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Granulocyte colony-stimulating factor attenuates spinal cord injury-induced mechanical allodynia in adult rats

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ABSTRACT

Spinal cord injury (SCI) can cause neuropathic pain (NeP), often reducing a patient's quality of life. We recently reported that granulocyte colony-stimulating factor (G-CSF) could attenuate NeP in several SCI patients. However, the mechanism of action underlying G-CSF-mediated attenuation of SCI-NeP remains to be elucidated. The purpose of the present study was to elucidate the therapeutic effect and mechanism of action of granulocyte colony-stimulating factor for SCI-induced NeP.

T9 level contusive SCI was introduced to adult male Sprague Dawley rats. Three weeks after injury, rats received intraperitoneal recombinant human G-CSF (15.0 μg/kg) for 5 days. Mechanical allodynia was assessed using von Frey filaments. Immunohistochemistry and western blot analysis were performed in spinal cord lumbar enlargement samples.

Testing with von Frey filaments showed significant increase in the paw withdrawal threshold in the G-CSF group compared with the vehicle group 4 weeks, 5 weeks, 6 weeks and 7 weeks after injury. Immunohistochemistry for CD11b (clone OX-42) revealed that the number of OX-42-positive activated microglia was significantly smaller in the G-CSF group than that in the vehicle rats. Western blot analysis indicated that phosphorylated-p38 mitogen-activated protein kinase (p38MAPK) and interleukin-1β expression in spinal cord lumbar enlargement were attenuated in the G-CSF-treated rats compared with that in the vehicle-treated rats.

The present results demonstrate a therapeutic effect of G-CSF treatment for SCI-induced NeP, possibly through the inhibition of microglial activation and the suppression of p38MAPK phosphorylation and the upregulation of interleukin-1β.

1. Introduction

Neuropathic pain (NeP) occurs as a result of alterations in neuronal activity in susceptible individuals produced by damage to the central or peripheral nervous system. Symptoms of NeP frequently include uncomfori, spontaneous sensations, dysesthesia, and exaggerated perception of non-noxious (allodynia) or noxious (hyperalgesia or hyperpathia) stimuli. Both abnormal sensations are described by patients as unrelenting, burning, biting, tingling, shooting and shock-like sensations.

Currently, the precise pathomechanism of NeP remains unclear. Extensive laboratory and clinical exploration to establish novel therapeutics for NeP is ongoing worldwide [1].

Spinal cord injury (SCI) is one of the major causes of central NeP. It is reported that the prevalence of NeP after SCI is 40% or more [2,3]. In SCI patients, NeP is one of the main causes of a patient's poor quality of life (QOL) [4].

Granulocyte colony-stimulating factor (G-CSF) is a 19.6 kDa glycoprotein initially identified as a serum factor that induces differentiation of a murine myelomonocytic leukemic cell line [5]. It is widely known as a hematopoietic cytokine that promotes survival, proliferation, and differentiation of cells of neutrophilic lineage [6]. G-CSF is used clinically for patients with leukocytopenia to increase white blood cell number and for donors of peripheral blood-derived hematopoietic progenitor cells prior to their collection for transplantation to mobilize bone marrow-derived hematopoietic progenitor cells into the peripheral blood [6]. Recently, nonhematopoietic effects of G-CSF have been reported, including effects on the central nervous system. G-CSF was found to protect neurons from ischemia-induced cell death and to promote neurogenesis in a rat model of brain ischemia [7,8]. Recently

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we reported that G-CSF protects neurons and oligodendrocytes from apoptosis in mouse and rat SCI models [9,10]. Moreover, it was reported that G-CSF attenuates peripheral NeP [11,12]. We recently conducted early-phase clinical trials to prove neuroprotective effects of G-CSF for SCI and acute aggravation of compressive myelopathy [13,14]. In the trial for compressive myelopathy, NeP was attenuated after G-CSF administration in 14/17 patients for several months. The ratio of pain reduction was 30–50% in average at 1 month after G-CSF treatment [15]. Therefore, we hypothesized that G-CSF can potentially attenuate neuropathic pain. However, the mechanism of action underlying G-CSF-mediated attenuation of SCI-NeP remains to be elucidated.

In this study, we administered G-CSF after induction of NeP following SCI to elucidate the mechanism of G-CSF treatment for SCI-NeP.

2. Materials and methods

2.1. Animals

All animals were treated and cared for in accordance with the Chiba University School of Medicine guidelines pertaining to the treatment of experimental animals. The study was approved by the Animal Care and Use Committee of Chiba University Graduate School of Medicine (approval number 2572). We used a total of 46 (22 for locomotor and von Frey assessments, 12 for immunohistochemistry and 12 for western blot analysis) adult male Sprague Dawley rats (10 to 12 weeks old; 200 to 240 g; Japan SLC, Hamamatsu, Japan), which were housed in individual cages and allowed free access to food and water. Rats were anesthetized with 1.5% halothane inhaled with 0.5 L/min oxygen. The T9 spinal cord was exposed by a T8–T9 laminectomy, leaving the dura intact. Contusive SCI was introduced using a New York University Impactor (10 g weight was dropped from 6.25 mm height). Upon awakening, rats were evaluated neurologically and were monitored for food and water intake, and urine output. Manual bladder expression was performed twice a day until the rats regained their bladder reflex (usually one week after SCI).

2.2. Locomotor assessment

The recovery of rat hind limb function in either group ($n = 4$ in sham-operated group, $n = 10$ in the G-CSF group and $n = 8$ in the vehicle group) was determined by measuring the hind limb motor function score with the Basso, Beattie and Bresnahan locomotor scale (BBB scale [16]). Rats were allowed to move freely on an open field with a rough surface for 5 min at each time tested. The hind limb movement of rats was videotaped and scored by two independent observers who were unaware of the treatment. If there were differences in the scores between observers, score was determined by discussion between observers. Measurement of motor function was performed weekly for six weeks after surgery.

2.3. G-CSF treatment and assessment for mechanical allodynia

Three weeks after SCI, 40% of rats showed mechanical allodynia as revealed by hypersensitivity to von Frey filament stimulation. Rats exhibiting no mechanical allodynia were euthanized and excluded from further experiments. The rats showing mechanical allodynia were randomized to one of two groups. Those in the G-CSF group received intraperitoneal recombinant human G-CSF dissolved in normal saline (15.0 μg/kg; Kyowa Kirin Pharma, Tokyo, Japan) for 5 consecutive days. Rats in the vehicle group received an equivalent volume of normal saline at the same time points. We followed the drug-administration regimen described in our previous report of our rat model of SCI [10]. On the day following the final administration of G-CSF, peripheral blood samples were collected for leukocyte counts. Blood leukocyte counts for rats in the vehicle and G-CSF groups were $3800 \pm 500/mm^3$ and $9700 \pm 700/mm^3$ respectively. Mechanical allodynia in rats from the vehicle and G-CSF groups was assessed using von Frey filaments, according to a previously described protocol [17] by blinded observer who did not know the experimental groups. In brief, von Frey filaments were applied to the central region of the plantar surface of a hind paw in ascending order of force (0.7, 1.2, 1.5, 2.0, 3.6, 5.5, 8.5, 11.7, 15.1, and 29 g). Each filament was applied 5 times. When the rats showed 1 withdrawal response to a given filament, the bending force of that filament was defined as the paw withdrawal threshold intensity. The median threshold intensity was calculated from the values following 1 descending and 2 ascending trials. The experimental conditions were identical for both groups of rats. Behavioral testing commenced one day after the operations and continued for 7 consecutive weeks.

2.4. Sample preparation

Tissues from a subset of rats ($n = 4$/group) were prepared for histological evaluation 3 weeks plus 6 days after surgery (the next day of last G-CSF administration). Animals were euthanized by pentobarbital overdose and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). Tissue blocks of the spinal lumbar enlargement were removed, postfixed overnight in 4% paraformaldehyde, stored for two days at 4 °C in 20% sucrose in PBS, and then embedded in Tissue-Tek O.C.T. Compound (optimum cutting temperature formulation; SAKURA Finetechnical Co., Ltd. Tokyo, Japan). The cryoprotected samples were frozen and stored at −80 °C until use. The samples were cut into serial 20 μm transverse sections.

2.5. Immunohistochemistry

For immunofluorescent labeling, sections were permeated with 0.3% Triton X-100 in PBS and treated for 1 h in blocking solution containing 1% bovine serum albumin and Block Ace (Dainippon Pharma, Japan). Sections were then incubated with the following primary antibodies: mouse monoclonal anti-glial fibrillary acidic protein antibody (GFAP, 1:400, Sigma, St Louis, MO) for astrocytes; or anti-CD11b mouse monoclonal antibody (clone OX-42, 1: 400, Serotec AbD, Kidlington, UK) for activated microglia. The sections were incubated with primary antibodies overnight at 4 °C, after which they were washed in PBS and then incubated for 1 h at room temperature with secondary antibodies: Alexa 488-labeled anti-rabbit or anti-mouse IgG (1:800, Invitrogen, Eugene, OR). Finally, the sections were washed twice in PBS and protected with coverslips. Positive labeling was observed using fluorescence microscopy (Eclipse E600; Nikon, Tokyo, Japan). To determine the specificity of staining, procedures were performed on control sections with the omission of primary or secondary antibodies. Positive immunofluorescent signals were counted for every fifth 20-μm transverse section (i.e., at intervals of 100 μm) from the spinal lumbar enlargement using Scion Image computer analysis software (version beta 4.0.3, Scion Corporation, Frederick, MA). At least ten sections from each animal were counted, covering a 1-mm length of spinal cord.

2.6. Western blotting

Three weeks plus 6 days after SCI (the day following the last administration of G-CSF), 10-mm sections of the spinal lumbar enlargement were removed from rats in both the vehicle and G-CSF-treated groups ($n = 4$/group). The tissues were homogenized in homogenization buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl and 1% Triton X-100) containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The homogenates were centrifuged at 100,000 *g* for 10 min at 4 °C to remove cellular debris. Protein concentrations of the supernatants were measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA) and were adjusted to 1 mg/mL by dilution with homogenization buffer. Protein samples were mixed with an equal volume of concentrated (2×) sample buffer: 250 mM Tris–HCl, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.02% bromophenol