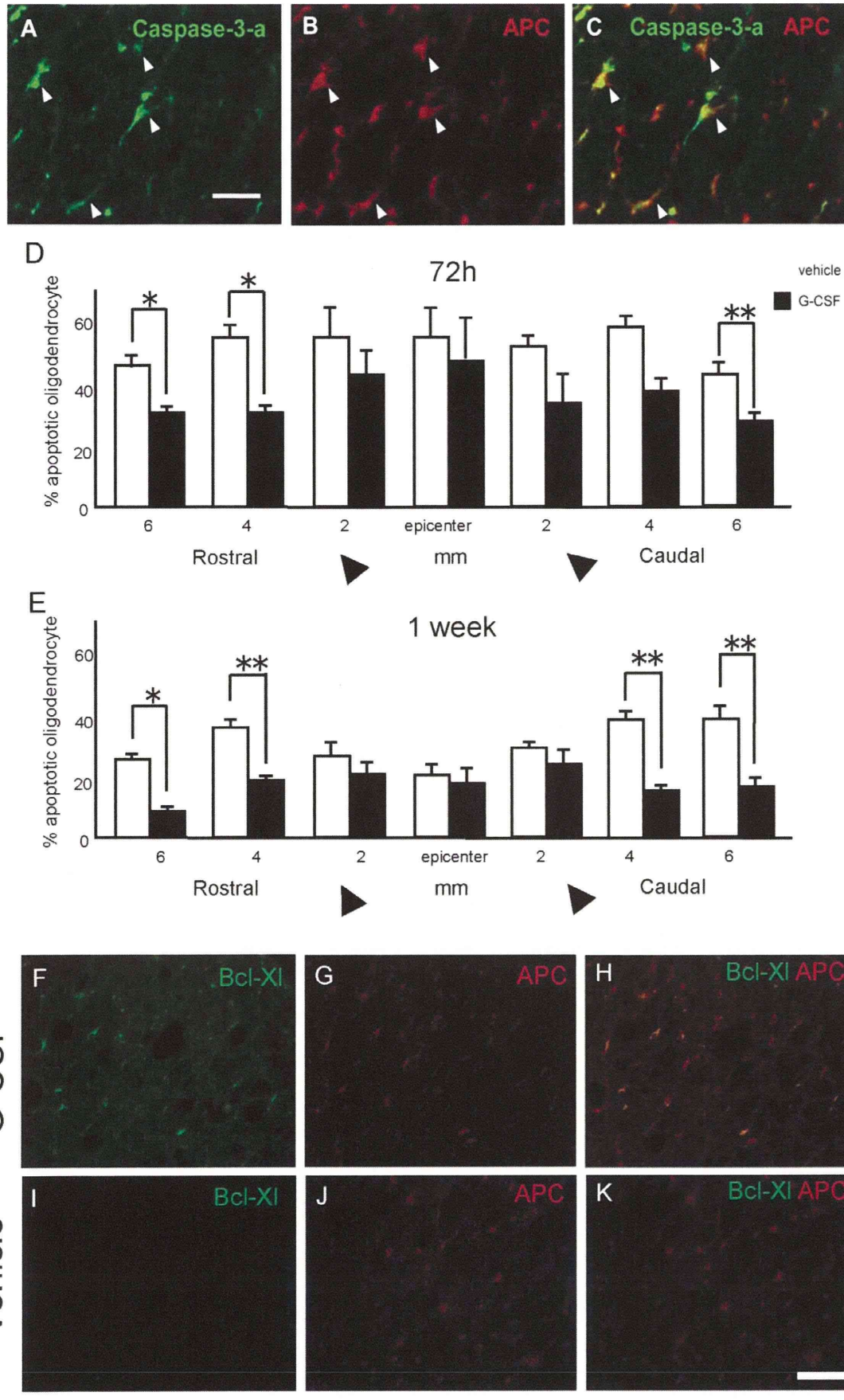
A
Caspase-3-a
B
APC
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Caspase-3-a APC
D
72h
vehicle
G-CSF
% apoptotic oligodendrocyte
0
20
40
60
*
*
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6
4
2
epicenter
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Rostral
mm
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1 week
% apoptotic oligodendrocyte
0
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*
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6
4
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epicenter
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mm
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G-CSF
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Bcl-Xl
G
APC
H
Bcl-Xl APC
Vehicle
I
Bcl-Xl
J
APC
K
Bcl-Xl APC

Figure 4. G-CSF suppressed apoptosis of oligodendrocytes ***and promoted anti-apoptotic protein Bcl-Xl on oligodendrocytes.*** Immunohistochemistry for adenomatous polyposis coli (APC; a marker for oligodendrocytes) and activated caspase 3 (a marker for apoptotic cells) in the acute phase of spinal cord injury. Representative figure of cells double-positive for APC and activated caspase 3 in the vehicle group 4 mm rostral to the lesion epicenter 1 week after injury is shown (A–C, arrowheads). The percentage of apoptotic oligoendrocytes was significantly smaller in the G-CSF group (closed column) than that in the vehicle group (open column) 72 hours (D) and one week (E) after surgery. *In vehice-treated control rats, Bcl-Xl/APC doubler-positive cells were not detected (I–K). In contrast, Bcl-Xl/APC doubler-positive Bcl-Xl expressing oligodendrocytes were observed in the spinal cord of G-CSF-treated rats (F–H).* Bars = 50 μm (A–C) and 100 μm (F–K). Values are mean±SEM. *p<0.05, **p<0.01.
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number of activated microglia/macrophages. Microglia/macrophages can involve inflammatory reaction in injured spinal cord. Therefore this data might suggest the other possible anti-inflammatory action of G-CSF.

G-CSF has direct neuroprotective effects against glutamate-induced neuronal death and stroke [11–15,28]. G-CSF elicits its anti-apoptotic effects on neurons via activation of proteins of the STAT family or the PI3-K/Akt pathway, similar to anti-apoptotic effects on neutrophils [13]. In this study of spinal cord injury in the rat, G-CSFR was expressed in neurons, astrocytes and oligodendrocytes, but not in microglia. SCI promoted up-regulation of G-CSFR expression in astrocytes and oligodendrocytes. Moreover, G-CSF treatment did not alter the expression pattern of G-CSFR. Thus, the reduction of oligodendrocyte apoptosis by G-CSF was possibly achieved by several mechanisms as followings. Firstly, G-CSF-mediated suppression of inflammatory cytokine expression might attenuate apoptosis of oligodendrocytes because these inflammatory cytokines are potentially toxic for oligodendrocytes [29,30]. Secondly, G-CSF-mediated up-regulation of anti-apoptotic proteins in oligodendrocytes might suppress apoptosis. The present results of immunofluorescence double labelling for APC and Bcl-Xl revealed G-CSF-mediated up-regulation of Bcl-Xl in oligodendrocytes. This data is in line with the G-CSF-mediated up-regulation of Bcl-Xl gene expression in spinal cord partial transection model [21]. Finally, G-CSF-mediated attenuation of neuronal death, which action we previously reported [19], and/or promotion of neurite outgrowth [21] could enhance survival of oligodendrocytes because the survival of oligodendrocytes depends on stimuli from axons [31]. The present data of G-CSF-mediated attenuation of loss of MAP-2- positive neurons might support that hypothesis.

The present results showed that G-CSF suppresses apoptosis of oligodendrocytes and white matter degeneration after SCI. There was a direct correlation between the area of spared myelin and final motor function score. This result is in line with previous reports [32,33] and explains how G-CSF promotes functional recovery. In the murine SCI model, we previously reported that G-CSF attenuates apoptosis of neurons via the activation of signaling pathways downstream from the G-CSFR [19]. Taken together, G-CSF exerts neuroprotective effects for neurons and oligodendrocytes via anti-inflammatory and anti-apoptotic actions, resulting in reduced white matter degeneration and promotion of functional recovery. Other than the anti-inflammatory or anti-apoptotic effects on the CNS, G-CSF can promote mobilization of bone marrow-derived stem cells and their migration into injured spinal cord tissues and promote functional recovery [18,20]. Furthermore, G-CSF has several actions on the vascular system. For example, G-CSF suppresses brain edema formation after stroke [11] and promotes angiogenesis in stroke [28] and SCI [17] models. Finally, G-CSF stimulates neurogenesis both directly [13] or via the up-regulation of vascular endothelial growth factor [34] All of those mechanisms could potentially contribute to the neuroprotective effects of G-CSF following SCI.

In terms of safety and efficacy, G-CSF has an excellent record in clinical use. In the case of stroke, STAIR presents criteria for drug development [35] and G-CSF fulfills those criteria well. Briefly, those criteria include the following: i) blood-brain barrier penetration, ii) neuroprotective activity in different stroke models including permanent ischemia demonstrated by independent groups, iii) activity shown in different species, iv) well-known pharmacokinetics, and v) functional outcome data. In this context, G-CSF may be a candidate for clinical application in the setting of acute SCI. In that regard, the therapeutic time window is important. In the rat photothrombotic stroke model, G-CSF administrated 72 h after induction of ischemia for ten days improved recovery [36]. G-CSF administration might be effective if delayed even further. Additional studies are needed to define the therapeutic time window of G-CSF treatment for SCI. G-CSF is an attractive candidate for treatment of acute SCI. While greater understanding of the optimal dosage, therapeutic time window and precise mechanism of action is needed, the present results are encouraging. The feasibility of conducting clinical trials of G-CSF treatment for acute SCI patients should be considered.

Conclusions

G-CSF protects oligodendrocyte from SCI-induced cell death via the suppression of inflammatory cytokines and up-regulation of anti-apoptotic protein. As a result, G-CSF attenuates white matter loss and promotes hindlimb functional recovery.

Materials and Methods

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 present study was approved by Animal Care and Use committee of Chiba University Graduate School of Medicine (the approval number was 20060017). Ninety adult female Sprague-Dawley rats (10–12 weeks old; weight 200–240 g; Japan SLC, Inc. Hamamatsu, Japan) were housed in individual cages and given food and water *ad lib*. Rats were anesthetized with 1.5% halothane in 0.5 L/min oxygen and contused on T9 spinal cord exposed by a T8/9 laminectomy. Contusive SCI was introduced using the Infinite Horizon Impactor (Precision Systems and Instrumentation, Lexington, KY, magnitude: 200 kilo dyne). Upon awakening, rats were evaluated neurologically and were monitored for food and water uptake and urine output. As prophylactic, Bactramin (Chugai, Japan) was added to drinking water.

Rats were randomly assigned to two groups. G-CSF group received recombinant human G-CSF (15.0 μg/kg; kindly provided by Kirin Pharma Co. Ltd., Tokyo, Japan), which was dissolved in normal saline. Vehicle group rats received equal volumes of normal saline at the same time points. All compounds were administered by tail vein injection at 1 h after surgery and daily the next four days. We followed the regimen of drug administration previously reported in the rat brain infarction model [13]. In addition, we performed preliminary experiments in other dose regimen (5, 15, 50 μg/kg/d for 5 days after injury). Those preliminary data suggested that 15 μg/kg/d G-CSF exerts most strong effects. Thus we employed 15 μg/kg/d dose regimen. We collected peripheral blood 1 day after injury and counted

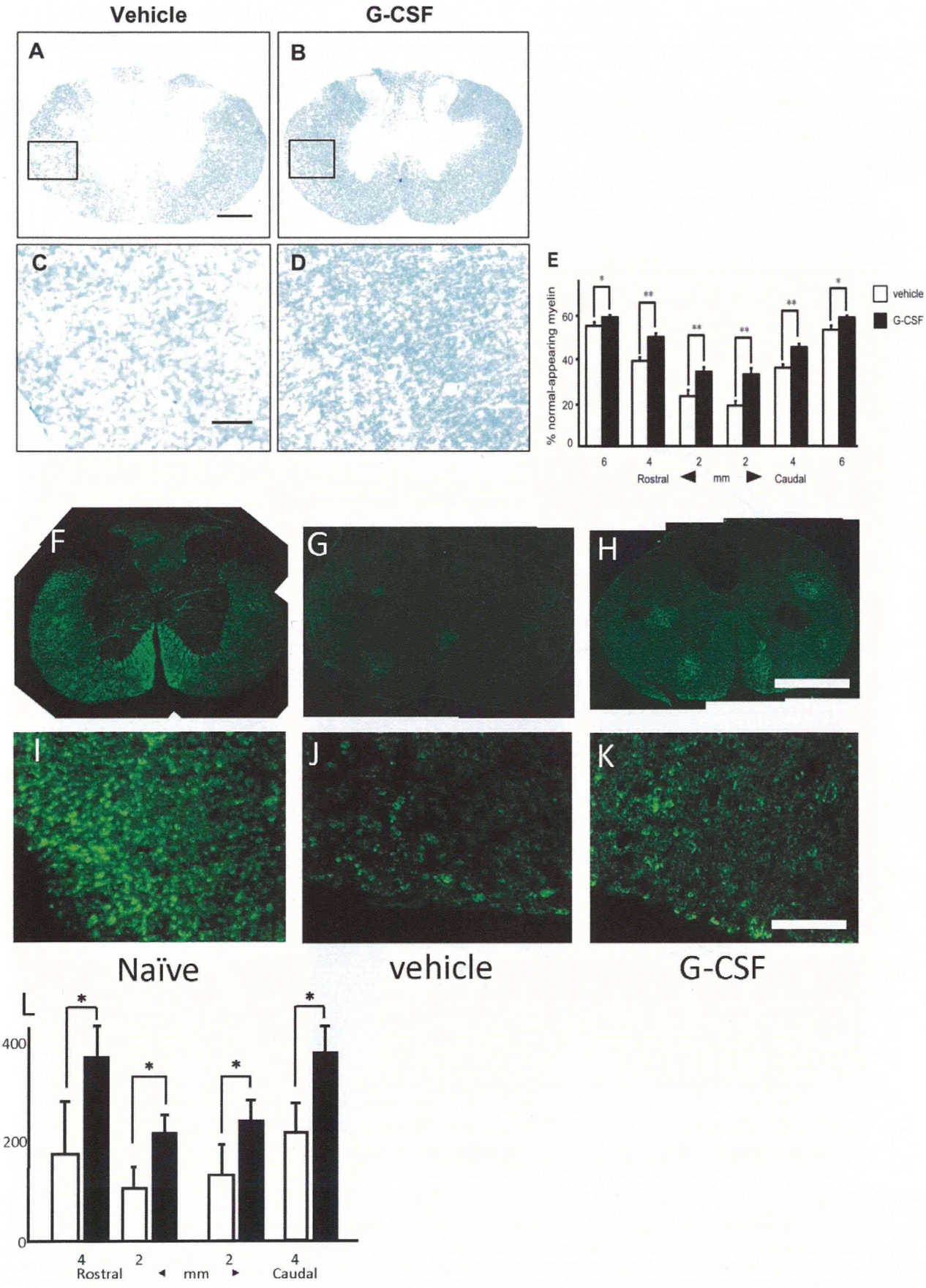
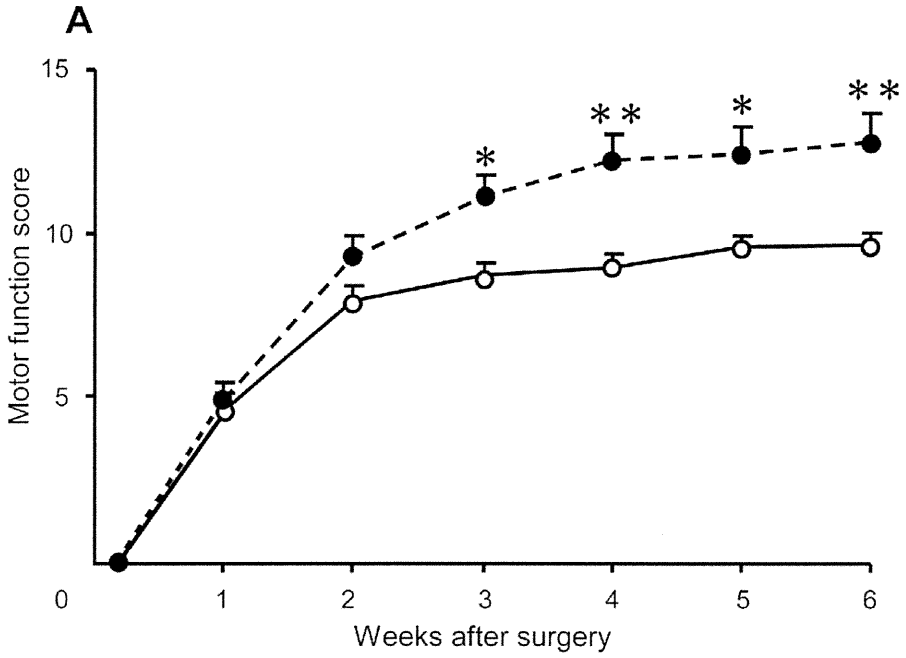

Vehicle
G-CSF
A
B
C
D
E
% normal-appearing myelin
vehicle
G-CSF
6
4
2
2
4
6
Rostral
mm
Caudal
F
G
H
I
J
K
Naïve
vehicle
G-CSF
L
Number of MBP+ myelin sheath
400
200
0
4
2
2
4
Rostral
mm
Caudal

Figure 5. G-CSF attenuated degeneration of white matter myelin. Luxol Fast Blue (LFB) staining and immunofluoresent staining for myelin basic protein (MBP) were performed to quantify spared myelin in the chronic phase of SCI. Figures show LFB staining of the spinal cord 4 mm caudal to the epicenter. Higher magnification revealed better myelin integrity in rats from the G-CSF group (D) than the vehicle group (C). The extent of loss of myelin in the chronic phase of injury (six weeks after surgery) was analyzed (Fig. 4–E). Gray matter of each sample was subtracted from the whole area of the slice to reveal the area of white matter. The actual area of normal-appearing myelin was divided by the white matter to determine the percentage of normal-appearing myelin. The percentage of normal appearing myelin was significantly larger in the G-CSF group (E; closed column) than the vehicle group (E; open column). Immunofluorescence showed that the number of MBP-positive myelin sheath was larger in the G-CSF group than that in the vehicle group (L). Bars = 1 mm (F–H), 500 μm (A, B) and 100 μm (C, D, I–K), values are mean±SEM. *p<0.05, **p<0.01. Values are mean±SEM. *p<0.05.
doi:10.1371/journal.pone.0050391.g005



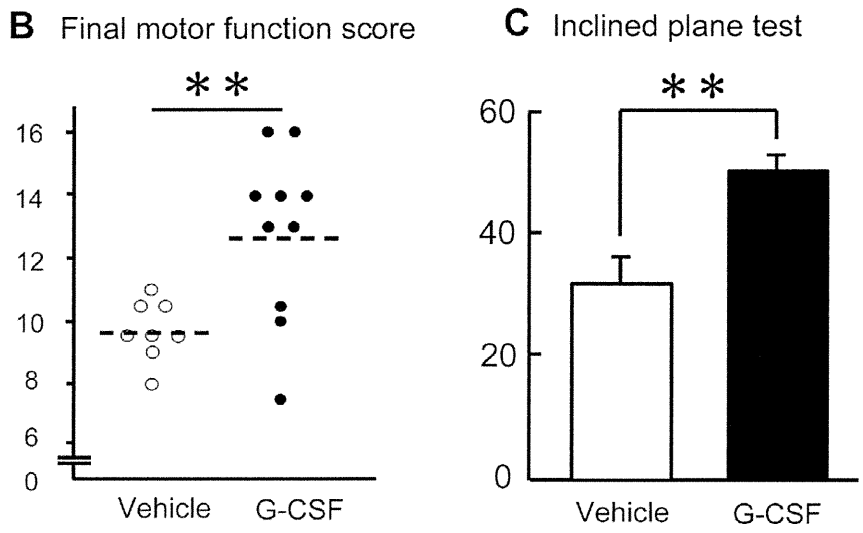


Figure 6. Locomotor recovery. Assessment of hind limb function with the Basso, Beattie and Bresnahan (BBB) locomotor scale (A, B). Time course of recovery of hindlimb function (A) and comparison of final motor function scores (B). Rats from the G-CSF group (closed circle) showed significant recovery compared to rats from the vehicle group (open circle) (A; Repeated measures ANOVA, $p<0.05$). The final motor function score six weeks after surgery in the G-CSF group (average 12.8; B, closed circle) was significantly higher than that in the vehicle group (average 9.7; B, open circle, $p<0.01$). The average score in the inclined plane test ten weeks after surgery in the G-CSF group (average 50 degrees; C, closed column) was significantly higher than that in the vehicle group (average 31.7 degrees; C, open column, $p<0.01$). Values are mean±S.E.M. *p<0.05, **p<0.01.
doi:10.1371/journal.pone.0050391.g006

leukocyte number, the average of which was 3800 in the vehicle control group and 9700 in the G-CSF group.

Real-Time Polymerase Chain Reaction (PCR) (Relative Quantitation)

We performed quantitative real-time PCR to determine expression of inflammatory cytokines. First-strand cDNA synthesis and Real-Time PCR were performed as previously described [11]. Briely, for gene analysis, animals of each group were euthanized under pentobarbital anesthesia 12, 24 h and 72h after surgery (n = 5/group) and 10 mm of spinal cord segment including lesion were removed, snap-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was isolated from spinal cord samples using the RNeasy RNA isolation system (Qiagen Inc.) according to the manufacturer's instructions. Taqman technology (Model 7500 sequence detector, Applied Biosystems, Warrington, UK) was used for quantitative real-time PCR. All samples were run in duplicate. TaqMan probes (labeled with the fluorescent reporter FAM) for interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), FAS, FAS ligand, interleukin-6, interferon-gamma (IFN-γ), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) were designed by Applied Biosystems. Samples were tested in duplicate, and the average values of the threshold cycle (Ct) were used for quantification. To quantify the relative expression of each specific gene, the Ct values were normalized for endogenous 18S ribosomal RNA, and compared with a calibrator using the ΔΔCt method ($Ct = Ct_{Sample} - Ct_{Calibrator}$) and converted to a logarithmic value. As calibrator, we used expression in the G-CSF group 12 h after surgery because the expression level of those cytokines in the normal spinal cord was very low. The mean result was further subjected to statistical analysis and expressed as fold–increase.

Western Blot Analysis

Injured spinal cords (10-mm segments) were homogenized in a homogenization buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (complete, Roche Diagnostics, Basel, Switzerland). Homogenates were cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Protein concentration of supernatants was measured with Bio-Rad Dc Protein Assay Reagents (Bio-Rad Laboratories, Hercules, CA, USA), and the protein concentration was adjusted to 1 mg/mL by diluting supernatants with a homogenization buffer. Protein samples were mixed with an equal volume of a 2×sample buffer (250 mM Tris-HCl, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.02% bromophenol blue, and 10% b-mercaptoethanol). After boiling for 5 min, equal volumes of samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA, USA). After blocking of the membrane with PBS containing 5% skim milk and 0.05% Tween 20, the membrane was reacted with an anti- IL-1β (BD Biosciences, Franklin lakes, NJ), an anti- TNF-α (BD Biosciences) and an anti-β-actin antibody as a loading control (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. For detection, a horse radish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and an ECL chemiluminescence system (GE Healthcare, Piscataway, NJ, USA) were used. Quantification of protein bands was performed using image J software.

Tissue Preparation

For histological evaluation, animals were perfused transcardially with 4% paraformaldehyde in PBS (pH 7.4) under pentobarbital anesthesia 12, 24, 72 h and one week after surgery (n = 4/group) and six weeks after surgery (n = 10/group). Spinal cord tissue blocks including the lesion epicenter were removed and postfixed in the same fixative overnight, stored in 20% sucrose in PBS at 4°C, and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). The cryoprotected samples were frozen and kept at −80°C until use. The samples were cut into serial 10 μm transverse sections with a cryostat and mounted on aminosilane-coated slides (Matsunami, Tokyo, Japan).

Immunofluorescent Labeling

For immunofluorescent labeling, sections were permeated with 0.3% Triton X 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 as indicated: mouse monoclonal anti-G-CSF receptor antibody (G-CSFR, 1:200, Abcam, Cambridge, UK), rabbit polyclonal anti-microtubule associated protein-2 (MAP-2) antibody (MAP-2, 1:400, Chemicon Inc, Temecula, CA) for neurons, rabbit polyclonal anti-glial fibrillary acidic protein antibody (GFAP, 1:400, Sigma, St Louis, MO) for astrocytes, mouse monoclonal anti-adenomatous polyposis coli antibody (APC, 1:800, Calbiochem, San Diego, CA) or mouse monoclonal anti-myelin oligodendrocyte specific protein IgM antibody (MOSP, 1:200, Chemicon Inc) for oligodendrocytes, goat polyclonal anti-ionized calcium-binding adaptor molecule 1 (Iba-1,1:500, Abcam) for microglia, rabbit polyclonal anti-activated caspase 3 antibody (Caspase 3-a, 1:400, R & D systems, Minneapolis, MN) for apoptotic cells, rabbit polyclonal anti-Bcl-Xl antibody (1:200, AbD Serotec, Kidlington, UK), mouse monoclonal anti-IL-1β antibody (IL-1β, 1:200, AbD Serotec), rabbit polyclonal anti-myeloperoxidase antibody (MPO, prediluted, Abcam, Cambridge, UK) for infiltrating inflammatory leukocytes, mouse monoclonal anti-inducible nitric oxide synthase antibody (iNOS, 1:100, Chemicon Inc.), mouse monoclonal anti-arginase 1 antibody (1:100, Chemicon Inc.) and mouse anti-myelin basic protein antibody (MBP, 1:100, Chemicon Inc.) for myelin sheath. The sections were reacted with primary antibodies overnight at 4°C. After incubation with the primary antibodies, sections were washed in PBS and then incubated with secondary antibodies at room temperature for 1 h: Alexa 488- or 594- labeled anti-mouse, anti-rabbit IgG, anti-goat IgG or anti-mouse IgM antibodies (1:800, Molecular Probes, Eugene, OR). Finally, sections were washed twice in PBS and coverslips were added. The positive signals were observed by fluorescence microscopy (ECLIPSE E600; Nikon, Tokyo, Japan). In case of double staining for G-CSFR/GFAP and G-CSFR/MOSP, the positive signals were detected using confocal laser scanning microscopy (LSM5 PASCAL; Carl Zeiss, Germany). The specificity of the staining procedures was controlled by omitting primary or secondary antibodies.

On the sections from normal spinal cord and one week after surgery, immunofluorescence double labeling for G-CSFR and cell specific markers (MAP-2, GFAP, MOSP and Iba-1) was performed to elucidate the expression of G-CSFR. To detect the influence of G-CSF on G-CSFR expression in oligodendrocytes, quantification was performed by cell count of G-CSFR and MOSP-double positive cells one week after SCI.

To observe the effects of G-CSF on inflammatory cells, immunofluorescence for neutorphils and microglia were performed. Twelve and 24 h after surgery, IL-1β-expressing inflammatory leukocytes were counted as IL-1β- and MPO-double

positive cells. To quantitatively analyze the number of IL-1β- and MPO-double positive cells sections were picked from lesion epicenter and 2 mm rostral and caudal segments to the epicenter. Three samples from each segment were observed and a mean value of results was analyzed. The mean values of number of double-positive cells per section were compared between the groups. One week after the injury, phenotype of microglia/macrophage was determined by immunofluorescent double staining for Iba-1/iNOS or Iba-1/arginase-1. The number of total Iba-1-positive cells was counted, then the ratio between iNOS-positivity and arginase-positivity was caluculated.

Apoptotic oligodendrocytes were counted as activated caspase 3 and APC-double positive cells in samples collected 72 h and one week after surgery. For the quantitative analysis of the number of apoptotic oligodendrocytes, sections were picked from epicenter, segments 2 mm, 4 mm and 6 mm rostral and caudal to the epicenter. The percentage of apoptotic oligodendrocyte was calculated by dividing the number of activated caspase 3 and APC-double positive cells by total APC-positive cell number. We also preformed immunefluorescence double labelling for APC and one of the anti-apoptotic protein Bcl-Xl on spinal cord histological sections one week after injury.

To determine the influence of neuronal death on oligodendrocyte survival or death, we performed immunofluorescence for MAP-2 one week after the injury and counted the number of MAP-2-positive neurons in both the vehicle and G-CSF-treated rats.

For quantification of immunofluorescence data, every fifth ten-μm transverse section (50 μm apart) was picked from epicenter, 2 mm, 4 mm and 6 mm rostral or caudal to the lesion epicenter. At least ten sections per each animal were counted, resulting in coverage for 500 μm area of spinal cord in each segment. To count positive cells, we used Scion Image computer analysis software (version beta 4.0.3, Scion Corporation, Frederick, MA).

Myelin Sparing and Oligodendrocyte Cell Count

We performed luxol fast blue (LFB) staining and immunofluorescence for MBP to measure an area of spared white matter myelin six weeks after surgery. The LFB-positive area and total area of white matter of the same section was calculated using Scion Image computer analysis software. Gray matter was subtracted from the whole area of the slice to reveal the area of white matter. To determine the percentage of normal-appearing myelin, the area of the LFB stain was divided by the area of the white matter (Fig. 5 E). Immunofluorescent labeling for MBP was performed to quantify the number of MBP-positive myelin sheath six weeks after surgery as described above. The number of MBP-positive myelin sheath in white matter was counted (Fig. 5 F). Quantification was done as described above.

Assessment of Locomotor Activity

The recovery of rat hindlimb function in both groups (n = 10 in the G-CSF group and n = 8 in the vehicle group) was determined by measuring the hindlimb motor function score with Basso, Beattie and Bresnahan locomotor scale (BBB scale [37]). Rats were allowed to move freely on the open field with a rough surface for five min at each time tested. The hindlimb movement of rats was scored by two independent observers who were unaware of the treatment. Measurement of motor function was performed weekly following the sixth week after surgery.

In another subset of rats treated the same as above, the inclined plane test was performed ten weeks after surgery as previously described [38] (n = 5 in each group). The highest degree of inclination was defined as that at which the animal could maintain its position for five seconds on two separate trials.

Statistical Analysis

Results of immunohistochemical studies, percentages of normal-appearing myelin, final motor function scores and inclined plane tests were subjected to Student's t-test. Recovery of motor function scores was subjected to Repeated Measures ANOVA followed by post hoc test using Fisher's Protected Least Signicicant Difference test. Percentage of normal myelin (average values of sections) and final motor function scores were analyzed for the Pearson product-moment correlation coefficient. Results are presented as mean values±S.E. Values of $p<0.05$ were considered statistically significant.

Supporting Information

Figure S1 G-CSFR expression in naïve spinal cord. GFAP-positive astrocytes (A), MOSP-positive oligodendrocytes (B) and MAP-2-positive neurons (C) expressed G-CSFR. Although the number of MOSP-positive oligodendrocytes was significantly different between the vehicle (D, open columns) and G-CSF groups (D, closed columns), all of the MOSP-positive oligodendrocytes expressed G-CSFR after SCI. Bar = 100 μm. Values are mean±S.E.M. *$p<0.05$.
(TIF)

Figure S2 The number of MAP-2-positive neurons after SCI. The number of MAP-2-positive neurons was significantly larger in the G-CSF group in the rostral and caudal segments (closed columns). Values are mean±S.E.M. *$p<0.05$.
(TIF)

Figure S3 The influence of G-CSF on microglia\macrophages. Double immunofluorescence study for ionized calcium-binding adaptor molecule 1 (Iba-1, as a marker for activated microglia and macrophages) and inducible nitric oxide synthase (iNOS, as a marker for Th1-driven activation of microglia/macrophages) or arginase-1 (a marker for Th2-driven activation of microglia/macrophages) was performed to elucidate G-CSF-mediated reaction and phenotypic alteration of macrophage/microglia. The number of Iba-1-positive cells in the G-CSF group was significantly smaller than that in the vehicle group in the rostral and caudal segments, whereas the ratios between iNOS (open columns) and arginase-1 (hatched or dotted columns) did not change in both the vehicle and G-CSF groups in lesioned spinal cord at any segments observed. Values are mean±S.E.M. *$p<0.01$.
(TIF)

Figure S4 Apoptotic oligodendrocytes. To further confirm the results of immunohistochemistry for apoptotic oligodendriocytes, we performed double immunofluorescence study for MOSP as another marker for oligodendrocytes and activated caspase-3 as a marker for apoptotic cells (A-C). The staining pattern was similar to that of the double fluorescence study for APC and activated caspase-3. Co-localization of MOSP and activated caspase-3 was further confirmed with orthogonal imaging obtained by laser confocal microscopy (D).
(TIF)

Author Contributions

Conceived and designed the experiments: RK MK KT MY. Performed the experiments: RK MK JK MH YN CM TM TF AO. Analyzed the data: RK MK KT MY. Contributed reagents/materials/analysis tools: RK MK JK MH YN CM. Wrote the paper: RK MK MY.

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ORIGINAL ARTICLE

Intravenous administration of granulocyte colony-stimulating factor for treating neuropathic pain associated with compression myelopathy: a phase I and IIa clinical trial

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Abstract
Objective To confirm the feasibility and safety of granulocyte colony-stimulating factor (G-CSF) for treating spinal neuropathic pain associated with compression myelopathy, we have initiated an open-label single-center prospective clinical trial.
Methods Between January 2009 and February 2011, 17 patients were accrued and were divided into two groups. One group included 7 patients who complained of pain associated with worsening symptoms of myelopathy (progressing myelopathy-related pain group). The other group included 10 patients who complained of pain that persisted after surgery for compression myelopathy (post-operative persistent pain group). All patients underwent intravenous administration of G-CSF (10 μg/kg/day) for 5 consecutive days. Pain severity was evaluated using a visual analog scale (VAS) before and after G-CSF administration.
Results In 14 of the 17 patients, pain was relieved within several days after G-CSF administration. Pain disappeared completely in 3 patients. In the progressing myelopathy-related pain group, the mean VAS score was 71.4/100 before G-CSF administration, and decreased to 35.9/100 at 1 week after G-CSF administration ($p < 0.05$). In the post-operative persistent pain group, the mean VAS score was 72.0/100 before G-CSF administration, and decreased to 51.7/100 at 1 week after G-CSF administration ($p < 0.05$). No severe adverse events occurred during or after G-CSF administration.
Conclusions The present results provide us with the possibility that G-CSF has a pain-relieving effect for neuropathic pain in patients with compression myelopathy.

Keywords Neuroprotective therapy · Granulocyte colony-stimulating factor · Myelopathy · Neuropathic pain · Clinical trial

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Introduction

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that promotes survival, proliferation, and differentiation of cells in the neutrophil lineage [11, 16]. Furthermore, G-CSF can mobilize both immature and mature bone marrow cells into the peripheral blood. As a result, it is used clinically for patients with leukocytopenia and for donors of peripheral blood-derived hematopoietic stem cells for transplantation. Recent studies have indicated that G-CSF also has non-hematopoietic activity and can potentially be used for the treatment of neuronal injury, including stroke and neurodegenerative diseases [3, 5, 7, 18, 19]. We previously demonstrated that G-CSF promoted the restoration of damaged spinal cord tissue and the recovery of neural function in experimental spinal cord injury in both mice and rats [4, 6, 12]. In addition, we showed that G-CSF promoted the migration of bone marrow-derived cells into the damaged spinal cord, suppressed apoptosis of neuronal cells and oligodendrocytes, protected myelin, decreased inflammation, and promoted angiogenesis [4, 6, 12]. Based on these findings, we initiated a clinical trial that evaluated the safety and efficacy of neuroprotective therapy using G-CSF for patients with

Table 1 Patient data (progressing myelopathy-related pain group)

Case no.	Age (years)/gender	Diagnosis	Most stenotic level	Surgical procedure[a]	Time of surgery after G-CSF administration (weeks)
1	46/M	OPLL	T6–7	PDF (T2–11)	8
2	75/M	OPLL	C4–5	PDF (C2–7)	7
3	64/M	OPLL	C4–5	PDF (C2–7)	2
4	32/M	OPLL	T9–10	PDF (T7–12)	4
5	67/M	OLF	T11–12	PD (T10–12)	5
6	36/M	OPLL	T5–6	PDF (T2–10)	7
7	72/F	OPLL	T11–12	PDF (T6–L3)	23

OPLL ossification of posterior longitudinal ligament, *OLF* ossification of ligamentum flavum, *PDF* posterior decompression with instrumented fusion, *PD* posterior decompression

[a] Surgery after G-CSF administration

worsening symptoms of compression myelopathy [17]. In this clinical trial, we intravenously administered G-CSF (5 or 10 μg/kg/day) to 17 patients for 5 consecutive days. G-CSF administration suppressed progression of myelopathy in all patients, and no serious adverse events occurred during or after treatment [17].

During this trial, several cases unexpectedly experienced a dramatic reduction in neuropathic pain associated with thoracic myelopathy after G-CSF administration [22]. Such a pain-relieving effect of G-CSF was not specified as an endpoint of this trial. However, this effect has important implications for future clinical use of G-CSF for compression myelopathy. Thus, we initiated a new clinical trial to verify the feasibility and safety of using G-CSF for spinal neuropathic pain. In the present study, G-CSF was administered to patients who complained of pain associated with compression myelopathy, and the pain-relieving effect of G-CSF for spinal neuropathic pain was analyzed.

Materials and methods

We performed a phase I and IIa clinical trial evaluating G-CSF administration in patients who complained of neuropathic pain associated with compression myelopathy. The trial was initiated following the approval of the Institutional Review Board of our university. According to the inclusion criteria, patients of 20–85 years of age were recruited. Patients in the following categories were excluded: (1) those with intracranial pathologies (e.g., tumors, infection, or ischemia), (2) those with a history of major bleeding requiring blood transfusion or a history of leukopenia, thrombocytopenia, hepatic or renal dysfunction, severe heart failure, or splenomegaly, and (3) those with evidence of malignant disease within the last 5 years. We also excluded patients who were pregnant or nursing. Eligible patients gave informed consent for participation in the trial.

Granulocyte colony-stimulating factor (10 μg/kg/day) was intravenously administered for 5 consecutive days. This was an open-label study; thus, there was no control group. Spinal neuropathic pain of patients analyzed in the present clinical trial was classified into two categories: at-level pain and below-level pain [1]. At-level pain is characterized as pain located within two or three spinal segments below the neurological level of a spinal cord lesion. In contrast, below-level pain presents diffusely caudal to the level of a spinal cord lesion. The severity of pain was evaluated before and after G-CSF administration using a visual analogue scale (VAS) ranging from 0 to 100. We also evaluated severity of myelopathy using the Japanese Orthopaedic Association (JOA) score (cervical myelopathy scores range from 0 to 17, thoracic myelopathy scores range from 0 to 11) [9]. In the present study, two orthopedic spine surgeons specializing in cervical and thoracic spine surgery evaluated neurological status independently every month until 6 months after G-CSF administration, and calculated the mean data. Hematological data from treated patients were analyzed. Adverse events using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events, version 3.0 were also evaluated.

Statistical analysis was performed using a Mann–Whitney U test. A p value less than 0.05 was considered statistically significant. Results are presented as mean ± SD.

Results

Patient data

Between January, 2009 and February, 2011, a total of 18 patients were enrolled in this trial. In one patient, however, fever developed 3 days after the initiation of G-CSF administration, and the administration was discontinued. This patient was excluded from the study. Thus, 17 patients

Table 2 Patient data (post-operative persistent pain group)

Case no.	Age (years)/gender	Diagnosis	Most stenotic level	Procedure of previous surgery[a]	Time of previous surgery before G-CSF administration (years)
8	58/M	OPLL	C5–6	PD (C3–6)	2
9	72/M	DH	T12–L1	PDF (T9–L3)	0.5
10	71/M	OPLL	C5–6	PD (C3–7)	3
11	78/M	OLF	T10–11	PDF (T10–12)	189
12	70/M	OPLL	C4–5	PD (C3–5)	30
13	70/F	OPLL	C5–6	PDF (C2–7)	1
14	81/F	OLF	T10–11	PD (T9–11)	5
15	69/M	CSM	C4–5	PDF (C4–5)	4
16	62/M	CSM	C5–6	PD (C3–7)	10
17	63/M	OPLL	C5–6	PDF (C3–7)	8

DH disc herniation, *CSM* cervical spondylotic myelopathy

[a] Surgery before G-CSF administration

Table 3 Neuropathic pain data

Case no.	Type of pain	VAS before G-CSF administration	Duration of pain
1	At-level	60	0.8
2	Below-level	50	3
3	At-level	50	0.3
4	At-level	80	4
5	At-level	90	0.2
6	At-level	100	0.2
7	At-level	70	5
8	At-level	50	1
9	Below-level	90	3
10	At-level	80	3
11	Below-level	60	19
12	At-level	60	27
13	At-level	60	1
14	Below-level	80	5
15	Below-level	80	4
16	Below-level	70	11
17	At-level	90	8

VAS visual analogue scale (0–100)

received G-CSF administration and were followed-up for ≥6 months (Tables 1, 2). These 17 patients were divided into two groups. One group included 7 patients (Cases 1–7) who complained of pain associated with worsening symptoms of myelopathy (progressing myelopathy-related pain group) (Table 1). The other group included 10 patients (Cases 8–17) who complained of pain that persisted after surgery for compression myelopathy (post-operative persistent pain group) (Table 2).

In the progressing myelopathy-related pain group, worsening of myelopathy occurred due to compression of the spinal cord by ossification of the posterior longitudinal ligament (OPLL) or ossification of the ligamentum flavum (OLF) (Table 1). The mean JOA score for cervical or thoracic myelopathy decreased ≥2 points or more during a recent 1-month period. Of the 7 patients in this group, 6 patients (Cases 1, 3, 4, 5, 6, and 7) complained of at-level pain and 1 patient (Case 2) complained of below-level pain (Table 3). The duration of pain was 0.2–5 years (mean, 1.9 years). In all 7 patients, surgery for myelopathy was performed 2–23 weeks after initial G-CSF administration (Table 1).

In the post-operative persistent pain group, pain caused by compression to the spinal cord persisted even after myelopathy surgery (Tables 2, 3). Of these 10 patients, 5 patients (Cases 8, 10, 12, 13, and 17) complained of at-level pain and 5 patients (Cases 9, 11, 14, 15, and 16) complained of below-level pain (Table 3). The duration of pain in all 10 patients in this group was 1–27 years (mean, 8.2 years), which was significantly longer than that of the progressing myelopathy-related pain group ($p < 0.01$).

VAS

In the progressing myelopathy-related pain group, a decrease in VAS score of >10 was obtained in all 7 patients within 1 week after initial G-CSF administration. In 1 patient (Case 4), pain completely disappeared. The mean VAS score immediately before G-CSF administration was 71.4, and it significantly decreased to 35.9 at 1 week after initial G-CSF administration ($p < 0.05$) (Fig. 1a). The pain-relieving effect of G-CSF was attenuated at 3 months after administration in 3 patients (Cases 2, 3, and 5), and the VAS score returned to the pre-administration level in 1 patient (Case 2). However, 3 and 6 months after G-CSF administration, mean VAS scores were still lower than those before G-CSF administration ($p < 0.05$) (Fig. 1a).

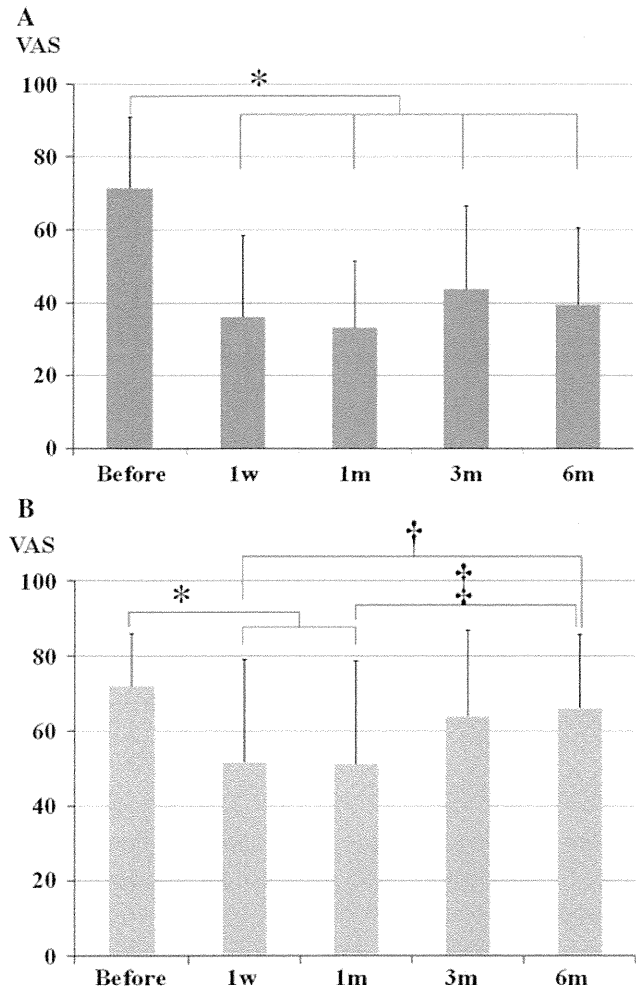

Fig. 1 Visual analogue scale before and after G-CSF administration in progressing myelopathy-related pain group (**a**) and post-operative persistent pain group (**b**). *VAS* visual analogue scale, *before* immediately before G-CSF administration, *1w* 1 week after initial G-CSF administration, *1 m* 1 month after initial G-CSF administration, *3 m* 3 months after initial G-CSF administration, *6 m* 6 months after initial G-CSF administration. $^{*}p < 0.05$ compared with that before G-CSF administration. $^{\dagger}p < 0.05$ compared with that 1 week after G-CSF administration. $^{\ddagger}p < 0.05$ compared with that 1 month after G-CSF administration

Figure 2 shows the change of VAS before and after surgery in seven cases of the progressing myelopathy-related pain group. After surgery, VAS was not altered in four cases (Cases 1, 3, 4 and 6), increased in two cases (Cases 2 and 5), and decreased in one case (Case 7) (Fig. 2).

In the post-operative persistent pain group, a decrease in VAS score of $\geq$10 was obtained in seven out of ten patients within 10 week after initial G-CSF administration. In three patients (Cases 9, 14, and 16), G-CSF did not show any pain-relieving effect. The mean VAS score immediately before G-CSF administration was 72.0, and it significantly decreased to 51.7 at 1 week after initial G-CSF administration ($p < 0.05$) (Fig. 1b). The pain-relieving effect of

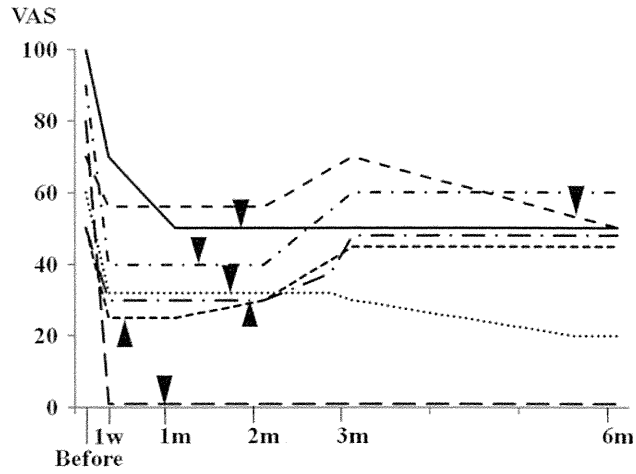

Fig. 2 Change of visual analogue scale after G-CSF administration in each case of progressing myelopathy-related pain group. *VAS* visual analogue scale, *before* immediately before G-CSF administration, *1 w* 1 week after initial G-CSF administration, *1 m* 1 month after initial G-CSF administration, *3 m* 3 months after initial G-CSF administration, *6 m* 6 months after initial G-CSF administration. *Arrowheads* indicate the time of surgery

G-CSF was attenuated at 3 months in 4 patients (Cases 11, 13, 15, and 17) and at 6 months in 1 patient (Case 12), and VAS scores returned to pre-administration levels in 4 patients (Cases 11, 13, 15, and 17). The mean VAS score increased to 64.0 at 3 months after G-CSF administration (Fig. 1b).

JOA score

In all 7 patients in the progressing myelopathy-related pain group, the JOA score increased after G-CSF administration. The mean JOA recovery rate at 1 and 6 months after G-CSF administration was 32.3 and 54.2 %, respectively (Table 4).

In the post-operative persistent pain group, an increase in JOA score was observed in only 3 patients (Cases 8, 10, and 11). Three patients (Cases 9, 14, and 16), in whom no pain-relieving effect was observed after G-CSF administration, also did not show any increase in JOA score. The mean JOA recovery rate at 1 month and 6 months after G-CSF administration was 7.3 and 7.3 %, respectively (Table 4). Thus, the neurological improvement after administration of G-CSF in the post-operative persistent pain group was inferior to that in the progressing myelopathy-related pain group (Table 4).

Blood data

White blood cell count dramatically increased the day after G-CSF administration; during G-CSF administration, it increased to 31.2 ± 8.3 ($\times 10^3/mm^3$) (Table 5). G-CSF mobilized cells of the neutrophil lineage, but lymphocytes

Table 4 Recovery rate of JOA score after G-CSF administration

Group	Time after G-CSF administration	
	1 month	6 months
Progressing myelopathy-related pain group	32.3 ± 27.7* (0–70.6)	54.2 ± 21.2** (28.6–81.8)
Post-operative persistent pain group	7.3 ± 12.2 (0–28.6)	7.3 ± 12.2 (0–28.6)

Data are expressed as the mean ± standard deviation, with the range in parentheses. Recovery rate = (post-operative JOA score − preoperative JOA score/full score − preoperative JOA score) × 100 (%)

JOA score Japanese Orthopaedic Association score (cervical myelopathy 1–17 points, thoracic myelopathy 0–11 points)

* $p < 0.05$ compared with that of the post-operative persistent pain group

** $p < 0.01$ compared with that of the post-operative persistent pain group

Table 5 Blood data before and after G-CSF administration

	Normal range	Before	Peak value after G-CSF administration[a]	*p*
WBC ($\times 10^3/mm^3$)	4.0–9.0	6.2 ± 2.0 (3.3–12.5)	31.2 ± 8.3 (19.2–47.3)	<0.01
Neutrophil ($\times 10^3/mm^3$)	1.8–5.0	3.6 ± 1.2 (2.0–6.6)	25.8 ± 5.4 (16.6–34.1)	<0.01
Lymphocyte ($\times 10^3/mm^3$)	1.0–4.1	2.0 ± 1.0 (0.9–5.4)	2.1 ± 1.1 (0.7–5.9)	0.25
Monocyte ($\times 10^3/mm^3$)	0.1–0.6	0.4 ± 0.1 (0.2–0.6)	1.2 ± 0.9 (0.3–3.4)	<0.01
CRP ($\times 10^3/mm^3$)	<0.5	0.1 ± 0.1 (0.0–0.4)	0.3 ± 0.4 (0.0–1.3)	<0.01

WBC white blood cell, *CRP* C-reactive protein

[a] Peak value within 7 days after initiating G-CSF administration

were not affected. G-CSF also caused an increase of monocytes. C-reactive protein levels slightly increased, but this did not appear to be related to any clinical events.

Adverse events

In this series, no patient experienced bone pain or hepatic dysfunction after G-CSF administration. No other severe adverse events occurred during or after G-CSF administration.

Case presentation

Case 6 (progressing myelopathy-related pain group)

A 36-year-old man was admitted to our hospital complaining of progressive motor weakness of his lower extremities and gait disturbance. On admission, his JOA score for thoracic myelopathy was 3/11 points. He also showed spontaneous severe back pain (at-level pain). Magnetic resonance (MR) and computed tomography (CT) images showed beak-type OPLL and OLF that compressed his spinal cord anteriorly and posteriorly at T5–6 (Fig. 3a, b). Beginning on the day of admission, he received G-CSF. Six days after initial G-CSF administration, he felt relief of his back pain. His pain VAS score was 100 before G-CSF administration, and it decreased to 70 1 week after initial treatment. At 1 month after initial administration, his VAS score further decreased to 50. He also felt improved muscle strength of his legs, and his JOA score increased to 3.5 points. At 7 weeks after G-CSF administration, he underwent surgery for spinal cord decompression using a posterior approach and T2–T10 posterior instrumented fusion. At 6 months after G-CSF administration, he showed recovery from myelopathy (JOA score = 8 points) and his VAS score was 50.

Case 11 (post-operative persistent pain group)

A 78-year-old man was admitted to our hospital complaining of motor weakness of his lower extremities and gait disturbance. Nineteen years prior, he had undergone T10–12 laminectomy for thoracic myelopathy due to OLF. After surgery, pain persisted in his lower extremities. On admission, his JOA score was 4/11 points. In addition to myelopathy symptoms, he complained of spontaneous severe bilateral pain at the level of his thigh and leg (below-level pain). MR images showed that his spinal cord was decompressed, but was atrophic at T10–11 (Fig. 4a, b). Beginning on the day of admission, he received G-CSF. One day after initial G-CSF administration, he felt pain relief in his bilateral thigh and leg. His VAS score for pain was 60 before G-CSF administration. At 1 week after initial administration, the VAS score was reduced to zero and his pain was diminished. His myelopathy also improved,

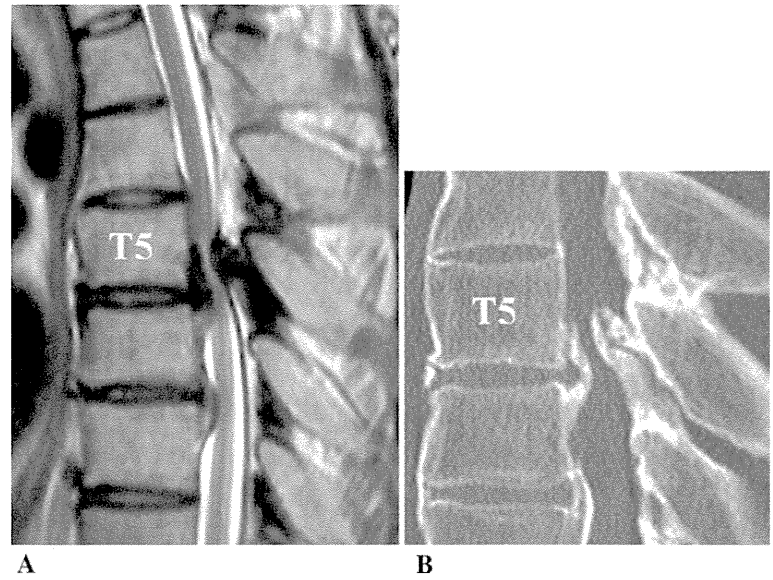

Fig. 3 Case 6. T2-weighted midsagittal MR image (**a**) and CT midsagittal reconstruction plane (**b**) showing anterior and posterior compression of the spinal cord by beak-type OPLL and OLF at T5–T6

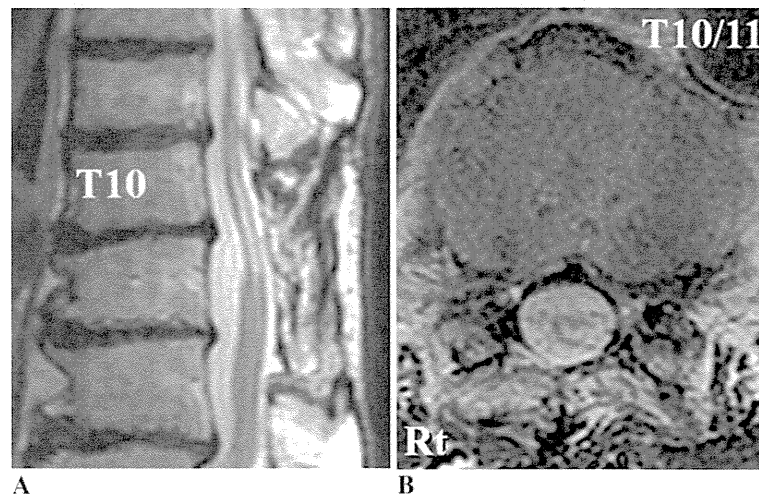

Fig. 4 Case 11. T2-weighted MR midsagittal image (**a**) and axial image at T10–T11 (**b**) showing that the spinal cord was decompressed but was atrophic at T10–T11

and his JOA score increased to 6/11 points 1 month after G-CSF administration. At 3 months after administration, however, he felt recurrence of his pain and his VAS score returned to 60.

Discussion

Neuropathic pain has been defined as a type of pain arising as a direct consequence of a lesion affecting parts of the somatosensory system, such as the brain, spinal cord, and peripheral nerves [1, 10, 21]. Among numerous diseases of the spinal cord, neuropathic pain following spinal cord injury (SCI) has been studied most extensively [10, 21]. Baastrup and Finnerup [1] reviewed pharmacological management of neuropathic pain following SCI. Based on the data from several randomized controlled trials, these investigators suggested that pregabalin, gabapentin, and tricyclic antidepressants (TCAs) are optimal first-line treatments for neuropathic pain associated with SCI. Furthermore, they considered that serotonin–norepinephrine reuptake inhibitors (SNRIs) are second-line choices, and that tramadol, opioids, and lamotrigine are third-line options. However, these researchers concluded that such oral pharmacological intervention is often inadequate, commonly resulting in a reduction of only 20–30 % in pain intensity. Thus, no established cure for spinal neuropathic pain currently exists.

The present study is the first to report the results of a clinical trial that evaluated the therapeutic effect of G-CSF on neuropathic pain associated with compression myelopathy. G-CSF was administered to two distinct groups of spinal neuropathic pain patients: the progressing myelopathy-related pain group and the post-operative persistent pain group. In the 7 patients in the progressing myelopathy-related pain group, G-CSF administration reduced

neuropathic pain within several days in all patients. The mean VAS score was 71.4/100 before G-CSF administration, and it significantly decreased to 35.9/100 at 1 week after administration, indicating that the severity of pain decreased to 50 % of the pre-administration level. In all 7 patients, surgery for compression myelopathy was performed ≥2 weeks after initial G-CSF administration. Thus, the pain-relieving effect of G-CSF occurred prior to surgery. We suggest that the pain reduction observed within 1 week of administration in these seven cases was caused by the pharmacological effect of G-CSF and not by surgery.

To the best of our knowledge, no report has fully determined the effect of surgery itself on spinal neuropathic pain associated with compression myelopathy. In the present study, all seven patients in the progressing myelopathy-related pain group underwent surgery ≥2 weeks after the initial G-CSF administration. At the time of surgery, pain reduction had already been achieved in all the patients receiving G-CSF. After surgery, further decreases of pain were not obtained. These findings suggest the possibility that surgery itself does not have pain-relieving effects exceeding G-CSF. However, there are several limitations to this hypothesis. In this study, surgeries were performed a rather long time after myelopathy worsening. In addition, the number of patients analyzed in the present study was too small for definitive conclusions. Further studies with a larger number of patients will be required. We will determine the effect of much earlier times of surgery on the reduction of neuropathic pain due to compression myelopathy.

In the present study, we employed only one pain measure, VAS, to evaluate the severity of pain before and after the G-CSF administration. A number of pain measures have been reported for evaluating the intensity and quality of pain in patients with spinal neuropathic pain [2, 8, 14, 20]. Previous clinical trials analyzing the effect of amitriptyline [2, 14], gabapentin [8, 14], and pregabalin [20] on neuropathic pain associated with SCI employed multiple pain measures in addition to VAS, such as the McGill Pain Questionnaire (MPQ) and the Center for Epidemiologic Studies Depression Scale (CESD). They combined several pain measures based on the characteristics of each tool, and adequately evaluated the efficacy of the drugs for neuropathic pain. Since our present study was a phase I and IIa clinical trial, we utilized only VAS. In the subsequent phase IIb clinical trial of G-CSF neuroprotective therapy, we are planning to employ multiple pain measures in addition to VAS to evaluate the details of the effect of G-CSF on spinal neuropathic pain.

In the 10 patients in the post-operative persistent pain group, G-CSF administration did not have a pain-relieving effect in 3 patients. Since no improvement of myelopathy was observed in these 3 patients, we speculate that the pain-relieving and neuroprotective effects with respect to improvement of motor and sensory deficits of G-CSF are correlated. However, a pain-relieving effect was observed in the other 7 patients within 1 week after initial G-CSF administration. The mean VAS score of all 10 patients in the post-operative persistent pain group was 72.0/100 prior to G-CSF administration, and it significantly decreased to 51.7/100 at 1 week after administration. This indicates that the severity of pain decreased to 72 % of the pre-administration level. Based on this finding, we suggest that G-CSF may have a certain pain-relieving effect in patients who complain of post-operative persistent pain, although this effect is not as pronounced as that for patients with worsening symptoms of compression myelopathy.

Of the 17 patients analyzed in the present study, a pain-relieving effect associated with G-CSF was detected in 14 patients. However, recurrence of pain occurred in 8 out of these 14 patients during the follow-up period. Notably, pain returned to pre-administration levels in 5 patients. The recurrence of pain was detected at 3 months after G-CSF administration in 7 patients and at 6 months after G-CSF administration in 1 patient. This finding suggests that the pain-relieving effect by G-CSF only lasts for at most 3–4 months. Therefore, when the clinical utility of G-CSF for spinal neuropathic pain is evaluated in the future, administration every 3–4 months should be considered.

Previous studies reported the presence of placebo effects in patients suffering from neuropathic pain, although the duration of the placebo effect was not fully established [13]. In the present study, the pain-relieving effect of G-CSF continued for 3–4 months. Because the study design was open label, we cannot deny the contribution of the placebo effect of injection for reducing the spinal neuropathic pain. To verify the pharmaceutical pain-relieving effect of G-CSF on spinal neuropathic pain, a subsequent clinical trial with double-blind placebo-controlled study design will be necessary.

To the best of our knowledge, no reports of experimental studies of G-CSF administration in an animal model of spinal neuropathic pain have been published. In our studies using animal models of compression-induced and contusive SCI, intravenously administered G-CSF resulted in functional recovery by (1) promoting the migration of bone marrow-derived cells into the damaged spinal cord, (2) directly suppressing the neural apoptosis that occurs via G-CSF receptors at the injured spinal cord, and (3) decreasing the expression of inflammatory cytokines such as IL-1β and TNF-α [4, 6, 12]. Ro et al. [15] administered G-CSF to animal models of peripheral neuropathic pain, and demonstrated that G-CSF increased the number of opioid-contained polymorphonuclear cells and relieved neuropathic pain. We suggest that such mechanisms may

participate in the pain-relieving effect of G-CSF on spinal neuropathic pain, although further studies are required to fully clarify all of the underlying mechanisms.

To date, no effective therapies for spinal neuropathic pain have been established. To the best of our knowledge, this is the first report showing the possibility of a therapeutic effect of G-CSF on neuropathic pain associated with compression myelopathy. The biggest limitation of the present study was that this was an open-label study, so no comparison with a control group was performed. We cannot deny the possibility that a placebo effect of injection and surgical intervention contributed to pain relief. Based on the experience of the present findings, however, we intend to advance to a further clinical trial to verify the feasibility of using G-CSF for relief of spinal neuropathic pain. This will be a multi-center, double-blind, controlled clinical trial; the control group will receive placebo injection. If the efficacy and safety of G-CSF treatment for spinal neuropathic pain is confirmed and clinical use of G-CSF therapy is approved, a novel and effective approach for the treatment of this disorder will be available.

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BASIC SCIENCE

Delayed Granulocyte Colony-Stimulating Factor Treatment in Rats Attenuates Mechanical Allodynia Induced by Chronic Constriction Injury of the Sciatic Nerve

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Study Design. Animal experimental study with intervention.

Objective. The aim of this study was to elucidate therapeutic effects of delayed granulocyte colony-stimulating factor treatment for mechanical allodynia induced by chronic constriction injury (CCI) of the sciatic nerve in rats.

Summary of Background Data. Granulocyte colony-stimulating factor (G-CSF) is used clinically for patients with hematological disorders. Previous reports showed that immediate G-CSF attenuates neuropathic pain in CCI of the sciatic nerve. However, the acute treatment for neuropathic pain prior to accurate diagnosis is not realistic in clinical settings.

Methods. Adult, female Sprague-Dawley rats were subjected to the CCI model. This model induces mechanical allodynia on the ipsilateral hind paw within the first week after the injury. One week after CCI, rats received intraperitoneal G-CSF (15.0 μg/kg) for 5 consecutive days. Mechanical allodynia was assessed using the von Frey hair test. Immunohistochemistry for phosphorylated p38 mitogen-activated kinase (p-p38MAPK) and OX-42 (a marker for activated microglia) on tissue slides from a subset of rats 2 weeks after surgery. Western blot analyses were carried out to determine protein expression level of p-p38MAPK and interleukin-1 β on spinal cord homogenates 2 weeks after CCI.

Results. Results of the von Frey filament test showed that G-CSF significantly attenuates mechanical allodynia induced by the CCI model. Immunohistochemistry revealed that G-CSF reduced the number of p-p38MAPK–positive cells in the ipsilateral dorsal horn compared with that in the vehicle group rats. Immunofluorescent double staining revealed that p-p38MAPK–expressing cells in the spinal cord dorsal horn are mainly microglia. Western blot analysis indicated that G-CSF decreased the expression levels of both p-p38MAPK and interleukin-1 β in the ipsilateral dorsal horn compared with that in the vehicle group rats.

Conclusion. The present results indicate a beneficial effect of delayed G-CSF treatment in an animal model of peripheral nerve injury-induced neuropathic pain.

Key words: neuropathic pain, G-CSF, animal model.

Level of Evidence: N/A

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Neuropathic pain is caused by damage to or dysfunction of the central or peripheral nervous system. In most cases, it cannot be explained by a single disease process or a locus of damage. It may be associated with dysesthesia or allodynia, spontaneously occurring sensations characterized by abnormal or hypersensitive responses to external stimuli, often limiting a patient's quality of life. Currently, neuropathic pain is difficult to treat, and patients frequently experience poor clinical outcomes, in large part because the precise pathophysiology of neuropathic pain still remains unclear. The search for novel therapeutic agents for the treatment of neuropathic pain is an area of intense laboratory and clinical research.[1]

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.[2] It is widely known as a hematopoietic cytokine that promotes survival, proliferation, and differentiation of cells

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of neutrophilic lineage.[2,3] G-CSF is used clinically for patients with leukocytopenia and for donors of peripheral blood-derived hematopoietic progenitor cells prior to their collection for transplantation.[3] 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 model.[4,5] It was also reported that G-CSF protects neurons and oligodendrocytes from apoptosis in mouse and rat spinal cord injury models.[6,7] We recently conducted early-phase clinical trials of G-CSF for spinal cord injury and acute aggravation of compressive myelopathy.[8,9] In those trials, we unexpectedly observed pain relief in several patients.[10] As a result, we hypothesized that G-CSF can attenuate neuropathic pain and tested this hypothesis in a phase 1/2a clinical trial for compression myelopathy-related neuropathic pain.[10] As for its effects in cases of peripheral nerve injury, it has been reported that the immediate administration of G-CSF attenuates neuropathic pain in the Bennett model *via* suppression of inflammatory cytokines, including tumor necrosis factor-α and interleukin-6, and upregulation of endorphins.[11] However, the acute administration of any treatment for neuropathic pain prior to accurate diagnosis of the condition is not realistic in clinical settings.

In this study, we have designed a protocol to better reflect the clinical need to treat neuropathic pain sometime after the initial nerve injury. To elucidate the effects of delayed G-CSF treatment, we administered G-CSF 1 week after the induction of neuropathic pain by sciatic nerve constriction, a time when neuropathic pain characterized by allodynia is obvious and measurable.

MATERIALS AND METHODS

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 24-276). We used 44 adult female Sprague-Dawley rats (10–12 wk, 200–240 g; Japan SLC, Inc., Hamamatsu, Japan), which were housed in individual cages and given food and water *ad libitum*.

Rats were anesthetized with 1.5% of halothane in oxygen, delivered at 0.5 L/min. Sciatic nerve injury was induced using the Bennett chronic constriction injury (CCI) model,[12] with slight modification. The left side biceps femoris and the gluteus muscles were divided to expose the sciatic nerve, around which 4 loose ligatures (6-0 nylon suture) were placed at 1-mm intervals. This model induces mechanical allodynia on the ipsilateral hind paw within the first week after the injury. Upon awakening, rats were evaluated neurologically, and their food and water consumption and urine output were monitored.

One week after CCI, the majority of rats showed mechanical allodynia as revealed by hypersensitivity to von Frey hair stimulation. Eight rats exhibited no mechanical allodynia and were excluded from further experiments. The remaining rats were assigned randomly to 1 of the 2 groups. Those in the G-CSF group received intraperitoneal recombinant human G-CSF (15.0 μg/kg; Kyowa Kirin Pharma, Tokyo, Japan) dissolved in normal saline 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 on the rat spinal cord injury model.[13] 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 control and G-CSF groups were 3800 ± 500/μL and 9700 ± 700/μL, respectively.

Mechanical allodynia in rats from the vehicle and G-CSF groups (n = 10 each) was assessed using the von Frey hair, according to a previously described protocol.[14] The von Frey hair were applied 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) to the central plantar surface of the ipsilateral hind paw. Contralateral hind paw was served as control. Each filament was applied 5 times. When a rat showed a single withdrawal response to a given filament, the bending force for 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 1 day after the operations and continued for 6 consecutive weeks.

Tissue Preparation

Tissues from a subset of rats (n = 4/group) were prepared for histological evaluation 2 weeks after surgery. Animals were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS, 7.4 pH). Tissue blocks of the spinal lumbar enlargement were removed, postfixed overnight in 4% paraformaldehyde, stored for time at 4°C in 20% sucrose in PBS, and then embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). The cryoprotected samples were frozen and stored at −80°C until use. The samples were cut into serial 20-μm transverse sections with a cryostat and mounted on aminosilane-coated slides (Matsunami, Tokyo, Japan).

Immunofluorescent Labeling

For immunofluorescent labeling, sections were permeated with 0.3% Triton X in PBS and treated for 1 hour in blocking solution containing 1% bovine serum albumin and Block Ace (Dainippon Pharma, Japan). Sections were then incubated with the following primary antibodies: rabbit polyclonal anti-phosphorylated p38 mitogen-activated kinase antibody (p-p38MAPK, 1:400; Cell Signaling Technology, Beverly, MA); mouse monoclonal anti-Neu-N antibody (1:400; Chemicon Inc., Temecula, CA) for neurons; 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; AbD Serotec, Oxford,

United Kingdom) for microglia. The sections were incubated with primary antibodies overnight at 4°C, after which they were washed in PBS and then incubated for 1 hour at room temperature with secondary antibodies: Alexa 488- labeled anti-rabbit IgG (1:800; Invitrogen, Eugene, OR) and Alexa 594–labeled anti-mouse IgG (1:800; Invitrogen). Finally, the sections were washed twice in PBS and protected with coverslips. Positive labeling was observed using fluorescence microscopy (ECLIPSE E600; Nikon, Tokyo, Japan), or, in the case of double staining for p-p38MAPK/cell markers, positive signals were detected using confocal laser scanning microscopy (LSM5 PASCAL; Carl Zeiss, Jena, Germany). 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 10 sections from each animal were counted, covering a 1-mm length of spinal cord.

Western Blot Analysis

Two weeks after CCI, 10-mm sections of the spinal lumbar enlargement ipsilateral and contralateral to the injury were removed from rats in the control and G-CSF groups (n = 4/group). The tissues were homogenized in 50 mM Tris-HCl (7.4 pH), 150 mM NaCl, and 1% Triton X-100 (homogenization buffer) containing a protease inhibitor cocktail (cOmplete; Roche Diagnostics, Basel, Switzerland). The homogenates were centrifuged at 100,000*g* for 10 minutes at 4°C to remove cellular debris. Protein concentrations of the supernatants were measured using the Bradford method (Bio-Rad Dc Protein Assay Reagents; 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 of Tris-HCl, 4% sodium dodecyl sulfate, 20% glycerol, 0.02% bromophenol blue, and 10% β-mercaptoethanol. After boiling for 5 minutes, equal volumes of samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). After blocking the membrane with PBS containing 0.3% skim milk and 0.05% Tween 20, the membrane was reacted with an anti- IL-1β (BD Biosciences, Franklin Lakes, NJ), anti-p-p38MAPK (Cell Signaling Technology), and an anti-β-actin antibody as a loading control (Santa Cruz Biotechnology, Santa Cruz, CA). For detection, a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) and an ECL chemiluminescence system (GE Healthcare, Piscataway, NJ) were used. Western blot analysis was performed in triplicate for each sample. Protein bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Mechanical allodynia data from the von Frey hair test were analyzed using repeated measures ANOVA followed by a *post hoc* Fisher protected least significant difference test. Immunohistochemical results were analyzed using the Student *t* test. Results are presented as mean values ± standard error values of $P < 0.05$ were considered statistically significant.

RESULTS

Results of the von Frey filament test showed that G-CSF attenuates mechanical allodynia induced by sciatic nerve injury in the CCI model. One week after the injury, there were no significant differences between the average paw withdrawal threshold for rats in the control and G-CSF groups (controls: 7.2 ± 3.9 g; G-CSF: 9.0 ± 3.9 g, Figure 1). The administration of G-CSF caused a marked attenuation of mechanical allodynia (*i.e.*, increase in paw-withdrawal threshold) relative to that seen in the control group (Figure 1). *Post hoc* analysis with Fisher protected least significant difference (PSLD) revealed a significant increase in the paw withdrawal threshold in the G-CSF group compared with the threshold in the control group 2 weeks after injury (G-CSF 14.3 ± 3.9 g; control 6.3 ± 3.7 g), 3 weeks after injury (G-CSF 12.1 ± 2.9 g, control 6.3 ± 3.7 g), and 4 weeks after injury (G-CSF 12.7 ± 3.1 g, control 8.0 ± 6.4 g). The average paw withdrawal threshold slightly decreased in the nonaffected hind paw in both the groups; however, there were no statistical differences between both groups (Figure 1).

Immunohistochemistry for OX-42 (a marker for activated microglia) in rats from the control group revealed that the number of OX-42–positive cells was larger in the dorsal horn from the ipsilateral spinal cord lumbar enlargement than the contralateral dorsal horn (Figure 2A, B, E). In the ipsilateral dorsal horn of rats from the G-CSF group, the number of OX-42–positive cells was significantly smaller than that in control rats (Figure 2A, C, E). However, for rats in the G-CSF group, the number of OX-42–positive cells in the ipsilateral dorsal horn was larger than that in the contralateral dorsal horn (Figure 2C–E).

Immunohistochemistry for phosphorylated p38 MAPK (p-p38MAPK) showed a greater number of p-p38MAPK–

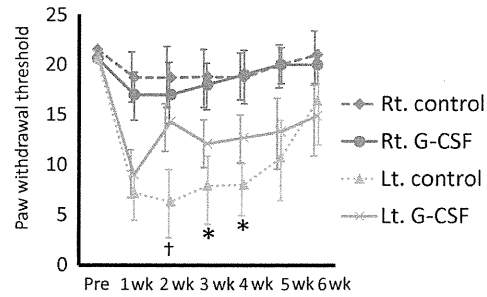

Figure 1. Mechanical allodynia data from the von Frey hair test. One week after the surgery, both groups showed decreased paw withdrawal threshold indicating mechanical allodynia. G-CSF-treated rats (circle) showed significant attenuation of paw withdrawal threshold compared with that of the control rats (square, dotted line) at 2, 3, and 4 weeks after the surgery (1, 2, and 3 weeks after G-CSF treatment). *$P < 0.05$. †$P < 0.01$. Error bar denotes standard error. G-CSF indicates granulocyte colony-stimulating factor; Rt, right; Lt, left.

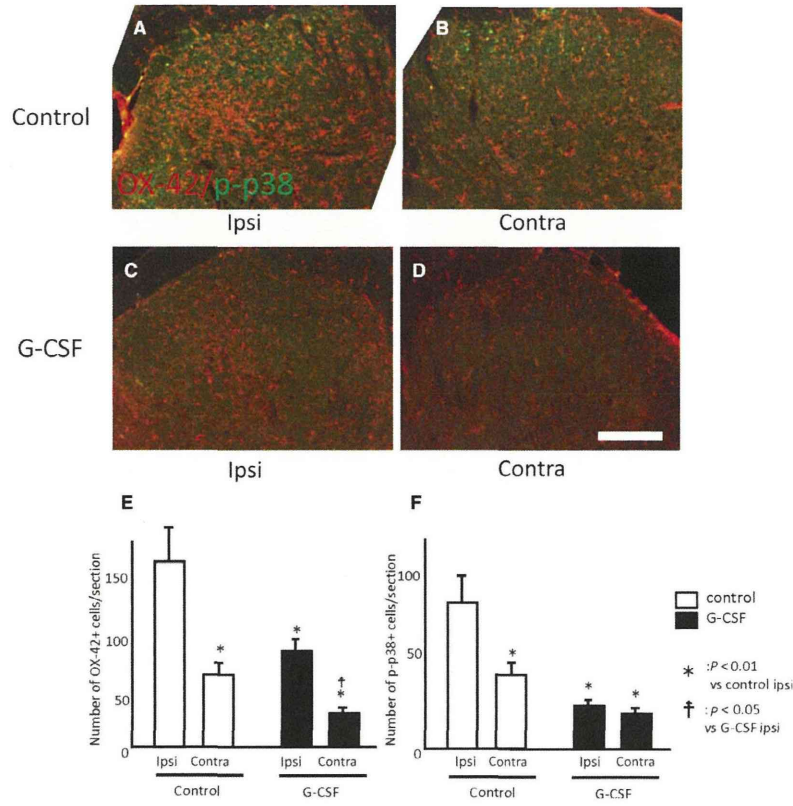

Figure 2. Immunohistochemistry for OX-42 (a marker for microglia) and p-p38MAPK. The number of OX-42–positive microglia decreased in the G-CSF group compared with that in the control group (**E**). A greater number of p-p38MAPK–positive cells in the ipsilateral dorsal horn of the spinal lumbar enlargement compared with the contralateral dorsal horn of control rats was observed (**A**, **B**, **F**). In the G-CSF group, the number of p-p38MAPK–positive cells in the ipsilateral dorsal horn was significantly smaller than that seen in sections from control rats (**A**, **C**, **F**). The number of p-p38MAPK–positive cells was larger in the ipsilateral dorsal horn than that in the contralateral dorsal horn in the G-CSF group (**C**, **D**, **F**). Scale bar = 200 μm. G-CSF indicates granulocyte colony-stimulating factor; p-p38MAPK, phosphorylated p38 mitogen-activated kinase; ipsi, ipsilateral; contra, contralateral.

positive cells in the ipsilateral dorsal horn of the spinal lumbar enlargement than the contralateral dorsal horn of control rats (Figure 2A, B, F). In the G-CSF group, the number of p-p38MAPK–positive cells in the ipsilateral dorsal horn was significantly smaller than that seen in sections from control rats (Figure 2A, C, F). The number of p-p38MAPK–positive cells was larger in the ipsilateral dorsal horn than that in the contralateral dorsal horn in the G-CSF group (Figure 2C, D, F). Immunofluorescent double staining for OX-42 and p-p38MAPK revealed that p-p38MAPK–positive cells were also positive for OX-42, indicating that a large part of p-p38MAPK–expressing cells in the spinal cord dorsal horn are microglia (Figure 3A–D). However, there were several p-p38MAPK–positive/OX-42–negative cells, indicating that p-p38MAPK was also expressed in nonmicroglial cells. Immunofluorescent double staining showed several double positive cells for GFAP and p-p38MAPK, whereas there were no double-positive cells for Neu-N and p-p38MAPK.

Western blot analysis indicated that the expression of p-p38MAPK and IL-1β protein was higher in the ipsilateral dorsal horn than in the contralateral dorsal horn (Figure 4A–D). G-CSF decreased the expression levels of both proteins in the ipsilateral dorsal horn compared with that in the control group.

DISCUSSION

The present results indicate a beneficial effect of delayed G-CSF treatment in an animal model of peripheral nerve injury-induced neuropathic pain. When administered 1 week after peripheral nerve injury, G-CSF significantly suppressed injury-induced phosphorylation of p38MAPK and upregulation of IL-1β expression, reduced the number of activated microglia, and significantly attenuated subsequent mechanical allodynia.

Our results show that CCI injury to the sciatic nerve induces allodynia and causes an increase in the number of microglia in the dorsal horn of the spinal cord on the injured side. This suggests that CCI nerve injury induces microglial activation and that the activation of spinal microglia is highly correlated with pain hypersensitivity. Many authors have reported microglia in response to nerve injury and inflammatory neuropathy in both the central and peripheral nervous systems, and it is thought that microglia may be responsible for the initiation of pain hypersensitivity induced by peripheral nerve injury.[15,16]

p38MAPK is widely known as a key signal mediator that serves as a "hub" in intracellular molecular networks related to inflammatory cytokines. Activation of p38MAPK leads to the upregulation of several inflammatory cytokines, including IL-1β.[17] Our results show that G-CSF suppresses the phosphorylation of p38MAPK and the upregulation of IL-1β. G-CSF also decreased the number of activated microglia, which are a main source of p38 in the spinal cord dorsal horn. Whether G-CSF-mediated suppression of p-p38MAPK and IL-1β results from the suppression of microglial activation, itself, or from a reduction in the number of activated

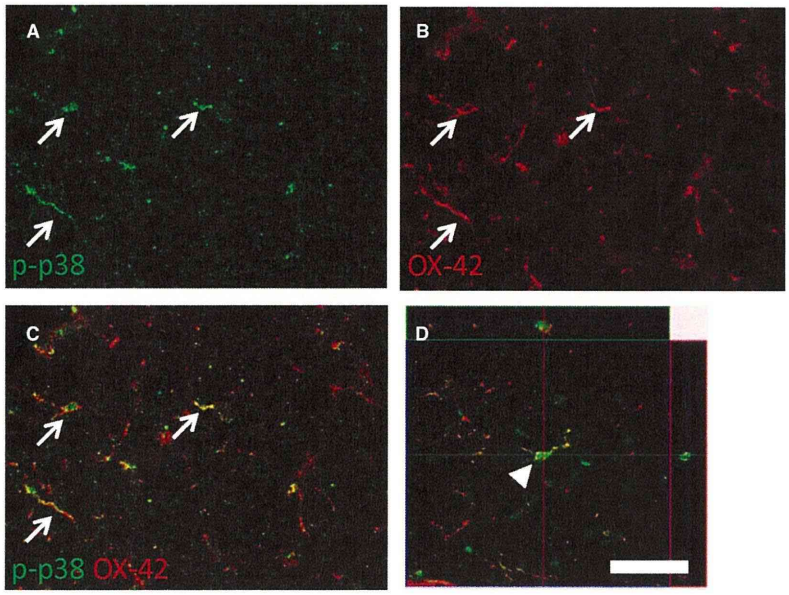

Figure 3. Immunofluorescent double staining for OX-42 and p-p38MAPK confocal laser scanning microscope was used to obtain the images. p-p38MAPK–positive cells (**A**) were also positive for OX-42 (**B**), indicating that p-p38MAPK–expressing cells in the spinal cord dorsal horn are mainly microglia. Scale bar = 100 μm. p-p38MAPK indicates phosphorylated p38 mitogen-activated kinase.

microglia remains unclear. It is also possible that an overall suppression of IL-1β upregulation might attenuate neuropathic pain, and several previous reports have indicated that IL-1β can exacerbate neuropathic pain in various animal models.[18,19] Thus, the suppression of IL-1β protein expression by G-CSF may be directly related to the alleviation of neuropathic pain.

IL-1β is known to modulate neuronal excitability by affecting neuronal receptors such as TRPV1, sodium channels, GABA receptors, and NMDA receptors. In various animal models of neuropathic pain, IL-1β expression is increased in the injured sciatic nerve, dorsal root ganglion, and spinal cord.[20,21,22,23]

In the CCI model in mice, sciatic nerve epineural injections of IL-1R1 neutralizing antibodies have been shown to reduce both thermal hyperalgesia and mechanical allodynia, suggesting a role for the upregulated IL-1β in the induction of neuropathic pain.[24,25] Additionally, in the same CCI model, mechanical allodynia was reduced by intrathecally administered IL-1β neutralizing antibody.[26]

The most important finding of this study is that delayed treatment with G-CSF effectively attenuated CCI-induced mechanical allodynia, extending previous reports showing that immediate G-CSF administration can suppress the onset of allodynia. We are not able to conclude which treatment is more effective, because we did not directly compare the therapeutic effects of immediate and delayed G-CSF treatments. In addition, not all of the animals that experience CCI surgery developed allodynia (82% in our laboratory). Thus, there is a potential to overestimate the beneficial effects of immediate treatment in experimental settings using the CCI model. In most clinical cases involving neuropathic pain, it is not realistic to treat the patient immediately after a nerve injury is sustained. By using a delayed-treatment paradigm, we have more closely approximated a clinical application and reduced the potential for measurement errors.

There were several major limitations of this study in clinical relevance. First, we used intraperitoneal injection for G-CSF administration, of which method cannot be applied for human subjects. There is significant difference in pharmacokynetics between intraperitoneal injection and intravenous injection.[27] Therefore, there might be a difference in antineuropathic effects between both methods of G-CSF administration. Next, we assessed mechanical allodynia as an indicator for neuropathic pain status. Spontaneous pain called dysesthesia, which is one of the characteristics of neuropathic pain

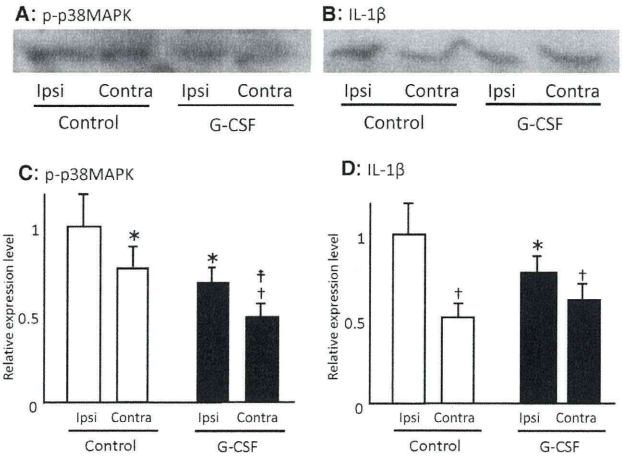

Figure 4. Western blot analysis for p-p38MAPK and IL-1β. G-CSF reduced the protein expression level of p-p38MAPK compared with that in the control group (**A**, **C**). G-CSF also reduced protein expression level of IL-1β (**B**, **D**). G-CSF indicates granulocyte colony-stimulating factor; IL-1β, interleukin-1β; p-p38MAPK, phosphorylated p38 mitogen-activated kinase; ipsi, ipsilateral; contra, contralateral.