Upregulation of RIP3 Expression in Various Neural Cells after Spinal Cord Injury in Mice

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Disclosures:

Introduction: Apoptosis, characterized by the activation of caspases and DNA fragmentation, was once considered the sole form of programmed cell death. In contrast, necrosis was originally considered a nonspecific mode of cell death. To date, it has been considered that the secondary damage after spinal cord injury is caused by apoptosis. Most researches related to the cell death in the injured spinal cord focused on apoptosis. “Necroptosis” is a newly identified type of programmed necrosis. Necroptosis is another form of programmed cell death regulated by caspase-independent pathway and has the morphological features of necrosis (early membrane and organelle swelling followed by cell lysis). [1] Receptor-interacting protein 3 (RIP3), a member of RIP family proteins, is known as a key mediator of necroptosis. [2] The amount of RIP3 protein expression correlates with necroptosis induction in various types of cells. Recent studies demonstrated that the increased expression of RIP3 in lesions and the induction of necroptosis in various disease models [3]. However, to our knowledge, there has been no study to investigate the expression of RIP3 and the involvement of necroptosis in damaged neural tissue after spinal cord injury. In the present study, we used a spinal cord hemisection model in mice to study the alterations of RIP3 protein expression and the involvement of necroptosis in spinal cord injury.

Methods: Animals: Adult female C57BL/6J mice between 8 and 10 weeks of age were used in this study.
Surgical procedures: The T10 vertebra was laminectomized to expose the spinal cord. With a scalpel, the cord was transected on the left side only [4].

Immunohistochemistry: At different time points (4, 24 hours, 3, 7 and 21 days) after hemisection and at 24 hours after sham operation, the spinal cords at the lesion were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned. Tissue sections were incubated with rabbit anti-RIP3 antibody (1:100; Sigma) and visualized by Alexa Fluor 594 goat anti-rabbit IgG antibody (1:500; Molecular Probes).

Counting of RIP3-positive cells: The number of RIP3-positive cells was counted in the injured and the contralateral sides of transverse sections at the different time points (4, 24 hours, 3, 7 and 21 days) after hemisection, and compared with those of the sham control.

Double staining of RIP3 and various cell type markers: To examine the expression of RIP3 in a specific population of cells, the transverse sections at 3 days after hemisection were co-stained for RIP3 and various cell type markers: NeuN for neurons, GFAP for astrocytes and Olig2 for oligodendrocytes.

Results: Immunohistochemical staining of RIP3: Representative pictures showed the RIP3-expressing cells on the injured side obviously increased compared to on the contralateral side at 3 days after hemisection (Fig. 1).

Counting of RIP3-positive cells: The number of RIP3-positive cells on the injured side was significantly higher than those on the contralateral side and sham control at 24 hours, 3, 7 and 21 days (Fig. 2). The maximum number of RIP3-positive cells in the injured side was observed at 3 days, and it decreased after 7 days (n = 3 at each time point).

Double staining of RIP3 and various cell type markers: Double staining of RIP3 and various cell type markers demonstrated the increased expression of RIP3 in neurons, astrocytes and oligodendrocytes on the injured side at 3 days after hemisection (Fig. 3).

Discussion: In the present study, we have demonstrated that the levels of RIP3 protein were dramatically increased at the lesion site after spinal cord hemisection. Our results first provided the evidence to support the involvement of necroptosis in neural tissue damage after spinal cord injury. To date, it has been considered that the secondary damage of the neural tissue following spinal cord injury is caused by apoptosis but not necrosis. Most previous researches related to the cell death in the injured spinal cord focused on apoptosis. In our study, the upregulation of RIP3 expression was observed starting from 24 hours, peaked at 3 days, and lasted for 21 days after injury. Importantly, the time course of RIP3 expression is quite similar to that of the secondary damage following spinal cord injury. [5] These results suggest necroptosis contributes to the secondary damage in the injured spinal cord. In our results, the expression of RIP3 was observed in neurons, astrocytes, and oligodendrocytes. These findings indicated necroptosis occurs in various neural cells and is related to multiple pathological mechanisms after spinal cord injury. Further studies to clarify molecular and biological mechanism of necroptosis can lead to novel therapeutic strategy for the treatment of spinal cord injury.
Significance: The expression of RIP3 was dramatically increased in neurons, astrocytes, and oligodendrocytes in the injured spinal cord. Necroptosis can contribute to neural tissue damage following spinal cord injury.

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Fig. 1. Immunohistochemical staining in transverse (A) and coronal (B) sections at 3 days after hemisection, stained RIP3 in red and nuclear maker DAPI in blue. The RIP3-expressing cells on the injured side was obviously increased compared to on the contralateral side (L: injured side, R: contralateral side, Scale bar: 500 μm).
Fig. 2. The number of RIP3-positive cells at different time points after hemisection, and in sham control. (A-F) Representative pictures showed the RIP3-expressing cells on the injured side obviously increased at 3 and 7 days after hemisection (Scale bar: 200 µm). (G) The number of RIP3-positive cells on the injured side was significantly higher than those on the contralateral side and sham control at 24 hours, 3, 7 and 21 days (L: injured, R: contralateral, Error bars indicate SD. * p<0.05).
Fig. 3: Double staining of RIP3 and various cell type markers. The RIP3 expression was observed in NeuN-, GFAP- and Olig2-labeled cells (Arrows indicate double stained cells. Scale bar: 50 μm).

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