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F. 健康危険情報

該当なし

G. 研究発表

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該当なし

H. 知的財産権の出願・登録状況

（予定を含む）

１．特許取得

該当なし

２．実用新案登録

該当なし

３．その他

該当なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書　籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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Ⅳ．研究成果の刊行物・別刷

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.

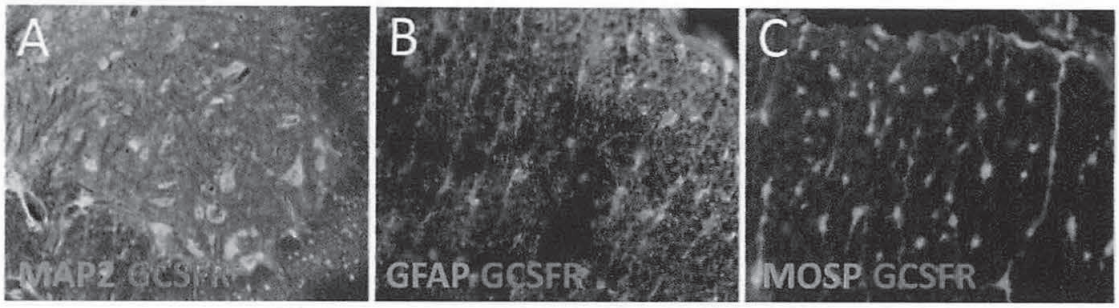

Fig. 13.1 Expression of granulocyte colony-stimulating factor receptor (G-CSFR) in normal spinal cord. Immunohistochemistry for G-CSFR and cell type markers was performed. G-CSFR was expressed by MAP2+ neurons (**a**), GFAP+ astrocytes (**b**), and MOSP+ oligodendrocytes (**c**)

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

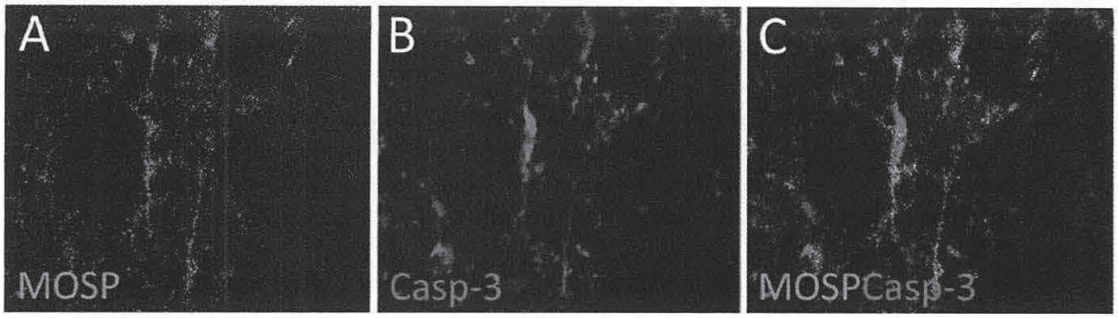

Fig. 13.2 Apoptosis of oligodendrocytes. Immunohistochemistry for oligodendrocyte marker MOSP (**a**) and apoptosis marker cleaved caspase-3 (Casp-3) (**b**) was performed. There were a lot of (**c**) MOSP- and Casp-3 double-positive apoptotic oligodendrocytes in injured spinal cord 1 week after SCI. 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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Chapter 27
Neuroprotective Therapy Using Granulocyte Colony-Stimulating Factor for Acute Spinal Cord Injury: A Multicenter Prospective Controlled Clinical Trial

Masashi Yamazaki, Hiroshi Takahashi, Takeo Furuya, and Masao Koda

Abstract We conducted a multicenter prospective controlled clinical trial to assess the feasibility of neuroprotective therapy using granulocyte colony-stimulating factor (G-CSF) for patients with acute spinal cord injury (SCI). The trial ran from August 2009 to March 2011 and included 45 SCI patients treated within 48 h of onset. Informed consent was obtained from all patients. After providing consent, patients were divided into two groups. In the G-CSF group (19 patients), G-CSF (10 μg/kg/day) was intravenously administered for five consecutive days, and in the control group (26 patients), patients were similarly treated except for the G-CSF administration. We evaluated motor functions using the American Spinal Cord Injury Association (ASIA) score 3 months after onset. The increase in ASIA motor score was significantly higher in the G-CSF group (26.1 ± 18.9) than in the control group (12.2 ± 14.7) ($P < 0.01$). In cases of incomplete paralysis (18 patients in the G-CSF group and 19 patients in the control group), the increase in motor score was also significantly higher ($P < 0.05$) in the G-CSF group (27.1 ± 18.9) than in the control group (15.1 ± 15.9). The present results suggest the possibility that G-CSF administration has beneficial effects on neurological recovery in patients with acute SCI. We believe that neuroprotection using G-CSF is an effective therapeutic strategy for acute SCI treatment.

Keywords Clinical trial • G-CSF • Neuroprotective therapy • Spinal cord injury

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27.1 Introduction

In Japan, there are currently more than 200,000 people with spinal cord injuries (SCI), and about 50,000 new SCI patients are diagnosed every year. Thus far, however, there has been no treatment to cure SCI, and neurological recovery has been determined according to the degree of injury at the onset. When SCI occurs, the primary injury is mechanical stress to the spinal cord. After that, secondary injury occurs, i.e., an inflammatory reaction caused by the release of pro-inflammatory cytokines [1]. It is conceivable that methylprednisolone sodium succinate (MPSS) relieves secondary injury to the spinal cord [2, 3]. However, many have questioned the efficacy of MPSS [4–7], and the side effects in the respiratory system and digestive organs are often critical for patients [5, 8]. Because of these reports, development of new therapeutic drugs for SCI has been expected.

Granulocyte colony-stimulating factor (G-CSF) is a 19.6-kDa glycoprotein. It is best known as a growth factor for hematopoietic progenitor cells and is commonly used to treat neutropenia and to mobilize peripheral blood-derived hematopoietic stem cells for transplantation [9, 10]. Several recent reports have indicated that G-CSF also has non-hematopoietic functions and can potentially be used for the treatment of neuronal injury, including stroke and neurodegenerative diseases [11–15]. Thus, we hypothesized that administration of G-CSF might have beneficial neuroprotective effects for acute SCI and examined this hypothesis using a compression-induced SCI model in rodents. We previously reported that G-CSF promotes functional recovery after compression-induced SCI and contusive SCI in mice [16–19]. The mechanisms by which G-CSF enhances recovery after SCI are as follows: G-CSF mobilizes bone marrow-derived cells to the injured spinal cord, where it directly suppresses neuronal apoptosis, suppresses the death of oligodendrocytes, protects myelin, suppresses the expression of inflammatory cytokines such as TNF-α and IL-1β, and facilitates arterialization [16–19].

Based on these findings, we conducted a multicenter prospective controlled clinical trial to assess the feasibility of neuroprotective therapy using G-CSF for patients with acute SCI.

27.2 Clinical Trial of G-CSF Neuroprotective Therapy for Acute Spinal Cord Injury

27.2.1 Study Design and Population

This clinical trial was an open-label, multicenter, prospective controlled study and was performed with the approval of the Institutional Review Board of each institute. Starting in August 2009, we recruited SCI patients within 48 h of onset. We restricted the age of the study patients to 16–85 years old. We excluded patients in the following categories: (1) those with intracranial pathologies (e.g., tumors, infection, or ischemia); (2) those having a history of major bleeding requiring blood transfusion

or a history of leukopenia, thrombocytopenia, or 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 provided informed consent for participation in the trial.

We assigned patients to a G-CSF group or a control group. The G-CSF group was given G-CSF (Gran®, Kyowa Hakko Kirin, Tokyo) intravenously at 10 μg/kg/day for five consecutive days. The control group received similar treatments as the G-CSF group except for the G-CSF administration. Regarding patient selection, G-CSF therapy was performed only at Chiba University Hospital. At the institution, all SCI patients fulfilling the inclusion criteria described above underwent G-CSF administration during the study period. At the other institutes, patients were treated without G-CSF administration. No patient in either the G-CSF or control group was given MPSS in the follow-up period. Surgeries for spinal decompression and stabilization were performed as soon as possible after injury in both groups.

27.2.2 Evaluation of Safety and Feasibility

We evaluated motor and sensory functions of the patients using the American Spinal Cord Injury Association (ASIA) score (motor score; range, 0–100) [20] and ASIA impairment scale (AIS grade; range, A to E). In the present study, two orthopedic spine surgeons specializing in cervical spine surgery evaluated patients' neurological status independently at 1 week and 3 months after onset and calculated the mean data. The administration of G-CSF was kept unknown to the investigators until the end of the study. In addition, we analyzed hematological data from the treated patients. We also evaluated adverse events related to G-CSF therapy. Patients were asked daily to describe their responses regarding common G-CSF side effects.

Results are presented as means ± standard deviation of the mean. Statistical analysis was performed using the Mann–Whitney *U* test for the improvement of ASIA scores, Fisher's exact probability test for the improvement of AIS grade, and Student's t-test for the blood data. A *P* value less than 0.05 was considered statistically significant.

27.2.3 Patient Data

From August 2009 to March 2011, a total of 56 patients were enrolled in this trial. Among them, 45 patients were followed up after G-CSF administration or initial treatment for 3 months and were evaluated. Twenty-six patients were enrolled in the G-CSF group. In one patient, however, fever developed the day after initiation of G-CSF administration, so administration was discontinued. Five cases dropped out during follow-up, and one case demonstrated postoperative paralysis. Thus, we excluded these seven patients from the study. Finally, 19 patients who received

Table 27.1 Patient data for G-CSF and control groups

	G-CSF	Control	*P*
Number of cases	19	26	
Gender			
Male	13	20	
Female	6	6	
Age (years)			
	57.7 ± 9.34 (38–68)	58.5 ± 18.0 (23–85)	0.871
Cause of injury			
Fall	11	16	
Road trauma	6	8	
Sports	1	1	
Falling object	1	0	
Others	0	1	
Level of injury			
C2/3	0	2	
C3/4	5	7	
C4/5	8	7	
C5/6	4	8	
C6/7	2	2	
ASIA impairment scale (AIS)			
A	1	7	
B	2	2	
C	5	5	
D	11	12	
Time of first examination after injury (h)	3.95 ± 2.74 (1–12)	10.3 ± 14.6 (1–48)	0.068
Time of G-CSF administration after injury (h)	32.4 ± 16.6 (6–48)		

G-CSF were evaluated (Table 27.1). Thirty patients were enrolled in the control group. Among them, three cases dropped out during follow-up. In one additional case, no motor paralysis was present. Thus, we excluded these four cases, leaving 26 patients in the control group for evaluation (Table 27.1).

27.2.4 AIS

The change of AIS grade between the first examination and 3 months after onset is shown in Table 27.2. In the analysis of total cases, AIS grade improved at least one step in 11 of 19 (57.9 %) patients in the G-CSF group and in 9 of 26 (34.6 %) patients in the control group (Table 27.3). In cases of incomplete paralysis (AIS grade at first examination: B, C, or D), AIS grade improved at least one step in 11 of 18 (61.1 %) patients in the G-CSF group and in 6 of 15 (31.6 %) patients in the control group (Table 27.3). Furthermore, when we restricted the subjects to incomplete paralysis cases with severe and moderate symptoms (AIS grade at first

Table 27.2 ASIA impairment scale (AIS)

G-CSF group						Control group					
Grade at first examination	Grade at 3 months after onset					Grade at first examination	Grade at 3 months after onset				
	A	B	C	D	E		A	B	C	D	E
A	1					A	4	2	1		
B			1	1		B		1	1		
C				4	1	C		1	2	2	
D				7	4	D				9	3

AIS grade
A: complete paralysis
B: sensory incomplete paralysis, motor complete paralysis
C: motor incomplete paralysis (muscle grading <3/5)
D: motor incomplete paralysis (muscle grading >3/5)
E: normal

Table 27.3 Improvement of AIS

	G-CSF (%)	Control (%)	*P*
Total cases	11/19 (57.9)	9/26 (34.6)	0.106
Incomplete paralysis cases (AIS: B, C, D)	11/18 (61.1)	6/15 (31.6)	0.07
Incomplete paralysis cases (AIS: B, C)	7/7 (100)	3/7 (42.9)	0.035*

*$P<0.05$
Data are expressed as number of neurologically improved patients/number of examined patients (neurological improvement was defined as an increase in at least one clinical grade)

examination: B or C), AIS grade improved at least one step in all 7 patients in the G-CSF group and in 3 of 7 (42.9 %) patients in the control group ($P<0.05$) (Table 27.3).

27.2.5 ASIA Motor Score

In the analysis of total cases, the ASIA motor score at the first examination was 61.6±27.3 in the G-CSF group and 51.3±36.6 in the control group; thus, scores were higher in the G-CSF group, although this difference was not statistically significant (Table 27.4). At 1 week after onset, the improvement in motor score was greater in the G-CSF group (increased points: 13.6±11.3) than in the control group (increased points: 2.69±7.9) ($P<0.01$). Still greater improvement in scores was observed at the three-month follow-up in the G-CSF group (increased points: 26.1±18.9) compared to the control group (increased points: 12.2±14.7) ($P<0.01$) (Table 27.4).

In cases of incomplete paralysis (AIS grade at first examination: B, C, or D), the ASIA motor score at the first examination was 63.6±26.8 in the G-CSF group and 65.0±32.6 in the control group; the difference between groups was not statistically significant (Table 27.5). At 1 week after onset, greater improvement in motor score

Table 27.4 ASIA motor score (total cases)

	G-CSF ($n=19$)	Control ($n=26$)	P
At first examination	61.6±27.3 (14–98)	51.3±36.6 (0–97)	0.497
1 week after onset	75.2±23.4 (27–100)	54.0±37.3 (0–100)	0.075
Increased points for 1 week	13.6±11.3 (1–50)	2.69±7.9 (−11–24)	0.0011*
3 months after onset	87.7±17.5 (35–100)	63.7±36.5 (0–100)	0.052
Increased points for 3 months	26.1±18.9 (1–73)	12.2±14.7 (−7–48)	0.0067*

Patients whose AIS grade at the first examination was A, B, C, or D were analyzed
*$P<0.01$

Table 27.5 ASIA motor score (incomplete paralysis cases)

	G-CSF ($n=18$)	Control ($n=19$)	p
At first examination	63.6±26.8 (14–98)	65.1±32.6 (7–97)	0.475
1 week after onset	77.4±21.9 (27–100)	68.7±31.9 (6–100)	0.574
Increased points for 1 week	13.9±11.6 (1–50)	3.58±9.0 (−11–24)	0.0049*
3 months after onset	90.6±12.3 (51–100)	80.2±26.3 (19–100)	0.417
Increased points for 3 months	27.1±18.9	15.1±15.9	0.044**

Patients whose AIS grade at the first examination was B, C, or D were analyzed
*$P<0.01$
**$P<0.05$

was observed in the G-CSF group (increased points: 13.9±11.6) than in the control group (increased points: 3.58±9.0) ($P<0.01$). The improvement was still observed at the three-month follow-up in the G-CSF group (increased points: 27.1±18.9) compared with the control group (increased points: 15.1±15.9) ($P<0.05$) (Table 27.5).

27.2.6 Blood Data

In the G-CSF group, white blood cell (WBC) counts immediately before G-CSF administration averaged 9.55±3.58 ($\times 10^3/mm^3$). During the administration, WBC counts increased to 34.2±10.9 ($\times 10^3/mm^3$), ranging from 12.4 to 56.9 ($\times 10^3/mm^3$) (Table 27.6). G-CSF mobilized cells of the neutrophil lineage, while lymphocytes were unaffected (Table 27.6). G-CSF also increased the level of circulating monocytes and basophils. There was no significant change in inflammation during G-CSF administration, as indicated by C-reactive protein levels (Table 27.6).

27.2.7 Adverse Events

In one patient in the G-CSF group, fever >40 °C developed the day after the start of G-CSF administration, and administration was discontinued. The cause of the fever proved to be from a urinary tract infection, and the infection was relieved by administration of antibiotics. No relationship was found between the infection and G-CSF