Original Article
Up-regulation of TUBA1B promotes astrocyte proliferation after spinal cord injury in adult rats

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Abstract: α-tubulin1b (TUBA1B) is one of five β Tubulin isoforms, which was high expression in proliferating HCC cells. However, the expression profiles of TUBA1B in spinal cord injury are still unclear. Hence, an acute spinal cord contusion injury (SCI) model in adult rats was established to probe the certain expression and biological function in central nervous system. Western blot analysis and immunohistochemistry both prompted us that TUBA1B expression was increased and got a peak at day 5 after spinal cord injury (SCI). TUBA1B immunoreactivity was found in astrocytes and neurons by double immunofluorescence. But we found the expression of TUBA1B was only increased predominantly in astrocytes. The colocalization of TUBA1B with PCNA was also detected in astrocytes. To further understand the role of TUBA1B in proliferation, the model of astrocyte proliferation induced by LPS was used in this experiment. Being similar to expression in vivo, TUBA1B expression was positively correlated with PCNA following LPS stimulation. Knocking TUBA1B down with siRNA can decrease PCNA expression in proliferation of astrocytes. These results suggest that TUBA1B can regulate proliferation of astrocytes after SCI. To summarize, we first uncover expression changes of TUBA1B in astrocyte after spinal cord injury, suggesting TUBA1B might be implicated in CNS pathophysiology after SCI.

Keywords: Spinal cord injury, α-tubulin1b, astrocyte proliferation, rats

Introduction
Spinal cord injury (SCI) is one of the most devastating of all traumatic events affecting young males, leading to sensation disability and paralysis in the world [1-3]. It is generally recognized that acute spinal cord injury is a two-step process containing primary and secondary injury mechanisms [4, 5]. Primary spinal cord injury is caused by external mechanical force, while secondary injury results from spinal cord edema, ischemia, electrolyte imbalance, free radical damage, ischemia, apoptosis, and inflammation [6-9]. These factors not only extend the range of lesion and aggravate the injury degree, influencing the prognosis of spinal cord severely, it also cause astrocyte hyperproliferative proliferation, resulting in the formation of the dense scar. Moreover, the formation of glial scars which provides a physical and biochemical barrier to regeneration are a source of multiple inhibitory factors affecting axon regeneration [11, 12]. Thus, it is very important for the treatment of SCI to detect the molecular mechanisms of glial cell activation and proliferation.

Tubulin is a major cytoskeleton component with 5 distinct forms designated α-, β-, γ-, δ- and ε-tubulin. α- and β-tubulin heterodimers form the microtubules, involving in cell movement, replication, adhesion, and division [13]. α-tubulin1b (TUBA1B) is one of microtubule proteins, which are in a dynamic process of polymerization and depolymerization during cell replication and division. Microtubules have important functions on the regulation of the mitotic apparatus. The mitotic spindle, polymerized by microtubules in the process of cell division, pulls chromosomes into two daughter
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cells during cell mitosis. Furthermore, microtu-
bule disruption can cause a cell cycle arrest in
G2/M phase and result in the formation of
abnormal mitotic spindles [14]. Hence, whether
the dynamic process of microtubules is broken
will affect cell proliferation, apoptosis and dif-
ferentiation directly [15]. Recently, it has shown
that TUBA1B is upregulated in HCC tumor tis-
ues and proliferating HCC cells, suggesting
TUBA1B may play a vital role in regulating cell
proliferation. However, its dynamic expression
patterns are still unknown in spinal cord injury.

In this study, we first investigated temporal-
spatial patterns of TUBA1B and it can regulate
PCNA expression in proliferative astrocytes in
vivo and vitro. Our results were conducted to
gain greater insight into the functions of
TUBA1B in spinal cord injury and repair.

Materials and methods

Animals and surgery

Experiments were performed on the basis of
National Institutes of Health Guidelines for the
Care and Use of Laboratory Animals; all animal
protocols were approved by the Department of
Animal Center, Medical College of Nantong
University. We used male Sprague-Dawley rats
(n = 64) with about body weight of 250 g (220-
275 g) in this study. Under anesthetized with
Ketamine (90 mg/kg)/xylazine (10 mg/kg),
Dorsal laminotomies at the level of the ninth
thoracic vertebra (T9) were carried out and the
surgery was performed in aseptic conditions.
To minimize postsurgical pain and discomfort,
the Ketoprofen (5 mg/kg) was administered in
rat. Contusion injuries groups (n = 56) were per-
fomed using the NYU impactor [16], the spinal
cord was exposed and further contused by
dropping a rod 2.0 mm in diameter and 10 g in
weight from a height of 75 mm. Sham-operated
animals (n = 8) were also anesthetized and sur-
gically prepared but did not receive spinal inju-
ry. The overlying muscles and skin were closed
in layers with 4-0 silk sutures and staples after
SCI. A 30°C heating pad was used for these
animals to recover. Postoperative treatments
including saline (2.0 ml, s.c.) for rehydration
and Baytril (0.3 ml, 22.7 mg/ml, s.c. once daily)
prevented urinary tract infection. Bladders
were manually expressed twice daily until reflex
bladder emptying returned. Animals were
housed under a 12 h light/dark cycle in a
pathogen-free area with free access to water
and food. Animals were killed at 6, 12 h, 1, 3, 5,
7 and 14 days after injury. Eight sham animals
were used as sham controls. All surgical inter-
ventions and post-operative animal care were
carried out in accordance with the Guide for the
Care and Use of Laboratory Animals (National
Research Council, 1996, USA) and were
approved by the Chinese National Committee
to the Use of Experimental Animals for Medical
Purposes, Jiangsu Branch. All efforts were
made to minimize the number of animals used
and their suffering.

Western blot analysis

The sham or injured spinal cords were excised
to obtain samples for western blot analysis.
The portion of spinal cord extending 5 mm rost-
ral and 5 mm caudal to the injury epicenter
was dissected out and immediately frozen at
-80°C until use. To prepare lysates, frozen spi-
cal cord samples were minced with eye scis-
sors in ice. The samples were then homoge-
nized in lysis buffer (1% NP-40, 50 mmol/L Tris,
pH 7.5, 5 mmol/L EDTA, 1% SDS, 1% sodium
deoxycholate, 1% Triton X-100, 1 mmol/L PMSF,
10 mg/ml aprotinin and 1 mg/ml leupeptin)
and clarified by centrifuging for 20 min in a
microcentrifuge at 4°C. After determination of
its protein concentration with the Bradford
assay (Bio-Rad), the resulting supernatant (50
μg of protein) was subjected to SDS–polyacryl-
amide gel electrophoresis (PAGE). The separat-
ed proteins were transferred to a polyvinylidine
difluoride membrane (Millipore) by a transfer
apparatus at 300 mA for 2 h. The membrane
was then blocked with 5% nonfat milk and incu-
bated with primary antibodies against TUBA1B
(anti-Rabbit, 1:500; Abcam), PCNA (anti-rabbit,
1:1000; Cell Signaling), or GAPDH (anti-Rabbit,
1:1000; Sigma) at 4°C. After incubating with
horseradish peroxidase-conjugated secondary
antibody, protein was visualized using an
enhanced chemiluminescence system (ECL,
Pierce Company, USA).

Immunohistochemistry

After defined survival times, sham and injured
rats were terminally anesthetized and perfused
through the ascending aorta with saline fol-
lowed by 4% paraformaldehyde. After perfu-
sion, the sham and injured spinal cords were
removed and post-fixed in the same fixative

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lowed by 4% paraformaldehyde. After perfu-
sion, the sham and injured spinal cords were
removed and post-fixed in the same fixative
solution for 3 h and cryoprotected with 20% sucrose for 2-3 days, followed by 30% sucrose for 2-3 days. After treatment with sucrose solutions, the tissues were embedded in O.C.T. compound. Then 8 mm frozen cross-sections at two spinal cord levels (2 mm rostral and caudal to the epicenter of injury) were prepared and examined. All of the sections were blocked with 10% donkey serum with 0.3% Triton X-100 and 1% (w/v) bovine serum albumin (BSA) for 2 h at room temperature and incubated overnight at 4°C with TUBA1B (anti-Rabbit, 1:500; Abcam) followed by incubation in biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 2 h. Staining was visualized with DAB (Vector Laboratories) and 0.5% H₂O₂ in 0.05 M Tris-HCl (pH = 7.6). After reactions, the sections were dehydrated, cleared, and coverslipped. Slides were examined with a Leica light microscope (Germany). Cells with strong or moderate brown staining were counted as positive, cells with no staining were counted as negative, while cells with weak staining were scored separately.

Double immunofluorescent staining

Slide-mounted sections were removed from the freezer and kept in an oven at 37°C for 30 min. Sections were blocked with 10% normal serum blocking solution, using normal serum from the same species as the secondary antibody, containing 3% BSA, 0.1% Triton X-100 and 0.05% Tween-20 for 2 h at RT to prevent nonspecific staining. Then the sections were incubated with TUBA1B (anti-Rabbit, 1:100; Abcam), anti-PCNA (anti-rabbit, 1:100; Merck) or anti-PCNA (anti-mouse, 1:100; Santa Cruz) antibodies. The cell-specific markers NeuN (neuronal marker, 1:500; Chemicon) and GFAP (astrocytic marker, 1:200; Sigma) were applied simultaneously. Sections were incubated with both primary antibodies overnight at 4°C, followed by a mixture of CY2- and CY3-conjugated secondary antibodies for 2.5 h at 4°C. The stained sections were examined with a Leica fluorescence microscope (Germany).

**Astrocyte cultures and cell treatment**

Astrocyte cultures were prepared from spinal cords of adult male Sprague-Dawley rats (P65-P70, weighing 220-275 g) using a previously described method with some modifications [17]. The spinal cords were ejected from the vertebral column using a saline-filled syringe. The tissue was chemically dissociated with 0.25% trypsin-EDTA for 10 min followed by mechanical trituration in modified essential medium (DMEM). After centrifugation at 1200 rpm for 5 min, the cells were suspended in DMEM with nutrient mixture F12 (1:1 v/v, Gibco, Grand Island, NY, USA), containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, and plated in a flask coated with poly-L-lysine (Sigma). The cultures were maintained in a humidified atmosphere of 95% O₂/5% CO₂ at 37°C for 10 days, with changes of the culture medium at days 4 and 7. Approximately on days 10 and 11, oligodendrocytes and microglial cells growing on top of the confluent astrocyte layer were removed by shaking at 200 rpm for 2 h at 37°C and replacing the culture medium. The next day, the cells
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were trypsinized and replanted in twelve-well plates (80,000 cells per well). Prior to experimental treatments, cultures of astrocytes were passaged twice. Cell culture medium was switched to serum-free DMEM/F12 culture medium. One group of six-well plates astrocytes were synchronized for 24 h in the absence of serum, and then incubated in the absence of serum and in the presence or absence of LPS with an incubation density of 100 μg/L for 1 h, 3 h, 6 h, 12 h, or 24 h [18]. LPS-induced astrocytes were then harvested for western blot analysis.

siRNA and transfection

For transient transfection, the TUBA1B siRNA vector, and the non-specific vector were carried out using lipofectamine 2,000 (Invitrogen) and plus reagent in OptiMEM (Invitrogen) as suggested by manufacturer. Transfected cells were used for the subsequent experiments 48 h after transfection.

Quantitative analysis

The numbers of TUBA1B -positive cells in the spinal cord 2 mm rostral to the epicenter were counted in a 500 μm × 500 μm measuring frame. For each animal, a measure was taken in a section through the dorsal horn, the lateral funiculus and the ventral horn. To avoid counting the same cell in more than one section, we counted every fifth section (50 μm apart). The cell counts were then used to determine the total number of TUBA1B -positive cells per square millimeter. We simultaneously quantified the percentage of cells with TUBA1B staining. Cells double-labeled for TUBA1B and the cell-specific markers NeuN and GFAP were also quantified. To identify the proportion of neurons and astrocytes expressing TUBA1B, a mini-

Figure 2. The expression of TUBA1B protein in rat spinal cord after injury. Spinal cord tissues from rats at various survival times after SCI were homogenized and subjected to immunoblot analysis. Samples immunoblots probed for TUBA1B and GAPDH are shown above (A). The ratio of TUBA1B to GAPDH for each time point (B). The data are means ± SEM (n = 3, *P<0.05, significantly different from the sham groups).
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maximum of 200 cells expressing a cell-specific marker were counted; GFAP-positive cells were counted in the white matter whereas NeuN-positive cells were counted only in the gray matter. We then recorded the number of cells double-labeled with TUBA1B and a cell-specific marker. Two or three adjacent sections per animal were sampled 2 mm from the epicenter.

Statistical analysis

The statistical methods for western blot depend on gray value, while the measurement for immunohistochemistry is determined by aspects such as positive cell areas, positive cell staining intensity. Both the distribution (the percentage of positive cells) and the intensity of staining in the immunohistochemistry analysis were assessed using the methods described by Shimizu [19]. The following system was used to score the distribution of positive cells: none (not stained) = 0, focal (less than one third of cells stained) = 1, multifocal (less than two thirds of cells stained) = 2, and diffuse (most cells stained) = 3. The intensity of staining was graded as follows: none (not stained) = 0, mild (between 0 and 2) = 1, and strong (clearly identified by ×40 magnification) = 2. The scores for distribution and intensity were added and graded as follows: 0 = (−), 2 = (+), 4 = (+), 6 = (+ ++), 8 = (+ + +). The method of immunofluorescent analysis is similar with the methods described above. All data were analyzed with Stata 7.0 statistical software. All values were expressed as the mean ± SEM. One-way ANOVA followed by the Tukey’s post-hoc multiple comparison tests and un-paired t test for double comparison were used for statistical analysis. P-values less than 0.05 were considered statistically significant. Each experiment consisted of at least three replicates per condition.

Results

Behavioral changes after traumatic spinal cord injury

The spontaneous recovery of locomotor function was tested following spinal cord injury by using the Basso, Beattie and Bresnahan (BBB) rating scale [20]. Scores were recorded and the averages are shown in Figure 1. All animals slacked hind-limb locomotion after suffering from contusion to spinal cord. Some spontaneous improvement in function occurred with time after SCI.

Expression profiles of TUBA1B following spinal cord injury

Western blot analysis was performed to investigate the expression pattern of TUBA1B after SCI. TUBA1B expression was low in sham-operated spinal cords, began to increase at day 1 after spinal cord injury, and got a top expression at day 5 (P<0.05), then returned to normal level gradually (Figure 2A, 2B).

Changes in expression and distribution of TUBA1B in the spinal cord

We performed immunohistochemistry with anti-TUBA1B polyclonal antibody on transverse
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cryosections of spinal cord to study the distribution of TUBA1B after SCI and expression changes in TUBA1B. Since TUBA1B appeared the maximal protein expression at day 5, this time point was chose for our immunohistochemistry work. We found that TUBA1B was expressed in white gray matter (Figure 3A-D), regardless of sham or injury. Notably, TUBA1B was increased significantly in white matter (Figure 3C, 3D), while the positively stained intensity in gray matter did not change obviously (Figure 3C, 3D).

Detection of TUBA1B with different cellular markers in spinal cord after SCI

To further address the expressions of TUBA1B in spinal cord, we performed double immunofluorescent microscopy studies in transverse cryosections of spinal cord tissues within 2 mm distance from the lesion site via co-labeling with NeuN and GFAP. We found TUBA1B expression was in neurons and astrocytes (Figure 4A-L). The expression of TUBA1B was upregulated significantly in astrocytes at day 5 after SCI compared with sham spinal cord (Figure 4G-L), while such change was not detected in neurons (Figure 4A-F).

Cellular proliferation in injured spinal cords

The following studies were performed to certify the relationship between TUBA1B and proliferative cells. As a general marker of dividing cells, Proliferating cell nuclear antigen (PCNA) [21], was examined in injured spinal cord. Its expression was increased gradually and got a peak at 3-5 days and decreased thereafter (Figure 5A, 5B). We performed double labeling immunofluorescent staining of TUBA1B with PCNA and GFAP with PCNA in injured spinal cords (Figure 5C-H). As shown in Figure 5F-H, the majority of activated astrocytes were PCNA-positive. Furthermore, co-localization of PCNA and TUBA1B was observed at day 5 after SCI (Figure 5C-E).

TUBA1B regulates astrocytes proliferation in vitro

Although we have found upregulation of TUBA1B might participate in astrocyte proliferation, the details of regulation are still unknown. To confirm the roles of TUBA1B in astrocyte, LPS was used to induce astrocyte proliferation in vitro. Primary astrocyte cultures were incubated in the absence of serum and in the absence or presence of LPS with an incubation...
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Discussion

Traumatic injury to the spinal cord leads to a big social burden of the whole world. Hence, it is a favorable way to examine the pathophysiology of the trauma and investigate the perplexing molecular and cellular mechanism about the secondary insult by using animal SCI model to recapitulation of the clinical scenario [22, 23]. Astrocyte is one of the main cell type involved in spinal cord injury following central nervous system injury. A critical mechanism is the activation of astrocyte proliferation in the pathophysiology of SCI, characterized by increasing expression of the astrocyte-specific marker GFAP. Although astrocytes secrete the important growth factors for neurons, overactivation of astrocytes can result in the formation of irreversible glial scarring, which presents a physical barrier to regeneration and plasticity [24-26]. Thus, better understanding of the reactive astrocytes and glial scar formation modulation after SCI will be indispensable and helpful in promoting the endogenous repair and regeneration after SCI. The identification of proteins involved in reactive astrocytes regulation in the microenvironment of SCI appears to be an effective therapeutic target.

In our study, we characterized that TUBA1B was upregulated after SCI and got a peak at day 5 by western blot and immunohistochemistry. The changes of TUBA1B were striking remarkably after treating TUBA1B-knock down astrocyte cells with LPS for 12 h (Figure 6D, 6E). These results suggest that TUBA1B can regulate the expression of PCNA and might play an important role in promoting astrocyte proliferation.

Figure 5. Association of TUBA1B with proliferation after SCI. Sample immunoblots probed for PCNA in spinal cord after injury and GAPDH are shown above. PCNA expression was obviously increased at day 5 after SCI (A, B). Double immunofluorescence stained for TUBA1B, PCNA and GFAP in spinal cord after injury (C, H). The majority of reactive astrocytes were PCNA-positive at 5 days after SCI (F-H). Moreover, there was co-localization between PCNA and TUBA1B (C-E). Scale bars: 20 μm (C-H).

Western blots showed a significant increase in TUBA1B and PCNA after LPS stimulation for 12 h (Figure 6A and 6B) (P<0.05). We employed siRNA to knock TUBA1B down in astrocyte to further investigate its function in proliferation. TUBA1B-siRNA, non-specific siRNA, and negative control were tested 48 h post-transfection. As predicted, TUBA1B-siRNA knocked down the protein expression of TUBA1B (Figure 6C). After TUBA1-B silencing, we examine PCNA protein expression by western blot with or without LPS treatment. The expression of PCNA was reduced density of 100 μg/L for 1 h, 3 h, 6 h, 12 h, or 24 h. LPS-induced astrocytes were then harvested for use in western blot analysis. Western blots showed a significant increase in TUBA1B and PCNA after LPS stimulation for 12 h (Figure 6A and 6B) (P<0.05). We employed siRNA to knock TUBA1B down in astrocyte to further investigate its function in proliferation. TUBA1B-siRNA, non-specific siRNA, and negative control were tested 48 h post-transfection. As predicted, TUBA1B-siRNA knocked down the protein expression of TUBA1B (Figure 6C). After TUBA1-B silencing, we examine PCNA protein expression by western blot with or without LPS treatment. The expression of PCNA was reduced remarkably after treating TUBA1B-knock down astrocyte cells with LPS for 12 h (Figure 6D, 6E). These results suggest that TUBA1B can regulate the expression of PCNA and might play an important role in promoting astrocyte proliferation.
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It has been known that TUBA1B (α-tubulin1b) is associated with the formation of microtubules [14], whereas microtubules exert a vital function on regulation of cell proliferation. During scarring after spinal cord injury, the key processes was tightly regulated by microtubule dynamics, including cell proliferation, migration and differentiation as well as intracellular trafficking and secretion of extracellular matrix (ECM) molecules [27, 28]. Moreover, microtubule stabilization reduces fibrotic scarring after spinal cord injury (SCI). Moderating microtubule stabilization can reduce scar formation and enhance the capacity of axons to grow after spinal cord injury by various cellular mechanisms in rodents [29]. Our results showed that spinal injury-evoked TUBA1B was expressed in reactive astrocytes. We found the GFAP-positive astrocytes surrounding the injured spinal cord also expressed PCNA, which indicated astrocytes proliferation. Therefore, we have a hypothesis that TUBA1B might play an important role in process of pathophysiological changes after spinal cord injury, the process being related to astrocytes proliferation. The hypothesis was further confirmed by the cell model of astrocyte proliferation in vitro. Astrocytes are triggered to become reactive and initiate astrogliosis after SCI, which is a critical mechanism in spinal cord injury. Overactivation of astrocytes can result in the formation of glial scarring, acting as regeneration and plasticity barrier for SCI. However, we can prevent the accumulation of chondroitin sulfate proteoglycans (CSPGs) and rendered the lesion site permissive for axon regeneration of growth competent sensory neurons by moderating various cellular mechanisms of microtubule stabilization. Therefore, we further infer that TUBA1B might participate in the process of astrocytes proliferation by manipulating microtubules after spinal cord injury, which may offer a new target for therapy after SCI.

In summary, we firstly report spatiotemporal expression of TUBA1B after spinal cord injury in adult rat. Furthermore, we also found the upregulation of TUBA1B exert an important effect on promoting astrocyte proliferation in vivo and vitro. Our results provided a novel molecular pathway to explore the endogenous responses of CNS after SCI and a novel strategy to the treatment of spinal cord injury. The further study should be performed to confirm the intrinsic mechanisms and functions of TUBA1B in central nervous system injury and repair.

Figure 6. TUBA1B regulates astrocyte proliferation. Western bolt analysis showed the expression of TUBA1B and PCNA was low in naive cell, and then gradually increased and got a peak at 12 h (P<0.05) (A). The ratio of TUBA1B/GAPDH, PCNA/GAPDH for each time point was demonstrated by the bar chart below (B). The data are means ± SEM (n = 3, P<0.05, significantly different from the naive group). Western bolt analysis showed the effect of silencing TUBA1B in astrocyte cells (C). Knocking TUBA1B down with siRNA decreased PCNA levels in primary astrocyte treated by LPS for 12 h (D). These data are means ± SEM. (*P<0.05, significantly different from the un-treated astrocyte cells; #P<0.05, significantly different from the LPS-treated non-specific siRNA transfected astrocyte cells (E).
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Disclosure of conflict of interest

None.

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