the initial injury. SCI is generally divided into 2 consecutive stages: the primary injury and the secondary inflammatory response. The primary injury is caused by a mechanical stress to the spinal cord. Subsequently, the secondary injury occurs, consisting of an inflammatory reaction caused by the release of proinflammatory cytokines.[2] The secondary injury is a potential target for pharmacological interventions. It has been suggested that methylprednisolone sodium succinate (MPSS) can relieve secondary injury to the spinal cord.[3,4] In recent years, however, questions regarding the efficacy of MPSS,[5–8] and its side effects on the respiratory system and digestive systems have arisen.[9] Hence, new pharmacological agents to replace MPSS would be desirable.

Granulocyte colony-stimulating factor (G-CSF) is a 19.6-kDa glycoprotein. It has been well characterized as a growth factor for hematopoietic progenitor cells and is commonly used to treat neutropenia and to mobilize bone marrow hematopoietic stem cells for transplantation.[9,10] Several recent reports have indicated that G-CSF also has nonhematopoietic functions and can potentially be used to treat neuronal injury in stroke and neurodegenerative diseases.[12–16] Thus, we hypothesized that administration of G-CSF might have neurotherapeutic effect on acute SCI. We initially examined this hypothesis in a rodent model of SCI. We previously reported that G-CSF promotes functional recovery after SCI in rodents by mobilizing bone marrow-derived cells to the injured spinal cord, suppressing neuronal apoptosis and oligodendrocyte death, protecting myelin, decreasing the expression of inflammatory cytokines such as TNF-α and IL-1β, and facilitating arterialization.[17–20]

On the basis of those findings, we initiated phase I and IIa clinical trials to confirm the safety and feasibility of neurotherapeutic G-CSF administration for patients with acute SCI. We observed no severe adverse events either during or after G-CSF administration. We determined that a dose of 10 μg/kg/d for 5 days could be safely administered, and that this dose improved the neurological American Spinal Cord Injury Association (ASIA) motor score in patients with SCI.[21] However, the long term effects of G-CSF therapy and the degree of neurological recovery after G-CSF administration remain unclear, in part because some neurological improvement is observed in untreated patients with incomplete SCI. We thus conducted a multicenter prospective nonrandomized controlled clinical trial to assess the neurotherapeutic effects of G-CSF in patients with acute SCI.

MATERIALS AND METHODS

Study Design and Exclusion Criteria

The clinical trial was designed as an open-label, multicenter, prospective, nonrandomized controlled study, and was performed with the approval of the institutional review board of each participating institute. This trial was performed in 4 centers in Japan; Chiba University Hospital, Hokkaido Chuo Rosai Hospital Spinal Cord Injury Center, Kobe Red Cross Hospital, and Japan LHWO Spinal Injuries Center. Starting in August 2009, patients with SCI were recruited within 48 hours of the primary injury. Patients younger than 16 or older than 85 years were excluded, as were patients with intracranial pathologies (*e.g.*, tumors, infection, or ischemia); patients with a history of major bleeding requiring blood transfusion or a history of leukopenia, thrombocytopenia, or hepatic or renal dysfunction, severe heart failure, or splenomegaly; patients diagnosed with a malignant disease within the previous 5 years; and patients who were pregnant or nursing. Written informed consent was obtained from each patient included in the study.

Patients were assigned to either the G-CSF group or the control group according to the institute in which the patients were treated without randomization. Both groups were treated similarly, but the G-CSF group was given a dosage of 10 μg/kg/d of G-CSF (Gran; Kyowa Hakko Kirin, Tokyo) intravenously for 5 consecutive days. The dosage of G-CSF was determined by preliminary open-label, nonrandomized, 1-arm dose-finding trial as we previously reported.[21] G-CSF therapy was performed only at the institute of corresponding author (M.K.). At this institution, all patients with SCI who were treated during the study period and who fulfilled the inclusion criteria received G-CSF treatment. At the other participating institutes, patients were treated similarly but did not receive G-CSF treatment. No patient in either the G-CSF or control group was given MPSS in the follow-up period. Patients received surgery for spinal decompression and stabilization according to each institute's criteria regardless of the patient's treatment group.

Evaluation of Feasibility

Motor and sensory functions were evaluated by the ASIA score (motor scores range from 0 to 100, pinprick scores range from 0 to 112)[22] and the ASIA impairment scale (AIS grade; range A–E). The increase in motor score over time was calculated as previously reported.[23] At least 2 orthopedic spine surgeons in each institute independently evaluated and the data were averaged in each patient's neurological status 1 week, 3 months, and 1 year after the primary injury.

Statistical Analysis

Results are presented as mean ± standard deviation. The Mann-Whitney *U* test was used to evaluate ASIA score improvement, Fisher exact probability test was used to evaluate AIS grade improvement, and Student *t* test was used to analyze hematological data. A *P* value less than 0.05 was considered statistically significant. The biostatistician in the corresponding author's institute reviewed the analysis and verified the statistical results.

RESULTS

Patient Characteristics

A total of 56 patients were enrolled between August 2009 and March 2011. Twenty-six patients were initially assigned to the G-CSF group. Among them, 9 patients did not complete the study; 1 patient developed a fever the day after initiation of G-CSF treatment, 6 patients dropped out during

TABLE 1. Patient Data of G-CSF and Control Groups

	G-CSF	Control	*P*
Number of cases (cases)	17	24	
Sex (cases)			
Male	11	19	
Female	6	5	
Age (yr)	57.1 ± 9.68 (38–68)	56.6 ± 17.9 (23–85)	0.925
Cause of injury (cases)			
Fall	9	13	
Road trauma	6	9	
Sports	1	1	
Falling object	1	0	
Others	0	1	
Level of injury (cases)			
C2–C3	0	2	
C3–C4	4	6	
C4–C5	8	7	
C5–C6	3	7	
C6–C7	2	2	
ASIA impairment scale (cases)			
A	1	7	
B	2	2	
C	5	5	
D	11	12	
Time of first examination after injury (hr)	3.76 ± 2.70 (1–12)	7.73 ± 10.8 (1–48)	0.127
Time of G-CSF administration after injury (hr)	32.7 ± 16.4 (6–48)		

G-CSF indicates granulocyte colony-stimulating factor; ASIA, American Spinal Cord Injury Association.

the follow-up period, 1 patient demonstrated postoperative paralysis and 1 patient died because of heart failure unrelated to the SCI or G-CSF administration. Hence, 17 patients in the G-CSF group completed the study (Table 1). Thirty patients were initially assigned to the control group. Among them, 4 patients dropped out during the follow-up period, 1 patient initially showed paralysis followed by rapid spontaneous recovery during transportation to the hospital, and 1 patient died because of pneumonia. Hence, 24 patients in the control group completed the study (Table 1). There was no significant difference in age, cause of injury, or level of injury between the 2 groups. The groups did, however, differ in the AIS grade because the control group tended to contain more AIS A patients with complete paralysis than the G-CSF group ($P = 0.061$). There was no statistical difference in the number of patient undergone surgery in both groups.

American Spinal Cord Injury Association Impairment Scale

The change in AIS grade between the initial examination and the examination 1 year after treatment is shown in Table 2. The AIS grade improved at least 1 step in 15 patients (88.2%) in the G-CSF group and in 8 patients (33.3%) in the control group, showing significant difference ($P = 0.0002$; Table 3). In patients with incomplete paralysis (AIS grade of B, C, or D at the initial examination; 16 patients in the G-CSF group, and 19 patients in the control group), the AIS grade improved at least 1 step in 15 patients (93.8%) in the G-CSF group and in 6 patients (31.6%) in the control group, still showing significant difference ($P = 0.0002$; Table 2).

ASIA Motor Score

The mean ASIA motor scores at the initial examination were 59.3 ± 28.0 in the G-CSF group and 57.2 ± 34.9 in the control group ($P = 0.979$; Table 3). One week later, the change in motor score was significantly greater in the G-CSF group (14.4 ± 11.6) than in the control group (2.58 ± 8.12) ($P = 0.0002$). Thereafter, patients in both groups continued to increase their motor scores, but the significant difference between the 2 groups was maintained at 1 year after treatment. The change in motor score at 1 year after treatment was significantly higher in the G-CSF group (30.5 ± 20.8) than in the control group (15.7 ± 16.4, $P = 0.013$, power = 0.77; Table 4).

The control group included more patients with AIS A complete paralysis, which might have influenced the results of the study. To exclude this possible bias, we narrowed our analysis to include only patients with incomplete paralysis (AIS grade at first examination: B, C, or D). In these patients, the ASIA motor score at the first examination was 61.3 ± 27.6 in the G-CSF group and 67.6 ± 30.9 in the control group, a difference that was not statistically significant ($P = 0.336$; Table 3). One week later, we found greater improvement in motor scores in the G-CSF group (14.8 ± 11.9) than in the control group (3.26 ± 8.94) ($P = 0.002$). Although motor scores improved in both groups during the next year, the difference in the degree of improvement between the G-CSF and control groups remained significant (31.8 ± 20.7 *vs.* 18.4 ± 17.3, respectively) ($P = 0.050$, power = 0.61; Table 4).

ASIA Pinprick Score

Patients in both the G-CSF and control group demonstrated improvement in their ASIA pinprick scores 1 year after treatment (increased points: 17.8 ± 23.2 and 10.6 ± 24.0, respectively). The difference was not statistically significant ($P = 0.486$; Table 5).

In those patients with incomplete paralysis, improvement was noted in both the G-CSF and control groups 1 year after

TABLE 2. ASIA Impairment Scale

G-CSF Group (17 Cases)						Control Group (24 Cases)					
	Grade at 1 yr After Onset (Cases)						Grade at 1 yr After Onset (Cases)				
First Examination (Cases)	A	B	C	D	E	First Examination (Cases)	A	B	C	D	E
A	1					A	3	2			
B			1	1		B		1	1		
C				4	1	C			2	3	
D				1	8	D				10	2

AIS grades: 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).

G-CSF indicates granulocyte colony-stimulating factor; AIS, ASIA impairment scale; ASIA, American Spinal Cord Injury Association.

treatment (increased points: 20.5 ± 23.0 and 13.8 ± 23.7, respectively). Again, the difference was not statistically significant ($P = 0.254$; Table 5).

Adverse Events

One patient in the G-CSF group developed a fever more than 40°C the day after initiation of G-CSF treatment, and the treatment was thus discontinued. The cause of the fever proved to be a urinary tract infection, which was successfully treated with antibiotics. No relationship was found between the infection and G-CSF administration. One patient developed mild hepatic dysfunction (glutamate oxaloacetate transaminase: 91 U/L [normal range: 13–33 U/L], glutamate-pyruvic transaminase: 99 U/L [normal range: 8–42 U/L]) 5 days after initiation of G-CSF treatment. The patient recovered without intervention. No other severe adverse events occurred during or after G-CSF administration.

DISCUSSION

Nonhematopoietic Effects of G-CSF

Experimental studies on acute myocardial infarction (AMI) have shown that G-CSF mobilizes stem cells into the myocardium, thereby protecting cardiac tissue from injury.[24] In models of ischemic stroke, G-CSF has been shown to protect the brain by suppressing neuronal apoptosis and the expression of inflammatory cytokines.[12–16] We previously made similar observations in preclinical rodent models of acute SCI.[17–20] On the basis of these results, clinical trials have been initiated for the treatment of AMI[25–30] and neurological disorders

TABLE 3. ASIA Motor Score

	G-CSF	Control	*P*
Total cases (G-CSF: n = 17, control: n = 24)			
At first examination	59.3 ± 28.0 (14–98)	57.2 ± 34.9 (4–97)	0.979
1 wk after onset	73.6 ± 24.2 (27–100)	59.8 ± 35.5 (4–100)	0.249
3 mo after onset	86.8 ± 18.6 (35–100)	69.8 ± 34.6 (6–100)	0.154
6 mo after onset	88.9 ± 18.7 (36–100)	71.3 ± 33.6 (6–100)	0.042*
1 yr after onset	89.8 ± 18.5 (36–100)	71.6 ± 14.7 (6–100)	0.025*
Incomplete paralysis cases (AIS: B, C, D) (G-CSF: n = 16, control: n = 19)			
At first examination	61.3 ± 27.6 (14–98)	67.6 ± 30.9 (7–97)	0.336
1 wk after onset	76.1 ± 22.8 (27–100)	70.8 ± 30.5 (6–100)	0.752
3 mo after onset	90.1 ± 13.4 (51–100)	82.8 ± 24.6 (19–100)	0.504
6 mo after onset	92.3 ± 13.2 (51–100)	84.3 ± 23.1 (19–100)	0.137
1 yr after onset	93.1 ± 12.7 (51–100)	84.4 ± 23.1 (19–100)	0.085

AIS grades: B, sensory incomplete paralysis, motor complete paralysis; C, motor incomplete paralysis (muscle grading <3/5); D, motor incomplete paralysis (muscle grading >3/5).

G-CSF indicates granulocyte colony-stimulating factor; AIS, ASIA impairment scale; ASIA, American Spinal Cord Injury Association.

**P < 0.05.*

TABLE 4. Increased Motor Score

	G-CSF	Control	*P*
Total cases (G-CSF: n = 17, control: n = 24)			
1 wk	14.4 ± 11.6 (1–50)	2.58 ± 8.12 (−11 to 24)	0.0002*
3 mo	27.5 ± 18.9 (1–73)	12.6 ± 14.9 (−7 to 48)	0.0005*
6 mo	29.6 ± 19.8 (2–77)	15.4 ± 17.2 (−5 to 64)	0.015†
1 yr	30.5 ± 20.8 (2–81)	15.7 ± 16.4 (−5 to 63)	0.013†
Incomplete paralysis cases (AIS: B, C, D) (G-CSF: n = 16, control: n = 19)			
1 wk	14.8 ± 11.9 (1–50)	3.26 ± 8.94 (−11 to 24)	0.002*
3 mo	28.8 ± 18.8 (1–73)	15.2 ± 15.7 (−7 to 48)	0.029†
6 mo	30.9 ± 19.7 (2–77)	18.3 ± 18.2 (−4 to 64)	0.063
1 yr	31.8 ± 20.7 (2–81)	18.4 ± 17.3 (0–63)	0.050†

AIS grades: B, sensory incomplete paralysis, motor complete paralysis; C, motor incomplete paralysis (muscle grading <3/5); D, motor incomplete paralysis (muscle grading >3/5).

G-CSF indicates granulocyte colony-stimulating factor; AIS, ASIA impairment scale; ASIA, American Spinal Cord Injury Association.

**P < 0.01.*

†P < 0.05.

such as cerebral infarction[31] and amyotrophic lateral sclerosis.[32,33] Many clinical trials in AMI have already reported the safety and feasibility of G-CSF administration.[25–30] A clinical trial in patients with cerebral infarction found that G-CSF administration improved neurological symptoms, although the sample size was small.[31] Furthermore, G-CSF has been shown to attenuate neuronal injury in patients with amyotrophic lateral sclerosis, delaying disease progression and improving quality of life.[33] Ours is the first clinical trial of G-CSF in patients with acute SCI.

Neurotherapeutic Effects of G-CSF for Acute SCI

In our preliminary study, we determined that 10 μg/kg/d dosage of G-CSF, administered by intravenous injection during 5 consecutive days, is the highest dose of G-CSF that can be safely administered to patients.[21,34] On the basis of those results, we conducted this multicenter prospective controlled clinical trial to verify the neurotherapeutic effect of G-CSF in patients with acute SCI. The pathophysiology and symptoms of acute SCI vary depending on the spinal level of injury (cervical, thoracic, or thoracolumbar) and the severity of the trauma. This variability can make data interpretation difficult. Thus, to increase the reliability of our data, we restricted our inclusion criteria to patients with cervical injury and excluded patients with thoracic or thoracolumbar injury.

We analyzed whether G-CSF administration improved muscle power in the upper and lower extremities using the ASIA motor score. One week after the primary injury, the motor score significantly increased in the G-CSF group compared with the control group, and this difference was maintained 1 year later. This result suggests that G-CSF administration may have a neurotherapeutic effect on the descending tracts of the white matter and on the gray matter within the injured spinal segments, resulting in earlier and more pronounced improvement in motor function.

Side Effects of G-CSF

Previous reports have described the side effects of G-CSF administration. Mild symptoms include low back and pelvic pain, fever, headache, nausea, and vomiting.[35–37] Symptoms were transient and disappeared 2 or 3 days after cessation of the drug. In this trial, 1 patient developed a fever 1 day after starting G-CSF treatment, but this was found to be due to a urinary tract infection and resolved with antibiotic treatment. One patient developed mild hepatic dysfunction that resolved spontaneously. Reported severe side effects of G-CSF include cerebral infarction, AMI, and splenic rupture.[38,39] The risk of severe side effects increases with high doses of G-CSF (*e.g.*, 20 μg/kg/d) and the risk of splenic rupture increases with white blood cell counts above 50 × 10^3/mm^3.[38] In this study, moderate doses of G-CSF were

TABLE 5. ASIA Pinprick Score

	G-CSF	Control	*P*
Total cases (G-CSF: n = 17, control: n = 24)			
At first examination	71.1 ± 30.0 (21–106)	66.1 ± 39.4 (12–112)	0.874
1 yr after onset	88.9 ± 24.9 (29–112)	77.7 ± 37.5 (16–112)	0.558
Increased points	17.8 ± 23.2 (−12 to 72)	11.6 ± 24.0 (−36 to 81)	0.486
Incomplete paralysis cases (AIS: B, C, D) (G-CSF: n = 16, control: n = 19)			
At first examination	73.6 ± 28.5 (21–106)	81.8 ± 34.6 (20–112)	0.251
1 yr after onset	92.2 ± 20.8 (62–112)	95.6 ± 27.7 (36–112)	0.354
Increased points	20.5 ± 23.0 (−12 to 72)	13.8 ± 23.7 (0–81)	0.254

AIS grades: B, sensory incomplete paralysis, motor complete paralysis; C, motor incomplete paralysis (muscle grading <3/5); D, motor incomplete paralysis (muscle grading >3/5).

G-CSF indicates granulocyte colony-stimulating factor; AIS, ASIA impairment scale; ASIA, American Spinal Cord Injury Association.

administered (10 μg/kg/d) and no severe side effects were observed. Hence, the dosage of 10 μg/kg/d is likely to be safe in patients with acute SCI.

MPSS has been used to treat secondary spinal cord injury (SCI) in patients with acute SCI.[3,4] However, the side effects of MPSS treatment, including pneumonia and gastric ulcer, are harmful to most patients.[5–9] This study suggests that G-CSF as a neurotherapeutic agent might be safer than MPSS in patients with acute SCI.

Future Investigation

This study has several limitations. First, the G-CSF group was treated at 1 institution and the control group was treated at the other institutions. Hence, treatment consistency between the 2 groups have been compromised because we could not control the difference among centers with respect to surgical indication, method of rehabilitation and nursing care, *etc.* As for surgical intervention, surgery itself was no apparent confounding factor shown by univariate analysis, of which result was confirmed by biostatistician. Second, the number of patients enrolled in the study was relatively small to obtain sufficient statistical power. Third, the initial AIS grade differed between the groups, possibly affecting the results. Fourth, this was an open-label study and assignment of patients to the 2 treatment groups was not randomized, resulting in selection bias. Finally, it is possible that evaluators were biased by their knowledge of which patients received the drug because of lacking of the blinding in this study design.

The next phase of the evaluation of G-CSF in acute SCI should be a randomized, double-blind placebo-controlled clinical trial. We are currently designing a phase IIb clinical trial that will include a relatively large number of patients. The results of this trial will provide a better understanding of the effectiveness of neurotherapeutic G-CSF in patients with acute SCI.

CONCLUSION

Despite its limitations, this study suggests that G-CSF administration may have beneficial effects on neurological recovery in patients with acute SCI and encourages the development of additional clinical trials.

➢ Key Points

- ❑ A multicenter prospective controlled clinical trial was performed to confirm the feasibility of G-CSF administration for acute SCI.
- ❑ In 17 acute patients with SCI, within 48 hours of onset, G-CSF (10 μg/kg/d) was intravenously administered for 5 consecutive days.
- ❑ The administration of G-CSF enhanced neurological recovery in 15 of 17 patients with acute SCI.
- ❑ Neurotherapeutic effects of G-CSF could be useful strategy for the treatment of SCI.

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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–

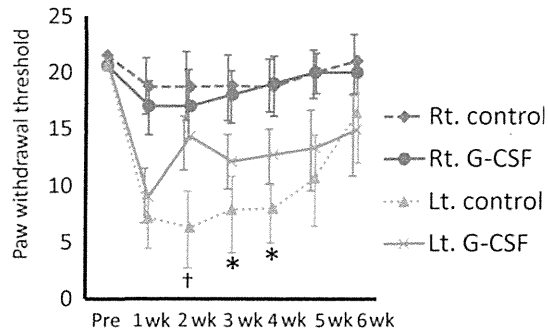

Figure 1. Mechanical allodynia data from the von Frey hair test. One week after the surgery, both groups showed decreased paw withdrawal threshold indicating mechanical allodynia. G-CSF-treated rats (circle) showed significant attenuation of paw withdrawal threshold compared with that of the control 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.

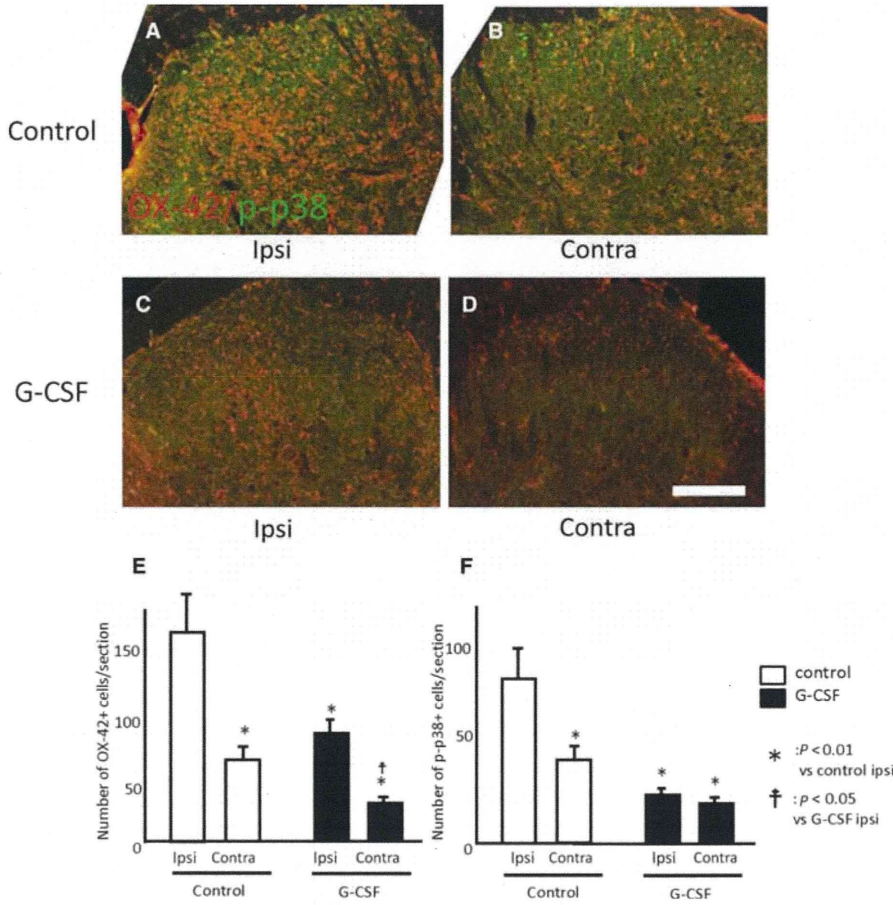

Figure 2. Immunohistochemistry for OX-42 (a marker for microglia) and p-p38MAPK. The number of OX-42–positive microglia decreased in the G-CSF group compared with that in the control group (**E**). A greater number of p-p38MAPK–positive cells in the ipsilateral dorsal horn of the spinal lumbar enlargement 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

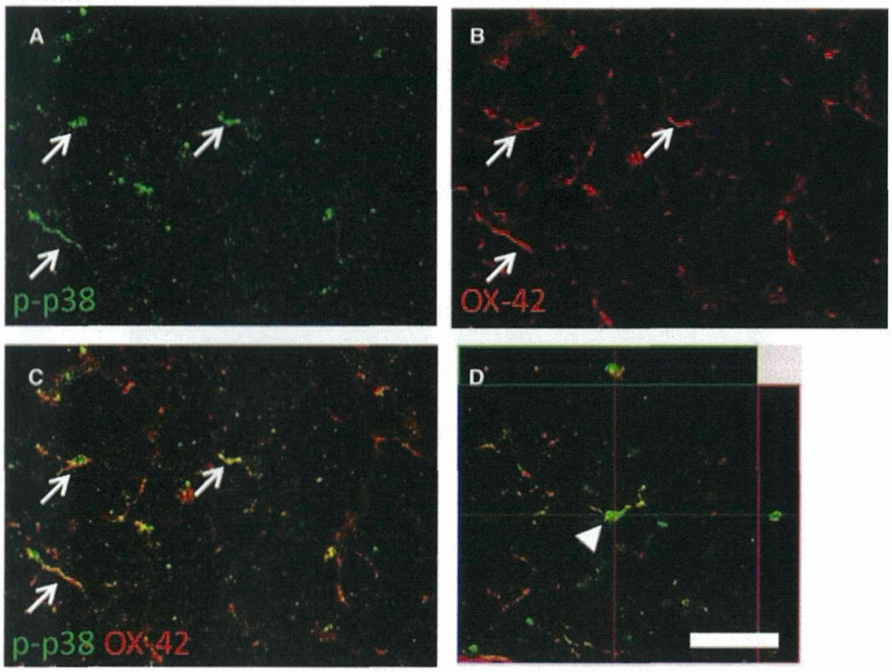

Figure 3. Immunofluorescent double staining for OX-42 and p-p38MAPK confocal laser scanning microscope was used to obtain the images. p-p38MAPK–positive cells (**A**) were also positive for OX-42 (**B**), indicating that p-p38MAPK–expressing cells in the spinal cord dorsal horn are mainly microglia. 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]

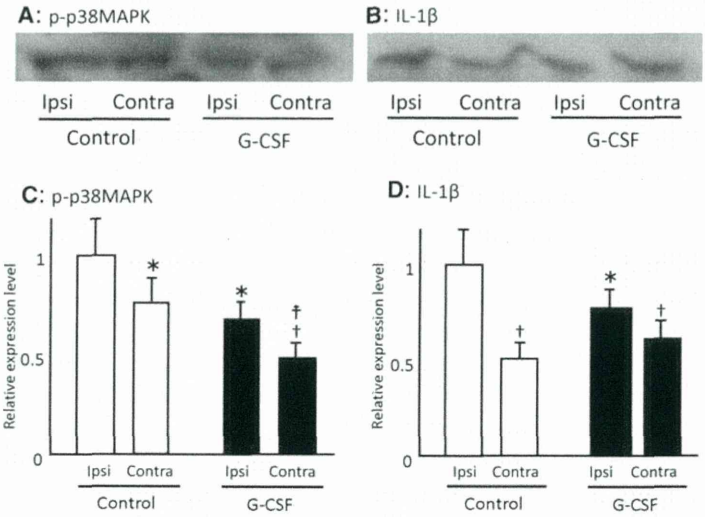

Figure 4. Western blot analysis for p-p38MAPK and IL-1β. G-CSF reduced the protein expression level of p-p38MAPK compared with that in the control group (**A**, **C**). G-CSF also reduced protein expression level of IL-1β (**B**, **D**). G-CSF indicates granulocyte colony-stimulating factor; IL-1β, interleukin-1β;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

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

in clinical settings, cannot be evaluated by assessment of allodynia. Therefore, we cannot show the effects of G-CSF for dysesthesia, resulting in limited clinical relevance of the present result. In near future, we better use the assessment tool for dysesthesia.[28] Finally, there was discrepancy between limited period of effectiveness of G-CSF for CCI-induced mechanical allodynia and histological changes. As we previously showed by preliminary human clinical trial of G-CSF for neuropathic pain related to spinal cord lesions,[10] the duration of G-CSF effect was approximately 3 to 4 months in average. The reason why the effects of G-CSF for neuropathic pain is transient although the histological change is obvious because precise mechanism of action of G-CSF for spinal cord lesion-related neuropathic pain is still unclear as same as that for CCI-induced mechanical allodynia. Further exploration is needed to clarify this issue.

CONCLUSION

The present results demonstrate a therapeutic effect of delayed G-CSF treatment for CCI-induced neuropathic pain. The data are relevant to the clinical treatment of neuropathic pain, because, in most cases, patients must be treated not in the acute stage but in the subacute/chronic stage. The elucidation of the therapeutic time window is especially important to guide the future clinical application of G-CSF treatment for peripheral nerve injury-induced neuropathic pain.

➢ Key Points

- ❑ Delayed G-CSF significantly attenuates mechanical allodynia induced by CCI of sciatic nerve.
- ❑ G-CSF reduced the number of activated microglia in the affected dorsal horn of the spinal cord lumbar enlargement.
- ❑ G-CSF reduced the expression level of p-p38MAPK and IL-1β.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Title: Identification of chimeric antigen receptors that mediate constitutive or inducible proliferation of T cells

Short Title: Constitutive CAR T-cell proliferation

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Key points:
1. Chimeric antigen receptors (CARs) have promise for a variety of cancers
2. An unexpected observation is that some CAR modified T cells that encode a CD28 domain have antigen independent proliferation and cytokine secretion, leading to inferior antitumor effects

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ABSTRACT

This study compared second generation chimeric antigen receptors encoding signaling domains composed of CD28, ICOS and 4-1BB. Here we report that certain CARs endow T cells with the ability to undergo long-term autonomous proliferation. Transduction of primary human T-cell with lentiviral vectors encoding some of the CARs resulted in sustained proliferation for up to three months following a single stimulation through the TCR. Sustained numeric expansion was independent of cognate antigen and did not require the addition of exogenous cytokines or feeder cells after a single stimulation of the TCR and CD28. Results from gene array and functional assays linked sustained cytokine secretion and expression of T-bet, EOMES and GATA-3 to the effect. Sustained expression of the endogenous IL2 locus has not been reported in primary T cells. Sustained proliferation was dependent on CAR structure and high expression, the latter of which was necessary but not sufficient. The mechanism involves constitutive signaling through NF-kB, Akt, Erk and NFAT. The propagated CAR T cells retained a diverse TCR repertoire and cellular transformation was not observed. The CARs with a constitutive growth phenotype displayed inferior antitumor effects and engraftment *in vivo*. Therefore the design of CARs that have a non-constitutive growth phenotype may be a strategy to improve efficacy and engraftment of CAR T cells. The identification of CARs that confer constitutive or non-constitutive growth patterns may explain observations that CAR T cells have differential survival patterns in clinical trials.

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INTRODUCTION

The creation of tumor-specific T lymphocytes by genetic modification to express chimeric antigen receptors (CAR) is gaining traction as a form of synthetic biology generating powerful antitumor effects (1-6). Because the specificity is conferred by antibody fragments, the CAR T cells are not MHC-restricted and are therefore more practical than approaches based on T cell receptors (TCR) that require MHC matching.

Clinical data from patients treated with CD19-specific CAR^+ T cells indicate that robust *in vivo* proliferation of the infused T cells is a key requirement for immunoablation of tumors (7, 8). Therefore, efforts have been made to incorporate the signaling endodomains of co-stimulatory molecules such as CD28, ICOS, OX40, and 4-1BB into CARs. It was first reported in 1998 that the use of gene-engineered T cells expressing chimeric single-chain (scFv) receptors capable of co-delivering CD28 costimulation and TCR/CD3 zeta chain (CD3ζ) activation signals increased the function and proliferation of CAR T cells (9). A number of laboratories have confirmed that incorporation of CD28 signaling domains enhances the function of CARs in pre-clinical studies compared to CD3ζ or FcεR1. In a study in patients with B-cell malignancies, CD28:CD3ζ CARs had improved survival compared to CARs endowed only with the CD3ζ signaling domain (5).

Here we report the unexpected finding that expression of some CARs containing CD28 and CD3ζ tandem signaling domains leads to constitutive activation and proliferation of the transduced primary human T cells. The CAR T cells that we have identified have constitutive secretion of large amounts of diverse cytokines and consequently do not require the addition of exogenous cytokine or feeder cells in order to maintain proliferation. This was surprising because in numerous previous reports that described CARs endowed with CD28 domains (9-28), the proliferation of such tandem CARs has been ligand-dependent, and required restimulation of the CAR T cells to maintain proliferation. Here we report that one mechanism that can result in the phenotype of CARs with continuous T-cell proliferation is the density of CARs at the cell surface.

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MATERIALS AND METHODS

Construction of lentiviral vectors with differing eukaryotic promoters and CARs

Supplemental figure 1A shows schematic diagrams of the CARs used in this study. All CARs contain an scFv that recognizes either human CD19, mesothelin or c-Met.

In vivo assessment of anti-c-Met CAR T cells

Xenograft tumors in NSG mice were established by intraperitoneal injection of $0.791x10^6$ SK-OV3 ovarian cancer cells or subcutaneous injection of $1x10^6$ L55 human lung adenocarcinoma cells, transduced to express click-beetle-green. Tumor growth was measured by bioluminescent imaging. Peripheral blood was obtained from retro-orbital bleeding or intracardiac puncture and was stained for the presence of human $CD45^+$ T cells. The human $CD45^+$ population was quantified using TruCount tubes (BD Biosciences). All experiments were performed in anonymized fashion.

Construction of deletion variants of PGK (phosphoglycerate kinase1) promoter

A series of 5' deletion mutations of the human PGK promoter was prepared by PCR using specific 5' primers with an incorporated PmeI site, indicated below, and a common 3' primer with an incorporated NheI site (5'-gtggctggagagaggggtgctagccgc-3'). The PCR product was digested and then inserted into the pELNS c-Met-IgG4-28z plasmid to substitute the EF-1α promoter with PGK promoter deletion mutants. PGK100, PGK200, PGK300 and PGK400 encompasses from nucleotides -38, -141, -243 and -341 of transcription start site of PGK to +84, respectively.

PGK100 5'- gcggtttaaacgtggggcggtagtgtgggccctg-3'

PGK200 5'- gcggtttaaacgcaatggcagcgcgccgaccg-3'

PGK300 5'-gcggtttaaacgcccctaagtcgggaaggttccttg-3'

PGK400 5'-gcggtttaaacgccgaccctgggtctcgcacattc-3'

Construction and characterization of chimeric antigen receptors

Lentiviral vectors from previously published work were used to express the anti-CD19 FMC63 CD8α (29), the anti-mesothelin SS1 CD8α, and the anti-mesothelin SS1 CD8α Δtail CAR constructs (30). The c-Met 5D5 IgG4 construct was used as a template to generate the SS1 IgG4 and CD19 IgG4 CAR

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constructs through PCR splicing and overlap extension. Restriction sites were introduced via PCR primers, which allowed for cloning into third generation self-inactivating lentiviral plasmids. The cytomegalovirus (CMV) and elongation factor-1α (EF-1α) promoter sequences were amplified via PCR from previously constructed plasmids and introduced into pre-existing CAR-containing constructs (29) using standard molecular biology techniques.

Microarray studies

Sample Collection. Human $CD4^+$ T cells from three healthy donors were stimulated and transduced with either the c-Met IgG4 or CD19 CD8α CAR construct. Cell pellets were collected and frozen on day 0 prior to stimulation, day 6 and day 11 at rest down for all samples and day 24 for the c-Met IgG4 CAR.

Microarray Target Preparation and Hybridization. Microarray services were provided by the UPenn Microarray Facility, including quality control tests of the total RNA samples by Agilent Bioanalyzer and Nanodrop spectrophotometry. All protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, 100ng of total RNA was converted into first-strand cDNA using reverse transcriptase primed by poly(T) and random oligomers that incorporated the T7 promoter sequence. Second-strand cDNA synthesis was followed by *in vitro* transcription with T7 RNA polymerase for linear amplification of each transcript, and the resulting cRNA was converted into cDNA, fragmented, assessed by Bioanalyzer, and biotinylated by terminal transferase end-labeling. cRNA yields ranged from 36-89μg, and cDNA was added to Affymetrix hybridization cocktails, heated at 99°C for 5 min and hybridized for 16 h at 45°C to Human Gene 1.0ST GeneChips (Affymetrix Inc., Santa Clara CA). The microarrays were then washed at low (6X SSPE) and high (100mM MES, 0.1M NaCl) stringency and stained with streptavidin-phycoerythrin. A confocal scanner was used to collect fluorescence signal after excitation at 570 nm.

Initial Data Analysis. Affymetrix Command Console and Expression Console were used to quantify expression levels for targeted genes; default values provided by Affymetrix were applied to all analysis parameters. Border pixels were removed, and the average intensity of pixels within the 75th percentile

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was computed for each probe. The average of the lowest 2% of probe intensities occurring in each of 16 microarray sectors was set as background and subtracted from all features in that sector. Probe sets for positive and negative controls were examined in Expression Console, and Facility quality control parameters were confirmed to fall within normal ranges. Probes for each targeted gene were averaged and inter-array normalization performed using the robust multichip average (RMA) algorithm.

Analysis of terminal telomeric restriction fragment lengths

Telomeric restriction fragment length analysis was performed essentially as described (31). Briefly, 2μg of genomic DNA was digested with RsaI + HinfI and resolved on a 0.5% agarose gel, which was then dried and probed with a ^{32}P- labeled (CCCTAA)4 oligonucleotide. After washing, the samples were visualized with a Phosphor imager.

Accession numbers

Micro array data will be deposited in the GEO repository upon acceptance of this manuscript for publication.

Statistical analysis

Raw data obtained from microarray core was normalized with RMA. Analysis was performed using a 3-way mixed model ANOVA with factors being sample date, treatment group and donor ID. An interaction term between sample and collection date was added. In conjunction with the multiple pair-wise comparisons the p-value and fold-change were determined. For all p-values we calculated the FDR corrected p-value using the method of Benjamini and Hochberg as implemented by Partek Genomic Suite (Partek). For transcription factor and cytokine dot plots the normalized absolute $\log_2$ gene expression intensities were plotted. Cluster analysis was performed using Euclidean distance of median normalized $\log_2$ gene expression intensities with average linkage. All growth curves, MFI and engraftment plots were plotted using Prism (GraphPad Software). All error bars are representative of standard deviation. A two tailed Mann-Whitney test was performed for the *in vivo* engraftment studies.

Additional methods are described in the **Supplementary Materials.**

RESULTS

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Construction and characterization of chimeric antigen receptors

A plethora of CARs have been generated that express CD28 and CD3ζ downstream of antibody fragments that mediate surrogate antigen recognition (12-18, 20-28). Given that these transgenes were constructed differently and by different investigators at different institutes, it remains unknown how these CARs would perform using a common expression system and a standardized culture system that has been optimized for clinical use. Therefore, a set of 12 CARs targeting c-Met, mesothelin and CD19 was expressed in primary human T cells (**Supplementary Fig S1A and C**). The CARs encoded IgG4 or CD8α hinge domains, CD28, ICOS or CD8α transmembrane domains and the signaling domains were composed of CD28, 4-1BB, ICOS and CD3ζ. A CAR with a truncated signaling domain, and CART19, a CD19 4-1BB:CD3ζ CAR used in a previous clinical trial (7) served as controls. All CARs were expressed constitutively using an EF-1α promoter, and in a typical experiment 50% of the cells initially expressed the CAR and had similar levels of expression on the surface by day 6 after transduction (**Supplementary Fig S1B**). The c-Met CAR T cells had specific and potent cytotoxicity (**Supplementary Fig S2**), and previous studies have shown that the CARs specific for CD19 and mesothelin have similarly potent effector functions (29, 30).

Chimeric antigen receptors with CD28 and CD3ζ can induce constitutive T-cell proliferation

Previous studies suggested that antitumor effects after CAR T-cell infusions require sustained expansion of CAR T cells *in vivo* after adoptive transfer (8). To determine the proliferative capacity of the CAR T cells, $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 beads, transduced with the lentiviral vector encoding the CAR and then propagated without further stimulation in the absence of exogenous cytokines or feeder cells. Unexpectedly, we observed constitutive proliferation of some of the CAR T-cell populations (**Fig 1A, left**). Exponential growth was observed for 60 to 90 days in CAR T cells transduced with the c-Met IgG4 construct encoding the CD28 and CD3ζ signaling domains (**Fig 1 A and B**). Similarly, the T cells expressing the anti-mesothelin SS1:IgG4 and SS1:CD8α CARs that signaled through chimeric CD28 and CD3ζ domains also had sustained proliferation that was independent of

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supplementation with exogenous growth factors. We also observed long-term proliferation of $CD8^+$ T cells that was independent of antigen stimulation and did not require the addition of exogenous cytokines or feeder cells (**Fig 1C**). To minimize experimental variables, we used bulk $CD4^+$ T cells for most of the experiments in this study.

The cultures with the non-continuous CAR T-cell populations had an initial period of exponential proliferation at the same rate, and after day 10, a decreasing rate of growth followed by death of the culture within 20 days (**Fig 1A and B**). Notably, in the absence of exogenous IL2, the CD19 CARs expressing the 4-1BB domain returned to a resting state with similar kinetics as that of the CD19:28ζ CAR T cells (**Fig 1E**). This expected pattern of initial growth followed by a return to a resting state by CD19 CARs and the mock transduced cells has been reported by our laboratory and others (15, 21, 24, 29, 32). For simplicity and clarity the CAR constructs that induce constitutive proliferation are henceforth referred to as "continuous CARs", while the CARs that exhibit inducible proliferation similar to that described in previous reports are referred to as "non-continuous CARs".

The mean cell volumes were monitored at frequent intervals as a measure of metabolic status and cell cycle (**Fig 1A, right**). T-cell cultures transduced with the various CAR constructs increased from a resting (G0) cell volume of ~160 fl to nearly 600 fl by day 6 of culture, consistent with the induction of DNA synthesis and the exponential increase in cell numbers. However, the non-continuous CAR T cells and non-transduced T cells rapidly returned to a resting cell volume, while the continuous CAR T cells (c-Met IgG4, SS1 IgG4 and SS1 CD8α) failed to return to a resting cell volume, consistent with continued cellular proliferation. On day 20 of culture, the mean cell volume in cultures of continuous CARs and non-continuous CARs was ~400 fl and 180 fl, respectively. Notably, the long-term proliferation of the CAR T cells was independent of cognate antigen, because the surrogate ligands c-Met and mesothelin are not expressed at detectable levels on the surface of activated human $CD4^+$ T cells (**Supplementary Fig S3**), consistent with previous reports (33). Q-PCR analysis did not detect transcripts for mesothelin or c-Met in resting CD4 T cells (**Supplementary Table S1**). However, activated T cells, either mock