Expression and role of PAK6 after spinal cord injury in adult rat

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【**Abstract**】**Objective:** To observe p21-activated kinase 6 (PAK6) expression and its possible role after spinal cord injury (SCI) in adult rat.

Methods: Sprague-Dawley rats were subjected to spinal cord injury. To explore the pathological and physiological significance of PAK6, the expression patterns and distribution of PAK6 were observed by Western blot, immunohistochemistry and immunofluorescence.

Results: Western blot analysis showed PAK6 protein level was significantly up-regulated on day 2 and day 4, then reduced and had no up-regulation till day 14. Immunohistochemistry analysis showed that the expression of PAK6 was significantly increased on day 4 compared with the control group. Besides, double immunofluorescence staining showed PAK6 was primarily expressed in the neurons and astrocytes in the control group. While after injury, the expression of PAK6 was increased significantly in the astrocytes and neurons, and the astrocytes were largely proliferated. We also examined the expression of proliferating cell nuclear antigen (PCNA) and found its change was correlated with the expression of PAK6. Importantly, double immunofluorescence staining revealed that cell proliferation evaluated by PCNA appeared in many PAK6-expressing cells on day 4 after injury.

Conclusion: The up-regulation of PAK6 in the injured spinal cord may be associated with glial proliferation.

Key words: ***PAK6 protein, human; p21-activated kinases; Spinal cord injury; Astrocytes***

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The pathology associated with spinal cord injury (SCI) often involves complex cascade of physiology and biochemistry mechanism. It is generally accepted that the overall pathology of SCI often associates with microglial activation and reactive gliosis, resulting in glial scar formation that may impair regeneration, and associated functional disabilities.[1,2] However, the molecular mechanisms of post-traumatic pathology of spinal cord are still poorly understood.

Currently, p21-activated protein kinases (PAKs) have been reported to regulate a wide variety of cellular functions, including regulation of cytoskeletal dynamics, cell survival, apoptosis, gene transcription and promoting cell proliferation.[3-5] Of the PAKs, high levels of PAK6 expression are detected in central nervous system (CNS). These data may suggest an important role for PAK6 in CNS.[6] However, to date its expression and possible roles in SCI are still unknown. The activation of PAK6 by both p38 MAP kinase and mitogen-activated protein kinase 6 (MKK6) suggests that PAK6 is a stress-induced kinase and may play a role in the pathology of SCI.[7]

In this study, we detected the expression and distribution of PAK6 in the spinal cord of rats after SCI. Our research is conducive to gain greater insight into the function of PAK6 and its association with the molecular mechanisms underlying the pathology of SCI.

METHODS

Animals and experimental protocol

Male Sprague-Dawley rats (*n*=48) with an average body weight of 250 g (220-275 g) were used in this study. Dorsal laminectomies at the level of the ninth thoracic vertebra were carried out under anesthesia with pentobarbital (50 mg/kg intraperitoneally). Contusion injuries (*n*=42) were implemented using the New York University (NYU) impactor.[8] The exposed spinal cord was contused by dropping a 10 g weight and 2.0-mm-diameter rod from a height of 10 cm. After SCI, the overlying muscles and skin were closed with 4-0 silk

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sutures and staples respectively, and the animals were allowed to recover on a 30°C heating pad. Postoperative treatments included saline (2.0 ml subcutaneously, twice daily) for rehydration, baytril (0.3 ml, 22.7 mg/ml subcutaneously, twice daily) for prevention of urinary tract infection and buprenorphine (0.05 mg/kg subcutaneously, thrice daily) for postoperative analgesia. Bladders were manually massaged twice daily until reflex bladder emptying returned. SCI animals were sacrificed at 6 h, 12 h, 1 d, 2 d, 4 d, 7 d and 14 d after injury. Six control animals were used as noninjured controls. All surgical interventions and postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals 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 extraction of samples for Western blot analysis was processed according to Shen et al's method.[9] The membrane was then blocked with 5% nonfat milk and incubated with primary antibody against PAK6 (anti-rabbit, 1:500; Santa Cruz, USA), proliferating cell nuclear antigen (PCNA, anti-mouse, 1:1000; Santa Cruz, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, anti-rabbit, 1:1000; Santa Cruz, USA). After incubating with an anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody, protein was visualized using an enhanced chemiluminescence system enhanced chemiluminescence (ECL, Pierce Company, USA).

Immunohistochemistry

After defined survival time, control and injured rats were terminally anesthetized and perfused through the ascending aorta with saline, followed by 4% paraformaldehyde. After perfusion, the control and injured spinal cords were removed and post-fixed in the same fixative for 3 h and then replaced with 20% sucrose for 2-3 d, followed by 30% sucrose for 2-3 d. After treatment with sucrose solutions, the tissues were embedded in OTC compound. Then, 10 μm frozen cross-sections at two spinal cord levels (3 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 anti-PAK6 antibody (anti-rabbit, 1:200; Santa Cruz, USA), followed by incubation in biotinylated secondary antibody (Vector Laboratories, Canada). Staining was visualized with DAB (Vector Laboratories, Canada). Cells with strong or moderate brown staining were counted as positive, cells with no staining were counted as negative, and cells with weak staining were scored separately. For double immunofluorescent staining, sections were firstly blocked with 10% normal serum blocking solution, containing 3% BSA and 0.1% Triton X-100 and 0.05% Tween-20 for 2 h at room temperature in order to avoid unspecific staining. Then the sections were incubated with both rabbit polyclonal primary antibodies for PAK6 (1:200; Santa Cruz, USA) and mouse monoclonal antibody for PCNA (1:100; Santa Cruz, USA) or different markers as follows: neuronal specific nuclear protein (NeuN, neuron marker, 1:600; Merck Millipore, Germany), glial fibrillary acidic protein (GFAP, astrocyte marker, 1:200; Sigma, USA). Briefly, sections were incubated with both primary antibodies overnight at 4°C, followed by a mixture of Cy3- and Cy2-conjugated secondary antibodies for 2 h at 4°C. The stained sections were examined with a Leica fluorescence microscope (Leica, Germany).

Quantitative analysis

The number of PAK6-positive cells in the spinal cord 3 mm caudal to the epicenter was 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. The cell counts in the three or four adjacent sections were then used to determine the total number of PAK6-positive cells per squared millimeter. Cells double-labeled for PAK6 and the other phenotypic markers used in the experiment were quantified. Sections were double-labeled for PAK6 and NeuN, or PAK6 and GFAP. To identify the proportion of each phenotype-specific marker-positive cells expressing PAK6, a minimum of 200 phenotype-specific marker-positive cells were counted in both the gray matter and white matter of each section, with the exception that only gray matter was conducted for NeuN. Then double-labeled cells for PAK6 and phenotype-specific markers were recorded. Two or three adjacent sections per animal were sampled 3 mm to the epicenter.

Statistical analysis

All data were analyzed with Stata 7.0 statistical