J Neuropathol Exp Neurol

Vol. 66, No. 8
August 2007
pp. 724–731

ORIGINAL ARTICLE

Granulocyte Colony-Stimulating Factor Attenuates Neuronal Death and Promotes Functional Recovery After Spinal Cord Injury in Mice

Yutaka Nishio, MD, PhD, Masao Koda, MD, PhD, Takahito Kamada, MD, PhD, Yukio Someya, MD, PhD, Ryo Kadota, MD, Chikato Mannoji, MD, Tomohiro Miyashita, MD, Seiji Okada, MD, PhD, Akihiko Okawa, MD, PhD, Hideshige Moriya, MD, PhD, and Masashi Yamazaki, MD, PhD

Abstract

Granulocyte colony-stimulating factor (G-CSF) is a protein that stimulates differentiation, proliferation, and survival of granulocytic lineage cells. Recently, a neuroprotective effect of G-CSF was reported in a model of cerebral infarction. The aim of the present study was to elucidate the potential therapeutic effect of G-CSF for spinal cord injury (SCI) in mice. We found that G-CSF is neuroprotective against glutamate-induced cell death of cerebellar granule neurons in vitro. Moreover, we used a mouse model of compressive SCI to examine the neuroprotective potential of G-CSF in vivo. Histologic 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 hindlimb functional recovery. Examination of signaling pathways downstream of the G-CSF receptor suggests that G-CSF might promote functional recovery by inhibiting neuronal apoptosis after SCI. G-CSF is currently used in the clinic for hematopoietic stimulation, and its ongoing clinical trial for brain infarction makes it an appealing molecule that could be rapidly placed into trials for patients with acute SCI.

Key Words: Apoptosis, Neuroprotection, Secondary injury.

From the Department of Orthopaedic Surgery (YN, TK, YS, RK, CM, TM, AO, HM, MY), Graduate School of Medicine, Chiba University, Chiba Japan; Department of Orthopaedic Surgery (MK), Togane Hospital, Chiba, Japan; and Division of Hematopoiesis Center for AIDS Research (SO), Kumamoto University, Kumamoto, Japan.

Send correspondence and reprint requests to: Masashi Yamazaki, MD, PhD, Department of Orthopaedic Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670 Japan; E-mail: masashiy@faculty.chiba-u.jp

This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (16591473) and by a medical research grant from of the General Insurance Association of Japan.

INTRODUCTION

The pathologic sequelae that follow acute spinal cord injury (SCI) are divided into 2 broad chronologic events: the primary injury and the secondary injury (1). The primary injury encompasses the focal destruction of neural tissue caused by direct mechanical trauma. This initial insult then instigates a progressive wave of secondary injury, which exacerbates the injury to the spinal cord via the activation of pathophysiologic mechanisms. Because this wave of secondary injury leads to the apoptotic death of neuronal and glial cells left intact by the initial trauma, it is a major impediment to functional recovery after SCI (1). Thus, apoptosis of neurons and glial cells after acute SCI is one of the main therapeutic targets for various kinds of drug therapies.

Granulocyte colony-stimulating factor (G-CSF) is a 19.6-kDa glycoprotein that was identified initially as a serum component that induces differentiation of the murine myelomonocytic leukemic cell line and is capable of inducing the survival, proliferation, and differentiation of cells of neutrophil granulocyte precursors (2, 3). In addition, G-CSF inhibits apoptosis of postmitotic neutrophil lineage cells. This effect results in increased numbers of circulating neutrophils, which is used for neutropenia in clinical situations. In addition to its antiapoptotic effect for neutrophil, it was recently reported that G-CSF has the potential to inhibit apoptosis of postmitotic cells of nonhematopoietic lineage. For instance, G-CSF suppresses apoptosis of cardiomyocytes in an acute myocardial infarction model (4). It is supposed that G-CSF activates common antiapoptotic machinery, which is conserved among the different lineage cells. In the CNS, apoptosis of neurons can also be suppressed by G-CSF. Recent reports showed that G-CSF attenuates glutamate-induced neuronal death in vitro and protects neurons after stroke in vivo (5–7). These lines of evidence show the antiapoptotic potential of G-CSF in the CNS, raising the possibility that G-CSF may also act as a neuroprotectant in acute SCI.

In the current study, we tested the hypothesis that G-CSF could attenuate neuronal apoptosis and promote functional recovery after SCI.

MATERIALS AND METHODS

Cell Culture

Cerebellar granule neurons (CGNs) were prepared from postnatal day 7 mice. Fresh cerebella were dissected, and the tissue was dissociated with trypsin (2.5 mg/mL; Invitrogen, Carlsbad, CA) and DNase I (0.3 mg/mL; Roche Applied Science, Indianapolis, IN). Cells were plated on poly-L-lysine-coated chamber slides (Lab-Tek Chamber Slides Permanox; Nalge Nunc International, Rochester, NY) or 6-cm dishes at a density of 7.0×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium Gibco BRL (Grand Island, NY), supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate; Invitrogen), and 0.02 M HEPES. After 16 hours in culture, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with penicillin-streptomycin, 20 mM HEPES, N2 supplement (0.01%; Invitrogen), KCl (20 mM), fibronectin (10 μg/mL), and cytosine arabinoside (1.0 μM). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Experiments on CGNs were performed after 7 days in culture.

Immunocytochemistry and Immunohistochemistry

To detect the G-CSF receptor (G-CSFR), immunocytochemical staining on cultured CGNs and immunohistochemistry on intact mouse spinal cord sections were performed. For histologic sections, animals were perfused transcardially with 4% paraformaldehyde in PBS under deep pentobarbital anesthesia. Spinal cord tissue was fixed overnight by immersion in 4% paraformaldehyde in PBS and then immersed for 48 hours in 20% sucrose in PBS at 4°C. The tissue was embedded in O.C.T. compound (Tissue-Tek; Sakura Finetech. Tokyo, Japan), frozen on dry ice, and sectioned on a cryostat. Axial sections (12 μm thick) were mounted onto poly-l-lysine-coated glass slides (Matsunami, Tokyo, Japan) and dried for 48 hours at room temperature. Immunocytochemistry and immunohistochemistry were performed as described previously (8). Briefly, cultured CGNs on chamber slides were washed 3 times with PBS, fixed with 4% paraformaldehyde for 10 minutes, and then washed 3 times with PBS. Histologic sections of mouse spinal cord were rehydrated with 0.3% Triton X in PBS for 1 hour and washed 3 times with PBS. Slides were then incubated with blocking solution (0.05 M Tris HCl, pH 7.6, 1% bovine serum albumin, Block Ace [Yukijirusi, Sapporo, Japan], 0.15 M NaCl, and 0.1% Tween 20) for 30 minutes at room temperature. After blocking, the slides were incubated with anti G-CSF receptor rabbit polyclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-neuronal nuclei mouse monoclonal antibody (Neu-N) (1:400 dilution; Chemicon International, Inc., Temecula, CA) overnight at 4°C. The slides were then washed 3 times for 5 minutes with PBS and incubated with secondary antibodies (goat anti-rabbit, Alexa Fluor 594, 1:800 dilution and goat anti-mouse, Alexa Fluor 388, 1:800 dilution; Molecular Probes, Eugene, OR) for 30 minutes at room temperature. The slides were then washed 3 times with PBS and analyzed by confocal laser scanning microscopy (LSM5 PASCAL; Carl Zeiss, Germany, Oberkochen).

Reverse Transcriptase-Polymerase Chain Reaction

To detect expression of G-CSFR mRNA in CGNs and spinal cord tissue, we performed reverse transcriptase (RT)-polymerase chain reaction (PCR). Total RNA was extracted from cultured CGNs or intact spinal cord tissue using TRIzol reagent (Gibco Life Technologies, Rockville, MD) according to the manufacturer's protocol. cDNAs were prepared by reverse transcription from 2.5 μg of total RNA using the Superscript II RT Preamplification System (Gibco Life Technologies) with an oligo $(dT)_{12\text{-}18}$ primer. PCR was performed with 2.0 μL of cDNA in a 50-μL reaction mixture containing 200 nM dNTP, 0.5 U of Extra Taq DNA polymerase (Takara, Tokyo, Japan), and forward and reverse PCR primers. Sequences of both primers were 5′-GTACTCTTGTCCACT ACCTGT-3′ and 5′-CAAGATA-CAAGGACCCCCAA-3′ for G-CSFR (accession number M58288). The following conditions were used for PCR amplification: after denaturation at 94°C for 5 minutes, the reaction was carried out at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute (40 cycles). The resulting 567-base pair product was analyzed on a 1% agarose gel.

Cell Culture Experiment

After 7 days in culture, CGNs were treated with glutamate (100 μM) for 6 hours to induce cell death. Recombinant human G-CSF was provided by KIRIN Brewery (Tokyo, Japan). Neuronal death was detected by double staining with propidium iodide and calcein using a Live/Dead Double Staining Kit (MBL, Nagoya, Japan). The ratio of dead cells to total cells was calculated (number of dead cells/total cells) and compared between each group. G-CSF was added to the culture medium of glutamate-treated CGN in different concentrations (i.e. 0, 10, and 100 ng/mL) to assess the dose dependency of its neuroprotective effects. G-CSFR activation was blocked by addition of anti G-CSFR antibody (30 minutes before glutamate treatment; 2 μg/mL of antibodies SC 9173 and SC694; Santa Cruz Biotechnology) to examine the specificity of G-CSF effects on attenuation of CGN cell death.

Signaling pathways downstream of G-CSFR activation were blocked with specific inhibitors as follows: AG490 (100 nM, a specific Janus kinase 2 [JAK2]/signal transducer and activator of transcription [STAT] inhibitor; Calbiochem, San Diego, CA), wortmannin (50 nM, a specific phosphatidylinositol 3-kinase [PI3K] inhibitor; Calbiochem), and PD98059 (0.2–20 μM), a specific inhibitor of Ras/mitogen-activated protein kinase. The inhibitors were added to the culture medium 30 minutes before glutamate treatment. After 6 hours of incubation, cell death was determined as described above.

Western Blot Analysis

To determine the precise mechanism of the neuroprotective action of G-CSF, Western blot analysis was

TABLE. Measurement of Motor Function Before and After Spinal Cord Injury

Score	Criteria
0	No noticeable movements of the hindlimbs.
1	Occasional, barely visible movements of any hindlimb joint (hip, knee, or ankle).
2	Obvious movements of 1 or more joints in 1 hindlimb but no forward propulsive, stepping movements.
3	Obvious movements of 1 or more joints in both hindlimbs but no forward propulsive, stepping movements.
4	Stepping and forward propulsive movement of 1 hindlimb. No weight-bearing. Often external rotation of the hindlimb.
5	Alternate stepping and forward propulsive movements of both hindlimbs but no weight-bearing ability. Often external rotation of hindlimbs.
6	Weight-bearing ability of hindlimbs but no normal walking (external rotation of 1 or both limbs and/or hip instability). The animals sweep 1 or both feet while walking (an obvious friction noise can be heard).
7	Weight-bearing ability of hindlimbs, walks with a mild deficit (slight external rotation of 1 or both limbs and/or hip instability).
8	Normal movements except for reduced speed of walking.
9	Normal movements, ability to walk on a 2-cm-wide bar.
10	Normal movements, ability to walk on a 1.5-cm-wide bar.
11	Normal movements, ability to walk on a 1-cm-wide bar.
12	Normal movements, ability to walk on a 7-mm-wide bar.
13	Normal movements, ability to walk on a 5-mm-wide bar.

performed as described previously (9). Briefly, after washing with PBS, cultured CGNs were lysed in homogenization buffer (0.05 M Tris-HCl, pH 7.6, 2% Triton X, and 0.5% protease inhibitor cocktail [Sigma, St. Louis, MO]) at 37°C for 15 minutes. Homogenates were centrifuged at 10,000 x *g* for 5 minutes at 4°C. Samples were stored at –80°C until use. The protein concentration of the samples was determined by the Bradford method. Samples containing 50 μg of protein were electrophoresed on 8% or 12% sodium dodecyl sulfate-polyacrylamide gels, followed by transfer to polyvinylidene difluoride membranes (Hybond-P; Amersham, Piscataway, NJ). Phosphorylated STAT3 (pSTAT3) and Bcl-2 were detected with the anti-pSTAT3 antibody (1:100; Cell Signaling Technology, Inc., Danvers, MA) and anti-Bcl-2 antibody (1:100; Santa Cruz Biotechnology) followed by a peroxidase-conjugated anti-rabbit IgG antibody and anti-mouse IgG antibody (1:2,500; Amersham), respectively. The signal was developed with chemiluminescence (ECL Plus Kit; Amersham). After signal detection, the blots were stripped and reblotted with anti-STAT 3 antibody (1:1,000; Cell Signaling) and anti-actin antibody (1:500, Santa-Cruz) as internal controls.

Surgery and Drug Treatment

Tissue samples were obtained from 9- to 10-week-old female BALBc/Cr mice (18–21 g, average weight 19.5 g; SLC, Hamamatsu, Japan). Animals were anesthetized with 1% to 1.2% halothane in 0.5 L/min oxygen. After laminectomy (T7–8 level), the animals were placed in a stereotaxic apparatus and their spines were fixed with forceps. Compressive SCI was produced at the T7–8 level using a compression rod. The tip of the weight was a 1-mm × 2-mm rectangular plastic plate (10), and the static load (20 g) was applied for 5 minutes. Food and water were given ad libitum. 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 mice were randomly divided into 2 groups (G-CSF group and control group). The G-CSF group mice were subcutaneously injected with recombinant human G-CSF (200 μg/kg/day) for 5 days. The control group was injected with vehicle (1% bovine serum albumin in PBS) only.

Tissue Preparation and Immunohistochemical and Histologic Assessments

Animals were killed at 1 day, 3 days, and 6 weeks after SCI. After transcardiac perfusion, spinal cords were dissected out and 12-μm-thick serial sections were made as described above. Every fifth sections (60 μm apart) were used for histologic (6 weeks after injury) and immunohistochemical (1 day and 3 days after injury) examinations.

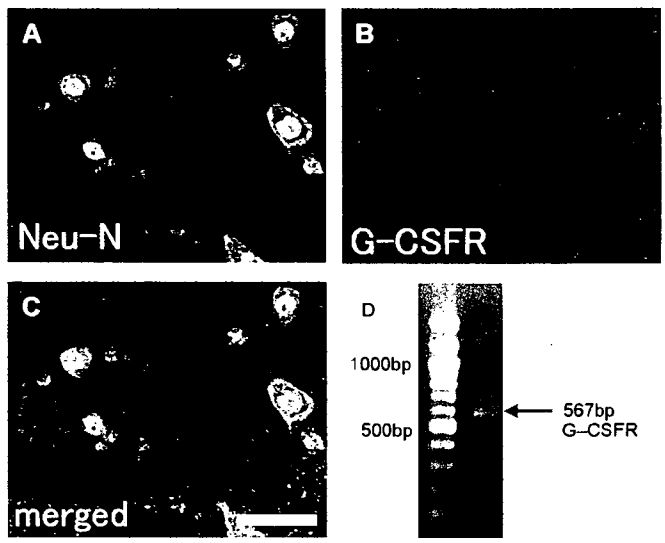


FIGURE 1. Granulocyte colony-stimulating factor receptor (G-CSFR) expression in mouse intact spinal cord. Immunofluorescence staining for **(A)** anti-neuronal nuclei mouse monoclonal antibody, a marker for neuron (green), **(B)** G-CSFR, and **(C)** merged view. G-CSFR expression was predominantly neuronal in mouse spinal cord. **(D)** Reverse transcriptase-polymerase chain reaction for mouse G-CSFR. Expression of G-CSFR mRNA was detected in mouse intact spinal cord with an expected size of 567 base pairs. Scale bar = **(A–C)** 50 μm.)

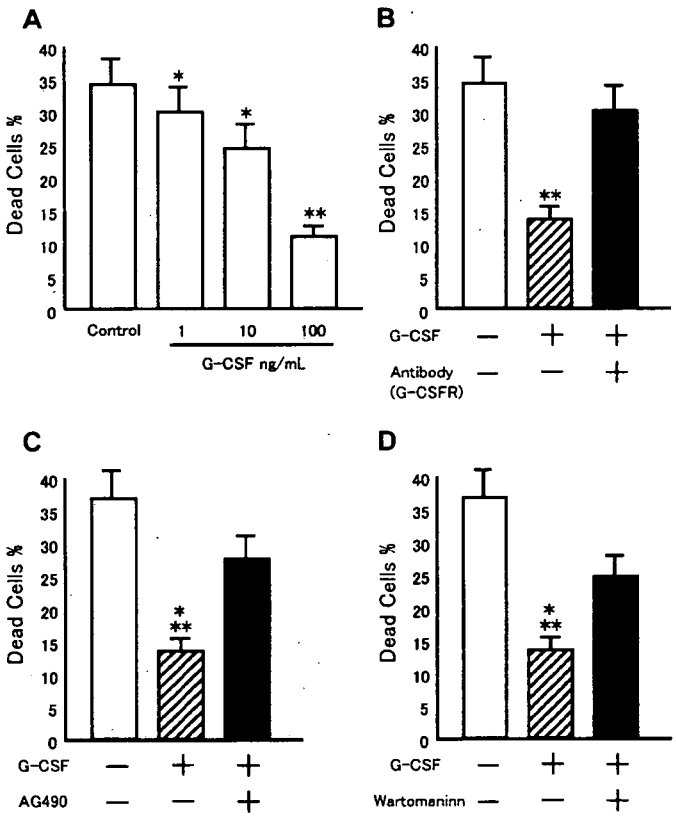


FIGURE 2. Granulocyte colony-stimulating factor (G-CSF) attenuates glutamate-induced neuronal death of cultured cerebellar granule neurons (CGNs). CGNs were exposed to glutamate (100 μM), and G-CSF was added to the culture medium. Viability of CGNs was evaluated with a Live/Dead Double Staining Kit. Granulocyte colony-stimulating factor attenuated glutamate-induced cell death of CGNs in a dose-dependent manner **(A)**. At the G-CSF concentration of 100 ng/mL, the neuroprotective effect was most apparent. Pretreatment of CGNs with anti G-CSFR antibody abolished G-CSF-mediated neuroprotection against glutamate-induced neuronal death **(B)**. Both the JAK2 inhibitor AG490 and the PI3K inhibitor wortmannin partially abolished G-CSF-mediated neuroprotection **(C, D)**. Data are expressed as means ± SE. *, significant difference compared with control ($p < 0.05$); **, significant difference compared with control ($p < 0.01$).

Then, 10 sections were picked up from both the rostral and caudal segments to the lesion epicenter and were used for histologic and immunohistochemical examinations. Sections near the lesion epicenter (from 300 μm rostral to 300 μm caudal) were excluded from immunohistochemical and histologic assessment because the tissue destruction was too severe to count neurons precisely in that area. Therefore, the analyses covered the area from either 300 to 900 μm rostral or 300 to 900 μm caudal to the lesion epicenter.

Immunohistochemistry for apoptotic cells was performed as described above to quantify neuronal apoptosis in the injured spinal cord. Mouse Neu-N antibody (1:400 dilution) was used as a neuronal specific marker, and rabbit anti-cleaved caspase-3 antibody (1:800 dilution; Genzyme/Techne, Minneapolis, MN) was used as a marker for apoptotic cells. Secondary antibodies were as follows: goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594 (1:800 dilution). The number of apoptotic neurons was determined by counting cells that were double positive for Neu-N and cleaved caspase-3. Neuronal survival was assessed 6 weeks after injury using cresyl violet staining.

Behavioral Testing for Recovery of Hindlimb Motor Function

The functional recovery of hindlimb was determined by measuring the hindlimb motor function score as described previously by Farooque et al (10). Mice were allowed to move freely in an open field with a rough surface for 5 minutes at each time tested. The hindlimb movements of mice were videotaped and scored by 2 independent observers who were unaware of the treatment group. Measurement of motor function was performed before surgery and 1 and 3 days and 1 to 6 weeks (once a week) after SCI. The scale ranged from 0 to 13, and scores are shown in the Table. In brief, a score of 0 indicates complete paralysis, a score of 1 to 3 indicates movement of hindlimbs without rhythmical stepping, a score of 4 to 5 indicates rhythmical motion of hindlimbs without weight-bearing ability, a score of 6 to 7 indicates weight-bearing ability, a score of 8 to 12 indicates walking ability with an increase in the hindlimb gait width, and a score of 13 indicates full recovery.

Statistical Analysis

Motor function scores were subjected to repeated measures analysis of variance followed by post hoc test using the Scheffé F-test. The final motor function score was statistically analyzed using the Mann-Whitney U-test. The neuron survival counts and apoptotic neuron counts were subjected to the Student *t*-test. Data are presented as mean

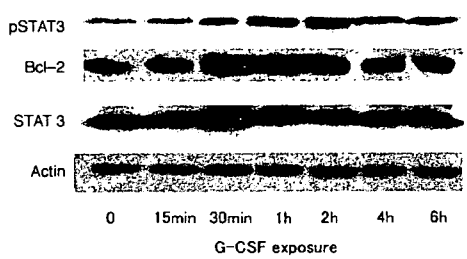


FIGURE 3. Western blot analysis for signaling molecules downstream of granulocyte colony-stimulating factor (G-CSF) receptor in cultured cerebellar granule neurons (CGNs). Cultured CGNs were exposed to G-CSF (100 ng/mL) and expression of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and Bcl-2 was detected by Western blot. The level of pSTAT3 increased by 15 minutes after G-CSF exposure, peaked 1 or 2 hours after exposure and then decreased after 6 hours. The level of Bcl-2 increased by 30 minutes after G-CSF exposure and remained elevated for 6 hours. There was no change in the expression levels of total STAT3 and actin, showing equal protein sample loading.

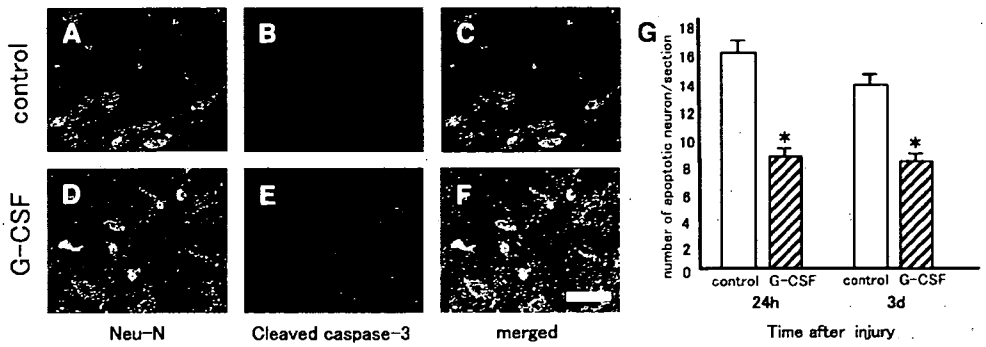


FIGURE 4. Immunohistochemical assessment of neuronal apoptosis in the acute phase after spinal cord injury. Double immunofluorescence staining for anti-neuronal nuclei mouse monoclonal antibody (Neu-N), a marker for neuron (green) (**(A, D)** and cleaved caspase-3 (red) **(B, E)** and the merged views **(C, F)**. **(A–C)** are the control group, and **(D–F)**) are the G-CSF-treated group. The number of apoptotic neurons (cells double positive for Neu-N and cleaved caspase-3) in the G-CSF group (hatched columns) was significantly smaller than that in the control group (open columns) at 24 hours and 3 days after injury. Data are expressed as means ± SE. *, significant difference ($p < 0.05$). Scale bar = 50 μm.

values ± SE. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Granulocyte Colony-Stimulating Factor Receptor Expression in Vitro and in Vivo

In cultured CGNs, G-CSFR was detected by immunocytochemistry (not shown) and G-CSFR mRNA was detected by RT-PCR (not shown). Similarly, immunohistochemistry on intact mouse spinal cord sections revealed G-CSFR expression on Neu-N-positive neurons (Fig. 1A–C), and RT-PCR on intact mouse spinal cord showed G-CSFR mRNA expression (Fig. 1D). These data indicate that G-CSFR is expressed by neurons in vitro and in vivo (in spinal cord).

Cell Culture Experiment

To examine neuroprotection against glutamate-induced neuronal death, G-CSF was administered to cultured CGNs simultaneously with glutamate. The results showed that G-CSF significantly decreased glutamate-induced neuronal death in a dose-dependent manner, with a concentration of 100 ng/mL exhibiting the optimal neuroprotective effect (Fig. 2A).

To examine the specificity of G-CSF-mediated neuroprotection, functional blocking of G-CSFR was performed. Pretreatment of CGNs with anti G-CSFR antibody 30 minutes before glutamate treatment abolished G-CSF-mediated neuroprotection against glutamate-induced neuronal death (Fig. 2B).

Next, signaling pathways downstream of G-CSFR were blocked with specific inhibitors to clarify the contribution of each signaling pathway to the neuroprotective effects of G-CSF. Both the JAK2/STAT inhibitor AG490 and the PI3K inhibitor wortmannin partially abolished G-CSF-mediated neuroprotection against glutamate-induced neuronal death (Fig. 2C, D), whereas the mitogen-activated protein kinase inhibitor PD98059 had no influence on the neuroprotective effects of G-CSF.

Western blot analysis was performed to examine signaling events after G-CSF treatment. In cultured CGNs,

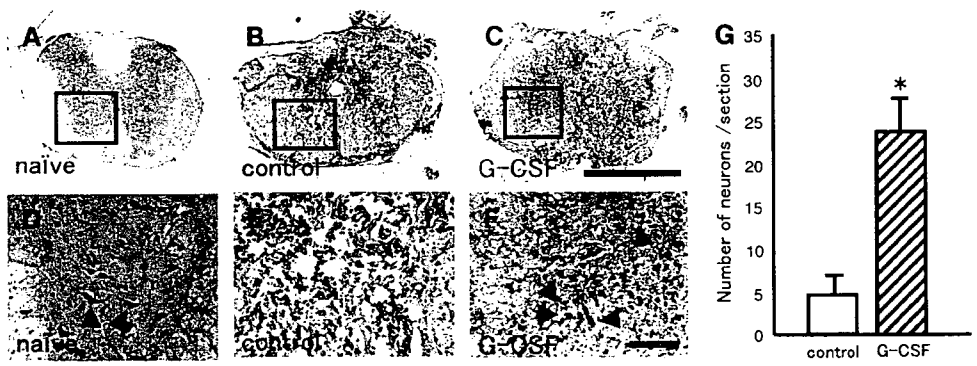


FIGURE 5. Histologic assessment with cresyl violet staining after spinal cord injury. Few neurons survived in the spinal cord of control mice 6 weeks after injury **(B, E)**. In the granulocyte colony-stimulating factor (G-CSF)-treated mice, increased neuronal survival was observed in the spinal cord 6 weeks after injury **(C, F**, arrowheads). Neurons in the anterior horn of the naive mouse spinal cords **(A, D**, arrowheads). Scale bars = **(A–C)** 1,000 μm; **(D–F)** = 100 μm. The number of surviving neurons in the G-CSF group was significantly larger than that of the control group **(G)**. The average number of the surviving neuron in the G-CSF group was 24.0/slice (hatched column), whereas neuronal survival in the control group was 4.6/slice (open column). Data are expressed as means ± SE. *, significant difference ($p < 0.05$).

the expression level of pSTAT3 increased by 15 minutes after G-CSF exposure, peaked 1 to 2 hours after exposure, and then decreased 6 hours after G-CSF treatment (Fig. 3). Levels of Bcl-2, an antiapoptotic target of STAT3, were elevated within 30 minutes of G-CSF exposure and remained elevated at 6 hours (Fig. 3). The expression level of total STAT 3 was unchanged during observation (Fig. 3). There was also no change in the expression level of actin, indicating equal protein loading (Fig. 3).

Immunohistochemical and Histologic Assessment

Our results using a model of compressive SCI demonstrate that G-CSF also exhibits neuroprotective effects in vivo. The number of cells double positive for Neu-N and cleaved caspase-3 was significantly smaller in the G-CSF-treated group than in the control group 1 day and 3 days after injury ($p < 0.05$) (Fig. 4). The average number of double positive cells for Neu-N and cleaved caspase-3 in the G-CSF group was 8.8 (5–13) 1 day after injury and 7.7 (3–11) 3 days after injury, whereas the number of apoptotic neurons in the control group was 16.3 (8–19) 1 day after injury and 14.1 (9–17) 3 days after injury, respectively.

Six weeks after injury, cresyl violet-positive neurons in the anterior horn of the spinal cord were counted as surviving neurons. The number of surviving neurons in the G-CSF group was significantly larger than that in the control group ($p < 0.05$) (Fig. 5). The average number of surviving neurons in the G-CSF group was 24.0/slice, whereas that in the control group was 4.6/slice.

Recovery of Hindlimb Motor Function

We assessed the recovery of hindlimb function using the motor function scale (10), in which the maximum hindlimb motor function scale score is 13. All mice had a score of 13 before surgery, and the score dropped to 0 immediately after the SCI. Significant recovery of hindlimb function was observed in mice from the G-CSF-treated group 4 weeks after injury compared with those of the vehicle-treated group ($p < 0.05$) (Fig. 6A). The average recovery score in the G-CSF group 6 weeks after injury was 4.0 (3–6), indicating stepping and forward propulsive movements of 1 hindlimb without weight bearing, whereas the average recovery score in the control group was 2.9 (2–5), indicating obvious movements of 1 or more joints in both hindlimbs without coordination, alternate stepping movements, or weight-bearing (Fig. 6B). The highest recovery score in the G-CSF group was 6, indicating weight-bearing ability of hindlimbs with abnormal walking (external rotation of 1 or both limbs and/or hip instability) whereas the highest recovery score in the control group was 5, indicating alternate stepping and forward propulsive movement of the hindlimbs without weight bearing.

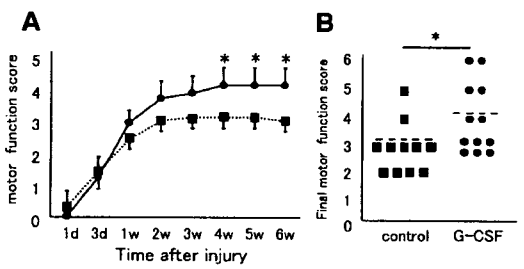


FIGURE 6. Hindlimb functional assessment using the motor functional scale (Farooque et al [10]). Time course of hindlimb functional recovery **(A)**, and comparison of final motor function score between the groups **(B)**. A significant recovery of hindlimb function was observed in granulocyte colony-stimulating factor (G-CSF)-treated mice (circles) 4 weeks after injury compared with the control group (**A**, squares) ($p < 0.05$). The average recovery score in the G-CSF-treated mice 6 weeks after injury was 4.0 (3–6) (**B**, circles), whereas the average recovery score in the control group was 2.9 (2–5) (**B**, squares). Data are expressed as means ± SE. *, significant difference ($p < 0.05$).

DISCUSSION

Our results from this study demonstrate that G-CSF prevented glutamate-induced neuronal death. Furthermore, administration of G-CSF prevented neuronal apoptosis during the acute phase after SCI, resulting in elevated neuronal survival in the spinal cord and improved hindlimb motor function 6 weeks after injury.

G-CSF regulates survival of postmitotic neutrophils by inhibition of apoptosis in the hematopoietic system with activation of the G-CSFR (11). Known signaling cascades downstream of G-CSFR are divided mainly into 2 classes, activation of the STAT family or the PI3K/Akt pathway. In hematopoietic lineage cells, G-CSF activates intracellular signaling pathways including STAT3 (12) and Akt (13), which are both linked to suppression of apoptosis and proliferation. The antiapoptotic function of STAT3 is mediated through its regulation of Bcl-2 expression (14). Akt promotes cell survival through multiple pathways, including the phosphorylation and inactivation of the proapoptotic protein BAD and through its induction of the antiapoptotic protein Bcl-2 (15–17). Recent reports have revealed that many basic cellular pathways are highly conserved between hematopoietic and neural cells. For instance, in addition to its expression in hemapoietic cells, G-CSFR is also expressed in neurons in the cerebral cortex (5), by Purkinje cells in the cerebellum and cerebellar nuclei (6), and by neurons and glial cells in the corpus callosum (5). G-CSF exerts antiapoptotic effects in these neurons through activation of pro-survival pathways similar to those in hemapoietic cells (18).

In the present study, we have confirmed that G-CSFR is expressed by cerebellar granule neurons in vitro and are the first to show that G-CSFR is also expressed by spinal cord neurons in vivo. Previous reports have shown that G-CSF treatment attenuates glutamate-induced neuronal death of CGNs (5), staurosporine-induced apoptosis of rat primary cortical neurons, and camptothecin-induced apoptosis of the human neuroblastoma cell line SHSY-5Y (6). Our results showed that simultaneous G-CSF administration had a partial neuroprotective effect against glutamate-induced neuronal death of CGNs. These results differ from those of Schäbitz et al (5), who reported that pretreatment with G-CSF completely suppressed glutamate-induced neuronal

death in CGNs. This discrepancy may be due to the difference in the time of G-CSF administration. Furthermore, we showed that pretreatment with anti-G-CSFR antibody completely abolished G-CSF-mediated neuroprotection against glutamate-induced neuronal death of CGNs, suggesting that G-CSF exerts its neuroprotective effect on CGNs via binding to G-CSFR. Schneider et al (6) have demonstrated that the antiapoptotic effects of G-CSF in rat primary cortical neurons treated with staurosporine and the human neuroblastoma cell line SHSY-5Y treated with camptothecin occurs via activation of PI3K/Akt pathway and could be abolished by the specific PI3K/Akt inhibitor LY294002. They also showed that activation of STAT3 and upregulation of its antiapoptotic target Bcl-xL were induced by G-CSF treatment in rat primary cortical neurons (6). Komine-Kobayashi et al (7) reported that G-CSF-induced activation of STAT3 and its antiapoptotic target Bcl-2 resulted in inhibition of apoptotic neuronal death after transient focal cerebral ischemia in mice. In the present study, G-CSF suppression of glutamate-induced cell death of cultured CGNs occurred via the activation of both the JAK/STAT and PI3K/Akt pathways, which could be abolished by their specific inhibitors. Moreover, the present results show that G-CSF promotes the activation of STAT3 and upregulation of Bcl-2 in cultured CGNs. These lines of evidence suggest that the neuroprotective effect of G-CSF is mediated at least partially via the activation of the JAK/STAT and PI3K/Akt pathways as previously reported (18).

Our in vivo results indicate that G-CSF suppressed neuronal apoptosis during the acute phase and increased the number of surviving neurons after the chronic phase of SCI, suggesting that G-CSF is also neuroprotective in vivo. In addition to its direct effects on spinal cord neurons, G-CSF has several actions in vivo that may contribute to neuroprotection. G-CSF suppresses inflammatory cytokines in experimental allergic encephalitis in mice (19) and tumor necrosis factor-α expression in lipopolysaccharide-stimulated human monocytes in vitro (20). Therefore, G-CSF may exert neuroprotective effects by suppressing inflammatory cytokine expression during the acute phase of SCI. G-CSF also can promote mobilization of bone marrow-derived cells and their migration into injured tissues including ischemic brain tissue (7, 21). Recently, Urdzikova et al (22) reported that subacutely (7–11 days postinjury) administered G-CSF promotes functional recovery after SCI in rats via mobilization of bone marrow cells.

Moreover, G-CSF has several actions on the vascular system, and, in a model of stroke, G-CSF suppresses brain edema formation (23) and promotes angiogenesis (24). Finally, G-CSF stimulates neurogenesis both directly (6) or via the upregulation of vascular endothelial growth factor (25). All of these mechanisms may contribute to the neuroprotective effects of G-CSF after SCI. Although this is the first report showing G-CSF-mediated neuroprotection that results in functional recovery, further exploration is needed to clarify the precise mechanism of action.

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 therapeutic agents 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, the currect use of G-CSF in the clinic for hematopoietic stimulation and its ongoing clinical trial for brain infarction (26) make it an appealing molecule that could be rapidly placed into trials for patients with acute SCI. Although many hurdles (e.g. optimal dosage, therapeutic time windows, and more precise mechanisms of action) still need to be resolved, the present results encourage us to pursue future clinical trials of G-CSF for patients with acute SCI.

ACKNOWLEDGMENT

The authors thank KIRIN Brewery (Tokyo, Japan) for kindly providing human recombinant G-CSF.

REFERENCES

1. Profyris C, Cheema SS, Zang DW, et al. Degenerative and regenerative mechanisms governing spinal cord injury. Neurobiol Dis 2004;15:415–36
2. Nicola NA, Metcalf D, Matsumoto M, et al. Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. Identification as granulocyte colony-stimulating factor. J Biol Chem 1983;258:9017–23
3. Roberts AW. G-CSF: A key regulator of neutrophil production, but that's not all! Growth Factors 2005;23:33–41
4. Harada M, Qin Y, Takano H, et al. G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. Nat Med 2005;11:305–11
5. Schäbitz WR, Kollmar R, Schwaninger M, et al. Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. Stroke 2003;34:745–51
6. Schneider A, Kruger C, Steigleder T, et al. The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. J Clin Invest 2005;115:2083–98
7. Komine-Kobayashi M, Zhang N, Liu M, et al. Neuroprotective effect of recombinant human granulocyte colony-stimulating factor in transient focal ischemia of mice. J Cereb Blood Flow Metab 2006;26:402–13
8. Kamada T, Koda M, Dezawa M, et al. Transplantation of bone marrow stromal cell-derived Schwann cells promotes axonal regeneration and functional recovery after complete transection of adult rat spinal cord. J Neuropathol Exp Neurol 2005;64:37–45
9. Koda M, Hashimoto M, Murakami M, et al. Adenovirus vector-mediated in vivo gene transfer of brain-derived neurotrophic factor (BDNF) promotes rubrospinal axonal regeneration and functional recovery after complete transection of the adult rat spinal cord. J Neurotrauma 2004;21:329–37
10. Farooque M, Isaksson J, Olsson Y. Improved recovery after spinal cord injury in neuronal nitric oxide synthase-deficient mice but not in TNF-α-deficient mice. J Neurotrauma 2001;18:105–14
11. Hu B, Yasui K. Effects of colony-stimulating factors (CSFs) on neutrophil apoptosis: Possible roles at inflammation site. Int J Hematol 1997;66:179–88
12. Chakraborty A, White SM, Schaefer TS, et al. Granulocyte colony-stimulating factor activation of Stat3α and Stat3β in immature normal and leukemic human myeloid cells. Blood 1996;88:2442–49
13. Dong F, Larner AC. Activation of Akt kinase by granulocyte colony-stimulating factor (G-CSF): Evidence for the role of a tyrosine kinase activity distinct from the Janus kinases. Blood 2000;95:1656–62

14. Fukada T, Hibi M, Yamanaka Y, et al. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: Involvement of STAT3 in anti-apoptosis. Immunity 1996;5:449–60
15. Datta SR, Brunet A, Greenberg ME. Cellular survival: A play in three Akts. Genes Dev 1999;13:2905–27
16. del Peso L, Gonzalez-Garcia M, Page C, et al. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 1997; 278:687–99
17. Ahmed NN, Grimes HL, Bellacosa A, et al. Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. Proc Natl Acad Sci U S A 1997;94:3627–32
18. Schneider A, Kuhn HG, Schäbitz WR. A role for G-CSF (granulocyte-colony stimulating factor) in the central nervous system. Cell Cycle 2005;4:1753–57
19. Zavala F, Abad S, Ezine S, et al. G-CSF therapy of ongoing experimental allergic encephalomyelitis via chemokine-and cytokine-based immune deviation. J Immunol 2002;168:2011–19
20. Nishiki S, Hato F, Kamata N, et al. Selective activation of STAT 3 in human monocytes stimulated by G-CSF: Implication in inhibition of LPS-induced TNF-α production. Am J Physiol Cell Physiol 2004;286: 1302–11
21. Kawada H, Takizawa S, Takanashi T, et al. Administration of hematopoietic cytokines in the subacute phase after cerebral infarction is effective for functional recovery facilitating proliferation of intrinsic neural stem/progenitor cells and transition of bone marrow-derived neuronal cells. Circulation 2006;113:701–10
22. Urdzikova L, Jendelova P, Glogarova K, et al. Transplantation of bone marrow stem cells as well as mobilization by granulocyte-colony stimulating factor promotes recovery after spinal cord injury in rats. J Neurotrauma 2006;23:1379–91
23. Gibson C, Jones C, Prior MLW, et al. G-CSF suppresses edema formation and reduces interleukin-1β expression after cerebral ischemia in mice. J Neuropathol Exp Neurol 2005;64:763–69
24. Lee ST, Chua K, Jung KH, et al. Granulocyte colony-stimulating factor enhances angiogenesis after focal cerebral ischemia. Brain Res 2005; 1058:120–28
25. Jung KH, Chu K, Lee ST, et al. Granulocyte colony-stimulating factor stimulates neurogenesis via vascular endothelial growth factor with STAT activation. Brain Res 2006;1073:190–201
26. Shyu WC, Lin SZ, Lee CC, et al. Granulocyte colony-stimulating factor for acute ischemic stroke: A randomized controlled trial. CMAJ 2006; 174:927–33

Eur Spine J (2007) 16:2206–2214
DOI 10.1007/s00586-007-0499-3

ORIGINAL ARTICLE

Adenovirus vector-mediated ex vivo gene transfer of brain-derived neurotrophic factor to bone marrow stromal cells promotes axonal regeneration after transplantation in completely transected adult rat spinal cord

Masao Koda · Takahito Kamada · Masayuki Hashimoto · Masazumi Murakami · Hiroshi Shirasawa · Seiichiro Sakao · Hidetoshi Ino · Katsunori Yoshinaga · Shuhei Koshizuka · Hideshige Moriya · Masashi Yamazaki

Received: 8 January 2007 / Revised: 1 June 2007 / Accepted: 28 August 2007 / Published online: 21 September 2007

Abstract The aim of this study was to evaluate the efficacy in adult rat completely transected spinal cord of adenovirus vector-mediated brain-derived neurotrophic factor (BDNF) ex vivo gene transfer to bone marrow stromal cells (BMSC). BMSC were infected with adenovirus vectors carrying β-galactosidase (AxCALacZ) or BDNF (AxCABDNF) genes. The T8 segment of spinal cord was removed and replaced by graft containing Matrigel alone (MG group) or Matrigel and BMSC infected by AxCALacZ (BMSC-LacZ group) or AxCABDNF (BMSC-BDNF group). Axons in the graft were evaluated by immunohistochemistry and functional recovery was assessed with BBB locomotor scale. In the BMSC-BDNF group, the number of fibers positive for growth associated protein-43, tyrosine hydroxylase, and calcitonin gene-related peptide was significantly larger than numbers found for the MG and BMSC-LacZ groups. Rats from BMSC-BDNF and BMSC-LacZ groups showed significant recovery of hind limb function compared with MG rats; however, there was no significant difference between groups in degree of functional recovery. These findings demonstrate that adenovirus vector-mediated ex vivo gene transfer of BDNF enhances the capacity of BMSC to promote axonal regeneration in this completely transected spinal cord model; however, BDNF failed to enhance hind limb functional recovery. Further investigation is needed to establish an optimal combination of cell therapy and neurotrophin gene transfer for cases of spinal cord injury.

Keywords Bone marrow stromal cell · Brain-derived neurotrophic factor · Adenovirus · Spinal cord injury · Gene therapy

M. Koda (✉) · T. Kamada · M. Hashimoto · M. Murakami · S. Koshizuka · H. Moriya · M. Yamazaki
Department of Orthopaedic Surgery,
Chiba University Graduate School of Medicine,
1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
e-mail: m-koda@bb.em-net.ne.jp

H. Shirasawa
Department of Molecular Virology,
Chiba University Graduate School of Medicine, Chiba, Japan

S. Sakao
Department of Respirology,
Chiba University Graduate School of Medicine, Chiba, Japan

H. Ino
Department of Neurobiology,
Chiba University Graduate School of Medicine, Chiba, Japan

K. Yoshinaga
Division of Rehabilitation Medicine,
Chiba University Graduate School of Medicine, Chiba, Japan

Introduction

It has been widely believed that axons in transected adult mammalian spinal cords cannot regenerate. This failure of regeneration is ascribed to the non-permissive environment in the damaged adult mammalian spinal cord, an environment marked by inhibitory molecules in scar tissue and myelin components that block axonal regeneration, a lack of trophic support for axotomized neurons, and post-axotomy intrinsic neuronal changes including cell atrophy and death [1]. Cell transplantation therapy has been attempted to overcome these problems and achieve restoration of damaged spinal cord [2]. Among various types of candidate cells, the bone marrow stromal cell (BMSC) is promising because it has shown potential to repair damaged spinal cord [3–7]. Moreover, BMSC can be easily obtained from

adult bone marrow and transplanted autologously, avoiding the immunological and ethical problems that are associated with transplantation of other types of stem cells such as embryonic stem cells [8] or neural stem cells [9].

Neurotrophic factors are keys in modulation of neuronal survival, neurite outgrowth, synaptic plasticity and neurotransmission. Exogenous administration of neurotrophic factors has been proposed as one potential therapeutic treatment for spinal cord injury. Among various neurotrophic factors, we and others have reported the effectiveness of brain-derived neurotrophic factor (BDNF) for spinal cord injury in adult rats. Exogenous administration of BDNF as a protein improves locomotor function after spinal cord injury [10, 11]. We have previously demonstrated that adenovirus vector-mediated in vivo gene transfer of BDNF in completely transected adult rat spinal cord is followed by regeneration of the descending tracts and partial functional recovery [12]. Thus, BDNF may have potential to enhance the regeneration that is promoted by cell transplantation therapy. In fact, retrovirus vector-mediated ex vivo gene transfer of BDNF to fibroblasts [13, 14] or BMSC [15] promotes axonal regeneration and functional recovery, and adenovirus vector-mediated ex vivo BDNF gene transfer enhances the regeneration promoting capacity of olfactory ensheathing glial cells [16].

In the current research, we tested the efficacy of adenovirus-mediated ex vivo BDNF gene therapy to BMSC in completely transected adult rat spinal cord as a candidate for combined therapy involving cell transplantation and gene transfer.

Materials and methods

Primary bone marrow stromal cell (BMSC) cultures

BMSC were collected from femurs of adult male Wistar rats (SLC, Hamamatsu, Japan). Rats were euthanized with pentobarbital overdose, and femurs were removed and placed in cold minimum essential medium with alpha modification (α-MEM, Sigma, St Louis, MO) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 ng/ml amphotericin B (Invitrogen, Carlsbad, CA). After removal of femoral epiphyses, marrow was flushed out with medium using a 25G needle attached to a syringe. Bone marrow cells were cultured with the same medium in a humidified 5% CO_2 atmosphere at 37°C under sterile conditions. Twenty-four hours after initial plating, non-adherent cells were removed by replacement of the medium. After the cells had grown to near confluency, they underwent two to five passages by detachment (0.25% trypsin/1 mM EDTA for 5 min), splitting into 1:3–4 dilution, and replating. At the sixth passage, the cells were transferred to Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 20% FBS without additional supplementation and were maintained beyond a total of 20 passages [17].

Preparation of the adenovirus vectors

We prepared first-generation, replication-deficient recombinant adenovirus vectors in which the E1 and E3 regions were deleted, encoding either *Escherichia coli* β-galactosidase (LacZ; AxCALacZ) or mouse BDNF (AxCABDNF), both under the control of CAG [cytomegalovirus IE enhancer, chicken β-actin promoter, and rabbit β-globin poly (A) signal] promoter using the adenovirus expression vector kit (TaKaRa, Tokyo, Japan) as previously described [12]. We obtained recombinant adenoviruses with a titer of 10^{11} order plaque-forming units (pfu)/ml after concentration and purification.

Transferred gene expression in vitro

To confirm transferred gene expession in vitro, BMSC were infected with adenovirus vectors. The vector dose was multiplicity of infection (MOI) 100 with AxCABDNF (BMSC-BDNF cells) and AxCALacZ (BMSC-LacZ cells). Twenty-four hours after inoculation, the infected BMSC were subjected to X-gal cytochemistry or conditioned medium collection for western blot analysis.

For X-gal cytochemistry, BMSC-LacZ cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, and incubated with X-gal reagents [1 mg/ml 5-bromo-4-chloro-3-indryl-D-galactoside, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$] for 3 h at 37°C.

For western blot analysis, BMSC with or without adenovirus infection were washed three times with cold PBS and the medium was changed to DMEM supplemented with 0.05% FBS. The conditioned medium was collected after 48 h of incubation and frozen until use. BDNF contained in the conditioned medium was detected with western blot analysis as described elsewhere [12]. Recombinant human BDNF (kindly provided by Sumitomo Pharmaceuticals Inc., Osaka, Japan) was used as a positive control.

Spinal cord injury and BMSC transplantation

Tissue samples were obtained from 8-week-old male Wistar rats (average weight 200 g; SLC). Animals were anesthetized with inhaled 1–1.2% halothane in 0.5 l/min

oxygen. Laminectomy was performed at the T8 level, leaving the dura intact. The T7 and T9 spinous processes were clamped to fix the spine. The spinal cord was completely transected with microsurgical scissors at the T7/8 and T8/9 levels, and the T8 spinal cord segment was removed. For transplantation, a 5-mm-long tube made from ultrafiltration membrane (NMWL: 10,000, Millipore, Bedford, MA) was filled with Matrigel (Becton-Dickinson Biosciences, Bedford, MA; MG rat group) or the mixture of Matrigel and BMSC-LacZ or Matrigel and BMSC-BDNF (BMSC-LacZ rat group and BMSC-BDNF rat group, respectively; density, 1×10^8 cells/ml in both groups). The grafts were transplanted into the gap between the rostral and caudal stumps. After transplantation, the T7 and T9 spinous processes were tightened with 1–0 silk suture to prevent kyphosis and to obtain contact between the graft and spinal cord stumps. Muscle and skin were sutured layer to layer, and the rats were placed in warm cages overnight. Food and water were given ad libitum. Manual bladder expression was performed twice a day until recovery of bladder reflex. The BMSC-transplanted animals showed no apparent abnormal behavior. All experimental procedures were performed in compliance with guidelines established by the Animal Care and Use Committee of Chiba University.

Tissue preparation

For histological evaluation, animals in all groups were perfused transcardially with 4% paraformaldehyde in PBS (pH 7.4) under deep pentobarbital anesthesia 6 weeks after transplantation. Three segments of spinal cords including the graft (T7–9) were removed and post-fixed in the same fixative overnight, stored in 20% sucrose in PBS at 4°C, and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). The cryoprotected samples were frozen and kept at –80°C until use. The samples were cut into serial 20-μm sagittal sections with a cryostat and mounted onto poly-L-lysine-coated slides (Matsunami, Tokyo, Japan).

Immunohistochemistry

To evaluate regenerating axons within the graft, we performed immunohistochemistry as previously described [18]. The following primary antibodies were used: anti-growth-associated protein-43 rabbit polyclonal antibody (GAP-43; 1:400; Santa-Cruz) as a marker for regenerating nerve fibers, anti-tyrosine hydroxylase monoclonal antibody (TH; 1:400; Chemicon Int.) as a marker for coeluro-spinal axons, anti-serotonin rabbit polyclonal antibody (5-HT; 1:5,000; Sigma) as a marker for raphe-spinal axons, and anti-calcitonin gene-related peptide rabbit polyclonal antibody (CGRP, 1:1,000; AFFINITI, Exeter, UK) as a marker for sensory afferent axons. Following reaction with primary antibodies, slides were incubated with biotinylated secondary antibodies (HISTOFINE kit, Nichirei, Tokyo, Japan). Then the slides underwent reaction with peroxidase-conjugated streptavidin (Nichirei). The color was developed with diaminobenzidine (DAB) and H_2O_2.

Every fourth section was reacted with each of the antibodies described above. At least three series were stained with each of the four antibodies, resulting in immunohistochemical examination of a tissue block 240 μm in width, approximately half the width of the graft. For each antibody, immuno-positive fibers that traversed lines perpendicular to the central axis of the graft at the rostral (500 μm caudal to the rostral graft-cord interface), central (center of the graft), and caudal (500 μm rostral to the caudal graft-cord interface) levels within the graft were counted and compared with values for other rats in the MG, BMSC-LacZ, and BMSC-BDNF groups. Statistical analysis between groups was performed with the Mann–Whitney *U* test.

Next, we performed immunofluorescent double staining to observe the relationship between regenerating fibers and grafted cells in the rats of the BMSC-BDNF group. Anti-fibronectin mouse monoclonal antibody (Chemicon Int.) was used as a marker for transplanted BMSCs and anti-GAP-43 rabbit polyclonal antibody (Santa-Cruz) was used as a marker for regenerating fibers. To detect a positive signal, each slide was incubated with Alexa 488-conjugated anti-mouse IgG and Alexa 594-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR).

Assessment of locomotor activity

Hind limb function of rats in the MG, BMSC-LacZ, and BMSC-BDNF groups was assessed with the BBB locomotor scale [19] before injury and at 1, 3 days, and 1–6 weeks (once a week) after injury. Statistical analysis of differences between groups was performed with repeated measure analysis of variance (ANOVA). To evaluate whether regenerated nerve fibers contributed to hind limb functional recovery, two rats in the BMSC-BDNF group received re-transection of the graft 6 weeks after transplantation.

Results

Transferred gene expression in vitro

Firstly, we confirmed the infection efficacy of adenovirus vector to BMSC in vitro with X-gal cytochemistry

(Fig. 1a). Approximately 80% of the AxCALacZ-infected BMSC were positive for X-gal staining, indicating the high infection efficacy of adenovirus vector to BMSC in this study.

Next, we examined levels of transferred gene expression of BDNF in vitro with western blot analysis (Fig. 1b). The conditioned medium from BMSC-BDNF cells contained a high level of BDNF, whereas there was no detectable level of BDNF in medium from plates with either uninfected BMSC or BMSC-LacZ cells.

Immunohistochemistry

We performed immunohistochemistry for GAP-43 to detect regenerating axons within the graft. Because the T8 spinal cord segment was removed and replaced by the graft, all GAP-43-positive nerve fibers within the graft could be considered regenerated axons. Numerous GAP-43-positive regenerating nerve fibers were detected within the grafts of both the BMSC-LacZ (Fig. 2d–f) and BMSC-BDNF group rats (Fig. 2g–i), whereas few GAP-43-positive fibers were detected within the grafts of the MG group (Fig. 2a–c). A large proportion of these axons were oriented longitudinally within the graft (Fig. 2f, i). The number of GAP-43-positive fibers was significantly larger at the central and caudal levels in rats of the BMSC-transplanted groups (both BMSC-BDNF and BMSC-LacZ groups) than in MG rats ($P < 0.05$, Fig. 3). Moreover, the number of GAP-43-positive fibers in the BMSC-BDNF group was significantly larger than that in both the BMSC-LacZ and MG groups at the caudal level ($P < 0.05$, Fig. 3).

Next we performed immunohistochemistry for nerve fiber markers to elucidate the subpopulation of regenerating axons. TH-positive nerve fibers are considered as coerulospinal adrenergic nerve fibers and 5-HT-positive nerve fibers are considered as raphe-spinal serotonergic fibers, both of which contribute to motor function [20]; CGRP is a marker for sensory nerve fibers. Numerous TH-positive nerve fibers were detected within the grafts of BMSC-BDNF rats (Fig. 4a), whereas only a few TH-positive nerve fibers were observed in grafts of MG rats (not shown). The number of TH-positive nerve fibers in the BMSC-BDNF group was significantly larger than numbers in the BMSC-LacZ and MG groups at the central level ($P < 0.05$, Fig. 5a). The number of TH-positive nerve fibers in both the BMSC-BDNF and BMSC-LacZ groups was significantly larger than that in the MG group at the caudal level ($P < 0.05$, Fig. 5a). Numerous CGRP-positive fibers were detected in the grafts of BMSC-BDNF and BMSC-LacZ rats (Fig. 4b), whereas few CGRP-positive fibers were observed in grafts of MG rats (not shown). The number of CGRP-positive nerve fibers in the BMSC-BDNF group was significantly larger than that in both the BMSC-LacZ and MG groups at the caudal level ($P < 0.05$, Fig. 5b); the number of CGRP-positive nerve fibers in the BMSC-BDNF and BMSC-LacZ groups was significantly larger than that in the MG group at the rostral and caudal levels ($P < 0.05$, Fig. 5b). Only a few 5-HT-positive nerve fibers were observed within the grafts of all three groups, although numerous 5-HT-positive nerve fibers were detected at the rostral stumps of host spinal cords (Fig. 4 c, d).

Immunofluorescent double staining for fibronectin (one of markers for BMSC) and GAP-43 (a marker for regenerating or sprouting axons) revealed close contact between GAP-43-positive fibers and grafted cells in the BMSC-BDNF group (Fig. 6a–c). In contrast, there were no fibronectin-positive cells in grafts of MG rats (not shown).

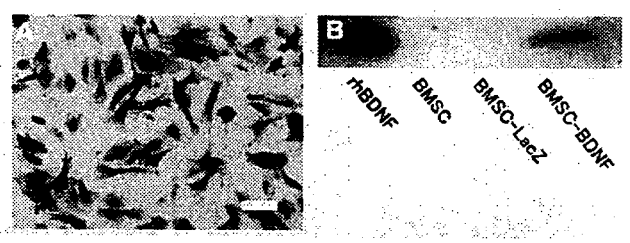


Fig. 1 X-gal histochemistry of AxCALacZ-infected BMSC. **a** AxCALacZ was infected to BMSC at multiplicity of infection (MOI) 100. Twenty-four hours after infection, cells were incubated with X-gal reagent at 37°C for 3 h; 80% of AxCALacZ-infected BMSC was positive for X-gal staining, indicating high infection efficacy. **b** Western blot analysis of BDNF produced by adenovirus AxCABDNF. Bands of BDNF were detected in conditioned medium taken from plates with AxCABDNF-infected BMSC, but not in medium from normal and AxCALacZ-infected BMSC. Recombinant human BDNF (rhBDNF) was used as a positive control. *Bar* 30 μm

Recovery of hind limb function

We assessed recovery of hind limb function with the BBB locomotor scale (Fig. 7). Both the BMSC-LacZ and BMSC-BDNF groups showed significant recovery compared with the MG group from 4 weeks after injury onward (Fig. 7, $P < 0.03$). The average recovery score 6 weeks after transplantation was 6.1 in the BMSC-BDNF group (range 4–8; Fig. 6, circle) and 6.0 in the BMSC-LacZ group (range 4–8; Fig. 7, square), which indicates that two joints of the hind limbs had extensive movement and the third joint had slight movement, whereas the average score in the MG group was 3.6 (range 2–5; Fig. 7, triangle), which indicates that two joints of the hind limbs had extensive movement. There was no significant difference between the BMSC-BDNF and the BMSC-LacZ groups at any time point, indicating that BDNF gene transfer to BMSC had no significant additional effect on the functional recovery promoted by transplantation of BMSC.

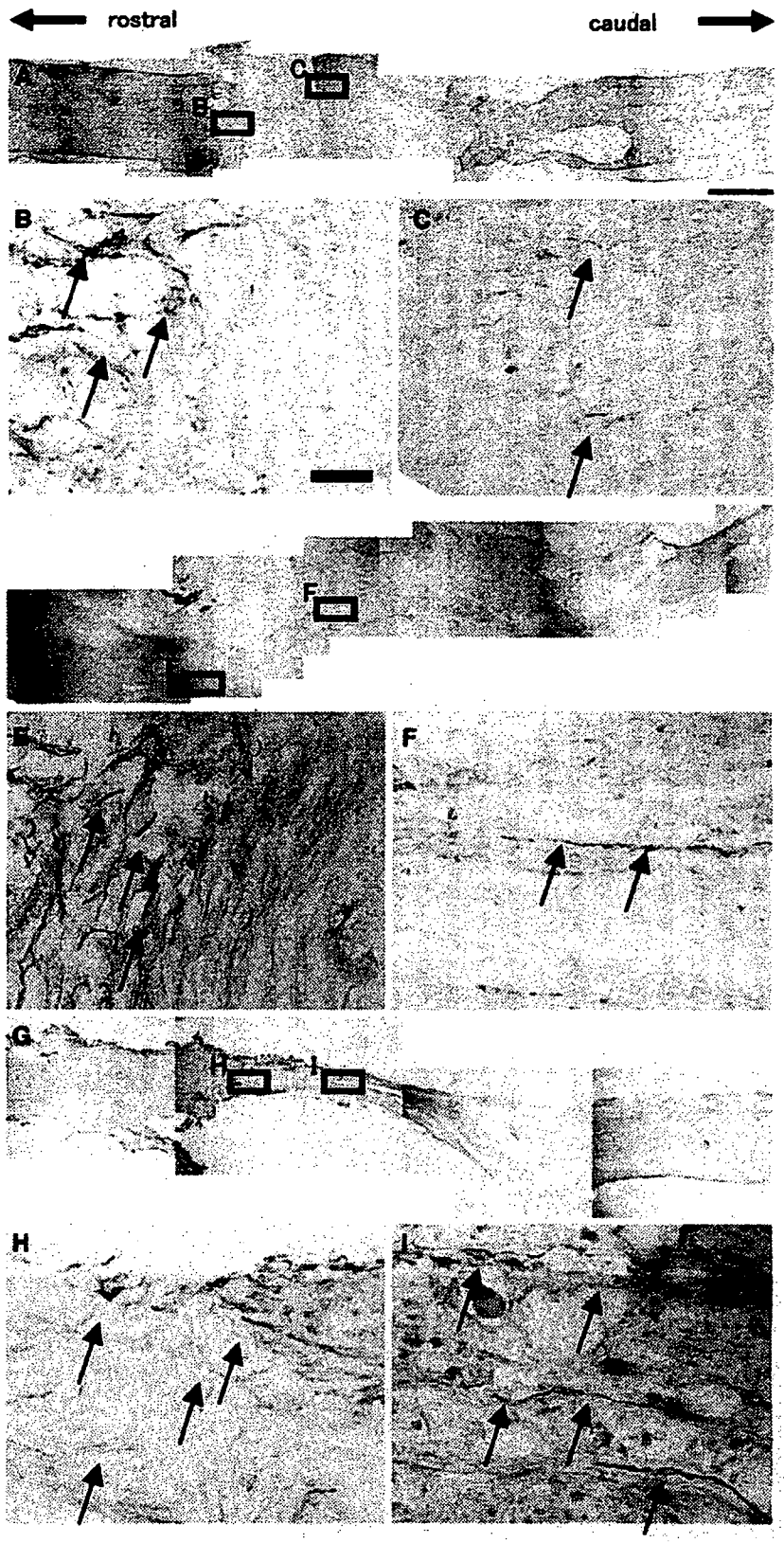


Fig. 2 Immunohistochemistry for GAP-43 in the MG group (**a** overview; **b**, **c** magnified), BMSC-LacZ group (**d** overview; **e**, **f** magnified), and BMSC-BDNF group (**g** overview; **h**, **i** magnified). **b** In the MG group, numerous GAP-43-positive fibers were detected at the interface between host spinal cord and graft (*arrows*). **c** However, few GAP-43-positive regenerating fibers were detected within the graft (*arrows*). In contrast, a number of GAP-43-positive regenerating fibers were found at the middle of grafts in the **f** BMSC-LacZ and **i** BMSC-BDNF groups (*arrows*) as well as at the interface between host spinal cord and graft (**e** BMSC-LacZ, **h** BMSC-BDNF, *arrows*). *Bars* 1 mm (**a**, **d**, **g**) and 30 μm (**b**, **c**, **e**, **f**, **h**, **i**)

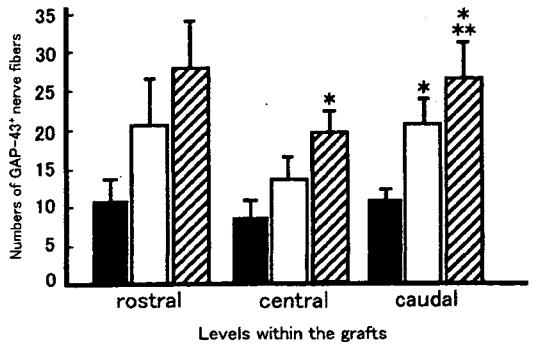


Fig. 3 Numbers of GAP-43-positive axons within the grafts. GAP-43-positive fibers that *traversed lines* perpendicular to the central axis of the grafts at rostral (500 μm caudal to the rostral graft-cord interface), central (center of the graft), and caudal (500 μm rostral to the caudal graft-cord interface) levels within each graft were counted and compared among the MG, BMSC-LacZ, and BMSC-BDNF groups. Statistical analysis of differences between groups was performed with the Mann–Whitney *U* test. At the central level in the graft, the number of GAP-43-positive axons in the BMSC-BDNF group (*hatched column*) was significantly larger than that of the MG group (*closed column*, $P < 0.05$). At the caudal level in the graft, the number of GAP-43-positive axons in the BMSC-BDNF group was significantly larger than that of the MG and BMSC-LacZ groups ($P < 0.05$), and the number of GAP-43-positive axons in the BMSC-LacZ group was significantly larger than that in the MG group ($P < 0.05$). Each value is a mean ± standard error of the mean (SEM). * Significant difference compared with the MG group ($P < 0.05$), ** significant difference compared with the BMSC-LacZ group ($P < 0.05$)

Two rats in the BMSC-BDNF group that showed functional recovery received re-transection of the graft 6 weeks after transplantation. Re-transection completely abolished the recovered function, and only a slight functional recovery was observed after re-transection (1–2 points in BBB score).

Discussion

In the present study, we demonstrated that transplantation of BMSC promoted axonal regeneration in completely transected adult rat spinal cord and that ex vivo BDNF gene transfer to BMSC enhanced axonal regeneration or sprouting.

Although the potential of BMSC to trans-differentiate into neural phenotypes is controversial [17, 21], BMSC have been raised as a candidate for cell transplantation therapy with central nervous system diseases because they have a potential to restore damaged neural tissue and they can be transplanted autologously and harvested easily [22]. In fact, some reports indicate the effectiveness of BMSC transplantation for spinal cord injury. Transplantation of BMSC significantly improves hind limb function in the rat spinal cord injury model [3]. BMSC form guiding strands in the injured spinal cord and promote hind limb functional recovery; in addition, transplanted BMSC differentiate toward neural lineage cells in vivo [4]. Finally, BMSC promote regeneration of injured spinal cord [6]. In contrast to previous reports that used contusion- or compression-induced spinal cord injury models, we used a segmental resection and replacement model to make clear the contribution of axonal regeneration to functional recovery. In the present study, the two BMSC-transplanted groups

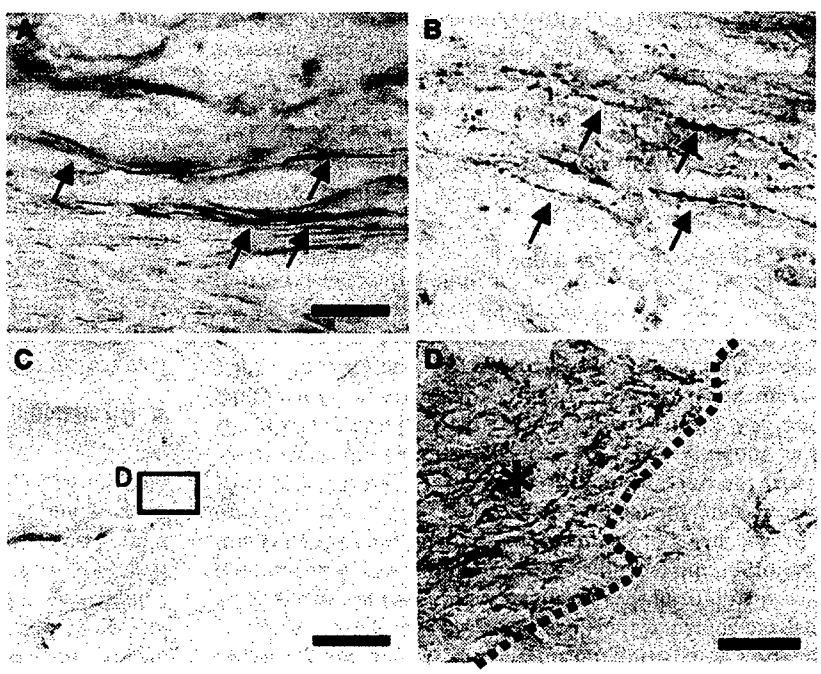


Fig. 4 Immunohistochemistry for nerve fiber markers in the grafts. **a** Numerous TH-positive fibers were detected at the middle of the graft in the BMSC-BDNF group (*arrows*). **b** Numerous CGRP-positive fibers were detected at the middle of the graft in the BMSC-BDNF group. **c**, **d** Only a few 5-HT-positive fibers extended into the graft in BMSC-BDNF rats, although numerous 5-HT-positive fibers were observed at the rostral stump of the host spinalspinal cord [**d**; higher magnification of *box* in **c**; *dotted line* indicates interface between host spinal cord (*asterisk*) and graft]. *Bars* 30 μm (**a**, **b**), 500 μm (**c**), and 50 μm (**d**)

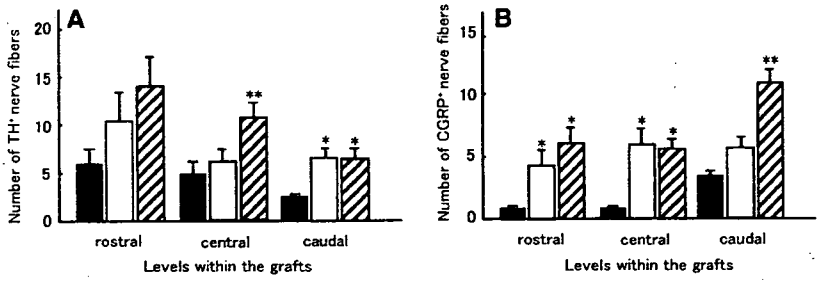


Fig. 5 Numbers of tyrosine hydroxylase-positive and calcitonin gene-related peptide-positive (CGRP-positive) fibers in the grafts. **a** The number of TH-positive axons in the BMSC-BDNF group (*hatched column*) was significantly larger than numbers in the MG (*closed column*) and BMSC-LacZ (*open column*) groups ($P < 0.05$) at the central level in the graft. At the caudal level, the numbers of TH-positive axons in the BMSC-BDNF (*hatched column*) and BMSC-LacZ (*open column*) groups were significantly larger than that in the MG group (*closed column*, $P < 0.05$). **b** The numbers of CGRP-positive axons in the BMSC-LacZ (*open column*) and BMSC-BDNF (*hatched column*) groups were significantly larger than that in the MG group (*closed column*) at the rostral and central levels ($P < 0.05$). At the caudal level, the number of CGRP-positive axons in the BMSC-BDNF group (*hatched column*) was significantly larger than those in the MG (*closed column*) and BMSC-LacZ (*open column*) groups ($P < 0.05$). * Significant difference compared with the MG group ($P < 0.05$), ** significant difference compared with the BMSC-LacZ group ($P < 0.05$)

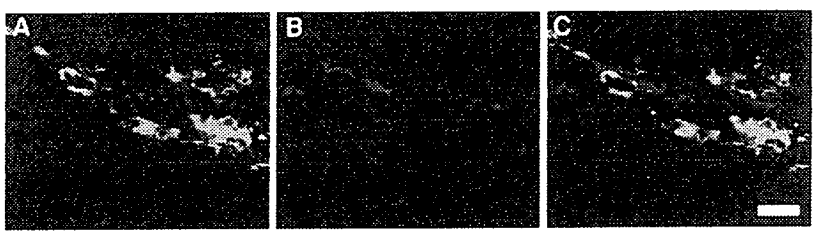


Fig. 6 Immunofluorescent double staining for fibronectin and GAP-43 in BMSC-BDNF rats. The slides were incubated with anti-fibronectin and anti-GAP-43 antibodies, followed by incubation with Alexa 488-conjugated (fibronectin; *green*) and Alexa 594-conjugated (GAP-43; *red*) secondary antibodies. **a** Fibronectin-positive transplanted BMSCs were longitudinally oriented within the graft. **b** In addition, some of the GAP-43-positive regenerating fibers were longitudinally oriented within the graft. **c** Merged view shows the close relationship between transplanted BMSCs and regenerating fibers. *Bar* 30 μm

(BMSC-LacZ and BMSC-BDNF) showed significant axonal regeneration and hind limb functional recovery compared with rats in the MG group, a finding that sheds light on the axonal regeneration-inducing properties of BMSC. Immunofluorescent double staining revealed a close relationship between transplanted cells and regenerating axons, suggesting that transplanted BMSC may act as an attractive cue for regenerating axons. Taken together, previous reports and the present results suggest that BMSC have great potential to repair damaged spinal cord.

It is well known that specific neurotrophic factors elicit regrowth of specific axonal populations after spinal cord injury [23]. BDNF promotes regeneration of CGRP-positive sensory fibers, 5-HT-positive serotonergic fibers, TH-positive coerulospinal fibers, as well as reticulospinal and rubrospinal tracts in various experimental paradigms [23]. It has been reported that transplantation of BMSC alone [4] or BMSC engineered to express BDNF by retrovirus vector [15] promote regrowth of 5HT-positive serotonergic axons. In the present study, adenovirus vector-mediated BDNF gene transfer to BMSC enhanced axonal regeneration of TH-positive coerulospinal fibers at the central level and CGRP-positive sensory fibers at the caudal level of grafts; however, BDNF did not promote regeneration of 5-HT-positive raphespinal fibers. The difference in regenerating axonal phenotype between the present results and previously reported ones may be caused by differences in experimental conditions (i.e., injury models, duration and amount of transferred gene expression, number of transplanted cells and scaffold).

In contrast to previous reports using retrovirus vector-mediated ex vivo gene transfer [13–15], we employed adenovirus vector to transfer therapeutic gene ex vivo in the present study, as Ruitenberg has reported [16]. The reasons are as follows. Firstly, adenovirus vector can infect various types of cells with high efficacy. In contrast, retrovirus vector requires selection with antibiotics, a time-consuming procedure, to obtain high transfection efficacy. Because the clinical therapeutic time window for cell therapy in spinal cord injury is limited to a few weeks after initial trauma [9], it is necessary to obtain transfectant within a relatively short time. Secondly, transient transfection of neurotrophic factor with adenovirus vector is sufficient to promote axonal regeneration, something we

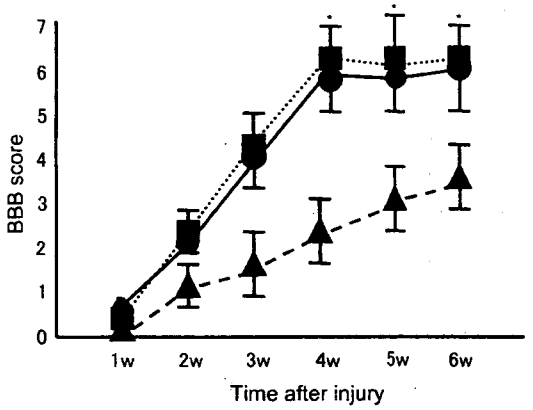


Fig. 7 Hind limb functional assessment with the BBB locomotor scale. Rats from the BMSC-BDNF group (*square*) and BMSC-LacZ group (*circle*) showed significant recovery from 4 weeks after injury onward compared with rats from the MG group (*triangle*). There was no significant difference between the recovery score of the BMSC-BDNF and BMSC-LacZ groups at any time point. Values represent mean ± SEM. * $P < 0.03$ compared with the MG group. *SEM* standard error of the mean

have previously reported [12]. It is not always necessary to transplant cells that have stably integrated the transferred gene to promote axonal regeneration.

In the present study, ex vivo BDNF gene transfer to BMSC failed to enhance hind limb functional recovery although it did significantly enhance axonal regeneration. Possible explanations for this discrepancy could be as followings; the observation period could be too short to detect significant functional recovery caused by axonal regeneration, greater numbers of regenerating axons are necessary to enhance hind limb functional recovery or regenerating axons cannot grow into the caudal spinal cord. We could not directly prove re-entrance of regenerating axons into the distal cord stump. However, re-transection of the graft in BMSC-BDNF rats completely abolished recovered hind limb function, suggesting that regenerating axons contributed to functional recovery to some extent.

In conclusion, ex vivo BDNF gene therapy to BMSC is a candidate for combined cell-neurotrophic factor therapy, although further investigation is needed to find an optimal condition.

Acknowledgments This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (16390427-00).

References

1. Profyris C, Cheema SS, Zang DW, Azari MF, Doyle K, Petratos S (2004) Degenerative and regenerative mechanisms governing spinal cord injury. Neurobiol Dis 15:415–436
2. Murray M (2004) Cellular transplantation: steps toward restoration of function in spinal injured animals. Prog Brain Res 143:133–146
3. Chopp M, Zhang XH, Li Y et al (2000) Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation. Neuroreport 11:3001–3005
4. Hofstetter CP, Schwarz EJ, Hess D et al (2002) Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proc Natl Acad Sci USA 19:2199–2204
5. Lee JB, Kuroda S, Shichinohe H et al (2003) Migration and differentiation of nuclear fluorescence-labeled bone marrow stromal cells after transplantation into cerebral infarct and spinal cord injury in mice. Neuropathology 23:169–180
6. Wu S, Suzuki Y, Ejiri Y et al (2003) Bone marrow stromal cells enhance differentiation of cocultured neurosphere cells and promote regeneration of injured spinal cord. J Neurosci Res 72:343–351
7. Koda M, Okada S, Nakayama T et al (2005) Hematopoietic stem cell and marrow stromal cell for spinal cord injury in mice. Neuroreport 16:1763–1767
8. McDonald JW, Liu XZ, Qu Y et al (1999) Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med 5:1410–1412
9. Ogawa Y, Sawamoto K, Miyata T et al (2002) Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. J Neurosci Res 69:925–933
10. Ikeda O, Murakami M, Ino H et al (2002) Effects of brain-derived neurotrophic factor (BDNF) on compression-induced spinal cord injury: BDNF attenuates down-regulation of superoxide dismutase expression and promotes up-regulation of myelin basic protein expression. J Neuropathol Exp Neurol 61:142–153
11. Jakeman LB, Wei P, Guan Z, Stokes BT (1998) Brain-derived neurotrophic factor stimulates hind limb stepping and sprouting of cholinergic fibers after spinal cord injury. Exp Neurol 154:170–182
12. Koda M, Hashimoto M, Murakami M et al (2004) Adenovirus vector-mediated in vivo gene transfer of brain-derived neurotrophic factor (BDNF) promotes rubrospinal axonal regeneration and functional recovery after complete transection of the adult rat spinal cord. J Neurotrauma 21:329–337
13. Jin Y, Fischer I, Tessler A, Houle JD (2002) Transplants of fibroblasts genetically modified to express BDNF promote axonal regeneration from supraspinal neurons following chronic spinal cord injury. Exp Neurol 177:265–275
14. Liu Y, Kim D, Himes BT et al (1999) Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. J Neurosci 19:4370–4387
15. Lu P, Jones LL, Tuszynski MH (2005) BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Exp Neurol 191:344–360
16. Ruitenberg MJ, Plant GW, Hamers FP et al (2003) Ex vivo adenoviral vector-mediated neurotrophin gene transfer to olfactory ensheathing glia: effects on rubrospinal tract regeneration, lesion size, and functional recovery after implantation in the injured rat spinal cord. J Neurosci 23:7045–7058
17. Woodbury DS, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370
18. Hashimoto M, Koda M, Ino H et al (2003) Upregulation of osteopontin expression in rat spinal cord microglia after traumatic injury. J Neurotrauma 20:287–296
19. Basso DM, Beattie MS, Bresnahan JC (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 12:1–21

20. Bregman BS, Kunkel-Bagden E, Reier PJ, Dai HN, McAtee M, Gao D (1993) Recovery of function after spinal cord injury: mechanisms underlying transplant-mediated recovery of function differ after spinal cord injury in newborn and adult rats. Exp Neurol 123:3–16
21. Neuhuber B, Gallo G, Howard L, Kosture L, Mackay A, Fscher I (2004) Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J Neurosci Res 77:192–204
22. Chopp M, Li Y (2002) Treatment of neural injury with marrow stromal cells. Lancet Neurol 1:92–100
23. Jones LL, Oudega M, Bunge MB, Tuszynski MH (2002) Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury. J Physiol 533:83–89

NO.1777

高齢者頸髄症の手術適応

千葉大学大学院医学研究院整形外科学講師
山崎正志

はじめに

高齢化社会の到来とともに，圧迫性頸髄症を有する高齢者が頸椎の手術を受ける機会が増えています。しかし，高齢者頸髄症例の手術を検討するにあたっては，高齢者に特有の脊柱の構築学的変化，合併症に伴う手術のリスクなど配慮すべき点が多々あります。したがって，青壮年者の頸髄症例に対する手術適応，術式選択の基準をそのまま高齢者に当てはめてもよいというものではありません。

本稿では，高齢者頸髄症の特徴を整理し，その手術治療に関する最近の進歩について解説します。

高齢者頸髄症の特徴

高齢者頸髄症として手術の対象となる疾患は，骨棘，椎間板を主な圧迫因子とする頸椎症性脊髄症と異所性骨化が圧迫因子である後縦靭帯骨化症が大部分です。しかし，両者の頸髄症発症の病態は必ずしも同一とはいえず，手術成績も異なります。

高齢者に特徴的な所見が顕著に現れるのは頸椎症性脊髄症です。高齢者の頸椎では加齢変化に伴い下位頸椎の椎間可動性が低下します。相対的に可動性が比較的保たれているC3/4，C4/5高位の椎間に不安定性，すべりが生じ，脊髄圧迫を来します。したがって，高齢者の頸椎症性脊髄症では，一般に，責任病巣が1あるいは2椎間に限局します。また，脊柱管が比較的広く，頸椎の前弯位が保たれている傾向があります。さらに，椎間腔の狭小化に伴い頸椎柱が頭尾側に短縮し，その結果として脊髄にゆるみが生じる現象が報告されています。これらの特徴は椎弓形成術に代表される後方法手術を施行するにあたっては有利な条件といえます。また，前方除圧固定術，いわゆる前方法を行ううえでも手術椎間が3椎間あるいは2椎間で済み，決して不利な条件ではありません。

これに対して，高齢者の後縦靭帯骨化症では先述のような高齢者頸椎に特徴的な所見が現れにくい傾向があります。すなわち，頸椎後弯を呈する例が少なくなく，脊髄圧迫も多椎間に及ぶ傾向があります。また，脊髄のゆるみが生じにくく，後方法による脊髄の後方移動があまり期待できません。前方法を選択する場合でも，大部分の例で多椎間の除圧固定が必要となります。したがって，後縦靭帯骨化症に対する術式選択に際しては，とくに注意を払う必要があります。

周術期合併症とリスク

ここで高齢者の周術期合併症と手術のリスクに

ついて触れます。高齢者では周術期の合併症が青壮年者に比べて高頻度に生じることが危惧され，患者さんが手術の侵襲に耐えられるかという点が問題となります。高齢者ではほぼ全例に複数の全身合併症があると認識しておく必要があり，他科との連携を密にして術前から周到に準備をすることが肝要です。とくに長範囲前方除圧固定術（通常はC3～C7）を行う際は周術期の合併症発生のリスクが高く，入念な術中・術後管理が必須です。

手術成績および術式選択の基準

一般に，高齢者頸髄症の手術成績は手術のタイミングを誤らなければ，青壮年者のそれと比較して成績が大きく劣るわけではないという報告が多くなされています。手術成績は罹病期間，術前日整会点数と相関するといわれています。とくに高齢者では歩行障害が患者さんの満足度に大きな影響を及ぼすというデータがあり，下肢症状が進行する前に手術を考慮すべきであるという意見が主流です。

疾患別にまとめてみます。頸椎症性脊髄症については，高齢者では後方法に有利な条件が多いことは先述のとおりです。したがって，著しい後弯を有するなどの特殊な例を除き大部分の例に対し，後方法で対処可能としている施設が多いようです。また，前方法を選択している施設も障害高位のみの単椎間あるいは2椎間の除圧固定で良好な成績が得られたとしており，前方法，後方法のいずれを選択するにしても術式選択の基準はほぼ確立しています。

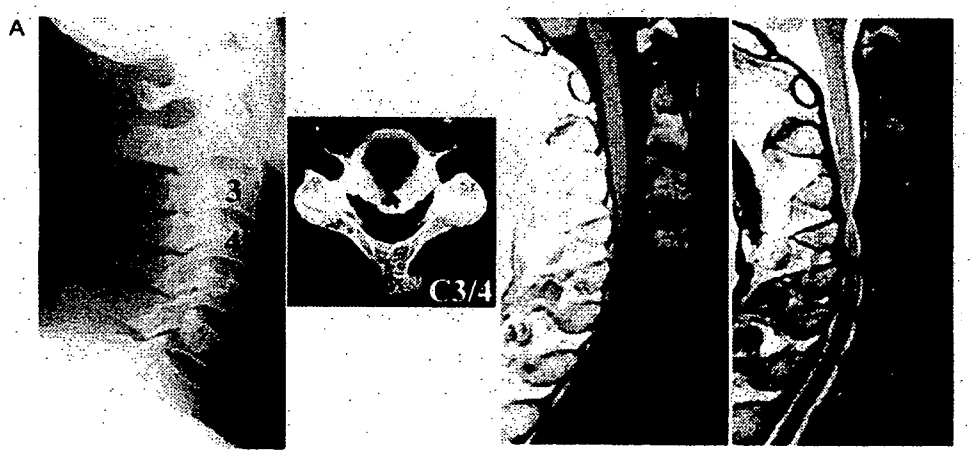
しかし，後縦靭帯骨化症については，とくに占拠率の大きな骨化を有する例に対して後方法を行った場合，成績が著しく劣るとの報告が散見されます。また，前方法が理論的には優れていることを理解しながらも，手術の難易度が高いこと，周術期の合併症のリスクが高いことなどの理由で前方法の採用をためらっている施設が少なくないようです。したがって，術式選択の基準は頸椎症性脊髄症ほど明確ではありません。

後縦靭帯骨化症に対する術後成績不良因子の解析

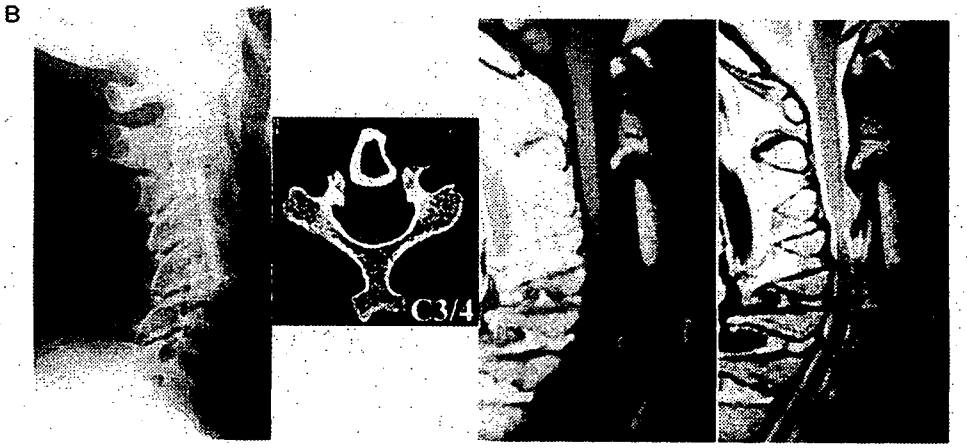
我々は後縦靭帯骨化症に対する術式選択の基準を明らかにすべく，最近10年間に経験した後縦靭帯骨化症手術例60例の成績を調査しました。

60例中，前方法は20例で選択され，1椎間から4椎間までの前方除圧固定術が行われました。一方，後方法は40例で選択され，C3～C7のen-block椎弓形成術が行われました。骨化占拠率，術前日整会点数に関しては前方法例と後方法例で差はありませんでした。しかし，手術時年齢は前方法例で平均52歳，後方法例で63歳，と後方法を選択した症例の年齢が有意に高いという結果でした。改善率は前方法例で平均65％，後方法例で54％であり，前方法の成績が優れていました（図1A，B）。改善率40％未満の改善不良例は前方法例では2例であったのに対し，後方法例では13例におよびました。また，前方法例では悪化例はありませんでしたが，後方法例では1例で術直後に症状が悪化しました。

続いて後方法例40例を改善良好例27例と改善不良例13例に分けて，成績不良因子を解析しました。手術時年齢は，良好例で平均60歳，不良例で68歳，と高齢者での成績が不良でした。術前最大圧迫高位での椎間可動域は良好例で平均6.7°，不良例で10°であり，改善不良例で有意に椎間可動性が大きいという結果でした。今回，新たに注目された後方法例の成績不良因子として最



52歳，男性：OPLL（C3/4が最大圧迫高位），cJOA＝11/17，前方除圧固定を選択



前方除圧固定術施行後，脊髄萎縮あるも症状改善良好，JOA＝15.5/17，改善率＝75％

図1．前方除圧固定術例

大圧迫高位での明瞭な椎間可動性の存在が挙げられました（図2A～C）．単純X線側面像では一見連続型の骨化として観察されるような例でも，前後屈の機能撮影を行うと最大圧迫高位で明瞭な椎間可動性を認める場合があります．

この意味づけについて考察すると，第1に術後に頸椎アライメントが変化する余地がある，すなわち，術後の後弯増強のおそれがある点が挙げられます．この場合，脊髄の前方圧迫が解除されないことになります．第2に術後も可動性が残存した場合，圧迫が解除されていない脊髄では脊髄への障害がくり返されることが考えられます．手術時年齢に焦点を当てますと，高齢者の後方法例で成績が不良となる傾向がありました．高齢者では前方法の利点を理解しながらも，周術期管理，後療法などの問題から後方法を選択せざるを得ない例が多く，このことが高齢者の成績不良につながると考えられます．