excitotoxicity and in patients with Alzheimer's disease or critical illness. Furthermore, the existence of a biochemical mechanism of crosstalk between apoptosis and autophagic cell death has been confirmed.[4,5] A number of proteins that intricately regulate autophagy have been reported including beclin-1, MAP-LC3[6] and Cathepsin D[7].

Spinal cord injury (SCI) is a significant cause of physical disability in young adults. There are two main types of cell death: necrosis and apoptosis. Both types have been found in the spinal cord after injuries. Recent studies have also focused on the alterations in the autophagy protein, expression proteins and involvement of autophagy following SCI.[8] Light chain 3 (LC3) has two forms: type I (LC3-I) is cytosolic and type II (LC3-II) is membrane-bound. During autophagy, LC3-II is increased by conversion from LC3-I. Cathepsin D is a protein

DOI: 10.3760/cma.j.issn.1008-1275.2014.04.002

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Chinese Journal of Traumatology 2014;17(4):193-197

known as mediate autophagy. Beclin-1 is a component of the Class III phosphatidylinositol-3-kinase (PI3K) complex which is essential for the formation of autophagosomes.[9] Beclin-1 interacts with B-cell lymphoma 2 (bcl-2) anti-apoptotic proteins.[4] However, little is known about the change of beclin-1, MAP-LC3, Cathepsin D and bcl-2 after SCI. The aim of this study is to investigate the profiles of autophagy-linked proteins on an experimental SCI rat model.

METHODS

Adult Sprague-Dawley rats weighing 200-250 g were used in this study. We minimized the number of animals used in this experiment and did great efforts to reduce their suffering. All surgical procedures were performed under sterile conditions and aseptic manners, and were allowed by the Research Animal Resources and Care Committee of the Soochow University.

Contusive SCI was induced using a New York University impactor, as described previously.[10] Rats were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and received a laminectomy at the T_8-T_{10} level. After the spinous processes of T_8 and T_{10} were clamped to stabilize the spine, the exposed dorsal surface of the cord was subjected to a weight-drop injury using a 10 g rod (2.5 mm in diameter) dropped from a height of 25 mm. Following surgery, 1.0 ml of saline was administered subcutaneously in order to replace the blood volume lost in the surgery. During the recovery from anesthesia, the rats were placed on a heating pad and covered with a warm towel. The rats were singly housed in a temperature-controlled room at 27°C for a survival period of 28 days. Food and water were provided adlibitum. During this time, the animals' bladders were manually voided twice a day until the rats were able to regain normal bladder function. In all injured groups, the spinal cord was compressed for 1 min. Sham-injured animals were only subjected to laminectomy.

Tissue collection and immunoblot analysis

At different time points (12 h, 24 h, 3 d, 7 d, 21 d, 28 d) after injury and immediately after the sham operation, the rats were killed by intraperitoneally injection of an overdose of sodium pentobarbital (100 mg/kg) and the spinal cord was rapidly collected. A section of tissue was frozen in liquid nitrogen and stored at -70°C until further use. Spinal cord was homogenized on ice in 10 mmol Tris-Hcl buffer (PH=7.4), 10 mmol EDTA, 30% Triton-1000, 10% SDS and NaCl using a homogenizer. The supernatant was collected and stored at -80°C. Samples (40 μg of total protein per well) were subjected to 10%-20% sodium dodecylsulfate polyacrylamide gel and then transferred onto polyvinylidene fluoride membrane on a semi-dry electrotransferring unit (Bio-Rad). Following the transfer, the membranes were blocked in 5% nonfat dry milk in 1×Tris-buffered saline with Tween 20 (TBST) and probed overnight with primary antibody at 4°C.

Antibodies and immunolabeling

Immunoblots were probed with anti-MAP-LC3 (Abcam, UK), anti-bcl-2 (Santa Cruz, USA, anti-beclin-1 (Abcam, UK) or Cathepsin D (Santa Cruz, USA) antibody overnight at 4°C. Following the overnight incubation with primary antibodies, the PVDF membranes were incubated with peroxidase-conjugated anti-rabbit (for beclin-1, Cathepsin D and MAP-LC3) and peroxidase-conjugated anti-mouse (for bcl-2) for one hour in 5% nonfat dry milk in TBST. Densitometric quantification of the bands was performed using Image J Software (version 1.29x; National Institutes of Health, USA).

Statistical analysis

Data were plotted as means±standard error of the mean (SEM, n=5). One-way ANOVA with Tukey post hoctest was used to draw comparisons among multiple groups at different time points. A value of P<0.05 was considered to be statistically significant.

RESULTS

SCI induced the conversion of LC3-I to LC3-II

Autophagy induction was determined through checking the expression levels of the autophagy protein MAP-LC3 by western blotting. Time course study of the LC3 demonstrated that the level of LC3-II increased (P<0.05) at 3 d, 7 d, and 14 d after injury compared with the sham-operated group. The ratio of LC3-II to LC3-I in the spinal cord was significantly increased at 3 d, peaked at 7 d, and decreased markedly at 21 d after SCI (Figure 1A).