recovery, a second comparison was performed in which patients with AIS grade A were excluded.

Assessment of adverse events

Adverse events were evaluated retrospectively by review of patient records and compared between treatment groups. Pneumonia was defined as respiratory distress accompanied by an infiltrating shadow on plain radiogram, positive sputum cultures and an elevated white blood cell count (WBC) or C-reactive protein. Urinary tract infection was defined as fever and elevated WBC in the context of positive urinary cultures. Notably, G-CSF treatment alone increases WBC, hence these criteria were excluded from the diagnosis of pneumonia and urinary tract infection in the G-CSF group. Gastric ulcers were defined as obvious ulcers of any stage observed by upper gastrointestinal fiber examination. Other adverse events were determined by review of patient records. The severity of each adverse event was assessed according to the Japanese version of the common terminology criteria for adverse events (CTCAE), version 4.0. The initial analysis of adverse events was performed on all patients, including those with AIS grade A. However, because these patients have complete paralysis which might increase the incidence of pneumonia and urinary tract infections, a second analysis was performed in which patients with severe incomplete paresis AIS grades B and C.

Statistical analysis

The ASIA motor score and the ΔASIA motor score were analyzed by the Mann–Whitney's U test. The extent of AIS grade elevation between the time of treatment and 3-month follow-up and the number of adverse events were compared between treatment groups using Fisher's exact test. A $p < 0.05$ was considered significant.

Results

Patient background data are shown in Table 1. No statistically significant differences in age, sex, mechanism of injury or injured vertebral level were observed between the groups. No statistically significant difference was observed in the baseline ASIA motor scores between the G-CSF and control groups (59.0 ± 29.6 and 50.3 ± 33.0, respectively). The ΔASIA motor score was significantly higher in the G-CSF group than in the MPSS group (27.7 ± 19.8 and 12.0 ± 11.0, respectively, $p < 0.01$) when all patients were included in the analysis.

The difference in patient background data between the groups, the MPSS group contained significantly larger number of AIS A patients who generally show poor neurological recovery, must influence the neurological outcome. Therefore, we excluded patients with AIS A complete paralysis and compared ΔASIA motor score in patients with severe incomplete paresis AIS grades B and C between both groups (12 patients in the G-CSF group and 14 patients in the MPSS group). Repeatedly, the ΔASIA motor score was also significantly higher in the G-CSF compared to the MPSS group (44.4 ± 17.2 and 17.4 ± 13.6, respectively, Fig. 1a, $p \leq 0.01$).

Next, the change in the AIS grade between the time of treatment and 3 months after treatment was compared between groups (Fig. 1b). We found that 67.9 % of patients in the G-CSF group had an AIS grade elevation of more than one step compared to 50.0 % of patients in the MPSS group, a difference that was not statistically significant. It is widely known that patients with AIS grade A complete paralysis demonstrate very little AIS grade elevation following injury. The MPSS group included more patients with AIS grade A paralysis, hence these results might underestimate the grade elevation in this group. To exclude the bias of patient background difference, we compared AIS grade change between both groups in AIS B/C patients, excluding AIS A complete paralysis patients and AIS D minor injury patients. We found that 91.7 % of patients in the G-CSF group had an

Table 1 Patient background data

	G-CSF	MPSS
Number	28	34
Male	21	27
Female	7	7
Age cause of injury	57.5 (38–72)	60.5 (18–85)
Over-turning	11	11
Falling	7	11
Road trauma	6	11
Falling Object	1	0
Sports	3	1
AIS		
A	2	9
B	4	3
C	8	11
D	14	11
Level of injury		
C2/3	0	3
C3/4	10	13
C4/5	9	5
C5/6	7	8
C6/7	2	4
Unclear case		1

The MPSS group contained significantly larger number of AIS A patients, whereas no statistically significant differences in age, sex, mechanism of injury, injured vertebral level or baseline ASIA motor score were observed between the groups

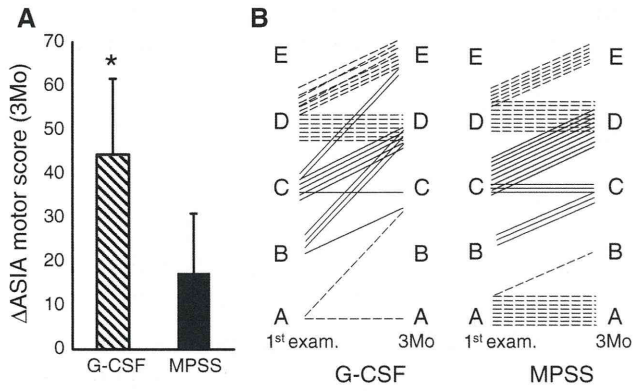

Fig. 1 Neurological recovery. The difference in patient background data between the groups, the MPSS group contained significantly larger number of AIS A patients who generally show poor neurological recovery, must influence the neurological outcome. Therefore, we excluded patients with AIS A complete paralysis and compared ΔASIA motor score in patients with severe incomplete paresis AIS grades B and C between both groups [12 patients in the G-CSF group and 14 patients in the MPSS group, (**a**)]. The ΔASIA motor score was also significantly higher in the G-CSF compared to the MPSS group [44.4 ± 17.2 and 17.4 ± 13.6, respectively, (**a**), $p < 0.01$]. Next, the change in the AIS grade between the baseline and 3 months after treatment was compared between groups (**b**). To exclude the bias of patient background difference, we compared AIS grade change between both groups in AIS B/C patients (*solid lines*), excluding AIS A complete paralysis patients and AIS D minor injury patients (*dashed line*). We found that 91.7 % of patients in the G-CSF group had an AIS grade elevation of more than one step compared to 78.6 % of patients in the MPSS group, a difference that was not statistically significant. However, we observed that 17.9 % of patients in the G-CSF group had an AIS grade elevation of two steps compared to 0 % of patients in the MPSS group ($p < 0.05$)

AIS grade elevation of more than one step compared to 78.6 % of patients in the MPSS group, a difference that was not statistically significant. However, we observed that 17.9 % of patients in the G-CSF group had an AIS grade elevation of two steps compared to 0 % of patients in the MPSS group ($p \leq 0.05$).

Finally, we compared the incidence of adverse events between treatment groups. The incidence of pneumonia was significantly higher in the MPSS group (44.1 %) compared to the G-CSF group (3.6 %). It has been shown that the severity of paralysis positively correlates with the incidence of pneumonia in patients with SCI. Hence, the fact that the MPSS group contained more patients with AIS grade A complete paralysis might have contributed to the higher incidence of pneumonia observed in the MPSS group. To exclude this bias, we analyzed the incidence of pneumonia in patients with AIS grades B/C incomplete paralysis. Again, we observed a significant difference in the incidence of pneumonia between treatment groups (42.9 % in the MPSS group and 8.3 % in the G-CSF group, Table 2, $p < 0.05$).

No significant difference in the incidence of urinary tract infections was observed between groups (35.7 % in the MPSS group and 16.7 % in the G-CSF group).

Table 2 Incidence of adverse events in AIS B/C incomplete paralysis patients

	G-CSF ($n = 12$)	MPSS ($n = 14$)	p-value
Pneumonia	1 (8.3 %)	6 (42.9 %)	$p < 0.05$
Urinary tract infection	2 (16.7 %)	5 (35.7 %)	$p = 0.17$
Gastric ulcer	0 (0 %)	2 (14.3 %)	$p = 0.27$

The difference in patient background data between the groups, the MPSS group contained significantly larger number of AIS A patients who can be easily affected with pneumonia, must influence the incidence of pneumonia. Therefore, we compared the incidence of pneumonia in incomplete paralysis patients of both groups, the result showed significant difference between G-CSF and MPSS groups

The incidence of gastric ulcers tended to be higher in the MPSS group compared to the G-CSF group (14.7 and 0 %, respectively, $p = 0.051$). When patients with AIS grade A and D were excluded from the analysis, no significant difference was observed between treatment groups.

Discussion

In the present study, the G-CSF group showed better neurological recovery compared to the MPSS group. Moreover, the incidence of severe adverse events is less frequent in patients treated with G-CSF than in patients treated with MPSS.

The MPSS group contained significantly larger number of AIS A patients who generally show poor neurological recovery, must influence the neurological outcome. Therefore, we assessed neurological outcome in severe incomplete paralysis patients (excluding AIS A and D patients) between both groups. Repeatedly, the G-CSF group showed better neurological recovery compared to the MPSS group, suggesting the superior neuroprotective potential of G-CSF treatment in SCI.

We observed that the incidence of pneumonia was significantly higher in patients treated with MPSS than in patients treated with G-CSF. The difference in patient background data between the groups, the MPSS group contained significantly larger number of AIS A patients who can be easily affected with pneumonia, must influence the incidence of pneumonia. Therefore, we compared the incidence of pneumonia in severe incomplete paralysis patients of both groups, the result repeatedly showed significant difference between G-CSF and MPSS groups (Table 2).

Methylprednisolone sodium succinate is a widely recognized immunosuppressant. In addition, spinal cord injury itself can induce systemic immunosuppression [14]. Hence, the immunosuppressive effects of SCI and MPSS may function in an additive or synergistic manner, increasing

the incidence of infections. In contrast, G-CSF increases the number of white blood cells in the peripheral blood. This feature of G-CSF is used clinically to treat neutropenia and prevent infectious complications. In this manner, G-CSF treatment might decrease the incidence of infections in SCI patients. Previous studies have reported a 31.4 % incidence of pneumonia in SCI patients with a Frankel Grade of A, B, or C [15]. Matsumoto reported a 30.4 % incidence of pneumonia and a 4.3 % incidence of urinary tract infections in SCI patients who received MPSS [16]. We cannot directly compare the incidence of infections between the present study and previous reports, and the anti-infection properties of G-CSF remain to be clarified, but our findings suggest that the incidence of pneumonia might be reduced in patients treated with G-CSF compared to those treated with MPSS.

We observed a lower incidence of gastric ulcers in patients treated with G-CSF than in patients treated MPSS. When we analyzed the incidence of ulcers among patients with severe incomplete paralysis (AIS grades B and C) to exclude the bias introduced by the difference in paralysis severity between the groups, no significant difference was observed. Treatment of gastric ulcers has been dramatically improved by the increased use of proton pump inhibitors. Our results might thus reflect this change in ulcer prophylaxis and treatment.

Those findings suggest that G-CSF treatment has a lower risk of severe adverse events than MPSS treatment. Hence, G-CSF may be a reasonable alternative to MPSS, but a direct comparison of the efficacy of each drug is needed.

As for the cost, the price of G-CSF (300 μg) in Japan is 24,926 yen (175.2 Euro in the rate of Jan. 26, 2014).We employed the G-CSF dose regimen of 10 μg/kg/d × 5 days. Therefore, the total cost of G-CSF therapy in patient with 60 kg body weight is 249,260 yen (1,752.05 Euro). MPSS (500 mg) costs 3,536 yen (24.85 Euro). The dose regimen of MPSS in NASCIS 2 is 5.4 mg/kg as a bolus injection followed by 5.4 mg/kg/h for 23 h. Therefore, the total cost of MPSS therapy protocol in patient with 60 kg body weight is 67,184 yen (472.17 Euro). The cost of G-CSF therapy is higher than that in the MPSS therapy, of which difference in total cost is 182,816 yen (1,284.83 Euro).

The present study has several major limitations. First, the patients were not randomly allocated to the treatment groups. Second, the control group was historical. Third, the number of patients was too small to prove the efficacy of G-CSF treatment with sufficient statistical power. Finally, the timing of treatment initiation differed between treatment groups (within 8 h after injury in the MPSS group and within 48 h after injury in the G-CSF group).

The results of the current study suggest that G-CSF administration is both safe and effective. Although we cannot draw conclusions about the efficacy of G-CSF without prospective randomized controlled trial, the present results encourage us to make step forward to perform next phase of clinical trial.

Conflict of interest None.

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Chapter 13
Granulocyte Colony-Stimulating Factor-Mediated Neuroprotective Therapy for Spinal Cord Injury

Masao Koda, Takeo Furuya, Taigo Ianada, Koshiro Kamiya, Mitsutoshi Ota, Satoshi Maki, Akihiko Okawa, Kazuhisa Takahashi, and Masashi Yamazaki

Abstract To prove the efficacy of granulocyte colony-stimulating factor (G-CSF) for spinal cord injury (SCI), we performed several animal experiments in rodent SCI models. Through those experiments, we showed G-CSF's mechanisms of action for SCI.

G-CSF showed efficacy for SCI through mobilization of bone marrow-derived cells. G-CSF attenuated neuronal cell death in vitro and in vivo, resulting in promotion of functional recovery after SCI. Expression of IL-1β and TNF-α was significantly suppressed by G-CSF in the acute phase of SCI. G-CSF promoted upregulation of anti-apoptotic protein Bcl-Xl on oligodendrocytes and suppressed apoptosis of oligodendrocytes after SCI. G-CSF exerted neuroprotective effects via promotion of angiogenesis after SCI.

G-CSF's current use in the clinic for hematopoietic stimulation and its ongoing clinical trial for brain infarction make it an appealing molecule that could be rapidly placed into trials for acute SCI patients. G-CSF is one of the hopeful candidates for clinical application.

Keywords G-CSF • Neuroprotection • Secondary injury

13.1 Introduction

The pathologies following acute spinal cord injury (SCI) are divided into two sequential events: the primary injury and the secondary injury [1]. Direct mechanical trauma induces the primary injury, which includes the spinal cord tissue damage.

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This initial insult then triggers a progressive wave of secondary injury, which exacerbates the injury to the spinal cord via the activation of pathophysiological mechanisms.

Known pathophysiological mechanisms of the secondary injury after SCI include ischemia, posttraumatic inflammatory response mediated by resident microglia and blood-derived inflammatory cells, release of excitatory amino acids, generation of reactive oxygen species, influx of Ca^{2+}, and so on [1]. Those multiple mechanisms instigate neuronal and glial cell death, resulting in exaggeration of tissue damage after SCI.

Secondary injury is the main therapeutic target for various kinds of drug therapies. Thus a huge effort has been expended by clinicians, basic scientists, and industry to discover effective neuroprotective agents which can act against mechanisms of the secondary injury following SCI [1].

Currently, high-dose methylprednisolone sodium succinate (MPSS) is the only clinically available treatment for acute SCI to reduce the secondary injury. In recent years, however, the use of high-dose MPSS in acute SCI has become controversial, largely based on the risk of serious adverse effects versus what is perceived to be a modest neurological benefit [2]. Therefore, development of new SCI drug therapies that could replace high-dose MPSS is an area of intense study.

Granulocyte colony-stimulating factor (G-CSF) is a 19.6 kDa glycoprotein that was identified initially as a serum component that induced differentiation of the murine myelomonocytic leukemic cell line and is capable of inducing the survival, proliferation, and differentiation of cells of neutrophil lineage [3, 4]. In addition to its effects as a hematopoietic cytokine, it was recently reported that G-CSF has the potential to promote the survival of other types of cells, including in ischemic myocardium [5]. In the central nervous system, G-CSF has direct neuroprotective effects against glutamate-induced neuronal death and stroke [6, 7]. Most recently, clinical trials have reported on the safety and feasibility of G-CSF administration following stroke, supporting the hypothesis that G-CSF may also be an effective therapeutic for SCI [8].

To prove the efficacy of G-CSF for SCI, we performed several animal experiments in rodent SCI models. Here we show the results of those experiments, indicating G-CSF's mechanism of action for SCI.

13.1.1 G-CSF Receptor Expression

To assess the expression of G-CSF receptor (G-CSFR), we performed immunofluorescence double staining on histological sections of spinal cords. The data revealed that G-CSFR was expressed on neurons, astrocytes, and oligodendrocytes in normal spinal cord (Fig. 13.1). According to the expression pattern of G-CSFR, we speculated that G-CSF can act on neuron, astrocyte, and oligodendrocyte.

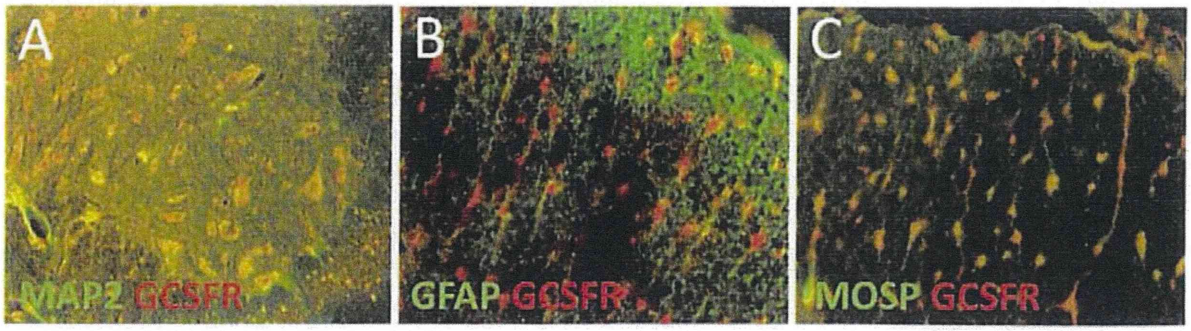

Fig. 13.1 Expression of granulocyte colony-stimulating factor receptor (G-CSFR) in normal spinal cord. Immunohistochemistry for G-CSFR and cell type markers was performed. G-CSFR was expressed by MAP2+ neurons (**a**), GFAP+ astrocytes (**b**), and MOSP+ oligodendrocytes (**c**)

13.1.2 G-CSF Promotes Migration of Bone Marrow-Derived Stem Cells into Injured Spinal Cord

To elucidate the effects of G-CSF-mediated mobilization of bone marrow-derived stem cells on the injured spinal cord, we constructed bone marrow chimera mice. Bone marrow cells of green fluorescent protein (GFP) transgenic mice were transplanted into lethally irradiated C57BL/6 mice. Four weeks after bone marrow transplantation, a large part of the bone marrow cells of those chimera mice was GFP-positive, enabling the tracking of bone marrow-derived cells by green fluorescence. SCI was produced by a static load (20 g, 5 min) at T8 level on those chimera mice. G-CSF (200 μg/kg/d) was injected subcutaneously for 5 days. Immunohistochemistry for GFP and cell lineage markers was performed to evaluate G-CSF-mediated mobilization of bone marrow-derived cells into injured spinal cord. Hind limb locomotor recovery was assessed for 6 weeks.

Immunohistochemistry revealed that G-CSF increased the number of GFP-positive cells in injured spinal cord, indicating that G-CSF promoted mobilization of bone marrow-derived cells and enhanced migration of those cells into injured spinal cord. The numbers of double-positive cells for GFP and glial markers were larger in the G-CSF-treated mice than in the control mice. G-CSF-treated mice showed significant recovery of hind limb function compared to that of the control mice. G-CSF showed efficacy for SCI treatment through mobilization of bone marrow-derived cells [9].

13.1.3 G-CSF Suppresses Apoptosis of Neurons After SCI

To elucidate the direct neuroprotective effect of G-CSF, we performed in vitro experiments using cultured neurons and in vivo experiments using mouse compressive SCI model. We found that G-CSF is neuroprotective against glutamate-induced cell death of cerebellar granule neurons in vitro.

Next, we used a mouse model of compressive SCI to examine the neuroprotective potential of G-CSF in vivo. Histological assessment with cresyl violet staining

revealed that the number of surviving neurons in the injured spinal cord was significantly increased in G-CSF-treated mice. Immunohistochemistry for neuronal apoptosis revealed that G-CSF suppressed neuronal apoptosis after SCI. Moreover, administration of G-CSF promoted hind limb functional recovery. G-CSF might promote functional recovery by inhibiting neuronal apoptosis after SCI [10].

13.1.4 G-CSF Suppresses Inflammatory Cytokine Expression After SCI

To elucidate the potential therapeutic effect of G-CSF for SCI in rats, rat contusive SCI was introduced using the infinite horizon impactor (magnitude, 200 kilodyne). Recombinant human G-CSF (15.0 μg/kg) was administered by tail vein injection for 5 days. To detect the anti-inflammatory effects of G-CSF in the SCI model, we performed real-time PCR for inflammatory cytokines on the spinal cord sample of G-CSF and control rats. Twelve hours after surgery, expression of IL-1β and TNF-α mRNAs was significantly suppressed in the G-CSF group compared to the vehicle control group. The results of real-time PCR for the other factors showed no significant difference between the vehicle and G-CSF-treated groups. According to these results, G-CSF suppresses inflammatory cytokine expression after SCI [11].

13.1.5 G-CSF Suppresses Apoptosis of Oligodendrocytes and Protects Myelin After SCI

To elucidate anti-apoptotic effect of G-CSF on oligodendrocyte, in vivo experiments using rat contusive SCI introduced by the IH impactor (200 kilodyne) were performed. Recombinant human G-CSF (15.0 μg/kg) was administered by tail vein injection for 5 days. Histological assessment with luxol fast blue staining revealed that the area of white matter spared in the injured spinal cord was significantly larger in G-CSF-treated rats. Immunohistochemical analysis showed that G-CSF promoted upregulation of anti-apoptotic protein Bcl-Xl on oligodendrocytes and suppressed apoptosis of oligodendrocytes after SCI (Fig. 13.2). Moreover, administration of G-CSF promoted better functional recovery of hind limbs assessed by BBB locomotor scale [11].

13.1.6 G-CSF Promotes Angiogenesis After SCI

Because the degree of angiogenesis in the subacute phase after SCI correlates with regenerative responses, it is possible that G-CSF's neuroprotective effects after SCI are due to enhancement of angiogenesis. We utilized the contusive SCI rat model using IH impactor and randomly divided subjects between a G-CSF-treated group and a control group. In the G-CSF-treated rats, the total number of vessels was

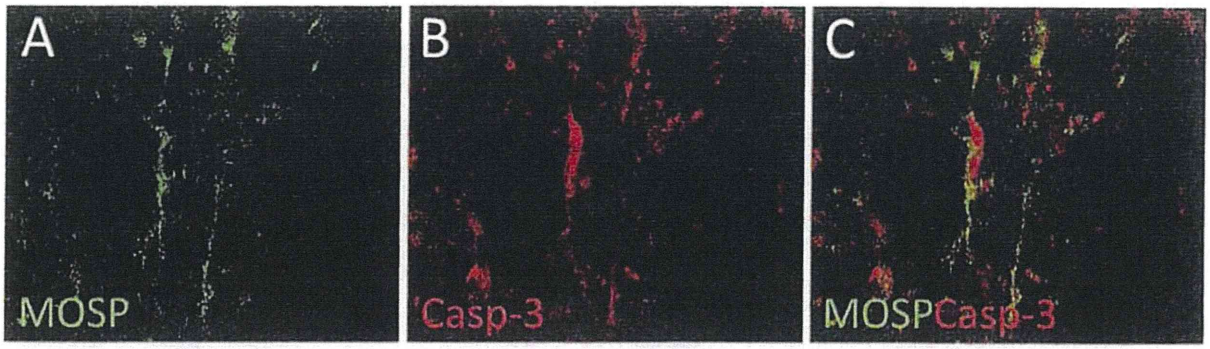

Fig. 13.2 Apoptosis of oligodendrocytes. Immunohistochemistry for oligodendrocyte marker MOSP (**a**) and apoptosis marker cleaved caspase-3 (Casp-3) (**b**) was performed. There were a lot of (c) MOSP- and Casp-3 double-positive apoptotic oligodendrocytes in injured spinal cord 1 week after SCI. In G-CSF-treated rats, the number of apoptotic oligodendrocytes decreased

significantly larger, and expression of angiogenic cytokines including bFGF, VEGF, and HGF was significantly higher than those in the control group. The G-CSF-treated group showed significant recovery of hind limb function compared to that of the control group. These results suggest that G-CSF exerts neuroprotective effects via promotion of angiogenesis after SCI [12].

13.2 Discussion

One of the major obstacles for conducting clinical trials for neuroprotective drugs is to first establish the safety and competency for use in human subjects. The complexity, size, and duration of clinical trials of novel drugs often make them quite costly to conduct and may impede the development of therapeutics that could have a significant impact in clinical practice. Therefore, although the efficacy of various drug therapies in models of SCI has been reported, few drugs have been practically carried into clinical trials. Thus, drugs with proven clinical exploitability have a significant advantage for clinical trials for novel therapeutic purposes. From this point of view, G-CSF's current use in the clinic for hematopoietic stimulation and its ongoing clinical trial for brain infarction make it an appealing molecule that could be rapidly placed into trials for acute SCI patients. Although many hurdles such as optimal dosage, therapeutic time window, and more precise mechanism of action still need to be resolved, the present results encourage us to make steps towards future clinical trials of G-CSF for acute SCI patients.

13.3 Conclusion

G-CSF exerts neuroprotective action for SCI via the abovementioned pleiotropic effects. Therefore G-CSF is one of the hopeful candidates for clinical application.

Acknowledgement Masao Koda declares that he has no conflict of interest.

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Multicenter Prospective Nonrandomized Controlled Clinical Trial to Prove Neurotherapeutic Effects of Granulocyte Colony-Stimulating Factor for Acute Spinal Cord Injury

Analyses of Follow-up Cases After at Least 1 Year

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Study Design. An open-labeled multicenter prospective nonrandomized controlled clinical trial.

Objective. To confirm the feasibility of using granulocyte colony-stimulating factor (G-CSF) for treatment of acute spinal cord injury (SCI).

Summary of Background Data. We previously reported that G-CSF promotes functional recovery after compression-induced SCI in mice. On the basis of these findings, we conducted a multicenter prospective controlled clinical trial to assess the feasibility of G-CSF therapy for patients with acute SCI.

Methods. The trial ran from August 2009 to March 2011, and included 41 patients with SCI treated within 48 hours of onset. Informed consent was obtained from all patients. After providing consent, patients were divided into 2 groups. In the G-CSF group (17 patients), G-CSF (10 μg/kg/d) was intravenously administered for 5 consecutive days, and in the control group (24 patients), patients were similarly treated except for the G-CSF administration. We evaluated motor and sensory functions using the American Spinal Cord Injury Association score and American Spinal Cord Injury Association impairment scale at 1 week, 3 months, 6 months, and 1 year after onset.

Results. Only 2 patients did not experience American Spinal Cord Injury Association impairment scale improvement in the G-CSF group. In contrast, 15 patients in the control group did not experience American Spinal Cord Injury Association impairment scale improvement. In the analysis of increased American Spinal Cord Injury Association motor score, a significant increase in G-CSF group was detected from 1 week after the administration compared with the control group. After that, some spontaneous increase of motor score was detected in control group, but the significant increase in G-CSF group was maintained until 1 year of follow-up.

Conclusion. Despite the limitation that patient selection was not randomized, the present results suggest the possibility that G-CSF administration has beneficial effects on neurological recovery in patients with acute SCI.

Key words: spinal cord injury, secondary injury, G-CSF, clinical trial.

Level of Evidence: 3

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There are currently more than 200,000 people with spinal cord injuries (SCI) in Japan, and about 5000 new cases are diagnosed every year.[1] Thus far, however, there has been no curative treatment of SCI, and the degree of neurological recovery has been dependent on the magnitude of