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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Cytokine array analysis of hBMSC-SC-conditioned media. hBMSC-SC-conditioned media was incubated with cytokine antibody arrays following manufacturer's instruction (RayBio® human cytokine antibody array 6 and 7 (RayBiotech, Inc., Norcross, GA, US). The following factors were detected in hBMSC-SC-conditioned media: macrophage chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), insulin-like growth factor binding protein 2 and 4 (IGFBP-2 and 4), tissue inhibitor of metalloproteinase-1 and 2 (TIMP1 and 2) vascular endotherial growth factor (VEGF), urokinase receptor (uPAR), soluble tumor necrosis factor alpha receptor-1 (sTNF-αR) and interleukin-8 (IL-8) (Supporting Fig. S1). Positive control spots were indicated by asterisks.

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Interferon-γ Decreases Chondroitin Sulfate Proteoglycan Expression and Enhances Hindlimb Function after Spinal Cord Injury in Mice

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Abstract

Glial cells, including astrocytes and macrophages/microglia, are thought to modulate pathological states following spinal cord injury (SCI). In the present study, we evaluated the therapeutic effects of interferon-γ (IFN-γ), which is one of the cytokines regulating glial function, in a mouse contusive SCI model. We found that intraperitoneal injection of IFN-γ significantly facilitated locomotor improvement following SCI. Immunohistochemistry demonstrated that IFN-γ decreased the accumulation of chondroitin sulfate proteoglycans (CSPGs), which are critical axon outgrowth inhibitors produced by reactive astrocytes in the injured central nervous system (CNS). Quantitative real-time polymerase chain reaction (RT-PCR) and Western blotting demonstrated that neurocan, one of several CSPGs, was reduced in the spinal cords of IFN-γ-treated mice compared to vehicle-treated mice. Consistently, IFN-γ inhibited the production of neurocan from activated astrocytes *in vitro*. In addition, IFN-γ treatment enhanced the number of serotonin-positive nerve fibers and myelinated nerve fibers around the lesion epicenter. We also found that glial cell line-derived neurotrophic factor (GDNF) and insulin-like growth factor-1 (IGF-1) were upregulated post-SCI following IFN-γ treatment. Our results indicate that IFN-γ exhibits therapeutic effects in mouse contusive SCI, presumably by reducing CSPG expression from reactive astrocytes and increasing the expression of neurotrophic factors.

Key words: astrocyte; interferon-γ; chondroitin sulfate proteoglycan; macrophage; microglia; neurotrophic factors; spinal cord injury

Introduction

INJURED AXONS in the central nervous system (CNS) regenerate poorly compared to those of the peripheral nervous system (PNS). Subsequent to spinal cord injury (SCI), the lack of axonal regeneration results in permanent functional deficits. Traumatic injury of the spinal cord causes the recruitment of glial cells including astrocytes and macrophages/microglia to the injured site, and these glial cells play positive and negative roles in axonal regeneration and functional recovery post-SCI (Donnelly and Popovich, 2008; Popovich and Longbrake, 2008; Yiu and He, 2006). Reactive astrocytes at the injured site are detrimental to axonal regeneration post-SCI, since they form a glial scar that constitutes a physical barrier to axon regeneration. They also produce chondroitin sulfate proteoglycans (CSPGs), that are potent axon outgrowth inhibitors (McKeon et al., 1991, 1999; Smith and Strunz, 2005; Yiu and He, 2006). It has been suggested that the blockade of the inhibitory effects of CSPGs is of potential therapeutic value in the treatment of SCI. Intrathecal infusion of chondroitinase ABC, which is a bacterial enzyme that cleaves glycosaminoglycan side chains on CSPGs, into the injured spinal cord has been demonstrated to overcome the inhibitory effect of CSPGs, and results in axon regrowth and functional recovery in rodents (Barritt et al., 2006; Bradbury et al., 2002).

Macrophages/microglia are also reported to play roles in the pathogenesis of CNS injuries. The beneficial effects of macrophages/microglia include the clearance of axon and myelin debris by phagocytic action/protease secretion, and the promotion of axon elongation/neuronal survival by the secretion of neurotrophic factors (Donnelly and Popovich, 2008; Jones et al., 2005; Popovich and Longbrake, 2008; Schwartz et al., 1999; Shechter et al., 2009). However, it has been suggested that the activation of macrophages/microglia after CNS injury is less efficient than that of macrophages after

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PNS injury, and that this might be partly attributable to the deficiency of appropriate regeneration of the injured CNS (Schwartz et al., 1999). It is of interest that the implantation of activated macrophages promotes axonal growth and functional recovery following CNS injury (Lazarov-Spiegler et al., 1996; Rapalino et al., 1998). Conversely, the excessive inflammation associated with tissue injuries mainly induced by macrophages/microglia can also be detrimental to recovery post-SCI (Donnelly and Popovich, 2008; Jones et al., 2005; Popovich and Longbrake, 2008). Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and/or interleukin-1 (IL-1) expressed by activated macrophages/microglia induce neurodegeneration following SCI (Genovese et al., 2006; Lee et al., 2000; Nesic et al., 2001). A recent report showed that inflammation itself is required for spinal cord repair (Stirling et al., 2009). Thus anti-inflammatory cytokines do not always promote recovery after SCI, and appropriate doses of proinflammatory cytokines might be helpful for the SCI repair process.

In this study, we demonstrate that a proinflammatory cytokine, interferon-γ (IFN-γ), which modulates the activities of both astrocytes and macrophages/microglia and is clinically available for the treatment of mycosis fungoides in humans, has therapeutic effects on an experimental mouse contusion model of SCI.

Methods

The mouse animal model of spinal cord injury

Adult female C57BL/6 mice (8–10 weeks old) were anesthetized with 1–1.2% halothane in oxygen. Following dorsal laminectomy (T9–T10 level), the spinal cord was contused (60 kdyn) using an Infinite Horizon Impactor (Precision Systems & Instrumentation, West Monroe, LA), as previously described (Koda et al., 2007; Nishio et al., 2007). The muscle and skin layers were then sutured. The bladder was expressed by manual abdominal pressure every day until 2 weeks post-injury. Food and water were provided *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.

IFN-γ treatment

Immediately following SCI, the mice received an IP injection of 1.0×10^4 U recombinant mouse IFN-γ (R&D Systems, Minneapolis, MN) diluted in 500 μL phosphate-buffered saline (PBS) or 500 μL vehicle (PBS) as a control, every day for 14 days. Prior to IP injection, we expressed their bladders manually, and measured body weight and the amount of residual urine.

Behavioral analysis

Hindlimb motor function was evaluated using the Basso mouse scale (BMS) open field locomotor test, in which the scores range from 0 to 9. BMS scores were recorded at 1, 3, 5, 7, 10, 14, 21, 28, 35, and 42 days following SCI by two independent examiners. We assessed hindlimb motion, mainly in order to determine whether the mouse could coordinately move and step. If there were differences in the BMS score between the right and left hindlimbs, we took the average of the two scores.

Tissue preparation and immunohistochemistry

For immunohistochemistry, the animals were sacrificed and perfused with 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) at 10 days (early phase) and 6 weeks (chronic phase) post-injury, and preserved spinal cord tissues were collected. The whole spine was dissected out and post-fixed in 4% paraformaldehyde for 24 h at 4°C. Next, the spinal cord was removed from the vertebral column and retained for 48 h in 30% sucrose at 4°C for cryoprotection. The spinal cord was embedded in Tissue-Tek OCT obtained from Sakura Finetechnical Co. Ltd. (Tokyo, Japan), and immediately frozen on dry ice at −80 °C. A series of 20-μm sagittal sections as well as cross-sections were cut on a cryostat and mounted on poly-L-lysine (PLL)-coated Superfrost-Plus slides purchased from Matsunami Glass (Osaka, Japan), and desiccated overnight. After washing three times with PBS, all sections were blocked in PBS containing 5% goat serum and 0.3% Triton X-100 for 1 h at room temperature. The sections were then incubated with primary antibodies overnight at 4°C, washed three times with PBS, and incubated with fluorescein-conjugated secondary antibodies for 1 h at room temperature. The sections were then rinsed three times in PBS and mounted. For primary antibodies, we used monoclonal anti-CD11b (1:400; BD Biosciences Pharmingen, San Diego, CA), monoclonal anti-GFAP (1:500; Sigma-Aldrich, St. Louis, MO), monoclonal anti-CSPG (1:400; Sigma-Aldrich), and monoclonal anti-serotonin (1:400; Sigma-Aldrich). Luxol fast blue (LFB) staining of spinal cord cross-sections was performed in order to measure the area of spared myelinated nerve fibers in the white matter. Immunohistochemistry using each antibody was performed simultaneously to equalize the variability of staining, and all of the slides underwent LFB staining simultaneously to equalize the variability of staining.

Histological assessment

Using spinal cord sections collected 10 days post-injury, we examined the distribution of macrophages/microglia, reactive astrocytes, and the expression of CSPGs. Every fifth section of the central portion of the spinal cords was serially mounted. At least four samples, each at 80-μm intervals within 320 μm of the center of the lesion site, were mounted on a slide and evaluated by immunohistochemistry as described above.

Using spinal cord sections collected 6 weeks post-injury, we determined the number of regenerated or spared neuronal fibers by staining serotonin fibers. Descending serotonergic fibers mainly derived from the raphe-spinal tract are thought to be important for locomotor control (Deumens et al., 2005). To confirm the axonal regeneration/sparing induced by IFN-γ treatment, we performed immunohistochemistry for serotonin to count the number of serotonin-positive fibers. Every fourth section of the central portion of the spinal cords was serially mounted. At least four samples, each at an 80-μm interval within 320 μm of the center of the lesion site, were mounted on a slide and evaluated for serotonin immunohistochemistry as described above. Lines were drawn perpendicular to the long axis of the spinal cord at the epicenter, and at 1, 2, and 3 mm rostral and caudal to the epicenter. The number of serotonin-positive fibers that traversed each line was counted.

We also assessed re-myelination or spared myelin by LFB staining in transverse sections. At least four samples, each at an 80-μm interval within 320 μm, were isolated from the epicenter, as were segments 0.6 mm, 1.2 mm, and 1.8 mm rostral or caudal to the lesion epicenter.

The number of cells was determined by immunoreactivity (fluorescence intensity) using Scion Image computer analysis software (Scion Corporation, Medford, MA), and Photoshop 7.0 software (Adobe Systems, San Jose, CA).

Real-time quantitative polymerase chain reaction

Total RNA from the injured spinal cords (6-mm segments including the lesion epicenter; the samples were near the region assessed by immunohistochemistry) was isolated 1 week after SCI using an RNeasy Kit (Qiagen, Hilden, Germany), and cDNA was obtained using reverse transcriptase (GE Healthcare, Buckinghamshire, U.K.). For the quantitative analysis of mRNA expression of neurotrophic factors, proinflammatory cytokines, and neurocan, the cDNA was used as the template in a TaqMan real-time PCR assay (ABI Prism 7500 Sequence Detection System; Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. Specific primers and probes for the TaqMan real-time PCR assay were purchased from Applied Biosystems. The following TaqMan probes were used in this study: MCP-1 (Applied Biosystems catalog no. Mm99999056_m1), CCR-2 (no. Mm99999051_gH), neurocan (no. Mm00484007_m1), GDNF (no. Mm00599849_m1), IGF-1 (no. Mm00439561_m1), BDNF (no. Mm00432069_m1), and NT-3 (no. Mm00435413_s1).

Enzyme-linked immunosorbent assay (ELISA)

Injured spinal cords (6-mm segments including the lesion epicenter; the sample was near the same region assessed by immunohistochemistry) were homogenized in homogenization buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 1% Triton X-100) containing a protease inhibitor cocktail (complete; Roche Diagnostics, Basel, Switzerland). Homogenates were cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Protein concentration of the supernatants was measured with Bio-Rad Dc Protein Assay Reagents (Bio-Rad Laboratories, Hercules, CA), and the protein concentration was adjusted to 1 mg/mL by diluting the supernatants with a homogenization buffer. IFN-γ in the supernatants was quantified with an immunoassay kit from Bender MedSystems (Vienna, Austria), following the manufacturer's protocol. In order to determine if IP-administrated IFN-γ reached the injured spinal cord, we examined IFN-γ concentration with ELISA at 5, 10, and 14 days post-SCI.

Western blotting

Homogenates of injured spinal cords were prepared as described above in the ELISA section. After centrifugation, protein concentration of the supernatants was adjusted to 1 mg/mL. For the detection of neurocan, the supernatants were digested by chondroitinase ABC (Seikagaku Corp., Tokyo, Japan) for 3 h at 4°C, and mixed with an equal volume of a 2×sample buffer (250 mM Tris-HCl, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.02% bromophenol blue, and 10% β-mercaptoethanol). For the detection of GFAP, Nogo A, and semaphorin 3A, the supernatants were mixed with an equal volume of a 2×sample buffer. After boiling for 5 min, equal volumes of the samples were subjected to 5% (neurocan) or 10% (GFAP, Nogo A, and semaphorin 3A) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Billerica, MA). After blocking of the membrane with PBS containing 5% skim milk and 0.05% Tween 20, the membrane was reacted with anti-neurocan (Seikagaku Corp.), anti-GFAP (Sigma-Aldrich), anti-Nogo A (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-semaphorin 3A (Santa Cruz Biotechnology) antibodies. For detection, a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA), and an ECL chemiluminescence system (GE Healthcare) were used. Quantification of protein bands was performed using Scion image software.

Astrocyte culture

Primary astrocyte cultures were prepared from newborn mice at post-natal day 1 (P1). The upper portion of the skull was opened and the meninges were carefully removed in order to minimize contamination of the cell culture with fibroblasts. The cerebral cortices were cut into small pieces and enzymatically dissociated using 0.025% trypsin in PBS for 10 min at 37°C. Following the addition of 10% FBS and 0.5 mg/mL DNase I, the dissociated cortices were triturated and then gravity filtered through a 70-μm cell strainer. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) plus GlutaMAX (Invitrogen Corp., Carlsbad, CA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and then seeded onto PLL-coated 75-cm^2 tissue culture flasks. The medium was changed every 3 days until the cells were confluent. Thereafter, the flasks were shaken at 200 rpm for 6 h in order to remove the contaminating non-astrocytic cells. The astrocytes were gently trypsinized, rinsed in fresh medium containing 10% FBS, and then plated on PLL-coated four-chamber glass slides. The astrocytes were treated with 10 ng/mL transforming growth factor-β (TGF-β), purchased from PeproTech, Inc. (Rocky Hill, NJ), or 10 ng/mL epidermal growth factor (EGF; PeproTech, Inc.), in the absence or presence of 2.0×10^3 U/mL IFN-γ (R&D Systems) for 24 h, before collection of total RNA using an RNeasy Kit (Qiagen).

Statistical analysis

For all experiments with the exception of the behavioral analyses, statistical analysis was performed using the Student's *t*-test. For the behavioral analyses, BMS scores were analyzed using repeated-measures analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) *post-hoc* test. For fractional BMS scores at each time point, one-way ANOVA followed by the Bonferroni/Dunn test were used. Statistical significance was set at $p < 0.05$ for fractional BMS scores. All values are the means ± standard error.

Results

Intraperitoneally-administered IFN-γ reaches the injured spinal cord

We performed ELISA for IFN-γ to determine whether intraperitoneally-administered IFN-γ can reach the injured

spinal cord. IFN-γ was almost undetectable in uninjured and injured spinal cords without IFN-γ treatment. In contrast, IFN-γ was clearly detected in the injured spinal cords with IFN-γ treatment at 5 and 10 days post-SCI, suggesting that the intraperitoneally-administered IFN-γ had reached the injured spinal cords at least until 10 days post-SCI (Fig. 1).

IFN-γ improves locomotor performance post-SCI

In order to assess the therapeutic effects of IFN-γ on a mouse contusion SCI model, IP administration of IFN-γ once a day for 14 days post-SCI was performed. We evaluated the locomotor function of hindlimbs by recording BMS scores for up to 6 weeks post-SCI. The overall BMS scores for IFN-γ-treated mice were significantly higher than those of the vehicle-treated mice, as calculated by repeated-measures ANOVA ($p < 0.01$); furthermore, at specific time points (10, 14, and 21 days) post-SCI, there were significant differences between the two groups ($p < 0.05$; Fig. 2). After 6 weeks, the vehicle-treated group's score leveled off at 2.8 ± 0.6 points, while the IFN-γ-treated group was at 4.4 ± 0.6 points, and its score appeared to continue to increase. Whereas the hindlimbs of the vehicle-treated mice exhibited only extensive ankle movement, IFN-γ-treated mice exhibited occasional plantar stepping or consistent dorsal stepping within 10 days post-SCI, suggesting that IFN-γ has therapeutic effects, even in the early phase post-injury. We also examined body weight and residual urine; however, there were no significant differences between the two groups (data not shown).

IFN-γ treatment increased the range of macrophage/microglia accumulation post-SCI

The distribution of macrophages/microglia in spinal cords following injury was examined immunohistochemically at 10 days post-SCI. At this time point, numerous CD11b-positive macrophages/microglia were seen densely clustered in the epicenter of the injured area in vehicle-treated mice, as previously reported (Kigerl et al., 2006; Sroga et al., 2003; Fig. 3A). However, the injured spinal cords of IFN-γ-treated mice exhibited a different distribution of these cells. In these mice, the accumulation of macrophages/microglia was less dense at the epicenter; instead, these cells were more widely distributed from the rostral to the caudal side of the epicenter (Fig. 3B). The mean range of the horizontal distribution of the infiltrated macrophages/microglia was 1.18 ± 0.03 mm in the vehicle-treated group, and 6.15 ± 0.96 mm in the IFN-γ-treated group (Fig. 3D), and the difference between the two groups was statistically significant ($p < 0.05$). There was a tendency for more macrophages/microglia to accumulate in the IFN-γ-treated group; however, the difference between the two groups was not statistically significant ($p = 0.08$; Fig. 3C). We attempted to determine the difference in macrophage/microglia distribution by examining the expression of chemokines that are known to affect macrophage migration. We examined the mRNA levels of MCP-1 and its receptor CCR-2 in the spinal cord at 1 week post-SCI by real-time PCR (Fig. 3E and F). The relative levels of MCP-1 and CCR-2 mRNAs were 1.9 and 3.8 times higher, respectively, in the IFN-γ-treated group than in the vehicle-treated group. To address the effects of IFN-γ treatment on the levels of proinflammatory cytokines, we checked mRNA levels of TNF-α, IL-1β, and IL-6 in the spinal cord at 1 week post-SCI by real-time PCR (Supplementary Fig. 1; see online supplementary material at http://www.liebertonline.com). There was no statistically significant difference in the levels of TNF-α, IL-1β, and IL-6 mRNA with vehicle or IFN-γ treatment, although there was a tendency toward an increase in IL-1β and IL-6 levels with IFN-γ treatment.

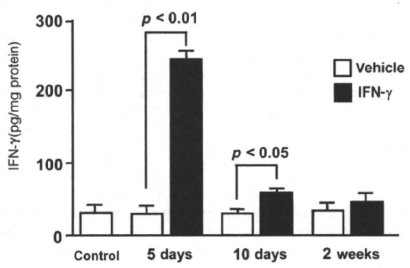

FIG. 1. Intraperitoneally-administered interferon-γ (IFN-γ) reached the injured spinal cord. We performed enzyme-linked immunosorbent assay (ELISA) for IFN-γ to determine whether intraperitoneally-administered IFN-γ could reach the injured spinal cord. IFN-γ was nearly undetectable in uninjured and injured spinal cords without IFN-γ treatment. In contrast, IFN-γ was clearly detected in the injured spinal cords with IFN-γ treatment at 5 and 10 days post-SCI, suggesting that the intraperitoneally-administered IFN-γ reached the injured spinal cords at least until 10 days post-SCI (SCI, spinal cord injury).

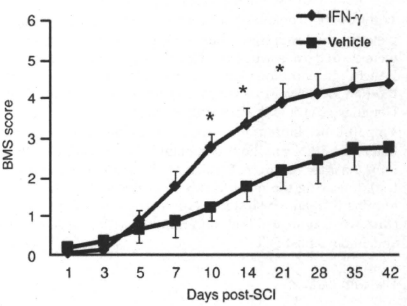

FIG. 2. Interferon-γ (IFN-γ) promotes functional recovery following spinal cord injury (SCI). Hindlimb function of mice following SCI was evaluated using the Basso mouse locomotor scale (BMS). Repetitive administration of IFN-γ for 14 days post-injury significantly enhanced hindlimb functional recovery, as assessed by repeated-measures analysis of variance (ANOVA; $p < 0.01$). After 10, 14, and 21 days post-SCI, IFN-γ-treated mice ($n = 15$) exhibited significantly better hindlimb function than vehicle-treated mice ($n = 13$; $^*p < 0.05$).

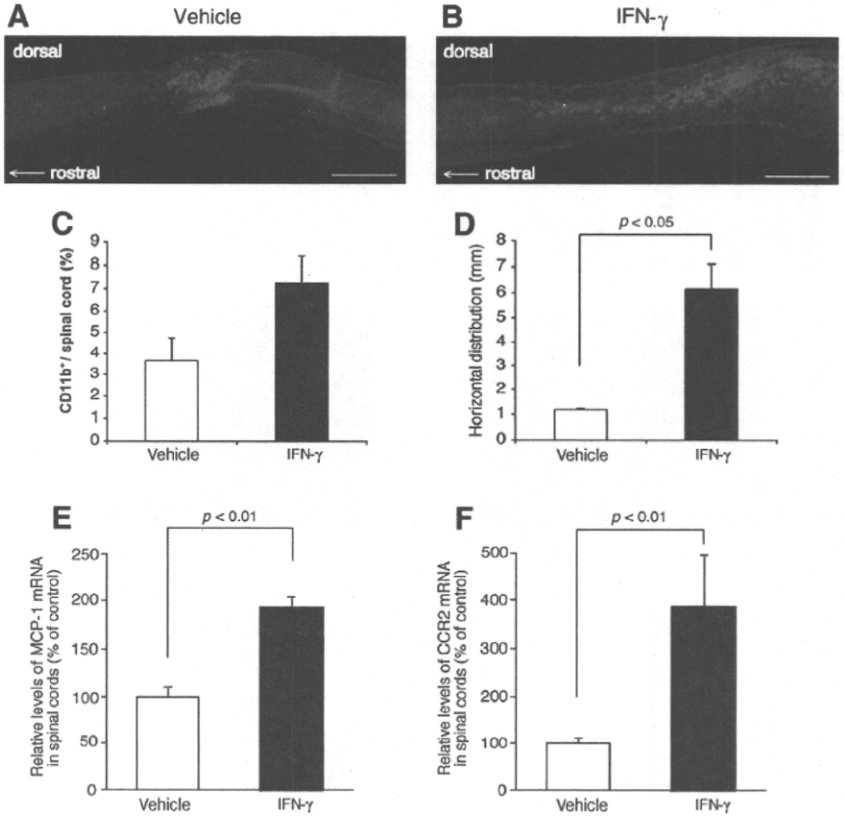

FIG. 3. Horizontal distribution of CD11b-positive macrophages/microglia in IFN-γ-treated mice post-SCI. Ten days post-SCI, the spinal cords of vehicle-treated (**A**) and IFN-γ-treated (**B**) mice were fixed and sagittal sections were stained with anti-CD11b antibody (scale bar = 1 mm). (**C**) The CD11b-positive area in a section including 5 mm rostral and caudal from the epicenter was measured, and is expressed as the percentage of CD11b-positive area relative to the total area examined. There was no statistically significant difference between the vehicle-treated group (3.7 ± 1.1%), and the IFN-γ-treated group (7.3 ± 1.1%; $p = 0.08$). (**D**) The length of the horizontal distribution of CD11b-positive cells was measured. Six spinal cord sections per animal and three animals from each group were included in the assessment. There was a statistically significant difference between the vehicle-treated group (1.18 ± 0.03 mm), and the IFN-γ-treated group (6.15 ± 0.96 mm; $p < 0.05$). Quantitative RT-PCR for MCP-1 (**E**) and CCR2 (**F**) was performed by using mRNA isolated from 6-mm-long sections of injured spinal cords including the lesion center at the middle ($n = 3$). Injured spinal cords were dissected 7 days post-SCI (IFN-γ, interferon-γ; SCI, spinal cord injury; RT-PCR, real-time polymerase chain reaction).

IFN-γ treatment decreases the accumulation of CSPG post-SCI

Following SCI, reactive astrocytes accumulate around the epicenter of the injured spinal cord and express molecules that inhibit axonal regeneration. Therefore, we examined the distribution of reactive astrocytes at 10 days post-SCI. Consistent with a previous report (Popovich et al., 1997), reactive astrocytes were detected in the tissue circumscribing the wound cavity at the lesion center (Fig. 4A and B). In the vehicle-treated group, GFAP staining was highest at the lesion edge and sharply decreased rostrally and caudally (Fig. 4A). In contrast, elevated GFAP staining in the IFN-γ-treated group had extended rostrally and caudally (Fig. 4B). Consistent with this observation, quantification of GFAP-positive immunoreactivity revealed that the accumulated reactive astrocytes were significantly higher rostrally and caudally in the IFN-γ-treated group than in the control group, except at the epicenter (Fig. 4C).

Next, we used immunohistochemistry to analyze the expression of CSPGs, which are the major axon growth inhibitors that accumulate following CNS injury, and which are mainly produced by reactive astrocytes (Fig. 5A–D). The CSPG signals were detected around the wound cavity at the epicenter. The distribution of immunoreactivity for CSPGs was similar to that of the GFAP-positive reactive astrocytes, as expected (McKeon et al., 1991, 1999; Smith and Strunz, 2005; Yiu and He, 2006). The mean density of CSPGs was significantly reduced in the IFN-γ-treated group (0.57 ± 0.05%), compared to the vehicle-treated group (1.16 ± 0.07%), suggesting that IFN-γ treatment decreased the accumulation of CSPGs following SCI in mice (data not shown). In addition, we measured the horizontal distribution of CSPG expression by quantification of immunoreactivity for CSPG. In the vehicle-treated group, CSPG staining was highest at the lesion edge, and sharply decreased rostrally and caudally (Fig. 5E). In contrast, elevated CSPG staining in the IFN-γ-treated group extended rostrally and caudally (Fig. 5E). Horizontal distribution of CSPG immunoreactivity was similar to that of GFAP (Fig. 4A–E). To directly examine whether the levels of CSPG production were reduced by IFN-γ treatment, we analyzed the mRNA

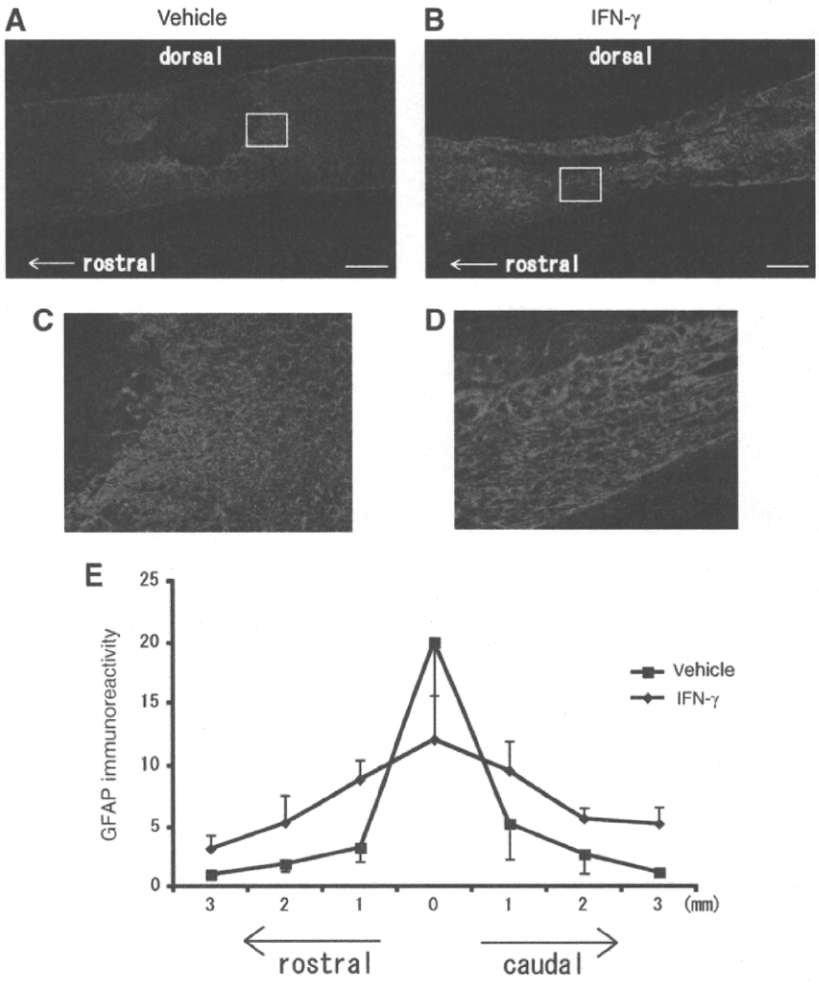

FIG. 4. Accumulation of glial fibrillary acidic protein (GFAP)-positive reactive astrocytes post-SCI. At 10 days post-SCI, the spinal cords of vehicle-treated (**A** and **C**), and IFN-γ-treated (**B** and **D**) mice were fixed and sagittal sections were immunolabeled with anti-GFAP antibody (scale bar = 400 μm). (**E**) The GFAP immunoreactivity was quantified rostrally and caudally from the lesion center at 1-mm intervals, and is expressed as the relative ratio to that of control animals at 3 mm rostral. There was a statistically significant difference between the control group and the IFN-γ-treated group, except at the epicenter ($p < 0.05$; IFN-γ, interferon-γ; SCI, SCI, spinal cord injury).

and protein levels of neurocan, one of the CSPGs that is typically upregulated following CNS injury, in injured spinal cords by quantitative RT-PCR and Western blotting, respectively. IFN-γ treatment significantly reduced the levels of neurocan in the tissues surrounding the lesion epicenter at 7 days post-SCI at both mRNA and protein levels (Fig. 6A and B). Since reactive astrocytes are considered to be a major source of CSPGs post-CNS injury, we evaluated the effects of IFN-γ on neurocan expression by stimulated astrocytes *in vitro* (Fig. 6C and D). It has been reported that TGF-β and EGF enhance the expression of GFAP and neurocan in cultured astrocytes as pathologically relevant stimulators, and mimic the molecular events occurring in reactive astrocytes in the injured spinal cord (Asher et al., 2000; Smith and Strunz, 2005). Therefore, we evaluated the effects of IFN-γ on TGF-β- or EGF-induced expression of neurocan in cultured astrocytes. IFN-γ significantly decreased the levels of neurocan mRNA in TGF-β- or EGF-treated astrocytes (Fig. 6C). In addition, IFN-γ cancelled the TGF-β-induced upregulation of neurocan protein expression, whereas IFN-γ treatment had no influence on TGF-β-induced GFAP expression enhancement (Fig. 6D). However, IFN-γ had no significant effect on EGF-induced neurocan upregulation (data not shown). These data suggest that IFN-γ treatment may block the upregulation of CSPGs in activated astrocytes at the transcriptional level.

IFN-γ had no effect on the levels of other axon growth inhibitors

We analyzed whether IFN-γ modulated the levels of other axon growth inhibitors such as semaphorin 3A and Nogo A, both of which were reported to be upregulated and involved in the deficit of axonal recovery post-SCI, by Western blotting. IFN-γ exhibited no effects on the levels of either protein in injured spinal cords at 5, 10, and 14 days post-SCI (data not shown).

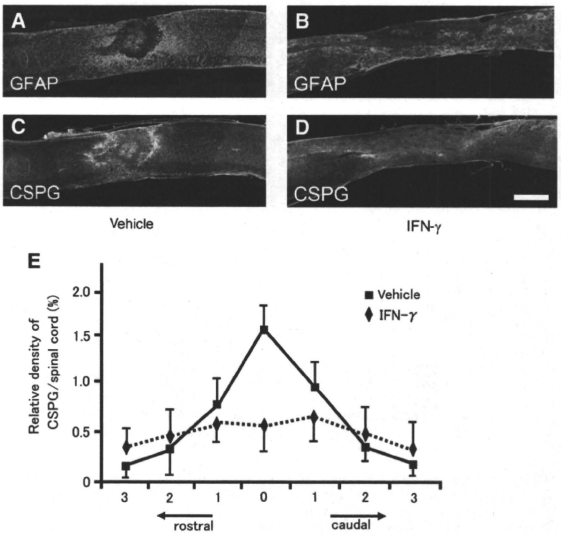

FIG. 5. Interferon-γ (IFN-γ) alters chondroitin sulfate proteoglycan (CSPG) distribution post-SCI. At 10 days post-SCI, the spinal cords of vehicle-treated (**A** and **C**), and IFN-γ-treated (**B** and **D**) mice, were fixed and parasagittal sections were stained with anti-GFAP antibody (**A** and **B**), and anti-CSPG antibody (**C** and **D**; scale bar = 500 μm). Note that the distribution of the CSPG signals (green) is similar to that of the GFAP signals (red). (**E**) Quantification of CSPG expression. The method of measurement was the same as for the quantification of GFAP expression shown in Figure 4E (GFAP, glial fibrillary acidic protein; SCI, spinal cord injury).

IFN-γ treatment increased 5-HT-positive fibers around the epicenter following SCI

Since the serotonergic raphe-spinal neuronal circuit contributes to locomotor function (Kim et al., 2004), we examined serotonergic fiber sprouting by 5-HT immunostaining at 6 weeks post-SCI. Numerous 5-HT-positive fibers were found close to the lesion cavity of injured spinal cords in IFN-γ-treated mice (Fig. 7C and D), whereas few positive fibers were detected in the vehicle-treated mice (Fig. 7A and B). The number of fibers was counted at 1-mm intervals rostrally or caudally from the lesion center. The number of 5-HT-positive fibers was significantly higher at the center and 1 mm rostral to the lesion center in the IFN-γ-treated group ($p < 0.05$) (Fig. 7E), suggesting that IFN-γ treatment either enhances the growth of serotonergic nerve fibers or was neuroprotective following SCI.

IFN-γ treatment increased the area of spared myelin

We performed Luxol fast blue (LFB) staining at 6 weeks post-SCI, and assessed the percentage of LFB-positive myelinated area relative to the total area of white matter. In both groups, the highest degree of demyelination occurred at the epicenter, and the area of spared myelin gradually increased rostrally and caudally (Fig. 8A). As shown in Figure 8B and C, IFN-γ treatment increased the myelinated area, and there was a significant difference between the two groups at 1.8 mm rostral and 0.6 mm caudal to the epicenter (Fig. 8A). The percentage of the myelinated area was 37.3 ± 1.5% in the vehicle-treated group, and 48.1 ± 1.6% in the IFN-γ-treated group, at 1.8 mm rostral to the epicenter, and 23.9 ± 2.0% in the vehicle-treated group, and 40.2 ± 2.6% in the IFN-γ-treated group, at 0.6 mm caudal to the epicenter (Fig. 8A). These data demonstrate that IFN-γ treatment increased the sparing of myelinated fibers post-SCI in mice.

IFN-γ treatment increased the expression of neurotrophic factors post-SCI

Since IFN-γ treatment increased the 5-HT-positive nerve fibers and enhanced sparing of myelinated fibers following SCI, we hypothesized that IFN-γ regulates the levels of certain neurotrophic factors post-SCI. We found that glial cell line-derived neurotrophic factor (GDNF) and insulin growth factor-1 (IGF-1) mRNA levels were significantly upregulated in the tissues surrounding the lesion epicenter in the IFN-γ-treated group, compared with those of the vehicle-treated group, whereas the levels of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) mRNA were about the same in the two groups (Fig. 9A).

Discussion

In the present study, we demonstrated that IFN-γ exhibits therapeutic effects in an experimental mouse contusion SCI

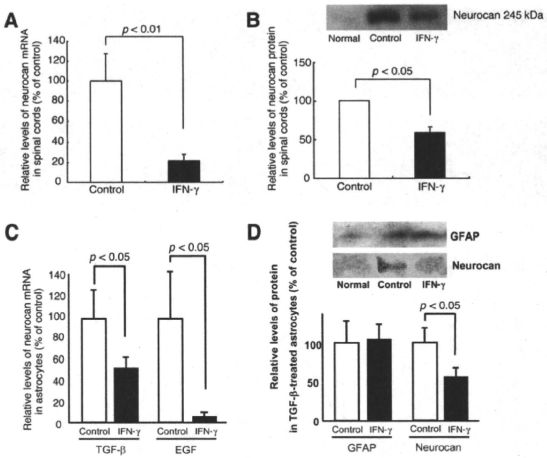

FIG. 6. Interferon-γ (IFN-γ) reduces chondroitin sulfate proteoglycan (CSPG) accumulation post-SCI. In order to analyze the levels of neurocan mRNA (**A**) and protein (**B**) in injured spinal cords with and without IFN-γ treatment, quantitative RT-PCR and Western blotting were performed ($n = 3$–6). (**C**) In the presence of TGF-β or EGF, cultured astrocytes were exposed to IFN-γ or vehicle (PBS) for 24 h, and the effects of IFN-γ on the levels of neurocan mRNA were analyzed by quantitative RT-PCR ($n = 3$–5). (**D**) TGF-β-treated astrocytes were exposed to IFN-γ or vehicle, protein was extracted, and Western blot analysis was performed to detect GFAP and neurocan. IFN-γ inhibited TGF-β-induced upregulation of neurocan protein expression (RT-PCR, real-time polymerase chain reaction; TFG-β, transforming growth factor-β; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; SCI, spinal cord injury).

model, which is one of the clinically relevant animal models of SCI. To the best of our knowledge, this is the first study to demonstrate the therapeutic effects of IFN-γ on SCI. The IP administration of IFN-γ promoted significant functional recovery as early as 10 days post-SCI, suggesting that IFN-γ therapy could be valuable in the clinical setting. Notably, the hindlimbs of IFN-γ-treated mice displayed plantar stepping, whereas vehicle-treated mice could only move their ankles. In order to identify the anatomical basis for the functional recovery seen in IFN-γ-treated mice post-SCI, we initially attempted to trace the corticospinal tract by biotin dextran amine labeling; however, we were unable to obtain stable labeling in our mouse contusion SCI model. Therefore, we assessed the growth of 5-HT-positive raphe-spinal fibers, which are associated with locomotor recovery post-SCI (Kim et al., 2004). Consistent with the functional recovery seen in the IFN-γ-treated mice, we detected an increase in 5-HT-positive neuronal fibers following IFN-γ administration. In addition, we detected an increase in spared myelinated fibers in the white matter around the epicenter of the injured spinal cord in these mice. It has been reported that an increase in 5-HT-positive fibers and myelinated fibers strongly correlates with functional recovery post-SCI (Fouad et al., 2005; Kim et al., 2004; Oatway et al., 2005).

Although it remains to be determined how IFN-γ treatment promotes the growth of the neuronal axons and myelin sparing seen post-SCI, the decreased accumulation of axon growth inhibitors and increase in neurotrophic factors around the injured spinal cords in IFN-γ-treated mice may, at least partly, contribute to the enhanced restoration. In the present study, we found that IFN-γ treatment post-SCI significantly reduced the levels of CSPGs around the epicenter of injured spinal cords, with almost no effect on the number of reactive astrocytes, which are considered to be cellular sources of CSPGs. It is well established that the degradation of CSPGs by exogenously-administered chondroitinase ABC enhances histological and functional recovery post-SCI (Barritt et al., 2006; Bradbury et al., 2002). Therefore, it has been suggested that the IFN-γ-induced reduction of CSPG accumulation seen post-SCI creates an environment within the injured spinal cord that is conducive to axon growth. In addition to the immunohistochemical analyses, quantitative RT-PCR and Western blotting demonstrated that IFN-γ treatment suppressed the upregulation of neurocan mRNA and protein, which is one of CSPGs that is upregulated post-SCI, in injured spinal cords. This result suggests that IFN-γ treatment downregulates the levels of neurocan post-SCI at the transcriptional level. Consistent with this observation, neurocan mRNA in TGF-β- and EGF-treated astrocytes *in vitro* was significantly reduced by IFN-γ treatment (Fig. 6C), as has been demonstrated in previous studies (Asher et al., 2000; Smith and Strunz, 2005). In addition, IFN-γ inhibited the TGF-β-induced upregulation of neurocan protein

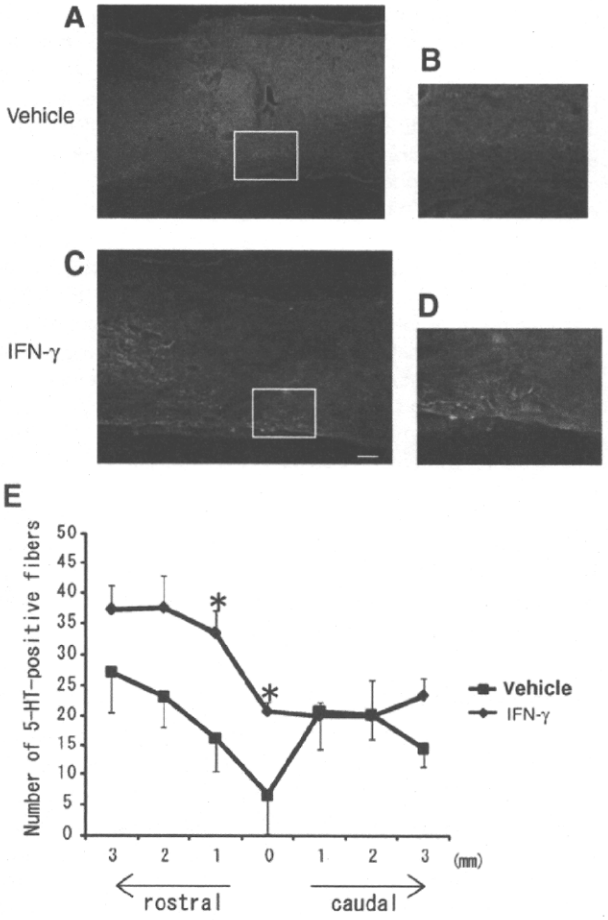

FIG. 7. Interferon-γ (IFN-γ) increases the number of 5-HT-positive fibers post-SCI. At 6 weeks post-SCI, the spinal cords of vehicle-treated (**A** and **B**) and IFN-γ-treated (**C** and **D**) mice were fixed, and sagittal sections were stained with anti-5-HT antibody. At the lesion center, higher-magnification images of the white boxes in **A** and **C** are shown in **B** and **D** (scale bar = 200 μm). (**E**) The numbers of 5-HT-positive fibers that crossed lines perpendicular to the central axis of the spinal cords were counted. The lines were positioned rostrally and caudally from the lesion center at 1-mm intervals. The number of 5-HT-positive fibers was significantly higher in IFN-γ-treated mice than in control mice at the lesion center and 1 mm rostral from the epicenter (*$p < 0.05$; 5-HT, serotonin; SCI, spinal cord injury).

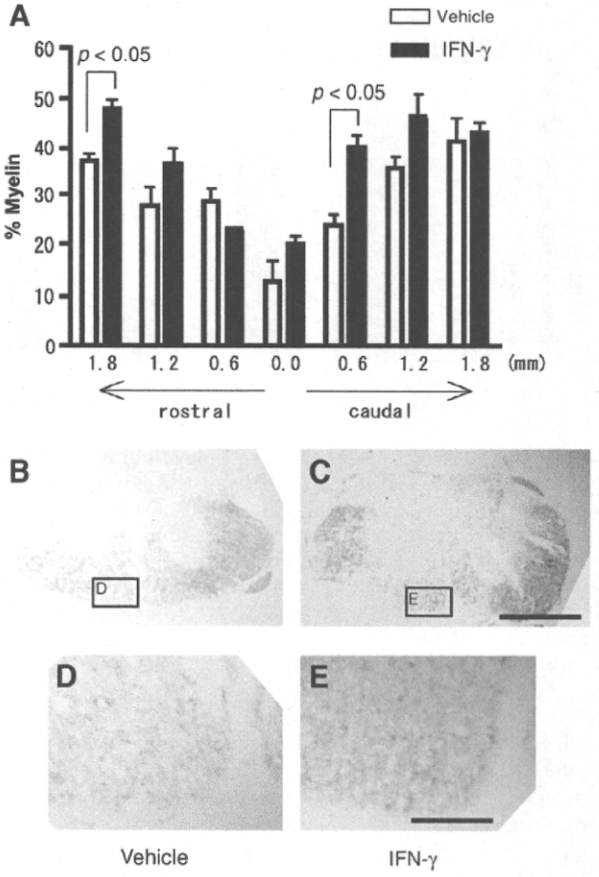

FIG. 8. Interferon-γ (IFN-γ) increases sparing of myelinated fibers post-SCI. At 6 weeks post-SCI, Luxol fast blue (LFB) staining of cross-sections of spinal cords was performed in order to analyze the effects of IFN-γ treatment on the levels of myelinated fibers seen post-SCI. (**A**) Graph showing the percentage of myelinated area relative to the total area of white matter in the cross-sections. We also assessed re-myelination or spared myelin by LFB staining in transverse sections. At least four samples, each at an 80-μm interval within 320 μm were isolated from the epicenter, and at 0.6 mm, 1.2 mm, and 1.8 mm rostral or caudal to the lesion epicenter. The number of myelinated fibers in the IFN-γ-treated group tended to be greater than in the control group, and the difference between the two groups at 1.8 mm rostral and 0.6 mm caudal from the center reached statistical significance ($p < 0.05$). (**B** and **C**) Representative LFB-stained cross sections at 400 μm caudal from the lesion center of vehicle-treated (**B** and **D**) and IFN-γ-treated (**C** and **E**) spinal cords are shown. The myelinated fibers were more dense in the IFN-γ-treated mice than in the vehicle-treated mice (SCI, spinal cord injury).

expression, whereas IFN-γ treatment had no influence on TGF-β-induced GFAP expression enhancement (Fig. 6D). GFAP upregulation is thought to be one of the manifestations of astrocyte activation. Thus IFN-γ treatment does not suppress the astrocyte activation itself, but suppresses CSPG upregulation. However, IFN-γ had no significant effect on EGF-induced neurocan upregulation. The precise mechanism underlying the discrepancy in the influence of IFN-γ between neurocan mRNA and protein expression in EGF-stimulated astrocytes is still unclear. These results suggest that IFN-γ treatment suppresses the upregulation of CSPGs at the transcriptional level post-SCI, by modulating the function of reactive astrocytes. The reduction of CSPGs by IFN-γ treatment in the injured spinal cord is relatively specific, since we observed that the levels of other axon growth inhibitors, such as semaphorin 3A and Nogo A, did not change with IFN-γ treatment.

IFN-γ also modulates the functions of macrophages/microglia post-SCI. We found that IFN-γ administration induced a widespread distribution of macrophages/microglia post-SCI. One possible mechanism by which IFN-γ alters macrophage/microglia distribution is via the IFN-γ-induced expression of chemokines such as IL-10, RANTES, and MCP-1,

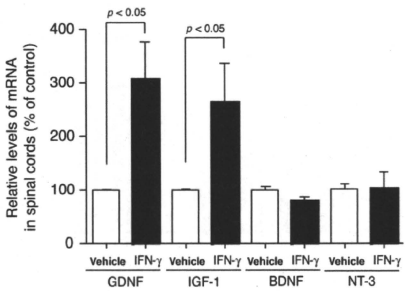

FIG. 9. Interferon-γ (IFN-γ) increases levels of neurotrophic factors post-SCI. The effects of IFN-γ on the expression levels of neurotrophic factors post-SCI were examined by quantitative RT-PCR for the indicated neurotrophic factors, as described in the legend to Figure 3 ($n = 3$; SCI, spinal cord injury; RT-PCR, real-time polymerase chain reaction; GDNF, glial cell line-derived neurotrophic factor (GDNF; IGF-1, insulin growth factor-1, BDNF, brain-derived neurotrophic factor; BNT-3, neurotrophin-3).

all of which promote the migration of macrophages into inflamed tissues (Schroder et al., 2004). In this context, we detected an upregulation of the mRNAs of MCP-1 and its receptor CCR2 in the IFN-γ-treated group at 7 days post-injury (Fig. 3E and F). Although the correlation between the IFN-γ-induced extensive accumulation of macrophages/microglia and the therapeutic effects of IFN-γ on SCI remain uncertain, the accumulation of macrophages/microglia induced by IFN-γ might produce effector molecules that support neuronal recovery post-SCI. Among these molecules, probable candidates for the promotion of neuronal recovery post-SCI are the IFN-γ-induced neurotrophic factors. It has been reported that activated macrophages/microglia produce several neurotrophic factors, such as NT-3, IGF-1, GDNF, and BDNF (Donnelly and Popovich, 2008; Elkabes et al., 1996; Hashimoto et al., 2005a, 2005b; Kaur et al., 2006; Nakajima et al., 2001). In this study, we detected an increase in the mRNAs of GDNF and IGF-1 with IFN-γ treatment post-SCI (Fig. 9). It is well established that these neurotrophic factors promote axon elongation and functional recovery post-SCI (Cheng et al., 2002; Grill et al., 1997; Iannotti et al., 2003; Sharma, 2005; Zhou et al., 2003). In this context, it has recently been proposed that IGF-1 is one of the axon-promoting factors of corticospinal motor neurons, the damage to which causes dysfunction of hindlimb movement in SCI models (Ozdinler and Macklis, 2006). In addition, these neurotrophic factors are reported to enhance the remyelination of PNS and CNS neuronal fibers in several experimental animal models (Blesch and Tuszynski, 2003; Girard et al., 2005; Mason et al., 2003; McTigue et al., 1998). These findings are consistent with our observation that IFN-γ treatment increases spared myelin post-SCI. At present, the cellular source of neurotrophic factors that are upregulated by IFN-γ treatment remains unclear. Our preliminary experiments showed that IFN-γ induces increased GDNF production by bone marrow-derived macrophages (BMDMs). In contrast, IFN-γ induced only a slight upregulation of GDNF expression by cultured microglia. Thus the main cellular source of GDNF may be macrophages. As for IGF-1, certain doses of IFN-γ can suppress IGF-1 expression in cultured microglia (Butovsky et al., 2006), and BMDMs. Therefore IFN-γ-mediated IGF-1 upregulation may be attributable to an interaction between IFN-γ-stimulated macrophages/microglia and the other cellular source within the spinal cord.

In the present study, we demonstrated that IFN-γ reduces the accumulation of CSPGs and enhances the production of GDNF and IGF-1 *in vivo* following SCI. Importantly, IFN-γ significantly promoted functional recovery following SCI in this experimental setting. The dose, timing, and duration of IFN-γ treatment should have a strong influence on its therapeutic effects. In a different setting, IFN-γ treatment might worsen the inflammatory reaction and exacerbate the paralysis resulting from SCI. Thus further exploration is needed to establish its optimal therapeutic regimen.

At present, the pharmacotherapeutic treatments available for human SCI are extremely limited. Since IFN-γ is currently used for the treatment of mycosis fungoides in humans, it would be of interest to test the therapeutic effects of clinically-available IFN-γ on patients with SCI.

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Author Disclosure Statement

No competing financial interests exist.

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BRAIN RESEARCH

Research Report

Treatment of rat spinal cord injury with a Rho-kinase inhibitor and bone marrow stromal cell transplantation

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ABSTRACT

In light of reports that the administration of fasudil, a Rho-kinase inhibitor, improved rats locomotor abilities following spinal cord injury, we hypothesized that combining fasudil with another type of therapy, such as stem cell transplantation, might further improve the level of locomotor recovery. Bone marrow stromal cells (BMSCs) are readily available for stem cell therapy. In the present study, we examined whether fasudil combined with BMSC transplantation would produce synergistic effects on recovery. Adult female Sprague–Dawley rats were subjected to spinal cord contusion injury at the T10 vertebral level using an IH impactor (200 Kdyn). Immediately after contusion, they were administrated fasudil intrathecally for 4 weeks. GFP rat-derived BMSCs (2.5×10^6) were injected into the lesion site 14 days after contusion. Locomotor recovery was assessed for 9 weeks with BBB scoring. Sensory tests were conducted at 8 weeks. Biotinylated dextran amine (BDA) was injected into the sensory-motor cortex at 9 weeks. In addition to an untreated control group, the study also included a fasudil-only group and a BMSC-only group in order to compare the effects of combined therapy vs. single-agent therapy. Animals were perfused transcardially 11 weeks after contusion, and histological examinations were performed. The combined therapy group showed statistically better locomotor recovery than the untreated control group at 8 and 9 weeks after contusion. Neither of the two single-agent treatments improved open field locomotor function. Sensory tests showed no statistically significant difference by treatment. Histological and immunohistochemical studies provided some supporting evidence for better locomotor recovery following combined therapy. The average area of the cystic cavity was significantly smaller in the fasudil+BMSC group than in the control group. The number of 5-HT nerve fibers was significantly higher in the fasudil+BMSC group than in the control group on the rostral side of the lesion site. BDA-labeled fibers on the caudal side of the lesion epicenter were observed only in the fasudil+BMSC group. On the other hand, only small numbers of GFP-labeled grafted cells remained 9 weeks after transplantation, and these were mainly localized at the site of injection. Double immunofluorescence studies showed no evidence of differentiation of grafted BMSCs into glial cells or neurons. The

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Rho-kinase inhibitor fasudil combined with BMSC transplantation resulted in better locomotor recovery than occurred in the untreated control group. However, the data failed to demonstrate significant synergism from combined therapy compared with the levels of recovery following single-agent treatment.

1. Introduction

Recent efforts to improve treatment for spinal cord injury (SCI) have included stem cell transplantation, drug therapy, and cytokine therapy in various model systems (Ackery et al., 2006; Nishio et al., 2007; Rossignol et al., 2007). However, SCI remains the most devastating type of trauma for patients due to the long lasting disability and the limited response to acute drug administration and efforts at rehabilitation. Although many studies have shown statistically significant recovery following single-agent drug treatment (Baptiste and Fehlings, 2006; Nishio et al., 2007), recovery is generally far from complete. We hypothesized that combined therapies for the appropriate time periods could yield better clinical recovery than a single-agent regimen.

Myelin-associated inhibitors often limit axonal regeneration after SCI (Mueller et al., 2005) as they stimulate intracellular Rho-ROCK kinase activation, leading to neuronal death and growth cone collapse. Targeting Rho-ROCK inhibition is a promising strategy to achieve neuroprotection and axonal regeneration. Fasudil is a ROCK inhibitor, marketed for the treatment of cerebral vasospasm after subarachnoid hemorrhage. Intraperitoneally administered fasudil has significantly improved locomotor recovery after spinal cord injury (Hara et al., 2000). In an experimental spinal cord injury rat study, fasudil significantly improved the BBB score and yielded better outcomes compared with C3 exozyme or Y-27632 (Sung et al., 2003).

Cell transplantation is another approach to neuroprotection and axonal regeneration because it compensates for tissue loss from the SCI. Many cell sources have been used and multiple types of stem cells are now recognized, including the following: ES cells, iPS cells, neural stem cells, adipose tissue-derived stem cells, hematopoietic stem cells, umbilical cord-derived stem cells, and bone marrow-derived stem cells. We previously demonstrated improved locomotion following transplantation of hematopoietic stem cells into mice (Koda et al., 2005; Koshizuka et al., 2004) and bone marrow stromal cell-derived Schwann cells into rats (Kamada et al., 2005).

With respect to stem cells, bone marrow stromal cells (BMSCs) are readily available as they can be collected from the patient and expanded. BMSC transplantation after SCI has resulted in improved locomotor recovery (BBB score) in several previous reports (Chopp et al., 2000; Hofstetter et al., 2002; Ohta et al., 2004; Wu et al., 2003). Some studies have indicated that BMSCs may express trophic factors which improve cell survival (Himes et al., 2006), while others have hypothesized that transplanted BMSCs possess neuroprotective effects (Chen et al., 2005; Chopp and Li, 2002; Neuhuber et al., 2005; Song et al., 2004). Moreover, BMSCs can differentiate to neurons and Schwann cells (Akiyama et al., 2002; Azizi et al., 1998; Chopp and Li, 2002; Deng et al., 2001; Dezawa et al., 2004; Hofstetter et al., 2002; Kim et al., 2002; Kopen et al., 1999; Lee et al., 2004; Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Woodbury et al., 2002). Neurons derived from clonal lines of MSCs have expressed synaptophysin (Woodbury et al., 2002). BMSC-derived neurons have action potentials compatible with those characteristic of functional neurons (Dezawa et al., 2004), suggesting that BMSCs may have the capacity to replace damaged neuronal cells in the spinal cord and form synapses with healthy neurons. On the other hand, transplanted BMSCs do not express neuronal markers (Koda et al., 2005; Lu et al., 2005; Yoshihara et al., 2006), and transplantation does not improve repair or recovery in rats with thoracic contusion injuries (Yoshihara et al., 2006). These discrepancies among spinal cord injury studies will likely require additional studies before they can be resolved.

In the present study, we combined fasudil treatment with BMSC transplantation in a rat spinal cord contusion model. Fasudil administration was started immediately after spinal cord contusion, and BMSCs were injected into the spinal cord 2 weeks later. We assessed the locomotor scale weekly and performed sensory tests 8 weeks after contusion. Histological studies were conducted after 11 weeks to assess tissue preservation and regeneration. In order to detect possible synergistic effects of fasudil and BMSC transplantation, we compared combined (fasudil+BMSC) treatment with single-agent treatments (fasudil alone, BMSCs alone) and with a control group of untreated rats.

2. Results

2.1. *BMSCs showed stem cell-like characteristics in vitro*

Primary cultured BMSCs from GFP-transgenic rats showed fibroblast-like morphology, and their characteristics were maintained over several passages. Because no cell-specific marker for BMSCs has yet been identified, we employed a combination of antibodies in the present study as an alternate approach to detect BMSCs. We based our choice of antibodies on the results of several previous studies (Vimentin and fibronectin, Someya et al., 2008; Nestin, Sauerzweig et al., 2009; CD44, Zhu et al., 2006). Immunocytochemistry showed that primary cultured BMSCs were positive for nestin, fibronectin, vimentin, and CD44 (Fig. 1).

2.2. *Grafted BMSCs survived but did not differentiate into glial cells or neurons in contused spinal cords*

GFP-labeled BMSC grafts survived both in the fasudil+BMSC group and in the BMSC-only group for 9 weeks after transplantation. The majority of the grafted cells remained close to the injection site. The number of donor cells was low in both the fasudil+BMSC group and the BMSC-only group: the mean number of survived transplanted cells was 48.6±44.1 in the Fasudil+BMSC group and 15.4±10.9 in the BMSC-only group. There was no statistically significant difference between these two groups ($p=0.52$). To determine whether grafted rat BMSCs differentiated

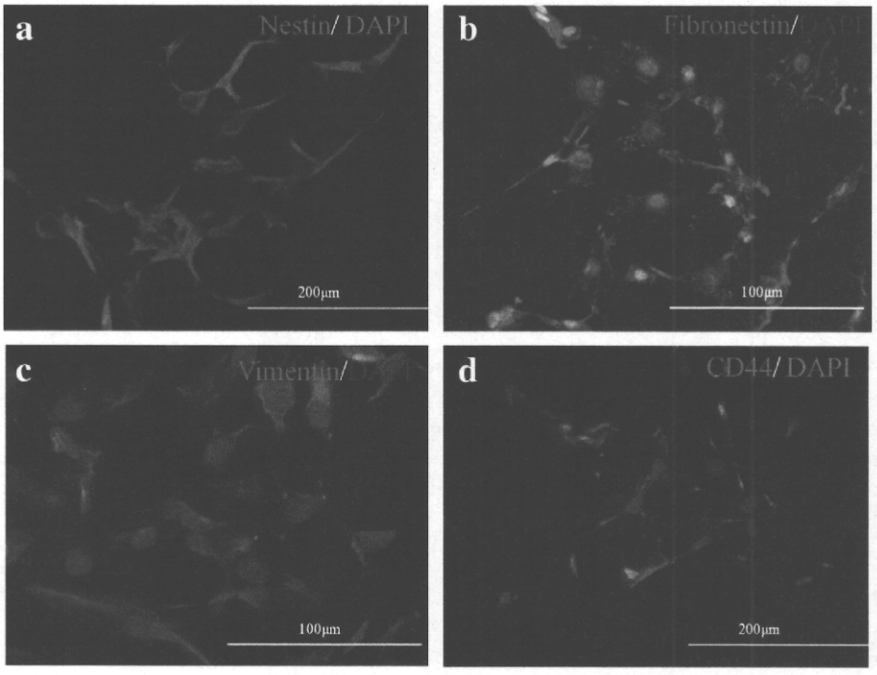

Fig. 1 – In vitro fluorescent immunocytochemical study of GFP-labeled BMSCs. Cells were positive for nestin (a), fibronectin (b), vimentin (c), and CD44 (d), four known markers for BMSC. Original magnification: ×200 for a and d, ×400 for b and c. Scale bar=200 μm for a and d, 100 μm for b and c.

into neural cells, we performed double immunofluorescence of cell-specific markers and GFP (Fig. 2a). GFP-labeled BMSCs did not co-localize with the astrocytic marker GFAP but were clearly separate from GFAP-positive astrocytes (Fig. 2b). GFP-labeled BMSCs also did not co-localize with two other neuronal markers, GSTπ (data not shown) and NeuN (data not shown).

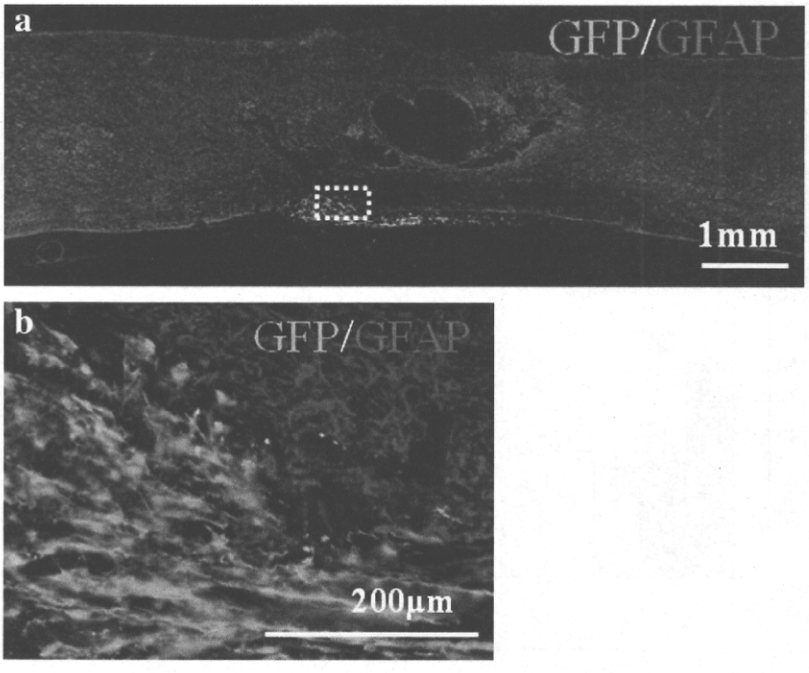

Fig. 2 – Transplanted BMSCs survived *in situ* and showed original markers for BMSC. GFP-labeled BMSCs in representative spinal cord section from the fasudil+BMSC group 9 weeks after transplantation. (a) Representative sagittal spinal cord section demonstrating double immunofluorescence of GFP (green) and GFAP (red). Rostral is to the left. Note that GFP positive cells remained close to the injection site. (b) None of the GFP-labeled cells expressed the astrocytic marker, GFAP. Scale bars=1 mm for a, 200 μm for b.

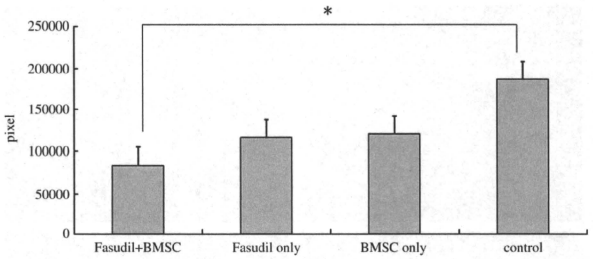

Fig. 3 – Multiple comparisons of cystic cavity formation among four groups. The mean cystic cavity size was smaller for the fasudil+BMSC group than for the control group. *$p<0.05$. The fasudil-only group and BMSC-only group also had smaller cavities than the control group, but these differences were not statistically significant.

2.3. *Combined fasudil infusion and BMSC transplantation reduced the size of the cystic cavity*

To elucidate the efficacy of fasudil treatment and BMSC transplantation for tissue protection or tissue sparing after SCI, we measured the area of the cystic cavity with cresyl violet staining 9 weeks after transplantation. The average area of the cystic cavity was significantly smaller in the fasudil+BMSC group than the control group ($p=0.023$; Fig. 3). The average cystic cavity area did not significantly differ between the other groups.

2.4. *The number of 5-HT nerve fibers was significantly higher following combined therapy with fasudil and BMSC transplantation*

To evaluate axonal survival and regeneration, we counted the number of immunoreactive fibers. We found no statistically significant difference in the number of neurofilament nerve fibers among the four groups (data not shown). The number of 5-HT nerve fibers was significantly higher in the fasudil+BMSC group than in the control group and BMSC-only groups on the rostral side of the lesion site ($p=0.012$, Fig. 4).

2.5. *Combined therapy with fasudil and BMSC transplantation improved CST regeneration/sprouting*

Representative images of traced axons on sagittal sections are shown in Fig. 5. While the groups did not significantly differ from each other in the number of BDA-labeled fibers at the levels of the lesion, BDA-labeled fibers on the caudal side of the lesion epicenter were observed only in the fasudil+BMSC group (Figs. 5a–c). The distance from the tip of the axons to the rostral edge of the scar was measured to assess the degree of axon sprouting; no significant differences among the groups were observed (data not shown).

2.6. *Combined therapy with fasudil and BMSC transplantation enhanced functional recovery in BBB score after SCI*

All groups showed an initial deficit in BBB score following contusion and showed recovery in hind limb function over the next 9 weeks (Fig. 6a). Transplantation surgery did not worsen the behavioral recovery. Hind limb function had recovered significantly in the fasudil +BMSC group at 8 weeks

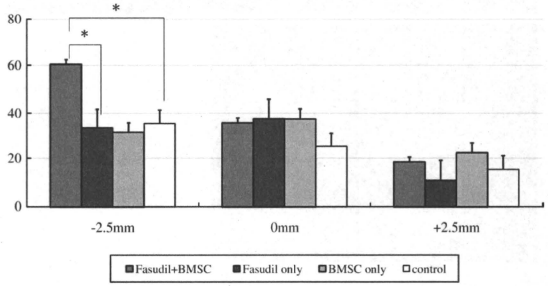

Fig. 4 – Serotonergic fiber counts at rostral, lesion epicenter, and caudal sites of injured spinal cord 11 weeks after SCI. The number of 5-HT nerve fibers in the fasudil+BMSC group was significantly higher than the number in the control and BMSC-only groups on the rostral side of the lesion. No statistically significant differences in 5-HT nerve fiber counts among the groups were observed on the caudal side or at the lesion epicenter. *$p<0.05$.

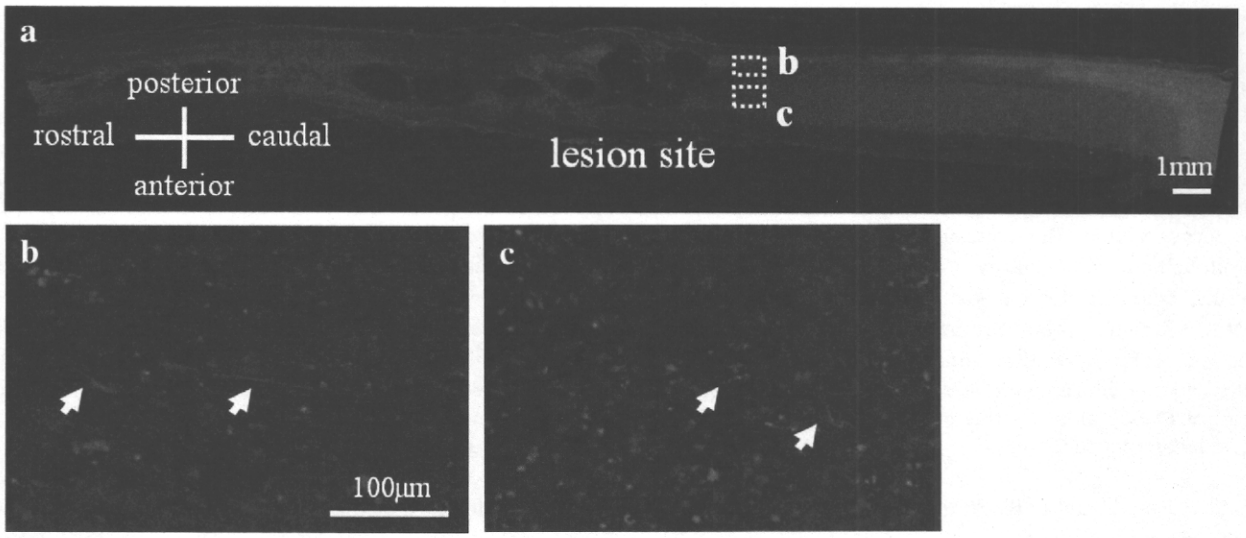

Fig. 5 – Fluorescent histochemistry of corticospinal tract tracing. Representative spinal cord sections of BDA-labeled corticospinal tracts from the fasudil+BMSC group 11 weeks after contusion. (a) Low magnification image of spinal cord sagittal sections. White dot boxes indicate white (b) and gray (c) matter on the caudal sites of the spinal cord lesion. (b, c) Residual or sprouted corticospinal tracts were stained for white (b) and gray (c) matter (arrows) on the caudal sites of the spinal cord lesion. No BDA-labeled corticospinal tracts caudal from the spinal cord lesion are seen in the other groups. Scale bar=1 mm for a, 100 μm for b and c.

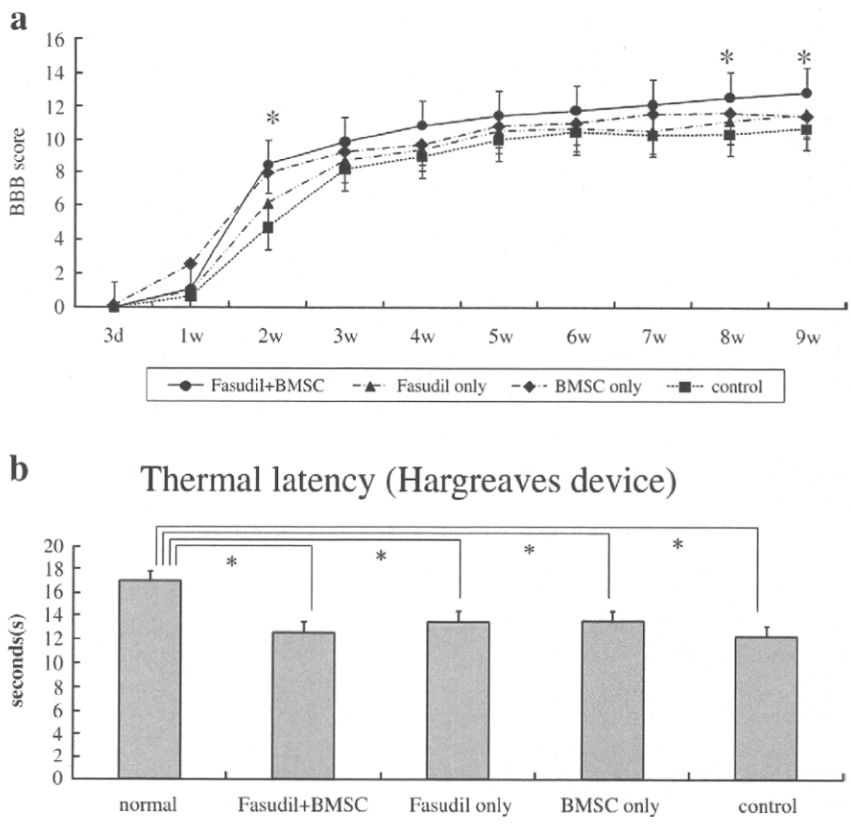

Fig. 6 – Locomotor and sensory recovery from SCI among the four groups. (a) Hind limb function recovered significantly in the fasudil+BMSC group compared with the control group 6 weeks after transplantation. The fasudil-only group and the BMSC-only group also showed better courses of recovery than the control group over time but these improvements did not reach statistical significance. (b) Thermal nociceptive thresholds in rat hind limbs were evaluated using a Hargreaves device. All contused rats showed statistically significant thermal hyperalgesia compared with normal rats. None of the differences in mean thermal latency among the four experimental groups were statistically significant. *$p<0.05$.

after transplantation compared with the control group (p=0.011 at 8 weeks; p=0.010 at 9 weeks). The average BBB score in the fasudil+BMSC group 9 weeks after transplantation reached 12.9±0.4, whereas the average BBB score in the control group was significantly lower (10.8±0.5). The best recovery score in the fasudil+BMSC group was 15. The fasudil-only group and the BMSC-only group showed better courses of recovery than the control group over time but these improvements did not reach statistical significance. These results demonstrate that fasudil treatment combined with BMSC transplantation promoted recovery of open field locomotor function after contusion of the spinal cord in rats. Neither single treatment with fasudil nor transplantation of BMSCs alone in this study protocol improved open field locomotor function.

2.7. *Fasudil and/or BMSC transplantation did not increase abnormal neurogenic pain*

Analysis of normal rats with a Hargreaves device revealed a mean thermal latency of 17.0±0.3 s (Fig. 6b) compared with mean values of 12.6±0.7 s in the fasudil+BMSC group, 13.5± 1.3 s in the fasudil-only group, 13.6±1.1 s in the BMSC-only group, and 12.3±1.1 s in the control group 8 weeks after contusion. Thus, while all contused rats showed significant thermal hyperalgesia compared with normal rats (p=0.00087), none of the differences in mean thermal latency among the four experimental groups were statistically significant.

Mechanical thresholds using a dynamic plantar aesthesiometer showed a mean of 32.6±1.2 g in normal rats, decreasing to 32.2±2.1 g in the fasudil+BMSC group, 31.5± 3.7 g in the fasudil-only group, 32.0±2.5 g in the BMSC-only group, and 31.2±5.8 g in the control group 8 weeks after contusion. All contused rats showed more mechanical allodynia compared to normal rats; however, no statistically significant increase in allodynia as a function of treatment was observed (data not shown). These two studies indicate that fasudil and/or BMSC transplantation did not increase abnormal neurogenic pain.

3. Discussion

In our study of rats with spinal cord contusion injury, we found that combined therapy with fasudil infusion and BMSC transplantation improved functional recovery and reduced cystic cavity size compared with controls.

3.1. *Fasudil infusion*

Rho and Rho-kinase exert their effects through regulation of the actin–myosin network (Amano et al., 2000; Dickson, 2001). Rho-kinase stimulates actin–myosin contractility by phosphorylating the myosin light chain and inhibiting myosin phosphatase. Following SCI, scar tissue containing myelin-derived inhibitors and chondroitin sulfate proteoglycans (CSPG) inhibits neurite outgrowth or induces growth cone collapse by activating the Rho/Rho-kinase signaling pathway (Mueller et al., 2005). The effects of myelin-derived inhibitors and CSPG are abolished when Rho-kinase is inhibited (Kubo and Yamashita, 2007; Monnier et al., 2003). Fasudil or Rho-kinase inhibition also has neuroprotective effects, as evidenced by studies showing that fasudil infusion reduced MPO activity in rats (Hara et al., 2000) and hindered ischemia-induced delayed neuronal death in gerbils (Satoh et al., 2007). Another study reported that fasudil application reversed Rho activation and reduced the number of TUNEL-labeled cells by approximately 50% in mice and rats with SCI (Dubreuil et al., 2003).

We hypothesized that fasudil infusion overcomes myelin-derived inhibition after spinal cord injury by inhibiting Rho-ROCK-kinase. We also hypothesized that a longer duration of fasudil administration would improve axonal regeneration or neuronal survival, which we tested in the present study by continuing fasudil administration for four weeks. However, our study found that the fasudil-only group did not show a statistically significant difference in locomotor recovery compared with the control group. One possible explanation for this observation is that after a certain point, further fasudil infusion becomes counterproductive and increases myelin inhibition, thereby slowing axonal regeneration for locomotor recovery. Support for this explanation comes from a recent study which discovered that another Rho-kinase inhibitor, Y27632, increased CSPG (Chan et al., 2007).

The extent of penetration of fasudil into the spinal cord is a critical concern. One report demonstrated that intraperitoneal administration of fasudil reduced MPO activity in injured spinal cords (Hara et al., 2000). Another study found that intrathecal fasudil treatment reduced thermal hyperalgesia in post-sciatic nerve ligation rats (Shiokawa et al., 2007). These two studies thus represent evidence that fasudil does permeate into the spinal cord.

3.2. *BMSC transplantation*

BMSC transplantation is a potential therapy for central nervous system (CNS) diseases because BMSCs secrete a variety of growth factors and cytokines that could contribute to the repair of CNS injuries. Following stimulation of TNF-alpha, ELISA assay findings indicate that BMSCs increase the release of IL-6, MCP-1, and BDNF (Himes et al., 2006). BMSCs trigger the endogenous survival signaling pathway Erk1/2 and also trigger Akt phosphorylation in neurons (Isele et al., 2007). BMSCs have the potential to support the survival of neurons in the marginal region of SCI (Yano et al., 2006).

BMSCs are also capable of differentiating into a variety of tissues. Some studies have suggested that BMSCs can differentiate into cells with neural phenotypes (Chopp et al., 2000). Others have reported that the apparent acquisition of a neuronal phenotype by BMSCs results from cell fusion or cell phagocytosis (Alvarez-Dolado et al., 2003; Hess et al., 2004; Terada et al., 2002; Ying et al., 2002). In an earlier experiment, we transplanted BMSC-derived Schwann cells to treat rat spinal cord defects and identified significantly improved locomotor recovery compared with controls (Kamada et al., 2005). Some studies by other researchers have reported significant functional recovery after transplanting BMSCs (Chopp et al., 2000; Hofstetter et al., 2002; Ohta et al., 2004; Wu et al., 2003), while others have

identified only modest or inconsistent recovery (Himes et al., 2006; Lu et al., 2005; Neuhuber et al., 2005; Yoshihara et al., 2006). In the present study, our assessments of behavior and histology following BMSC transplant-alone did not detect significantly different locomotor recovery compared with the control group.

Lack of a significant therapeutic effect following BMSC transplantation alone might be attributable to the timing of the transplantation. We chose to perform cell transplantation 2 weeks after SCI because some interval for cell culturing is required in actual clinical autograft transplantation. However, our treatment timing might have hindered efficacy, as evidenced by the low number of surviving cells. Furthermore, if our aim were to enhance host neural survival by BMSC transplantation, this 2-week delay would have caused the intervention to be too late to save neurons from consecutive death after SCI because neural death after spinal cord injury often happens within one day (Liu et al., 1997).

Another possible reason for the poor response was the site of the transplantation. We injected BMSCs into rostral and caudal sites to facilitate migration of BMSCs and re-myelination of regenerative fibers. However, histological examination found that residual BMSCs were scarce and that they did not migrate as we had expected they would. Thus, transplanting BMSCs directly into the site of injury might yield better outcomes.

3.3. *Combined therapy*

In our study, fasudil+BMSC therapy increased 5-HT-positive fibers on the rostral side of the lesion, enhanced CST regeneration and/or sprouting, and significantly reduced mean cystic cavity size compared with the control group. These histological results were consistent with a significant improvement in locomotor activity.

As we discussed previously, increased fasudil infusion beyond a certain point might become counterproductive and increase myelin inhibition. One study found that BMSCs stimulated neurite outgrowth over CSPG, myelin-associated glycoprotein, and Nogo-A in vitro (Wright et al., 2007). Thus, the sprouting of CST and serotonergic fibers observed under combined treatment might reflect the effect of BMSC transplantation, which can stimulate neurite outgrowth over inhibitory proteins. On the other hand, another study revealed that Rho-pathway inhibition after cervical dorsal rhizotomy in rats increased the density of serotonergic fibers in the dorsal horn (Ramer et al., 2004). Correspondingly in the present study, fasudil, a Rho-pathway inhibitor, likewise may have taken part in the regeneration of serotonergic fibers observed in the fasudil+BMSC group. Overall, Fasudil-induced Rho-kinase inhibition and BMSC transplantation each appear to provide some degree of neuroprotection against neuronal death (Chen et al., 2005; Chopp and Li, 2002; Dubreuil et al., 2003; Neuhuber et al., 2005; Satoh et al., 2007; Song et al., 2004).

In the present study, the mean cystic cavity area was significantly smaller in the fasudil+BMSC group than in the control group. Since neither fasudil alone nor BMSCs alone significantly changed the size of the cystic cavity compared with the control group, these findings together suggest it was the combination of fasudil infusion and BMSC transplantation that produced reductions in cystic areas that neither agent alone could achieve. These histological findings also suggest that fasudil and BMSC transplantation in combination had anti-inflammatory activity which suppressed local inflammatory reactions from SCI. Several published studies have also reported that fasudil exhibited anti-inflammatory activity. Intraperitoneal administration of fasudil in rats with collagen-induced arthritis significantly reduced synovial inflammation and ROCK activity (He et al., 2008). In vitro treatment with fasudil or Y27632 decreased production of tumor necrosis factor alpha (TNFalpha), interleukin-1beta (IL-1beta), and IL-6 by synovial membrane cells, peripheral blood mononuclear cells, and fibroblast-like synoviocytes from patients with active rheumatoid arthritis (He et al., 2008).

Because the number of residual transplanted BMSCs was low in all BMSC transplantation groups 9 weeks after transplantation, improved locomotor recovery is unlikely to have resulted from BMSCs alone. Also, the BMSC-only group did not show significantly greater recovery than the level of recovery in the control group. Thus, it appears that better locomotor recovery results from the combination of fasudil and BMSC, the effectiveness of which might arise from BMSCs acting to reverse the putative toxicity of high total dosage fasudil treatment.

The BBB score for combined therapy reached 12.9 while the control group BBB score reached 10.8. Although rats in both groups could perform weight-bearing plantar stepping, only the rats in the combined treatment group had coordination of fore limbs and hind limbs. These results indicate that combined therapy could enhance recovery of a central pattern generator.

In summary, combined therapy showed better locomotor recovery compared with controls, and histological studies of cavity volume and serotonergic fiber counts confirmed the locomotor results. Future studies are required to identify the effect of combined fasudil infusion and BMSC transplantation to clarify any potential clinical application to damaged spinal cords in humans.

4. Experimental procedures

4.1. *Bone marrow stromal cell culture*

Bone marrow stromal cells (BMSCs) were collected from the femurs and tibias of 8-week-old female GFP transgenic Sprague–Dawley rats (SLC, Hamamatsu, Japan). Briefly, animals received an overdose of pentobarbital to induce deep anesthesia and were then decapitated. Bone marrow was flushed with PBS and centrifuged at 1500 rpm for 5 min. Cells were plated in α-MEM (Sigma, St. Louis, MO) plus 10% fetal bovine serum supplemented with 100 U/mL penicillin G and 100 μg/mL streptomycin (Molecular Probes, Eugene, OR) and then incubated at 37 °C in 5.0% CO2. After non-adherent cells were removed just before confluency, adherent cells were detached using 0.25% trypsin with EDTA, centrifuged, and re-plated as BMSCs. BMSCs were used for transplantation and in vitro induction experiments between passages four to eight.

To characterize the BMSCs in vitro, we performed immunocytochemistry using the following primary antibodies: anti-