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Original Article

Aggravation of spinal cord injury by CCL5 via activating NK-κB signaling pathway

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Abstract: The prognosis of spinal cord injury (SCI) is still unfavorable even under rapid progression of medicine. Due to the loss of regeneration potency of mature neurons, lots of SCI patients suffer from severe motor and/or sensory dysfunctions. Chemokines are closely correlated with SCI as inflammation related factors. This study thus investigated the role and mechanism of chemokines in SCI. A total of 30 SD rats were prepared for SCI model, in parallel with 30 SD rats recruiting sham control group. The successful generation of SCI model was examined by motor dysfunction scale. Microarray assay screened out those cytokines with significant changes between groups, followed by qPCR confirmation. Primary neurons from both groups were treated with recombinant CCL5 protein. Pathway Finder was used to screen out those signal pathways with significant change, followed by confirmation by immunofluorescence assay. The generation of SCI model was evaluated by motor dysfunction scale, which showed significantly lowered motor score in model group compared to sham group, suggesting successful generation of SCI model. Both microarray chip and qPCR reveled over-expression of CCL5 in SCI tissues (P=0.025). Primary culture of neurons treated with CCL-5 revealed activation of NK-κB and nuclear translocation of pP65 by immunofluorescence assay, but not in DMSO control group. CCl5 is over-expressed after SCI, and may aggravate SCI via activating NK-κB signal pathway.

Keywords: CCL5, spinal cord injury, NK-κB

Introduction

The incidence of acute spinal cord injury (SCI) is increasing by years after traffic accidents or unexpected falling [1-3]. Due to the weak regeneration potency of central nervous cells and ganglion cells, slow repair and recovery process is needed after injury, or even causing irreversible change [4, 5]. Therefore, severe SCI leads to severe consequence or even life-long paralysis of patients. Other complications such as dysfunction of sensory, atrophy of skin or muscles may occur after SCI, affecting patients' life quality [6-8]. Early study has shown significant elevation of TNF-α and IL-1β after SCI. The occurrence of those cytokines also caused secondary injury of spinal cord [9, 10]. CCL5 is one secretory small molecule inflammatory cytokine, and is one inflammatory chemokine after T cell activation, and can induce a series of inflammatory response and secondary diseases via inducing leukocytes migration, with receptors including CCD1, CCR3 and CCR5 [11-13]. Study has found that low-expression of CCL5 could alleviate tissues injury and prevent disease recurrence [14]. Currently lots of studies have indicated the correlation between CCL5 and SCI, with its detailed mechanism unknown. NK-κB signal pathway can regulate multiple gene expression inside cells, thus regulating various biological activities including proliferation, differentiation, cell growth and apoptosis, inflammation and immune response [15, 16]. As one nuclear transcription factor, NK-κB plays crucial roles in the regulation of various genes especially for those related with inflammation and immune response [17]. As NK-κB signal pathway is closely related with inflammatory response, this study thus aimed to investigate the role and mechanism of CCL5 in SCI, in an attempt to provide evidences for clinical treatment.

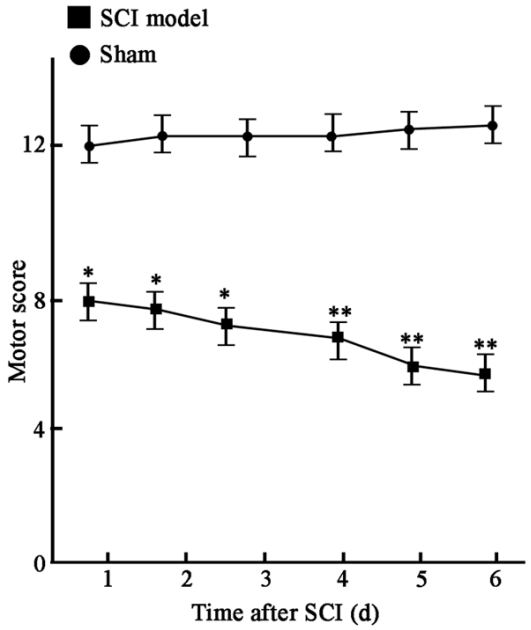

Figure 1. Establishment of SCI model. In model group, the motor score was significantly lowered than sham group. *, P<0.05, **, P<0.01 compared to sham group.

Materials and methods

SCI model and reagents

Rats were purchased from Shanghai Cell Biology Institute, Chinese Academy of Sciences. A total of 60 SD rats (30 males and 30 females) were randomly divided into SCI model group (group A) and sham group (group B). Rats were anesthetized and were exposed for 9 mm diameter region around T10 spine. The spinal cord was clipped for 3 sec to generate SCI. The successful generation Successful SCI model was deduced as the occurrence of spastic tail movement, retraction of hind limb and trunk, and paralysis of forelimbs as previously recorded [18]. Rats in group B received all treatment but not spinal cord clipping. Other chemical reagents were purchased locally.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Bethune International Peace Hospital of PLA.

Motor dyskinesia grading

The bladder was emptied before testing. After 5-10 min acclimation, rats were allowed to move freely in the test field. Those with normal body movement in stable had 9 points. Dysfunctions in the position of hind limb during movement, or dis-coordination between forelimbs and hind limbs were deduced as 5-8 points. Inability for hind limbs to stretch the trunk or forward movement was interpreted as 3-4 points. Leaving only ankle movement made the total score between 0 and 2 points.

Real-time qPCR

Reverse transcription kit was purchased from TaKaRa (Japan). Equal volume of tissues were mixed with aliquot Trizol, and were centrifuged at 12,000 rpm at 4°C for 10 min. Supernatants were saved to incubated for 5 min until complete lysis. 0.2 mL chloroform was added for 15 sec vortex, followed by 3 min incubation and 12,000 rpm centrifugation at 4°C for 15 min. The upper phase was saved to mix with equal volume of isopropanol, and was incubated at room temperature for 10 min to precipitate. The mixture was centrifuged at 12,000 rpm for 10 min under 4°C. Supernatants were discarded with adding 75% ethanol (in DPEC water). After mixture, 7,500 rpm centrifugation at 4°C was performed for 5 min. Supernatants were discarded to dry RNA pellet, which was re-suspended in 20 μL DEPC water. Nuclei acid dye was used to detect the expression level of CCL5 in spinal cord tissues, using primers: CCL5-F, 5'-GCGAG CTACA TTGTC TGCTG GGTT-3'; CCL5-R, 5'-GTCGA GGGTC CGAGG TATTC CG-3'; U6-F, 5'-CGGCG GTAGC TTATC AGACT GATG-3'; U6-R, 5'-CCAGT CGAGG GTCCG AGGTA TT-3'.

HE staining

Tissue samples were fixed in formalin overnight, and were dehydrated in 70%, 80%, 95% and 100% ethanol (3 h, 3 h, 2 h, and 1.5 h × 2 times). Xylene was used to treat tissues (1.5 h × 2 times), followed by paraffin immersion at 60°C (1 h + 2 h). Paraffin blocks were sectioned into 3 μm slices, which were de-waxed using routine methods (xylene, absolute ethanol, 95%, 90%, 85% and 80% ethanol). Tissues slides were then stained in hematoxylin for 1 min, and were rinsed in tap water. Eosin was then added to stain tissues for 10 sec, followed by washing under tap water. The slice was dried and mounted with coverslips. Under the microscope, 20 × filed in the middle was captured. Three independent pathologists performed the diagnosis of tissue injury.

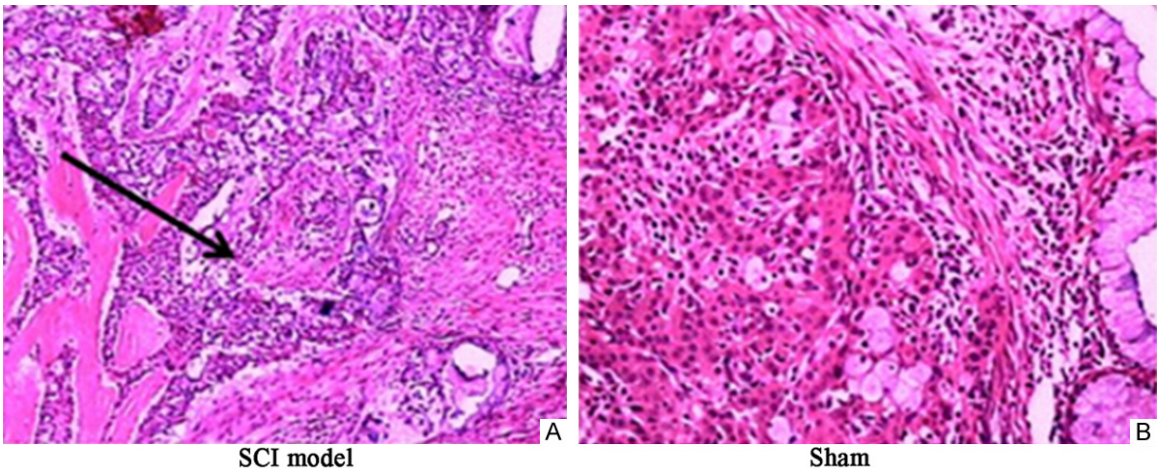

Figure 2. A. SCI model spinal cord tissues had severely damaged neurons with nuclear atrophy (arrows). B. Minor injury of neurons in sham group with barely any change of nucleus.

miRNA hybridization

Using small molecule extraction kit (Ambion, US) to purify microRNA, which was labelled by Cy3. Further hybridization assay was performed on miRNA microarray. Pathfinder chip was purchased from Qiagen (US).

Neuron cell extraction

After anesthetization, dura and white matter were all removed from the whole brain. Cerebral cortex was collected in HBSS-2 buffer for homogenization. Debris was removed into HBSS-2 buffer containing 0.025% trypsin at 37°C for 15 min. After digestion, cells were rinsed twice in HBSS-2 buffer containing 10% fetal bovine serum (FBS). Neural basic culture medium (replenishing with 0.5 mM L-glutamine, 25 μM L-glutamate, 2% B27 and 0.12 mg/mL gentamicin) was used to re-suspend cells, which were seeded into polylysine-coated culture dish at 1×10^5 cell/cm^2 density. Cells were cultured in a humidified chamber at 37°C with 5% CO_2. Culture medium was changed every 3 days. 8 days later cells can be used in further assays.

Immunofluorescence assay

After fixation, tissues were dehydrated in gradient sucrose, and were sectioned into 6 μm slices. Antigen retrieval was performed by 5-min heating. After cooling down, tissue slices were rinsed in PBS (5 min × 3 changes) and were blocked in 10% BSA for 50 min. DAPI dye (1:100, Cell signaling technology, US) was added for staining, followed by observation under a fluorescent microscope.

Statistical analysis

SPSS 11.0 software was used to process all data. Student t-test was employed to compare means, in addition to Pearson correlation analysis, and chi-square test. A statistical significance was defined when $P<0.05$. *, ** and *** represented $P<0.05$, $P<0.01$ and $P<0.001$. Each experiment was carried out in at least triplicates. The number of positive cells in IHC was calculated by ImageJ software.

Results

Successful establishment of SCI model

A total of 60 SD rats were randomly divided into SCI model group (group A, N=30) and sham group (group B, N=30). Using motor dyskinesia grading system to detect the phenotype of SCI model in both groups, we found significantly weakened motor behavior in SCI model group but not in sham control group (**Figure 1**).

Neuron morphology in SCI rats

After successful generation of SCI model, we extracted spinal cord tissues from both groups and examined tissue morphology using HE staining. Results showed significantly server tissue injury of neurons in SCI model group (**Figure 2A**). Sham group, however, had minor injury of neurons (**Figure 2B**).

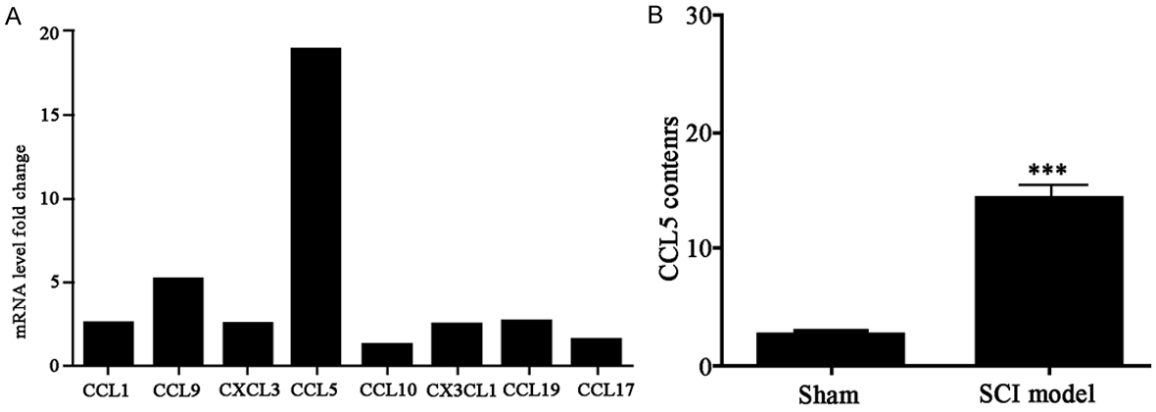

Figure 3. Elevated CCL5 in SCI tissues. A. Pathfinder screening showed significantly elevated CCL5 in SCI model rats. B. Real-time qPCR confirmed higher CCL5 contents in SCI model group. ***, P<0.001 compared to sham group.

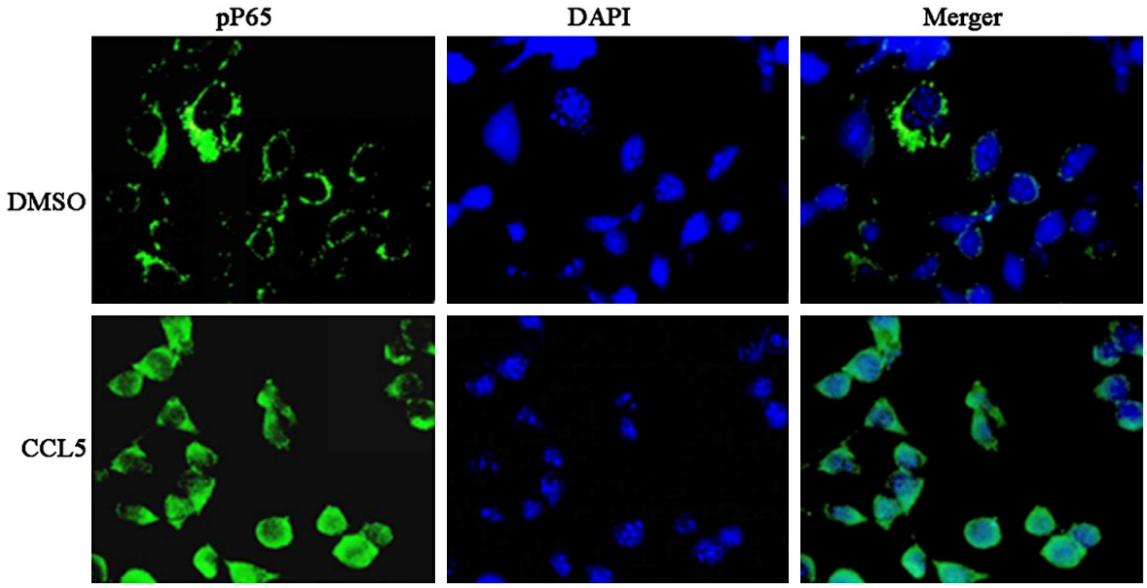

Figure 4. CCL5 activated NK-κB signal. After treating sham neurons with CCL5, nuclear translocation of pP65 was significantly elevated, indicating activation of NK-κB signal. With the addition of DMSO, barely any nuclear translocation was observed, suggesting inactivation of NK-κB signal.

Elevated CCL5 expression in SCI group

There were certain alternations of neurons in spinal cord tissues. After *in vitro* culture of primary neurons from both groups, and extraction of RNA in Pathfinder screening, results showed significantly elevation of CCL5 contents in SCI group (**Figure 3A**). Further real-time qPCR assay confirmed elevation of CCL5 level compared to sham group (P<0.001, **Figure 3B**).

CCL5 activated NK-κB signal

Using primary cultured neurons, we tested the change of NK-κB signals after replenishing recombinant CCL5 protein in sham group, in parallel with DMSO control group. Immunofluorescent staining showed significantly nuclear translocation of pP65 after treating with CCL5, indicating the activation of NK-κB signals. Such phenomena, however, did not occur in DMSO group (**Figure 4**).

Discussion

Spinal cord is one important component of central nervous system in vertebrates. It exerts reflexes, transduction, motor and regulatory functions, and thus plays a crucial role in normal body activity. Acute SCI can cause paraly-

sis of motor system, sensory disorder, limb numbness and other symptoms, severely affecting normal physiological activity [19, 20]. The dysfunction of micro-circulation after SCI cause aggravation of tissues edema; inflammation can also aggravate SCI, further leading to tissues ischemia and edema, forming a feedback loop for eventually irreversible denature and necrosis of spinal cord nerve tissues. In this process, over-inflammation induces higher vascular permeability, leukocytes recruitment and inflammatory cytokine release are key events. Therefore, the avoidance of aggravation of inflammation may help to decrease reinjury of spinal cord tissues [21, 22].

In this study we mimicked SCI in rat models. Gene microarray screening found elevated expression of chemokine CCL5 in spinal cord tissues with injury. qPCR assay confirmed such over-expression of CCL5 in SCI. As one small secretory inflammation related cytokine with small molecular weight, CCL5 can attract leukocytes toward disease site, thus playing an important role in inflammation. The over-expression of CCL5 suggested the wide-spread of inflammation, which further aggravates SCI. We then explored how CCL5 could aggravate SCI in molecular aspects. By treating primary cultured neurons with recombinant CCL5 proteins, Pathway Finder screening found significant change of NK-κB signal pathway. Further immunofluorescent assay detected nuclear translocation of pP65, suggesting that CCL5 might aggravate SCI via activating NK-κB. The pathway of NK-κB has been widely studies. pP65 protein is the key point for activation of NK-κB signal pathway as one nuclear localization signal for cytoplasm-nuclear translocation. NK-κB pathway can regulate hundreds of target genes' transcription, and exerts a wide array of biological effects such as cell division, proliferation and apoptosis, as well as regulation on inflammation or immune response. As one critical transcriptional factor, NK-κB also participates in various physiological or pathological processes in central nervous system, including synaptic plasticity, inflammation, synaptic transmission and pain. The up-regulation of inflammatory factors can also activate other inflammatory cytokines, forming a positive feedback loop, resulting in wide spread of inflammation [23-25]. After SCI, the effective management of inflammatory cytokine release can relieve inflammation response, and may impede further injury of spinal cord tissues in improve patient prognosis.

In summary, CCL5 may aggravate SCI via activating NK-κB signal pathway. Our study provides new insights for clinical treatment of SCI.

Disclosure of conflict of interest

None.

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