Original Article

Temporal and spatial pattern of RhoA expression in injured spinal cord of adult mice

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Abstract: Objective To quantitatively analyze the temporal and spatial pattern of RhoA expression in injured spinal cord of adult mice. Methods A spinal cord transection model was established in adult mice. At 1, 3, 7, 14, 28, 56 and 112 days after the surgery, the spinal cords were dissected and cryosectioned for RhoA/NF200, RhoA/GFAP, RhoA/CNPase or RhoA/IBA1 double fluorescent immunohistochemistry to visualize RhoA expressions in the neurons, astrocytes, oligodendrocytes and microglia. The percentages as well as the immunostaining intensities of RhoA-positive cells in the parenchymal cells were quantitatively analyzed. Results RhoA was weakly expressed in a few neurons and oligodendrocytes in normal spinal cord. After spinal cord injury, the percentage of RhoA-positive cells and RhoA expression intensity in the spinal cord increased and peaked at 7 days post injury (dpi) in neurons, oligodendrocytes and astrocytes, followed by a gradual decrease till reaching a low level at 112 dpi. In the microglia, both the RhoA-positive cells and RhoA expression intensity reached the maximum at 14 dpi and maintained a high level till 112 dpi. Conclusion Traumatic spinal cord injury can upregulate RhoA expression in the neurons as well as all the glial cells in the spinal cord. RhoA expression patterns vary with post-injury time, location and among different parenchymal cells in the injured spinal cord.

Key words: spinal cord injury; neurons; astrocytes; oligodendrocytes; microglia; RhoA expression; temporal and spatial pattern

INTRODUCTION

The neurons in the adult spinal cord possess rather limited capacity of axonal regeneration after spinal cord injury (SCI). As currently no effective treatment has been available, SCI often results in permanent neurological disability [¹]. One of the main obstacles that impede axonal regrowth is the presence of various inhibitors in the microenvironment of the injured spinal cord, such as chondroitin sulfate proteoglycans (CSPGs) from glial scars [²], Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) derived from myelin [³]. Blocking these inhibitors can potentially promote neural regeneration after SCI. However, targeting a single molecule may not be sufficient to achieve a satisfactory therapeutic efficiency [⁴]. Accumulating research data suggest that most of the extracellular inhibitors mediate axonal regeneration via an intracellular small GTP-binding protein RhoA [⁵], which identifies RhoA as a key factor in the pathology and recovery of SCI [⁶,⁷].

RhoA upregulation and activation are known to occur following SCI. However, most of the currently available studies examined RhoA expression only in the acute or subacute stage of SCI, and its expression pattern in the chronic stage remain poorly documented. So far no study has been reported to examine the cellular localization or the expression pattern of RhoA at different time points after SCI. In the present study, we investigated the temporal and spatial pattern of RhoA expression in the injured spinal cord of adult mice from 1 day up to 112 days after SCI. These detailed data may provide clues for future researches of therapies targeting RhoA for promoting spinal cord regeneration.

MATERIALS AND METHODS

Mouse models of SCI

The experiment was carried out in line with the Guidelines for Animal Care and Use of Southern Medical University. Adult wild-type female Kunming mice (48 in total) weighing 25 to 35 g (supplied by the Experimental Animal Center of Southern Medical University) were used in this study. The mice were randomized into 8 equal groups (n=6), including a sham-operated group and 7 SCI groups observed at 1, 3, 7, 14, 28, 56 or 112 days after SCI. To establish SCI models, dorsal laminectomy was performed on the mice to expose the T₁₀ and T₁₁ spinal cord under anesthesia induced by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). A full transection at the T₁₀ cord level was then performed, leaving a 1-mm-long defect in...
the spinal cord. The mice in the sham-operated group received only laminectomy without cord transection. The bladder of the mice was emptied manually twice a day until recovery of urinary reflex in the SCI groups.

Tissue preparation and double fluorescent immunohistochemistry

At the designed time points the mice were perfused transcardially with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) after an overdose of pentobarbital. The spinal cords were harvested and postfixed overnight, followed by cryoprotection in 30% sucrose for 72 h at 4 °C and embedding in optimum cutting temperature (OCT) medium for sectioning. The tissues of two mice from each group were randomly selected to prepare horizontal sections of the spinal cord tissue (1.5 cm in length) flanking the injury site, and the rest mice were used for preparing cross sections of T9 and T10 spinal cord segments. All the sections were processed with a cryostat (Leica) at the thickness of 15 μm. The sections were consecutively mounted onto gelatin-subbed slides and stored at −20 °C for further use.

Every tenth section of the spinal cord was double immunostained with Rhoa/NF200, Rhoa/GFAP, Rhoa/CNPase or Rhoa/IBA1 to detect the cellular Rhoa localization in neurons, astrocytes, oligodendrocytes and microglia. Briefly, the selected sections were washed with PBS (3×5 min) and blocked with 0.1 mol/L PBS containing 5% gelatin and 0.3% Triton X-100 for 1 h at room temperature. The sections were then incubated overnight at 4 °C with the primary antibodies of mouse anti-Rhoa (1:200, Santa Cruz) mixed with rabbit anti-NF200 (1:400; Sigma, marker of neuron), rabbit anti-GFAP (1:1000; Chemicon, marker of astrocyte), rabbit anti-CNPase (1:200; Bioworld, marker of oligodendrocyte), or rabbit anti-IBA1 (1:1000; Wako, marker of microglia) as appropriate. After washing in PBS for three times, the sections were incubated with the mixture of fluorescent Alexa 488-conjugated goat anti-rabbit and Alexa 568 goat anti-mouse secondary antibodies (1:400, Molecular Probes) for 2 h at room temperature. The sections slides were then covered with mounting medium (Dako) containing DAPI for counterstaining of the cell nuclei.

Rhoa quantification in spinal parenchymal cells

The fluorescence images of the immunostained sections were obtained using a fluorescence microscope (Leica). Under a constant exposure condition for all the sections, the red (Alexa 568), green (Alexa 488) and blue (DAPI) images of the same field were merged by DP-manager software. Four squares measuring 100 μm×100 μm were superimposed onto the merged image of each side of the spinal cord with two of them on the grey matter and rest on the white matter. The percentages of Rhoa+ cells in each subtype of parenchymal cells were calculated.

As Rhoa expression level varied in different cells and with time, we measured the relative immunohistochemical (IHC) intensity of the Rhoa+ cells. For each cell type, 5 brightest cells in the grey matter in each section were selected for cell counting using Image-Pro Plus software (version 6.0). The relative IHC intensity was defined as the intensity of a Rhoa+ cell minus the background intensity near the target cell.

Statistical analysis

SPSS software (version 13.0) was used for statistical comparisons by analysis of variance with repeated measures or by Student’s paired t-test for paired observations. A P value less than 0.05 was considered to indicate a statistically significant difference. The data are presented as Mean±SE where applicable.

RESULTS

Rhoa expression is upregulated in injured spinal cord

In the intact spinal cord, only a few neurons or oligodendrocytes showed weak Rhoa immunoreactivity. After SCI, Rhoa expression was upregulated dramatically especially in the area near the defect. One day post injury (dpi), Rhoa was expressed mainly in the area near the lesion covering about one segment from the lesion area on both the rostral and caudal sides. At 3 dpi, Rhoa was expressed in most of the cells in up to 5 segments on both sides. From 7 to 112 dpi, the Rhoa+ cells were confined mainly in the 2 segments near the lesion site. To understand the temporal and spatial pattern of Rhoa expression in different subtypes of spinal parenchymal cells after traumatic injury, we quantitatively analyzed the percentages of Rhoa+ cells as well as the IHC intensity of Rhoa+ cells on the cross sections of T9 and T10 segment. The overall number of Rhoa+ cells and the IHC intensity of Rhoa+ cells were increased at 1 dpi and peaked at 7 dpi, followed by a gradual decline till reaching a quite low level at 112 dpi (Fig.1). Fluorescent double immunostaining identified Rhoa immunoreactivities in the neurons, astrocytes, oligodendrocytes and microglia, but not all the cells were positive (Fig.2).

Rhoa expression in neurons

In the intact spinal cord, Rhoa was expressed at a low level in only a few of the neurons, as shown by Rhoa/NF200 double fluorescent immunostaining. At 1 day after SCI, both of the percentage and intensity of Rhoa+ neurons increased significantly and reached the peak level at 7 dpi. In T9 segment, Rhoa expression kept increasing till 112 dpi, whereas in T10 segment Rhoa expression almost recovered the level in the intact spinal cord at 56 and 112 dpi. Both the percentage of Rhoa+ neurons and Rhoa expression intensity were significantly higher in T10 segment than in T9 segment at each of the time points of measurement (P<0.05) except for Rhoa+ neuron percentage at 1 dpi and Rhoa intensity at 112 dpi (P>0.05, Fig.3).

Rhoa expression in glial cells

The majority of glial cells in intact spinal cord were
immunohistochemically negative for RhoA expression, and only a few of oligodendrocytes showed a weak expression. In the injured spinal cord, RhoA expression was detected in various glial cells. The overall pattern of RhoA expression in the astrocytes and oligodendrocytes was similar with that in the neurons. Both of percentage and intensity of RhoA expression increased after injury and peaked at 7 dpi, and then gradually decreased. The RhoA+ cell percentage showed no significant difference between T9 and T10 segments in either astrocytes or oligodendrocytes, but RhoA expression intensities were significantly higher in T10 than in T9 segment from 1 to 56 dpi ($P<0.05$, Fig.3). Interestingly, the RhoA+ microglia showed a different temporal expression pattern from the other cells in that its percentage reached a high level as early as 1 dpi and peaked at 14 dpi when RhoA expression intensity also reached the maximum. After that both the percentage and intensity of RhoA expression decreased gradually but maintained a significantly higher level than those in other cell types at 112 dpi ($P<0.05$, Fig.3).

**DISCUSSION**

Before assessing the data obtained from this study, we searched for the existing data pertaining to RhoA expression in the spinal cord published in English and Chinese, and found 12 literatures describing RhoA expression in intact and injured spinal cord [7-18]. All these published data support the notion that RhoA expression increases in injured spinal cord, but so far no study has been reported to address the temporal and spatial pattern of RhoA expression. The currently available data did not thoroughly document RhoA protein expression patterns in different parenchymal cells of the spinal cord or provide quantitative descriptions of the level of RhoA expression. Most of the previous studies examined only the mRNA and protein levels of RhoA expression in the acute or subacute stage of SCI, and the status of RhoA expression in the chronic stage remains unclear. Understanding the temporal and spatial patterns of RhoA expression in the injured spinal cord can provide valuable insights into the roles of RhoA in the pathophysiological processes, neurodegeneration and neuroregeneration in different stages of SCI.

The current data regarding RhoA expression in injured spinal cord are not consistent. Yune et al. [13] reported that the total RhoA level did not undergo significant variations after SCI while other researchers [6-12] demonstrated an increment of both mRNA and protein levels of RhoA after SCI. In the present study, we found only weak positive RhoA expression in the spinal cord published in English and Chinese, and found 12 literatures describing RhoA expression in intact and injured spinal cord [7-18]. All these published data support the notion that RhoA expression increases in injured spinal cord, but so far no study has been reported to address the temporal and spatial pattern of RhoA expression. The currently available data did not thoroughly document RhoA protein expression patterns in different parenchymal cells of the spinal cord or provide quantitative descriptions of the level of RhoA expression. Most of the previous studies examined only the mRNA and protein levels of RhoA expression in the acute or subacute stage of SCI, and the status of RhoA expression in the chronic stage remains unclear. Understanding the temporal and spatial patterns of RhoA expression in the injured spinal cord can provide valuable insights into the roles of RhoA in the pathophysiological processes, neurodegeneration and neuroregeneration in different stages of SCI.

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expression in a few neurons and oligodendrocytes in intact spinal cord, but in injured spinal cord, the number of RhoA⁺ cells increased drastically along with significantly increased RhoA expression intensity. The conclusion by Yune et al seems to be questionable as they did not compare the total RhoA level with the internal control proteins (actin for instance) in their study.

RhoA belongs to the Rho family of GTPases, which is a subfamily of the Ras superfamily. The members of Rho GTPase family have been shown to regulate many aspects of intracellular actin dynamics. As the "molecular switches" in all eukaryotic organisms, they play a crucial role in cell proliferation, apoptosis, gene expression, and multiple other common cellular functions. It has been well demonstrated that most axonal regrowth inhibitory molecules within scar tissues and myelin converge to the RhoA pathway. Numerous experiments including our previous work indicated that inhibiting RhoA could promote axonal regeneration, reduce apoptotic neuronal death and improve locomotor function recovery after SCI.

The results from the present study provide additional data to systematically elucidate the temporal and spatial pattern of RhoA expression after SCI. In neurons, oligodendrocytes and astrocytes in the injured spinal cord, the percentages of RhoA⁺ cells increased and RhoA expression intensity peaked at 7 dpi and gradually decreased after that to a very low level at 112 dpi. In the microglia, both the RhoA⁺ cell percentage and RhoA intensity reached the maximum at 14 dpi and maintained a high level at 112 dpi. As the microglia are derived from monocytes but not neural stem cells, and activated microglia perform mainly the function of

Fig.2 Double fluorescence immunohistochemical staining showing cellular RhoA localization after spinal cord injury. NF200 (A), GFAP (B), CNPase (C) and IBA1 (D) were used to identify the neurons, astrocytes, oligodendrocytes and microglia, respectively. A', B', C' and D' show the RhoA immunoreactivities, and A'', B'', C'' and D'' are merged images with DAPI staining of the cell nuclei. Scale bar=50 µm.
scavenging the debris of damaged cells and myelin in the event of SCI\(^{25}\), the delay of the peak and declination of RhoA expression in the microglia (compared to the other cell types) seem to be understandable, even if the exact mechanism still remains unknown.

In conclusion, our results indicate that RhoA up-regulation in the neurons, oligodendrocytes and astrocytes of the injured spinal cord plays crucial roles to obstruct neural regeneration in the first week after SCI. Early interventions following SCI to down-regulate RhoA expression by RNA interference or block RhoA pathway with inhibitors may promote the recovery of the injured spinal cord. Based on our data, the optimal time window for interventions targeting RhoA pathway is the first week after SCI.

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REFERENCES

RhoA

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关键词: 脊髓损伤; 神经元; 星形胶质细胞; 少突胶质细胞; 小胶质细胞; RhoA; 时空分布

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