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Myelotomy suppresses autophagic marker LC3-II expression and elevates mTORC1 expression and improves neurological function recovery in rats with spinal cord injury

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Abstract

Although previous studies have shown functional efficacy of myelotomy for the treatment of spinal cord injury (SCI), the underlying mechanism remained unknown. This study aimed to determine the relationship between myelotomy-mediated neuroprotection and autophagy following SCI by evaluating the expression of microtubule-associated protein light chain 3 (LC3-II) and mammalian target of rapamycin complex 1 (mTORC1). Ninety-nine adult female rats were randomly assigned to either sham-operated group (SG), model group (MG), or 24 h-myelotomy group (MTG). SCI at T10 was induced with a New York University impactor, and myelotomy was performed 24 h after SCI. Functional recovery was evaluated via the open-field test. The protein expression of LC3-II was analyzed by Western blot, and the mRNA expression of LC3-II and mTORC1 were detected by real-time quantitative reverse transcriptase polymerase chain reaction. Rats in the MTG exhibited significantly better performance in the hind limbs compared to those in the MG on day seven and fourteen post-injury. Myelotomy suppressed the protein and mRNA expression of LC3-II on day three, seven and fourteen post-injury and increased the mRNA expression of mTORC1 in the MTG on day three and seven post-injury. The LC3-II protein expression was significantly and negatively correlated with BBB scores at day seven and fourteen post-injury. These results showed that myelotomy-induced neuroprotection in a rat model of SCI was likely mediated by inhibition of autophagy by activation of the mTORC1 signaling pathway.

INTRODUCTION

Spinal cord injuries (SCI) are particularly devastating at both a personal and social level. Based on the pathophysiological mechanism, SCIs are classified as either primary or secondary injuries. To date, there is no evidence for central nervous system (CNS) regeneration following SCI.[1] Therefore, it is very crucial that the deleterious secondary injury mechanisms be halted. Secondary injury (including ischemia, inflammation, excitotoxicity, mitochondrial damage, energy supply reduction, apoptosis, autophagic cell death, and oxidative cell damage) is what ultimately causes the loss of neural tissue.[2,3] The two key initial factors contributing to secondary damage following SCI are hemorrhage and edema. Myelotomy is a surgical procedure where a portion of the spinal cord at the site of hemorrhage and necrosis is removed. It may prove to be a promising method to prevent secondary damage and aid in the preservation of more spinal cord tissue.[4-9]

Although decompressing the intradural elements is not a widely accepted therapeutic strategy for SCI, there is some pre-clinical and clinical research demonstrating the efficacy of myelotomy.[10,11] The molecular mechanism underlying this treatment for SCI remains unknown.

Following CNS trauma, cellular necrosis occurs immediately at the injury epicenter, while ensuing apoptosis and autophagy spread out into the lesion

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penumbra and exaggerate the primary lesion.[3,12,13] Autophagy is involved not only in the balance between protein synthesis and degradation, but also in the execution of cell death. Its aberrant activation can cause autophagic cell death, and this contributes to neural tissue damage in many diseases, such as several neurodegenerative disorders[14-16] traumatic brain injury, and stroke.[17-19] Recent studies have indicated that autophagy has an important role in neural tissue damage in spinal cord injury.[3,13,20,21] To date, there is no evidence that autophagy is involved in the protective effects of myelotomy in SCI.

There are three basic types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Bulk degradation of cytoplasmic organelles is largely mediated by macroautophagy, commonly referred to as autophagy. Autophagy starts with the formation of double-membrane cisternae that subsequently engulf cytoplasmic materials or whole organelles to become double-membrane bubble like vacuoles known as autophagosomes (APs). LC3, a mammalian homologue of yeast Atg8, is an essential marker for detecting APs. LC3 has two forms, LC3-I (cytosolic) and LC3-II (membrane bound). Upon activation of autophagy, LC3-I is conjugated with phosphatidylethanolamine and then converted to LC3-II.[22] Therefore, detecting LC3-II by immunoblotting or immunofluorescence is a reliable method for monitoring AP formation and autophagy-related processes, including autophagic cell death.[23]

The mammalian target of rapamycin complex 1 (mTORC1) pathway, which acts as a master regulator to control protein synthesis, can inhibit autophagy by phosphorylating and inactivating ULK1, an autophagy regulatory protein.[24] A decrease in TORC1 activity can lead to autophagy induction, whereas the activation of mTOR signaling can reduce autophagic activity.[21]

This study investigated the relationship between myelotomy-mediated neuroprotection and autophagy following SCI by observing the expression of LC3-II and mTORC1 and behavior.

METHODS

Ninety-nine Sprague-Dawley female rats (11 weeks old on surgery day) were randomly divided into three groups: sham group (SG), contusion-alone group (MG), and 24 h-myelotomy group (MTG). Rats in the SG (n = 33) received laminectomy, and rats in the MG (n = 33) received laminectomy plus SCI. Thirty-three rats in the MTG received laminectomy plus SCI and a myelotomy. Myelotomy was performed 24 h post SCI. All rats were housed in identical environments maintained at a constant temperature of 20-28^{0}C with standard rodent chow and water available *ad libitum*. Bladders were emptied manually every 8 h until recovery or sacrifice. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Capital Medical University (approval number 2011-D-007).

Rat model of spinal cord injury

Adult rats were anesthetized with chloral hydrate (400 mg/kg) given intraperitoneally (I.P.). Laminectomy was performed at the T9–T10 level to expose the cord without disrupting the dura mater. The spinous processes of T8 and T11 were clamped to stabilize the spine, and the exposed dorsal surface of the cord was subjected to contusion injury (10 g rod, 25 mm height) using a New York University (NYU) weight-drop device. The entire aseptic operation was performed under a Carl Zeiss operating microscope (73446, Oberkochen, Germany).

Microsurgical myelotomy

Rats were re-anesthetized at 24 h after SCI. Microsurgical myelotomy was performed under a Zeiss operating microscope. After the incision was disinfected and the stitches were removed, the spinal cord tissue with dark purple appearance was exposed. A one ml disposable syringe needle (27 G) was used to puncture a small hole in the dorsal dura mater, slightly away from midline. The dura mater was cut with microscissors (Micro Scissors Straight 22.5 cm, Daddy D Pro Surgical Company, USA), and the arachnoid exposed. A 3.5 mm long incision was made with a blunt microprobe (0.2 mm diameter tip size, modified from Kryenbuhl Nerve Hook, WI-GS-3382, Wrangler Instruments, Pakistan). The spinal cord was penetrated longitudinally into the posterolateral region approximately half way through the spinal cord. The incision in the spinal cord was washed gently twice with warm saline (37 ^{0}C, one ml at a time), and the necrotic cord and dark bloody clot were usually washed out. A piece of gelfoam was placed on the surface of the dura mater, and the muscles and skin layers were sutured.

Evaluation of motor function

Locomotor function was evaluated according to the open field test (Basso-Beattie-Bresnahan, BBB). Open-field test was tested before the operation, and then once a week until the end of the experiments (score 21=normal; Score 0=no hindlimb movement). The scores were averaged for each group at each time point. All behavioral tests were observed by two independent investigators blinded to experimental condition.

Western blot analysis

At three, seven, or fourteen days post-injury, six rats were randomly selected from each group and anesthetized. A 15 mm spinal cord segment containing the injury epicenter was removed for protein extraction 24 h following SCI. Equal protein concentration from each sample was loaded onto 8–10% polyacrylamide gels, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane by electrophoresis. The membranes were immunoblotted with the following primary antibodies: polyclonal rabbit anti-LC3-II (1∶1000; Abgent, San Diego, CA, USA) to assess autophagy activity and monoclonal mouse anti-GAPDH (1∶1000; Sigma-Aldrich, St Louis, MO, USA) as a loading control. Membranes were incubated with a goat anti-rabbit or goat anti-mouse fluorescent secondary antibody, fluorescing at either 680 nm or 800 nm wavelength (1∶10,000; Rockland Immunochemicals, Inc., Gilbersville, PA, USA). Bands were visualized using the LiCor Odyssey infrared imaging system and software Version 1.2 (LiCor Biosciences, Lincoln, NE, USA) per the manufacturer's instructions, and quantification of detected bands was performed by densitometry using quantity one software (Biorad, Hercules, CA, USA).

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis

At three, seven, or fourteen days post-injury, five rats randomly selected from each group were anesthetized and 15 mm spinal cord segments containing the injury site were removed. Total RNA was extracted from the spinal cord tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA), where 0.1 g of homogenized spinal cord was placed in 1 ml of Trizol reagent on ice. RNA content and purity were determined by AD260/280 ultraviolet spectrophotometry. Complementary DNA (cDNA) copies of two genes, including mTORC1 and LC3-II were synthesized using the TIANScript RT Reagent kit (Tiangen Biotech, Beijing, China) for a 20 μl reaction system. A two μl sample of each cDNA template was amplified in a 45 reaction system containing one μl of SYBR Premix Ex Taq II (Invitrogen) and 0.5 μl of each primer. The qRT-PCR was performed using an TL988 II System (Tianlong, Shanxi, China) under the following conditions: denaturation at 95 ^{0}C for two min, 45 cycles of denaturation at 94 ^{0}C for 30 s, and annealing at 54 ^{0}C 30 s ,and extension at 72 ^{0}C for 30 s, and annealing read fluorescence value at 72 ^{0}C. The primer sequences for the target genes used in this study were obtained from Leagene Biotechnology and are listed in Table 1. Each primer pair was tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve which was used to calculate the PCR efficiency. Specificity was determined by melting-curve analysis and gel electrophoresis of products. Each sample was assayed in triplicate and was compared to arbitrary values assigned to a standard curve generated from serial dilutions of myelonic RNA to obtain relative abundance of amplified product.

The result from each sample was normalized against that of glyceraldehydes 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Statistical analysis was performed using SPSS software 17.0. BBB scores of locomotor behavior were analyzed using the non-parametric Mann-Whitney U test. LC3 protein expression was analyzed using one-way ANOVA with post-hoc bonferrnoi test, and relative mRNA expression was compared using an independent T-test. The relationship between LC3 protein expression and BBB was analyzed using linear regression. All values were expressed as Mean ± S. D. where $P < 0.05$ was considered statistically significant.

RESULTS

Myelotomy inhibits protein expression of LC3-II in injured spinal cord tissues after SCI

In order to determine if myelotomy following spinal cord contusion alters LC3-II expression, we evaluated its protein levels via immunoblot. Band density analysis revealed that the level of LC3-II protein was significantly higher in the injured spinal cord in MG than SG on day three, seven and fourteen post-injury by 54%, 78% or 107%, respectively ($P < 0.001$; Figure1 A,B). The level of LC3-II expression was decreased in the

Table 1: Primer sequences used for real-time quantitative RT-PCR

Name of target gene	Primer sequence	Product size (bp)
mTORC1	Forward: 5′-ctgatcagaccacagacga-3′ Reverse: 5′-gtggaatttgaccacagag-3′	285bp
LC3-II	Forward: 5′-gagagcgagagagatgaag-3′ Reverse: 5′-cggatagtctagtttagatgag-3′	266bp
β-actin	Forward: 5′-acacccgccaccagttc-3′ Reverse: 5′-tgacccatacccaccatc-3′	155bp

LC3-II, microtubule-associated protein light chain 3; mTORC1, mammalian target of rapamycin complex 1; bp, base pairs

MTG compared with MG on day three, seven, and fourteen post-injury by 43.3%, 47.3% or 33.9%, respectively ($P < 0.001$; Figure 1 A,B).

Effects of myelotomy on expression of LC3-II and mTORC1 in injured spinal cord tissues at the mRNA level

First, we used qRT-PCR to detect the mRNA expression of LC3-II and mTORC1 after microsurgical myelotomy in the spinal cord contusion model. The mRNA expression of LC3-II in the MTG was decreased compared with the MG at day three, seven, and fourteen post-injury by 66%, 52.4% and 76.2%, respectively ($P < 0.05$; Figure 2). The mRNA expression of mTORC1 in the MTG was significantly higher than that in the MG at day three and seven post-injury by 126% and 83.9%, respectively ($P < 0.05$; Figure 3).

Myelotomy improves the recovery of motor function of rats after SCI

Before the operation, the BBB score in all groups was 21. Rats in the MTG showed significantly better performance in the hind limbs compared to those in the MG at day seven and fourteen post-injury by 126% or 128%, respectively ($P < 0.05$; Figure 4). BBB scores in the SG were 21 at day seven and fourteen post-injury.

The regression analysis of LC3-II protein expression and BBB in rats after injury

Linear regression analyses are summarized in Table 2. LC3-II protein expression at day seven and fourteen post-injury was significantly and negatively correlated with the functional deficit as measured by the BBB ($P < 0.01$).

DISCUSSION

Myelotomy has been used in preclinical and clinical research for the treatment of SCI for a century.[7] Myelotomy reduced the damaged area in a SCI rat model[4] and aided in the recovery of evoked potentials in a SCI dog model.[25] Although there is some evidence demonstrating the efficacy of this procedure[4-7,11], myelotomy has not been widely used as a therapeutic strategy for SCI. One

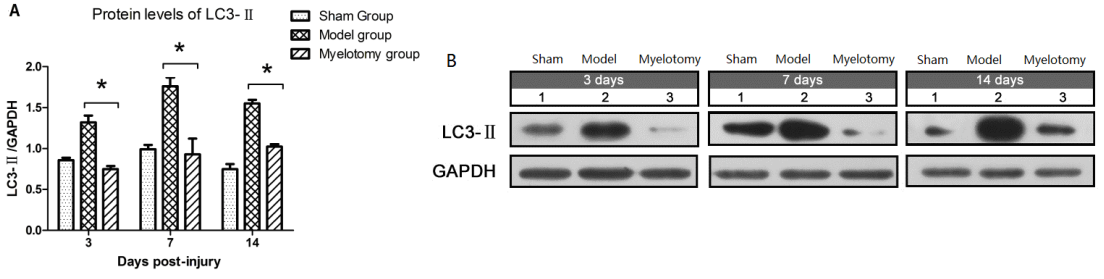

Figure 1. The expression of LC3-II protein in the injured spinal cord after sham operation, contusions and myelotomy in Western blot (A, B). (A) A quantitative analysis showed that the level of LC3-II expression was decreased in the MTG compared with MG on different time points (n = 6 per group, * $p < 0.001$). The quantities of the band densities were normalized by GAPDH. (B) The expression of LC3-II obviously increased in the model group.

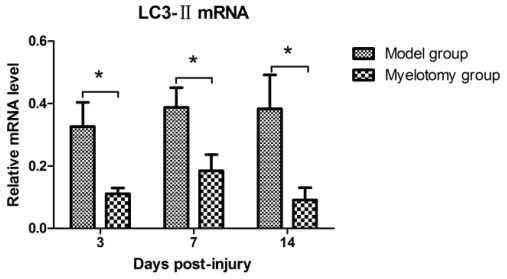

Figure 2. The mRNA expression of LC3-II in injured spinal cord tissues in model group and myelotomy group. n = 5 per group, * $p <$ 0.05 versus the model group.

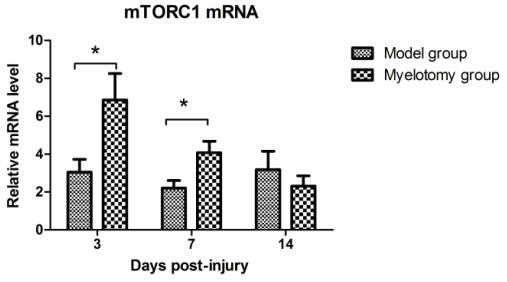

Figure 3. The mRNA expression of mTORC1 in injured spinal cord tissues in model group and myelotomy group. n = 5 per group, * p < 0.05 versus the model group.

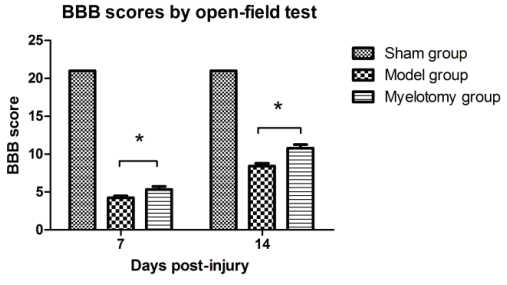

Figure 4. The BBB scores by open-field test in sham group, model group and myelotomy group. * $p < 0.05$ versus the model group.

important reason for this may be that there were no clear theoretical mechanisms available until now. Given the paucity of research regarding the neuroprotective effects of myelotomy, we aimed to determine the molecular mechanism underlying this clinically effective therapeutic strategy. Autophagy plays an important role in various nervous diseases, including neurodegenerative diseases and traumatic and ischemic brain injuries.[14-18] It is known that aberrant activation of autophagy can cause autophagic cell death, an important modality of cell death.[26] Indeed, some studies have shown autophagic cell death was induced by and contributed to neural tissue damage after brain trauma, cerebral ischemia, and SCI.[3,13,27,28] We hypothesized that myelotomy suppresses the aberrant activation of autophagy and reduces neural tissue damage to improve the neurological outcome.

Because the LC3-II protein, the mammalian homologues of the ATG8-PE conjugate, is an essential molecular marker of autophagy induction, many studies investigating autophagy use LC3-II protein as an autophagy marker.[3,24,26,29] In this study, the expression of LC3-II protein was elevated after spinal cord contusion when compared with sham group without contusion. Whether autophagy is protective or detrimental in neural tissues following traumatic brain or spinal cord injury remains controversial. In traumatic brain injury, autophagy is also involved in the induction of cell death. Activation of the Akt/mTOR/p70S6K signaling pathway in the injured spinal cord inhibited the activation of autophagy and produced beneficial effects on SCI-induced motor function defects and repair potential[21], suggesting that autophagy is harmful in SCI. However, autophagy is also cytoprotective in neurodegenerative disease, traumatic brain injury, and neonatal hypoxia- ischemia induced brain injury.[19] Consistent with previous studies, we found that myelotomy improved motor functions in SCI rats.[4-8] Concomitantly, the expression of LC3-II in the MTG was largely decreased, meaning that myelotomy inhibited the activation

Table 2: Linear regression analyses of LC3-II protein expression and BBB

Time after injury	Standardized Coefficients(LC3-II VS BBB)	*p*
7 days	-.752	0.005
14 days	-.742	0.006

LC3-II protein expression at day seven and fourteen post-injury was significantly and negatively correlated with the BBB scores ($p < 0.01$).

of autophagy in SCI. In addition, there was a significant and negative relationship of LC3- II protein expression and BBB scores by linear regression analysis, which further supports the hypothesis that the function of autophagy is detrimental in SCI. Therefore, we speculate that the neuroprotective function of myelotomy is partly due to the inhibition of the activation of autophagy.

Distinct from previous study results[3,13,29], the expression of LC3 II in protein and mRNA levels reached the peak at seven days post-injury. The difference in the time of LC3 expression is probably related to the difference in model design or investigated tissue size. In a spinal cord hemisection model study in mice, the increase of LC3-positive cells observed peaked at three days, and it lasted for at least 21 d after injury.[3] Furthermore, the level of LC3-II protein expression as detected by Western blot was significantly increased in the injured spinal cord at three d after injury.[3] Chen *et al*. found increased LC3-II expression within the epicenter of the injured spinal cord (5 mm length) at two h, four h, and one d, with the peak at two h, which then subsequently declined at later time points until one week after injury.[29] In the Chen study, the five mm length spinal cord sample within the epicenter lesion mainly included necrotic tissue and very little nervous tissue. In our study, the 15 mm length spinal cord segment containing the injury epicenter removed for investigation provided a more accurate reflection of the actual level of LC3 protein expression. Another study showed that LC3 II protein level was elevated three d post-SCI and its increase lasted for at least 28 d after spinal cord contusion in rats.[20] We observed the LC3 expression only within 14 d, so we do not know when myelotomy stopped regulating LC3. Since LC3-positive cells include neurons, astrocytes, and oligodendrocytes in the spinal cord hemisection model, the cell type in which autophagy was activated was not identified in this study.

The mechanisms of myelotomy associated inhibition of autophagy activation in SCI remain unclear. Signaling pathways regulating autophagy include PtdIns3K-Akt-mTORC1, AMPK, p53, Bcl-2 protein family and Beclin-1.[24] The serine/threonine protein kinase mTOR, a downstream signaling molecule of Akt, acts as a central controller of multiple cellular processes, including autophagy, and plays an important role in the pathophysiology of the nervous system.[30] TOR exists in two distinct complexes, TORC1 and TORC2, and they are conserved from yeast to mammals. The primary function of TORC1 is regulating autophagy. Mammalian TORC1 (mTORC1) also is sensitive to rapamycin and integrates upstream activating signals that inhibit autophagy via the class I PtdIns3K-protein kinase B (PKB, also known as Akt) pathway. Many interventions that activate mTORC1 reduced autophagy and promoted the improvement of local motor function after SCI in rats.[21] In our study, the level of mTORC1 mRNA was significantly increased in MTG at three and seven d post-injury, suggesting that the protective function of myelotomy in SCI is mediated by the mTORC1pathway. Here, myelotomy had no effect on mTORC1 mRNA expression by 14 d, indicating that myelotomy regulated mTORC1 expression only at early stages. At later stages in SCI, other mechanisms are probably involved in the regulation of LC3-II. It remains to be determined how myelotomy activated the mTORC1signaling pathway.

In conclusion, myelotomy-mediated neuroprotection in a rat model of SCI was likely mediated by the inhibition of autophagy by activation of mTORC1 signaling pathway.

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DISCLOSURE

Conflict of Interest: None

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