include the possibility of allograft rejection and the ethical problems raised by the use of fetal tissue; autologous transplantation may resolve these problems.

Adult bone marrow contains several different stem cell populations including HSCs and BMSCs, also called mesenchymal stem cells. Both HSCs and BMSCs are candidates for use in cell therapy for neurological disorders because they can be transplanted autologously. Indeed, it has been reported that bone marrow cells have the potential to restore injured spinal cord tissue and promote functional recovery. In fact, transplantation of HSCs promotes functional recovery after compression-induced SCI in mice[28,29] and transplantation of BMSCs significantly improves hindlimb function after SCI in mice and rats.[12,24,37,49] However, the therapeutic efficacy of BMSCs for SCI is still controversial. Several researchers have suggested that BMSC transplantation cannot promote functional recovery.[1,53] In addition, transplantation of the non–neural lineage cells has potential problems including differentiation of transplanted cells and compatibility with the host spinal cord. Differentiation into neural lineage cells prior to transplantation could enhance the therapeutic effect of BMSCs and offer a solution to these problems.

We recently reported that cells with SC properties could be derived from BMSCs in vitro, and that they effectively promoted regeneration of lesioned sciatic nerves.[17,34] Moreover, we have previously reported that transplantation of BMSC-SCs effectively promotes regeneration of damaged axons and hindlimb functional recovery in completely transacted adult rat spinal cord.[27]

The aim of the present study is to evaluate the therapeutic effects of BMSC-SC transplantation in adult rats with contusive SCIs, a more clinically relevant model than the spinal cord transection model. Additionally, we compared BMSC-SC transplantation with transplantation of untreated BMSCs and peripheral nerve–derived SCs, which are widely known to promote recovery of the injured spinal cord and aid in tissue restoration, survival of transplanted cells, axonal regeneration/sparing, and promotion of hindlimb functional recovery.

Methods

Cell Cultures and In Vitro Differentiation

Bone marrow stromal cells were cultured as previously described.[27] Briefly, total bone marrow cells were collected from the femurs of adult male GFP-transgenic Wistar rats (provided by the YS Institute, Inc.) and plated onto plastic culture dishes. The cells adherent to the dishes were cultured as BMSCs in alpha-MEM (Sigma) supplemented with 20% FBS. The BMSCs were used for transplantation and in vitro induction experiments between passages 4 to 6.

Peripheral SCs were cultured from dissected sciatic nerves of 2-day-old GFP-transgenic Wistar rats.[21,32] The dissected nerves were treated with 0.1% collagenase and 0.1% trypsin in PBS for 60 minutes at 37°C. Dissociated cells were cultured in Dulbecco modified Eagle medium containing 10% FBS and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). After 1 day of culturing, cells were exposed to 2 cycles of cytosine-arabinoside treatment (10 μM) for 3 days to prevent proliferation of fibroblasts. After 7 days of culturing, cells were plated on dishes coated with poly-L-lysine (Sigma) in Dulbecco modified Eagle medium containing 10% FBS, 1% antibiotics, and 10-μM forskolin (Sigma). Cells were maintained in a 37°C incubator containing 5% CO_2 and passaged when confluent. The medium was changed every 3 days. Bone marrow stromal cells were derived from SCs in vitro as previously described.[17,27] Briefly, the BMSCs were incubated with alpha-MEM containing 1-mM beta-mercaptoethanol for 24 hours. After washing the cells, the medium was replaced with alpha-MEM containing 10% FBS and 35 ng/ml all-transretinoic acid (Sigma) for 3 days. Cells were then transferred to alpha-MEM containing 10% FBS, 5-μM forskolin (Calbiochem), 10 ng/ml recombinant human basic fibroblast growth factor (Peprotech), 5 ng/ml platelet derived growth factor (Peprotech), and 200 ng/ml heregulin β1 (R&D Systems) for 7 days.

Immunohistochemical analysis was performed to characterize the BMSC-SCs in vitro. Mouse monoclonal anti-vimentin antibody (1:200; Dako Cytomation) and mouse monoclonal anti-fibronectin antibody (1:400; Chemicon International) were used as markers for BMSCs. Mouse monoclonal anti–protein zero antibody (P0, 1:300; Astexx), rabbit polyclonal anti-S100 antibody (1:100; Dako Cytomation), and rabbit polyclonal anti-p75NTR antibody (1:200; Chemicon) were used as markers for SCs. Cell nuclei were stained with 4'-6-diamidino-2-phenylindole (Molecular Probes). A negative control was performed by omitting the primary antibodies.

The RT-PCR Analysis

Total RNA from the cells was extracted with TRIzol Reagent (Invitrogen) and purified according to the manufacturer's instruction. From 5 μg of RNA, the first cDNA strand was generated using SuperScript II-RT (Invitrogen). The PCR reactions were performed using Ex Taq DNA polymerase (TaKaRa). The conditions for amplification were as follows: 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C for 30 cycles (25 cycles for beta-actin) and the final incubation at 72°C for an additional 4 minutes. We used the following primers specific to SC genes, designed by Primer3 software, and beta-actin was used as internal control. The Krox20 (Egr2) sense strand was 5'-CAGGAGTGACGAAAGGAAGC-3' and the antisense was 5'-ATCTCACGGTGTCCTGGTTC-3'; Krox24 (Egr1), sense: 5'-GACGAGTTATCCCAGCCAAA-3' and antisense: 5'-AGGCAGAGGAAGACGATGAA-3'; P0, sense: 5'-GATGGGCAGTCTGCAGTGTA -3' and antisense: 5'-TTTGGCAGGTGTCAAGTGAG -3'; beta-actin, sense: 5'-TAAAGACCTCTATGCCAACAC-3' and antisense: 5'-CTCCTGCTTGCTGATCCACAT-3'.

Spinal Cord Injury and Transplantation

For experimental SCI, we used 48 male Wistar rats 10–11 weeks old (weight 225–250 g) divided into 3 experimental and 1 control group of 12 animals each. A

laminectomy was performed at the T8–9 level. The moderate contusion injury was created with a New York University impactor (10-g rod and 25-mm height).[5,54] Afterwards the rats' muscles and skin were sutured in layers and they were placed in warm cages overnight; food and water were provided ad libitum. Manual bladder expression was performed twice daily until the rats recovered bladder reflexes. All animals were given antibiotic medication in their drinking water (1.0-ml bactramin in 500-ml acidified water) for 2 weeks after injury.

Seven days after injury, the injured site was reexposed and transplantation was performed. The BMSC group was injected with a mixture of matrigel and BMSCs (5×10^5/5 μl), the BMSC-SC group received BMSC-SCs and matrigel (5×10^5 cells/5 μl), the SC group received peripheral SCs and matrigel (5×10^5/5 μl), and the control group received matrigel alone (5 μl). The injections were delivered to the injured site with a glass micropipette attached to a Hamilton microsyringe.

All animals received immunosuppressive treatment with cyclosporine A (Novartis), because BMSC transplantation may cause a host immunological reaction resulting in rejection of transplanted cells.[13] Twenty-four hours before transplantation, cyclosporine A (20 mg/kg) was injected subcutaneously. After transplantation, cyclosporine A was also injected for the entire experimental period (20 mg/kg on Monday and Wednesday, and 40 mg/kg on Friday).[50] No animal showed abnormal behavior. All the experimental procedures were performed in compliance with the guidelines established by the Animal Care and Use Committee of Chiba University.

Tissue Preparation

At the end of the functional assessment period 6 weeks after injury, animals were perfused transcardially with 4% paraformaldehyde in PBS (pH 7.4) after an overdose of pentobarbital anesthesia. Three spinal cord segments (T8–10) including the injury epicenter were removed and postfixed in the same fixative overnight, stored in 20% sucrose in PBS at 4°C, and embedded in Optimal Cutting Temperature compound (Sakura Finetechnical). Sagittal cryosections (12-μm thickness) were cut using a cryostat and the sections were mounted onto poly-L-lysine–coated slides (Matsunami). Every fifth section from the central portion of the spinal cords was serially mounted. At least 4 samples from each animal, each 60-μm interval within a 240-μm width centered of the lesion site, were mounted on slides and evaluated for histochemical characteristics as described below.

Anterograde Tract Tracing

Four weeks postinjury, the cranial bone overlying the motor cortex was removed and 10% BDA was injected in 4 divided doses (1–3 mm posterior to the coronal suture and 2.5-mm lateral to the coronal suture; 2 each side, 4 × 1 μl aliquots) bilaterally into the motor cortex using a glass micropipette attached to a Hamilton microsyringe. Two weeks after tracer injection, animals were perfused as described, and the spinal cords were dissected for staining of labeled corticospinal axons.

Cystic Cavity Measurement

To measure the area of the cystic cavity, the sections were stained with cresyl violet, dehydrated, and sealed with Permount (Fisher Scientific). At least 4 samples, each 60-μm apart, were chosen for cavity measurements; the measurements therefore covered a 240-μm width in the central portion of spinal cord including the lesion site. We measured the area of the cystic cavity using Scion Image computer analysis software (Scion Corp.). The average cystic cavity area was compared among the 4 groups of rats.

Immunohistochemical and Electron Microscopy Studies

We performed immunohistochemical studies as previously described.[23,27] Briefly, the GFP-positive cells were counted to compare with the number of surviving transplanted cells; goat polyclonal anti-GFP antibody (1:100; SantaCruz) was used as the primary antibody. The average number of surviving GFP-positive cells in 4 samples obtained from each animal was compared among the BMSC, BMSC-SC, and SC groups. To evaluate the phenotypic changes of transplanted cells, a double immunofluorescence study for GFP and cell-specific markers were performed in the BMSC, BMSC-SC, and SC groups.

Immunohistochemical analysis for axonal markers was performed to evaluate the extent of axonal regeneration and sparing. The following primary antibodies were used to detect various types of nerve fibers: rabbit polyclonal anti-Gap43 antibody (1:400; Santa-Cruz), mouse monoclonal anti–Th antibody (1:400; Chemicon), and rabbit polyclonal anti-serotonin antibody (1:5000; Sigma). After incubation with primary antibodies, the sections were incubated with Alexa 594 dye–conjugated secondary antibodies (Molecular Probes) to detect positive signals. We used Gap43 as a general marker for regenerating/sprouting nerve fibers. Tyrosine hydroxylase–positive nerve fibers are mainly cerulospinal adrenergic, and serotonin-positive nerve fibers are mainly raphe spinal serotonergic; both fiber types contribute to motor function.[7,16,43,48] To evaluate regeneration/sparing of Gap43-, Th- or serotonin-positive nerve fibers, the number of immunoreactive fibers that traversed the lines perpendicular to the central axis of the spinal cord at rostral (5-mm rostral to the injury epicenter), epicenter, and caudal (5-mm caudal to the epicenter) levels were counted and compared between groups. For the BDA tracing study, sections were immunostained with streptavidin-conjugated Alexa 594 (1:800; Molecular Probes) and examined under laser scanning confocal microscopy. The negative control was performed by omitting the primary antibodies.

The spinal cord tissue samples from the BMSC-SC group rats were cut into 100-μm-thick sections and stained with rabbit polyclonal anti-GFP antibody (Molecular Probes). Positive signals were detected with avidin-biotin complex method using the Histofine kit (Nichirei). The positive signals were visualized with diaminobenzidine and hydrogen peroxide. The sections were then postfixed with 1% OsO_4 in PBS (pH 7.4) for 1-hour and then block-stained with 1% uranyl acetate in acetate buffer, dehydrated, and embedded in Epon 812 (Shell Chemi-

cal).[26] Ultrathin sections (90-nm thickness) were stained with lead citrate. These sections were examined with JEM 1200EX electron microscope (JEOL).

Assessment of Locomotor Activity

The hindlimb functioning of the animals in all groups was assessed with the BBB locomotor scale[4] before injury and 1 day, 3 days, and each week (for 6 weeks) after injury.

Statistical Analysis

Each statistical analysis was evaluated using multiple comparisons between groups. For histological studies, the 1-way ANOVA was used, followed by Bonferroni-Dunn post hoc test. For the locomotor scale scores, repeated-measures ANOVA, and Fisher protected least significant difference post hoc test was used. For fractional BBB scores at 8 time points, the 1-way ANOVA and Bonferroni-Dunn test was used. Statistical significance was set at $p < 0.05$ and $p < 0.01$, respectively.

Results

In Vitro Character of BMSC-SCs

Bone marrow stromal cells in primary culture show a fibroblast-like morphology, and these characteristics were kept for several passages (Fig. 1A). The BMSC-SCs were successfully derived from BMSCs and appeared short and spindle-shaped (Fig. 1B). The morphological characteristics of these cells were similar to that observed in normal peripheral SCs (Fig. 1C). The BMSCs stained positive for vimentin and fibronectin (not shown), while the BMSC-SCs were positive for P0, S100 protein, and p75NTR (Fig. 1D and E), widely known markers for SCs.

Results of RT-PCR revealed that expression level of P0—an important glycoprotein in peripheral myelination that is widely known as a specific marker for SCs—increased in BMSC-SCs[31] compared with BMSCs (Fig. 1F). There was no significant difference between BMSCs and BMSC-SCs in the expression level of Krox20 and Krox24 (Fig. 1F). These data show that BMSC-SCs have characteristics similar to SCs not only in their morphological characteristics, but also in their phenotype and genotype.

To elucidate the efficacy of BMSC-SC for tissue sparing after SCI, we measured the area of cystic cavity with cresyl violet staining 5 weeks after transplantation (Fig. 2A–D). The average area of the cystic cavity was significantly smaller in BMSC-SC and SC groups ($p < 0.01$ and $p < 0.05$, respectively) than that in control group indicating that transplantation of BMSC-SCs or peripheral SCs

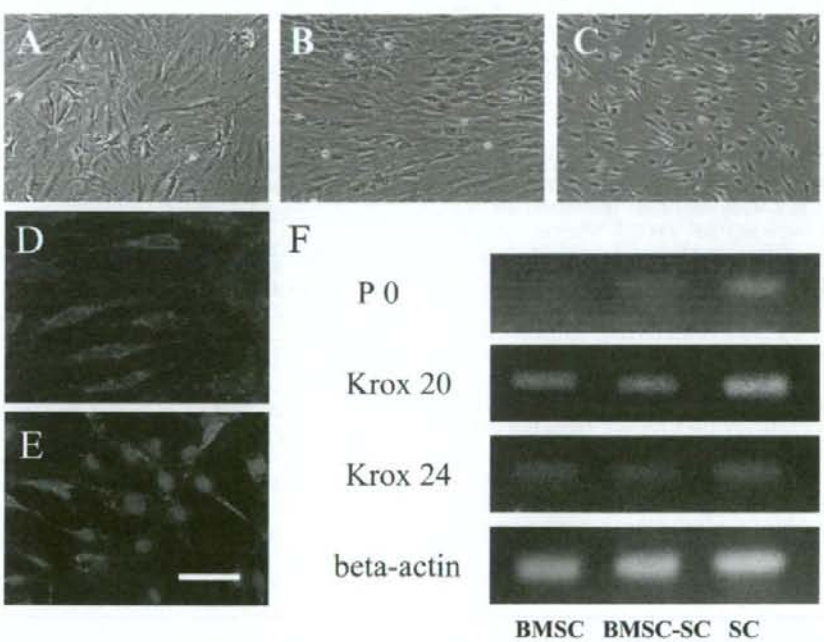


FIG. 1. Photomicrographs demonstrating the peripheral SC-like characteristics of BMSC-SCs. A: Phase-contrast microscopic image of BMSCs cultured from GFP-transgenic rats. B: Phase-contrast microscopic image of BMSC-SCs. The BMSC-SCs are morphologically and phenotypically similar to SCs. C: Phase-contrast microscopic image of peripheral SC cultured from a GFP-transgenic rat. D and E: Immunofluorescence images of BMSC-SCs stained for P0 (D) and p75NTR (E). The BMSC-SCs were positive for P0, S100, and p75NTR. Nuclei were stained with 4'-6-diamidino-2-phenylindole. Bar = 50 μm. F: Results of RT-PCR analysis showing that P0 mRNA was upregulated in BMSC-SC–like peripheral SCs. The expression level of P0 in BMSCs is shown in comparison to its expression in BMSC-SCs and SCs. No significant statistical difference was shown between BMSCs and BMSC-SCs in the expression of Krox20 and Krox24.

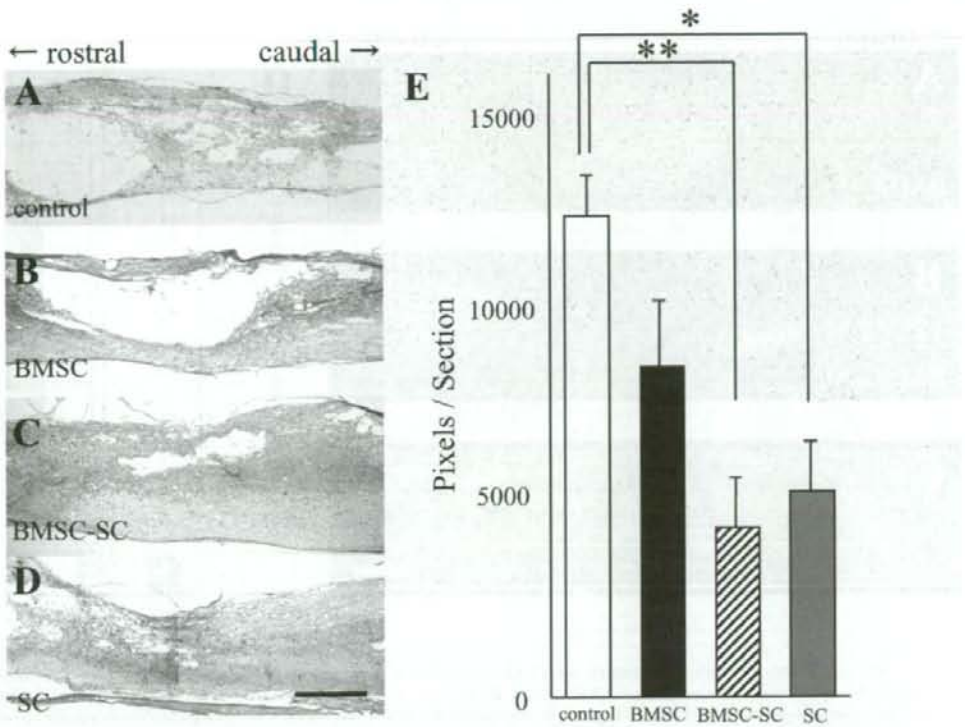


FIG. 2. A–D: Cresyl-violet–stained BMSC-SCs and SCs in preserved spinal cord tissue samples from all 4 groups. Representative image of cystic cavity in samples obtained in the control (A), BMSC (B), BMSC-SC (C), and SC (D) groups. The area of cystic cavity of BMSC-SC and SC groups were significantly smaller than that of the control group. Bar = 1 mm. E: Statistical analysis of area of cystic cavity (*white column*, control group; *black column*, BMSC group; *hatched column*, BMSC-SC group; and *gray column*, SC group). The area of cystic cavity in the BMSC-SC and SC groups was significantly smaller than that in the control group. *Bars* represent the means, and *whiskers* the SEMs. *$p < 0.05$, **$p < 0.01$.

helps to preserve spinal cord tissue (Fig. 2E). There was no significant difference in the average area of the cystic cavity between the other groups.

Phenotype of BMSC-SCs in the Injured Spinal Cord

The number of GFP-positive transplanted cells in the BMSC, BMSC-SC, and SC samples was 142.0 ± 18.0, 386.3 ± 28.6, and 1301.8 ± 310.8 per section, respectively (Fig. 3A–C). Note that the number of GFP-positive transplanted cells in the SC group was significantly larger than in the BMSC and BMSC-SC groups ($p < 0.01$; Fig. 3D). In addition, the number of GFP-positive transplanted cells in the BMSC-SC group was significantly larger than that in the BMSC group ($p < 0.05$).

A double immunofluorescence study showed that GFP-positive transplanted BMSCs were simultaneously positive for vimentin and fibronectin (data not shown). The BMSC-SCs and SCs were both positive for P0, S100, and p75NTR (not shown), indicating that the transplanted cells maintained the specific phenotypes observed in vitro even after transplantation into the injured spinal cord.

Adrenergic and Serotonergic Fibers in the Injured Spinal Cord

We next performed immunohistochemical analyses for axonal markers and anterograde axonal tracing to evaluate axonal the extent of regeneration and/or sparing. The number of Gap43-positive nerve fibers at the rostral level in the SC group was significantly larger than that in the other groups ($p < 0.01$; Figs. 4A–C and 5A). The number of Gap43-positive nerve fibers at the injury epicenter level in the BMSC-SC group (Fig. 4B) was significantly larger than that in the control ($p < 0.05$; Fig. 5A), and the number of Gap43-positive nerve fibers at the epicenter level in the SC group was significantly larger than in the control and BMSC groups ($p < 0.01$; Fig. 5A). There was no significant statistical difference in the number of Gap43-positive nerve fibers at the caudal level between the groups.

To characterize the phenotype of regenerated or spared axons, we performed immunohistochemical analysis for several nerve fiber markers. There were significantly more Th-positive fibers at the injury epicenter and caudal level in the BMSC-SC ($p < 0.01$, both locations;

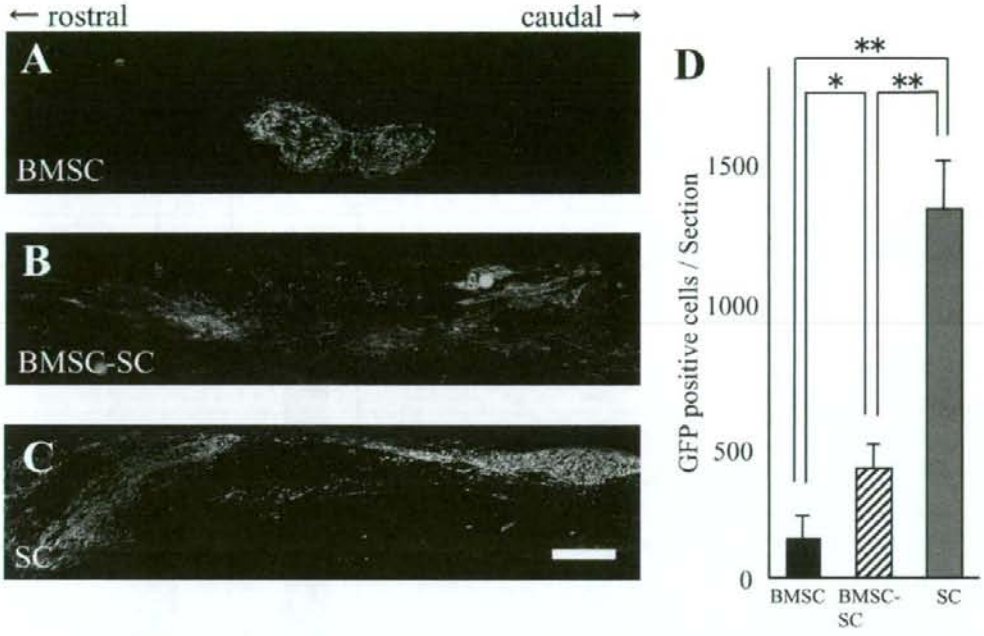


FIG. 3. Photomicrographs. Staining with GFP in the BMSC (A), BMSC-SC (B), and SC groups (C) 5 weeks after cell transplantation. Bar = 1 mm. D: Graph of the statistical analysis of number of GFP-positive transplanted cells. There were significantly more GFP-positive transplanted cells in the BMSC-SC group *(hatched column)* than in the BMSC *(black column)* group ($p < 0.05$). There were also significantly more GFP-positive transplanted cells in the SC group *(gray column)* than in the BMSC *(black column)* and BMSC-SC *(hatched column)* groups. *Bars* represent the means, and *whiskers* the SEMs. *$p < 0.05$, **$p < 0.01$.

Figs. 4D–F and 5B) and SC ($p < 0.05$ and $p < 0.01$, respectively) groups than in the control group. The number of serotonin-positive nerve fibers in the BMSC-SC group was significantly larger than that in the control and BMSC group at the caudal level ($p < 0.05$; Figs. 4I and 5C). Biotinylated dextrin amine–labeled corticospinal tract fibers were found in the rostral and epicenter levels, however there were no BDA-labeled fibers in caudal level any groups (data not shown).

In the immunoelectron microscopic study of rats from the BMSC-SC group, the GFP-positive transplanted cells (Fig. 6 *asterisks*) had several thin processes, which were immediate contact with host nerve tissues (Fig. 6 *arrows*).

Locomotor Recovery

Finally, we assessed the recovery of hindlimb functioning in all groups on a weekly basis for 6 weeks (Fig. 7). The repeated-measures ANOVA and post hoc Fisher protected least significant difference tests showed that hindlimb function recovered significantly in the BMSC-SC group compared with the control and BMSC group ($p = 0.024$ and $p = 0.047$, respectively). The average BBB recovery score in the BMSC-SC group 5 weeks after transplantation was 10.3 ± 0.8, indicating occasional weight-supported plantar steps without forelimb–hindlimb coordination, and in the BMSC group was 7.9 ± 0.6, indicating that all 3 joints of hindlimbs showed extensive movement. Five weeks after transplantation, the average recovery score 5 weeks after transplantation was 7.7 ± 0.6 in the control group, and 9.2 ± 0.8 in the SC group, indicating occasional plantar placement of the paws with weight support. Comparison among the groups at each time point revealed a statistically significant difference between the BMSC-SC and control and the BMSC-SC and BMSC groups in average BBB scores 3–5 weeks after transplantation (Fig. 7).

Discussion

In the present study, we derived SCs from BMSCs and confirmed that BMSC-SCs have SC-like characteristics in immunohistochemical analysis and RT-PCR study of P0. We transplanted BMSC-SCs into the injured rat spinal cord, and performed immunohistochemical studies 5 weeks after transplantation. Transplanted BMSC-SCs maintained the spindle shape and immunohistochemical characteristics of SCs (P0, S100, and p75NTR). In our previous report, we showed that BMSC-SC not only has phenotypic similarity with SCs, but also forms myelin in peripheral nerve tissue.[17,44] However, in the present study no myelin was detected on electron microscopy. A pos-

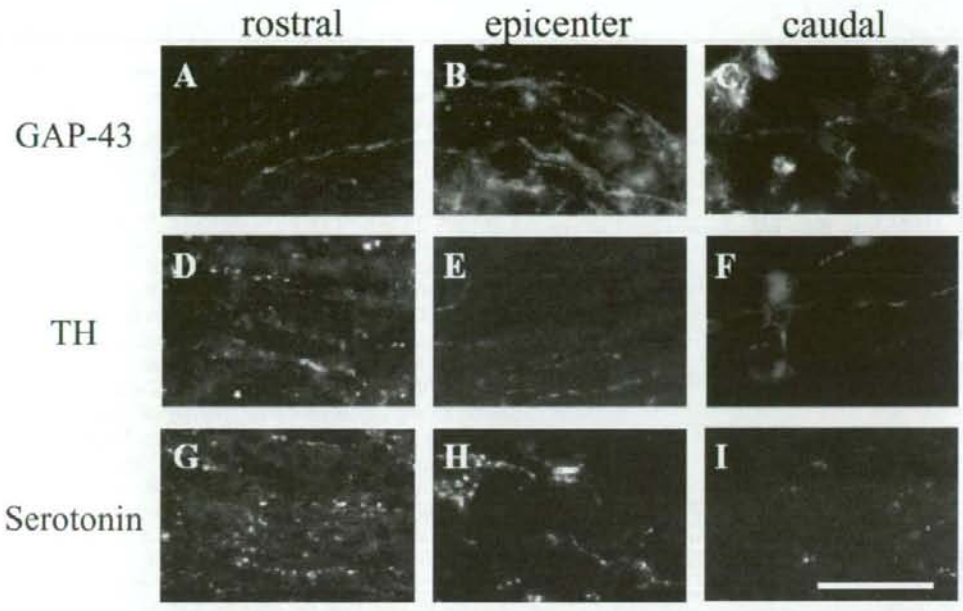


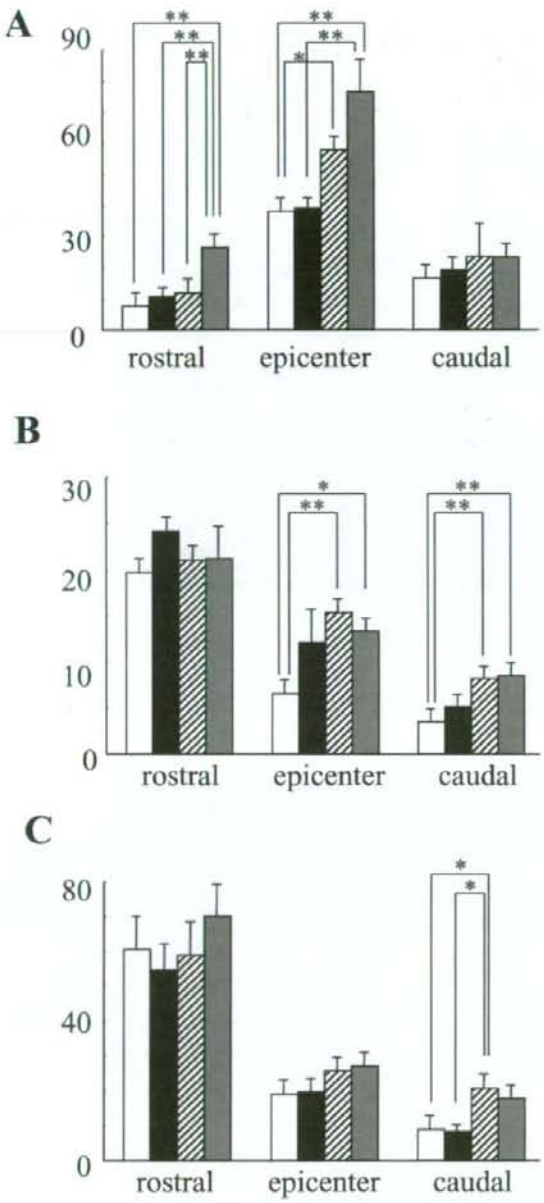
FIG. 4. Immunostaining for regenerated/spared nerve fibers after BMSC-SC transplantation. The Gap43-positive (A–C), Th-positive (D–F) and serotonin-positive (G–I) fibers were detected at the rostral (A, D, and G) epicenter (B, E, and H) and caudal (C, F, and I) levels. Bar = 50 μm.

sible explanation for this discrepancy in myelin formation between our previous results in sciatic nerve and the current findings in the spinal cord is that the experimental period in the present study might be too short for detectable myelin formation to be generated.

Although there are many reports that BMSCs can be differentiated into neural cells in vitro[17,18,40,46] and in vivo,[1,11,12,52] differentiation of BMSCs into neural lineage cells remains controversial. It was recently suggested that the differentiation of BMSCs into neurons in vitro is nothing more than a phenotypic change induced by structural changes in the cell.[35] In the present study, the transplanted BMSCs maintained their original phenotype 5 weeks after transplantation. Five weeks of locomotor assessment showed no improvement in BBB scores in the BMSC group compared to the control. We can conclude that transplantation of BMSC-SCs is better than BMSC transplantation for obtaining locomotor recovery.

We have presented some evidence as to why animals in the BMSC-SC group showed a better functional recovery than those in the BMSC or control groups. First of all, transplantation of BMSC-SCs reduced the area of the cystic cavity. We used reduction in cystic cavity as a measure of spinal cord tissue sparing because the correlation between the reduction of cystic cavity volume and hindlimb functional recovery has been reported previously.[22,37,49] Spinal cord tissue sparing might reflect neuroprotection, the action which suppresses progression of tissue destruction in the acute or subacute phase of injury. Bone marrow stromal cell–derived SCs may exert neuroprotective effects by secreting several growth factors or through cell-cell contact with host spinal cord cells. It has been reported that BMSCs secrete several neurotrophic factors including potential neuroregulatory molecules such as the neurotrophins BDNF, NGF, NT-3, and VEGF.[1,10,14,19,42] Isele et al.[25] reported on BMSC-mediated trophic effects and neuroprotection through stimulation of PI3-K/Akt and MAPK signaling pathways in neurons. In the present study, the average area of cystic cavity was smaller in the BMSC-SC group than in the BMSC group, suggesting that BMSC-SCs have a stronger tissue sparing effect than do BMSCs. The soluble factors secreted by BMSC-SCs to promote tissue sparing are still unclear. Further exploration is needed to determine the precise mechanism of tissue sparing action of BMSC-SC. There was no significant difference between the BMSC-SC and SC groups in average area of the cystic cavity, suggesting that BMSC-SCs and SCs have similar tissue sparing effects. However, the number of surviving cells was much smaller in the BMSC-SC group than in the SC group. This discrepancy suggests the possibility of a different mechanism of tissue sparing effects between 2 groups.

Another possibility that BMSC-SCs have the potential to promote axonal regeneration and/or sparing. In the present study we showed that transplanted BMSC-SCs increased the number of Th- and serotonin-positive fibers at the caudal level; both descending fibers may contribute to motor function.[7,16,43,48] Whether regeneration or sparing is the main cause of increased number of those descending fibers is unclear, because some of the long spinal fiber tracts are spared in the contusive SCI model. However, we have previously shown that BMSC-SCs successfully



promote axonal regeneration in completely transacted adult rat spinal cords.[27] Together with the present results, axonal regeneration/sparing of descending motor fibers promoted by BMSC-SCs might contribute to the functional recovery at least in part.

An increased number of Gap43-positive fiber at the epicenter in BMSC-SC-transplanted rats may reflect the sprouting or regeneration of propriospinal fibers, because the number of Gap43-positive fibers in the epicenter was significantly larger than that in the rostral and caudal levels and much larger than the number of Th-positive or serotonin-positive fibers. Bareyre et al.[2] reported that the formation of the new intraspinal circuit in the injured spinal cord promotes spontaneous functional recovery after incomplete SCI. Thus, sprouting or regenerating of propriospinal fibers may contribute to functional recovery in addition to the regeneration or sparing of descending fibers such as the raphe spinal tract and cerulospinal tract.

We previously reported that BMSC-SCs effectively promote axonal regeneration of completely transected rat spinal cords.[27] Before this can be applied clinically, the efficacy of this treatment must be shown in a compression- or contusion-induced model of SCI, both of which are more relevant to clinical SCIs than transection or hemisection models.[45] In the present study, we successfully proved the efficacy of BMSC-SC transplantation in a contusive SCI model in rats. In our experiments we found that transplantation with BMSC-SCs promoted better functional recovery than with BMSCs. To prove the difference in efficacy for SCI between these cell types, further exploration is needed in different experimental settings.

Schwann cells are a candidate cell source for transplantation into the injured spinal cord.[3,8,9,20,51] It has been reported that transplanted SCs can incorporate themselves into the lesioned cord, bridge defects, attract axonal growth to the graft,[31] and myelinate both regenerating and intact axons,[38] resulting in functional recovery. One of major obstacles to SC use in cell therapy is harvesting these cells from the peripheral nerves. Harvesting SCs potentially causes complications including anesthesia or allodynia at the harvesting site. In the present study, some of the rats who received SCs showed severe autophagia. In contrast, BMSCs are easily harvested because they can be obtained from the iliac crest or femur by bone marrow aspiration. Bone marrow aspiration is much less invasive, and can be performed in an outpatient setting. Bone marrow stromal cells are easily expanded in vitro because

FIG. 5. Comparisons of the average number of Gap43-positive (A), Th-positive (B), and serotonin-positive (C) fibers among the groups. Staining for Gap43, Th, and serotonin were performed, and the number of immunoreactive fibers that traversed the lines perpendicular to the central axis of the spinal cord at rostral level (5-mm rostral to the injury epicenter), epicenter, and caudal level (5-mm caudal to the epicenter) were counted. A: There were significantly more Gap43-positive fibers in the BMSC-SC group *(hatched column)* than in the control group *(white column)* at the epicenter level. The number of Gap43-positive fibers in the SC group was significantly larger at the rostral level than others. B: The numbers of Th-positive fibers were significantly larger in the BMSC-SC group *(hatched column)* than that in the control group *(white column)* at the epicenter and caudal level. There were similar numbers of Th-positive fibers in the SC and BMSC-SC groups. C: The numbers of serotonin-positive fibers of the BMSC-SC group *(hatched column)* was significantly larger than that of the BMSC *(black column)* and control *(white column)* groups at the caudal level. *Bars* represent the means, and *whiskers* the SEMs. *$p < 0.05$, **$p < 0.01$.

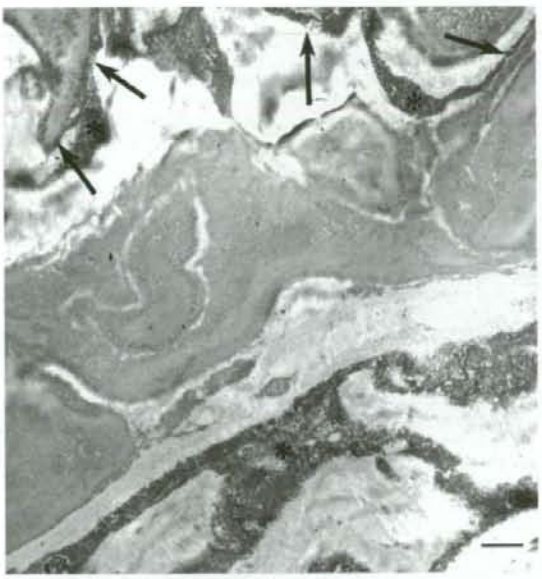


FIG. 6. Electron micrograph obtained in the BMSC-SC group. The GFP-positive transplanted cells *(asterisks)* had several thin processes, which were in immediate contact with host nerve tissues *(arrows)*. Bar = 2 μm.

they proliferate more vigorously than peripheral nerve–derived SCs.[6,30,41] However it is difficult to control the differentiation of transplanted BMSCs in vivo. To exclude the possibility that transplanted BMSCs might differentiate into aberrant cells in vivo, we induced production of BMSC-SCs prior to transplantation. To prove the safety of this technique and exclude dedifferentiation or tumor formation after transplantation a longer study period (> 6 months in rats) must be completed before advancement for clinical trial.

In addition, it has been reported previously that the survival rate of transplanted BMSCs in injured spinal cords is relatively low.[47] Our results demonstrate that the number of surviving cells in the BMSC-SC–transplanted rats was significantly higher than that in BMSC-transplanted rats. There are several possible explanations for the increased survival of transplanted BMSC-SC: perhaps differentiation into a neural lineage prior to implantation increases affinity to the host spinal cord, BMSC-SCs may have a higher durability against cell death, or BMSC-SCs may have a higher potential to proliferate after transplantation. Eventually, the higher survival rate of these transplanted cells might increase the efficacy of cell transplantation therapy.

In the present study we showed that BMSC-SCs have a higher potential than BMSCs in tissue sparing, survival of transplanted cells, axonal regeneration/sparing, and promotion of hindlimb functional recovery.

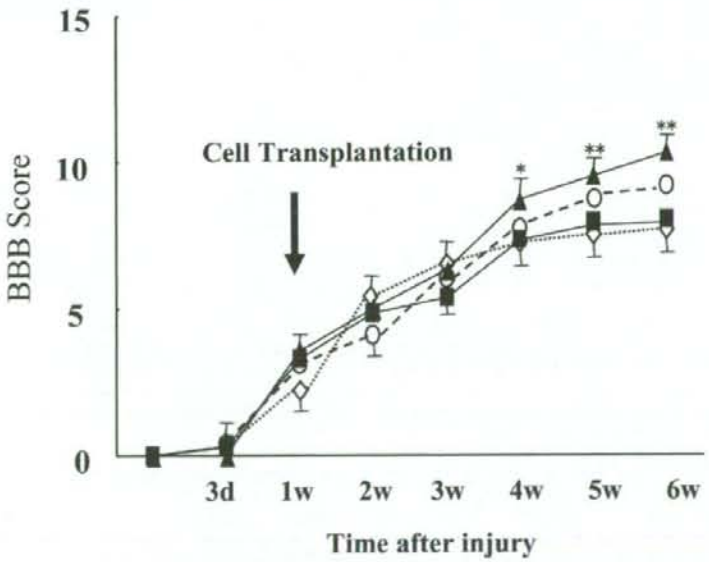


FIG. 7. Graph showing that rats with BMSC-SC transplantation showed better hindlimb functional recovery. The repeated measure ANOVA and post hoc test showed better functional recovery in BMSC-SC group *(triangle)* than in the control (*diamond* and *dotted line*) and BMSC group *(black square)*. There was no significant difference between the SC group (*circle* and *broken line*) and the other groups. Comparison among the groups at each time point revealed that there was significant statistical difference in average BBB scores in the BMSC-SC and control or BMSC-SC and BMSC groups 3–5 weeks (w) after transplantation. *Bars* represent the means, and *whiskers* the SEMs. *$p < 0.05$, **$p < 0.01$.

Moreover, BMSC-SCs have abilities similar to peripheral nerve–derived SCs in tissue repair. It is known that SC or BMSC-SC transplantation alone can promote only modest recovery of function. A combination with other kinds of therapies (such as drug therapy and rehabilitation) could enhance the therapeutic effects of BMSC-SC transplantation as shown by Pearse et al.[39] For example, SC transplantation could be combined with cAMP and phosphodiesterase inhibitor treatment.

A limitation of the present study is that the observation period is too short to fully clarify the behavior of transplanted BMSC-SCs. Moreover, long-term observation is needed to refine some issues including safety (the potential for tumor formation, for example), maintenance of differentiation (possibility of dedifferentiation) and long-term effects. Before clinical applications of BMSC-SC transplantation for SCI can proceed, we must resolve these issues and establish the best combination therapy.

Conclusions

Although further exploration is needed, our present results demonstrate that BMSC-SCs are an excellent potential candidate as a cell transplantation source for the treatment of SCI. These lines of evidence show that BMSC-SC transplantation reduces cystic cavity, promotes axonal regeneration/sparing, results in hindlimb functional recovery, and can be a useful tool in SCI as a substitute for SCs.

Disclosure

This study was supported by grants from the Ministry of Education, Science and Culture of Japan (Grant No. 16591473) and the General Insurance Association of Japan, to Masashi Yamazaki, M.D., Ph.D.; and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation and Research on Psychiatric and Neurological Diseases and Mental Health (H19-016) to Mari Dezawa, M.D., Ph.D.

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Manuscript submitted April 11, 2008.
Accepted September 18, 2008.
This work was previously presented as an abstract to the Annual meeting of International Neurotrauma Symposium 2006.

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Neuroscience Letters 444 (2008) 143–147

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Brain-derived neurotrophic factor suppresses anoikis-induced death of Schwann cells

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ARTICLE INFO

Article history:
Received 3 May 2007
Received in revised form 22 June 2008
Accepted 21 July 2008

Keywords:
Spinal cord injury
Cell therapy
Anoikis

ABSTRACT

Anoikis is a type of apoptosis due to the detachment from the extracellular matrix and neighboring cells. In case of cell transplantation therapy for spinal cord injury, preparation of graft cells includes dissociation of cultured cells, which may cause anoikis-induced cell death. Thus suppression of anoikis may increase survival of grafted cells. Here we tested the effect of brain-derived neurotrophic factor (BDNF) on anoikis-induced cell death of cultured Schwann cells. Schwann cells were collected and cultured from sciatic nerves of neonatal Wistar rats. Schwann cells were plated upon a non-adherent polyhydroxyethyl methacrylate substrate to induce anoikis. BDNF was added into the culture medium at various concentrations. Twenty-four hours after non-adherent culture, approximately 40% of Schwann cells died and BDNF significantly decreased the number of dead cells in that culture condition. Next, Schwann cells were transplanted with or without BDNF treatment into contused rat spinal cord 1 week after injury. Five weeks after transplantation, immunohistochemistry revealed that the number of transplanted cells was significantly larger in the BDNF-treated group than that of the non-treated group. Suppression of anoikis may increase survival of grafted cells in case of cell therapy for spinal cord injury.

It has been widely believed that axons in the lesioned adult mammalian spinal cord cannot regenerate. This failure of regeneration is mainly attributed to the non-permissive environment of the damaged adult mammalian spinal cord, whose milieu is formed of inhibitory molecules in the scar tissue and myelin components of oligodendrocytes interfering with the regeneration of axons [14]. In contrast to the central nervous system, the peripheral nervous system can regenerate lesioned axons. The difference of the glial environment may be the key to the dissimilarity of the regenerating capacity between the central and peripheral nervous systems. Peripheral glia are composed of Schwann cells that can express various types of neurotrophic factors and adhesion molecules supporting axonal regrowth and can reconstruct myelin sheath during the process of peripheral nervous system regeneration. Thus, Schwann cells may have the capacity to promote axonal regeneration of the central nervous system. Many authors have previously reported that Schwann cells can promote axonal regeneration of lesioned adult rat spinal cord. Therefore Schwann cell is one of strong candidates for cell transplantation therapy for spinal cord injury [3].

Recent findings show that the number of engrafted cells decreases shortly after transplantation. Okada et al. showed that engrafted neural stem cells decreases during the first week after transplantation into the injured spinal cord using *in vivo* bioluminescent imaging system [12]. Host environments including impaired vascularization to the engrafted cells and the non-specific inflammatory reaction at the site of transplantation contribute to apoptosis of engrafted cells and subsequent early graft loss. In addition, graft processing procedure itself may trigger the apoptosis of engrafted cells. Anoikis is a type of apoptosis due to the detachment from the extracellular matrix and neighboring cells [8]. In case of cell transplantation therapy for spinal cord injury, preparation of graft cells generally includes dissociation of cultured cells, which may cause anoikis-induced cell death. Thus suppression of anoikis may increase survival of grafted cells. It has been reported that BDNF can suppress anoikis in cultured epithelial cell line [5]. Thus BDNF might have a potential to suppress anoikis of other types of cells. Here we tested the effect of brain-derived neurotrophic factor (BDNF) on anoikis-induced cell death of cultured Schwann cells as a cell source of transplantation for SCI.

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doi:10.1016/j.neulet.2008.07.055

Schwann cells were collected from sciatic nerves of 2-day-old Wistar rats (postnatal day 2–4; SLC, Hamamatsu, Japan) as previously described [6]. Briefly, the dissected nerves were treated with 0.1% collagenase and 0.1% trypsin (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) for 60 min at 37 °C. Dissociated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. After 1 day of culturing, cells were exposed to two cycles of cytosine–arabinoside treatment (10 μM) for 3 days to prevent proliferation of fibroblasts. After 7 days of culturing, cells were plated on poly-D-lysine (PDL; Sigma) coated dishes in DMEM containing 10% FBS, 1% antibiotics, and 10 μM forskolin (Sigma). Cells were maintained in a 37 °C incubator containing 5% CO_2, fed every 3 days, and passaged when confluent. Schwann cells at passage 4–6 were used in this study. The purity of cultured Schwann cells was confirmed by immunocytochemistry as previously described [10] for p75 low affinity nerve growth factor receptor (p75) and S-100. The ratio of p75- or S-100-positive cells was calculated by dividing by the number of DAPI-stained cell nuclei. The purity of Schwann cell was approximately 95%~ in all the culture we used in the present study.

Cultured Schwann cells were detached from plastic dishes with 0.25% trypsin and then transferred to a non-adherent polyhydroxyethyl methacrylate (poly-HEMA; 40 mg/ml, Sigma)-coated 24 well multi-well plate (Thermo Fisher, Rochester, NY) at cell density of 4×10^5 cells/ml (2×10^5 cells/each well). Each treatment protocol was applied to three wells per each independent culture. We used poly-HEMA as non-adherent cell culture surface because poly-HEMA almost completely inhibits the attachment of various kinds of cells without cell toxicity by poly-HEMA itself. Twenty-four hours after non-adherent culture, apoptosis of Schwann cells was observed. To confirm apoptosis of Schwann cells, Giemsa staining and immunocytochemistry for cleaved caspase-3 were performed. Using Giemsa staining, condensation or fragmentation of cell nuclei were observed as one of a morphologic signs of apoptosis. Cleavage by caspase-9 induces the activation of caspase-3 and is known as one of the molecular marker of apoptosis. Immunocytochemistry was performed as previously described [10]. Non-adherent-cultured Schwann cells were collected with brief centrifugation. The primary antibodies used in the present study were as followings; anti p75 (Chemicon International, Temecula, CA), anti S-100 (Dako Cytomation, Copenhagen, Denmark) and anti-cleaved caspase-3 rabbit polyclonal antibody (1:100 dilution; Genzyme/Techne). The secondary antibodies were Alexa Fluor® 594-conjugated goat anti-rabbit or anti-mouse secondary antibody; 1:800 dilution; Molecular Probes, Inc., Eugene, OR) Cell nuclei were counterstained with DAPI (Molecular Probes). The positive signals were observed by fluorescence microscope (ECLIPSE E600; Nikon, Tokyo, Japan).

Our preliminary experiments suggests anti-anoikis effect of BDNF, thus we tested NGF family proteins and GDNF in the current study. To elucidate anti-anoikis effects of neurotrophic factors, NGF, BDNF, NT-3 or GDNF (Peprotech, London, UK) were added to the culture medium (100 ng/ml). Twenty-four hours after non-adherent culture, cell death was detected by double staining with using Live/Dead Double Staining Kit (MBL, Nagoya, Japan). We had already confirmed that most of Schwann cell death induced by detachment was apoptosis in the first experiments, thus we employed Live/Dead Double Staining Kit to evaluate anti-anoikis effects of neurotrophins because of its convenience. Non-adherent-cultured Schwann cells were collected by brief centrifugation and treated with 0.25% trypsin for 5 min. As for control, Schwann cells cultured on PDL-coated surface were detached by trypsin treatment. All the groups of Schwann cells were re-suspended in staining buffer containing propidium iodide and calcein for 15 min at 37 °C. After staining, all the cells collected from each well were mounted on slide glass, cover-slipped and observed using fluorescence microscopy. Ratio of dead cell number to the total cell number was calculated (number of dead cells/total cell) and compared between each group. Among the neurotrophic factors tested, only BDNF showed apparent anti-anoikis effect. Next, BDNF was added to the culture medium in different doses (0–100 ng/ml) to determine dose dependency of BDNF-mediated anti-anoikis effect. Because Schwann cells express only truncated form of TrkB, which is high affinity receptor for BDNF lacking intracellular transducing domain [7], BDNF may act via p75 low affinity nerve growth factor receptor (p75). Anti-p75 antibody (1:1000; Advanced Targeting Systems, San Diego, CA) was added to the culture medium to block BDNF-mediated anti-anoikis effect. Excessive amount of NGF was added to competitively bind to p75 receptor against BDNF. To exclude the possibility that BDNF and NGF (as a competitor against BDNF) act via activation of Trk receptors, the inhibitor of Trk receptors K252a was added to the culture medium.

All the *in vitro* experiments were triplicate (prepared from three independent cultures) and those results were averaged.

For *in vivo* survival study of engrafted Schwann cells, contusion injury was introduced to adult rat spinal cord. Ten-week-old female Wistar rats (average weight 225 g) were anesthetized with 1–1.2% halothane in 0.5 l/min oxygen and laminectomized at T9 level, then exposed spinal cord was injured using Infinite Horizons injury device (Precision Systems and Instrumentation, Lexington, KY) with injury force of 200 kdyn. Food and water were given *ad libitum*. Manual bladder expression was performed twice a day until recovery of the bladder reflex. All animals were treated and pertaining to the treatment of experimental animals. For transplantation experiment, Schwann cells were cultured from sciatic nerve of green fluorescent protein (GFP) transgenic rat (The YS institute, Utsunomiya, Japan) as described above. Prior to the transplantation, BDNF (100 ng/ml) was added to the medium for 1 h at 37 °C after detachment. BDNF-treated or untreated (control) GFP-positive Schwann cells were transplanted into injured spinal cord 1 week after injury. The number of transplanted cells was 3×10^5 cells in 3 μl of PBS/rat in both groups. Injury site was re-exposed under general anesthesia and cells were directly injected into injury site using a micro-glass-pipette attached to the 10-μl Hamilton syringe. The injection was performed slowly over a 5-min period to avoid additional trauma. After injection, the tip of micro-glass-pipette was left in place for 3 min to prevent leak of transplanted cells. Muscles and skin were then sutured layer by layer. All the animals were given antibiotics in their drinking water (1.0 ml Bactramin, [Roche, Basel, Switzerland] in 500 ml acidified water) for 2 weeks after transplantation. All animals were immunosuppressed with cyclosporine A. Cyclosporine A was administered as previously described [18]. Twenty-four hours before transplantation, cyclosporine A (10 mg/kg) was injected subcutaneously. After transplantation, cyclosporine A was also injected for the entire experimental period (20 mg/kg on Monday and Wednesday, 40 mg/kg on Friday).

The recovery of hind-limb motor function was assessed by measuring Basso, Beattie and Bresnahan (BBB) scores [2]. All behavioral tests were digitally videotaped and scored by two observers who were unaware of prior treatment. Measurement was performed before surgery, 1 and 3 days and 1–6 weeks (once a week) after spinal cord injury.

Five weeks after transplantation, animals were perfused transcardially with ice-cold 4% paraformaldehyde in PBS under deep pentobarbital anesthesia. Spinal cord tissue was fixed overnight by immersion in the same solution. The tissue was then immersed for 48 h in 20% sucrose in PBS at 4 °C and embedded in O.C.T. compound (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan) and frozen on dry

ice. The tissue was then sliced into 20 μm thickness sagittal sections on a cryostat, mounted onto poly-L-lysine-coated glass slides (Matsunami, Tokyo, Japan), and dried for 48 h at room temperature.

Immunohistochemistry for GFP was performed as previously described [10] to detect transplanted cells. Rabbit polyclonal anti-GFP antibody (Molecular Probes) was used as a primary antibody and Alexa488-conjugated anti-rabbit IgG antibody (Molecular Probes) was used as a secondary antibody. We counted the number of transplanted cells as GFP-positive cells in the sagittal sections. Every fifth slides (20 μm thickness each) were picked up and the cell count was performed on six slides including the mid sagittal section, resulting in the coverage of the middle 600 μm of the spinal cord in each animal.

Statistical analysis in the present study was performed as followings; for *in vitro* studies, one-way ANOVA followed by Bonferroni/Dunn post hoc test was used. Motor function scores were subjected to Repeated Measures ANOVA followed by post hoc test using Scheffe's *F*-test. The cell survival count was subjected to the Student's *t*-test. Data are presented as mean values ± S.E. Values of $p < 0.05$ were considered statistically significant.

As shown in Fig. 1, detachment induced apoptosis of Schwann cells. Twenty-four hours after non-adherent culture, a number of Schwann cells underwent apoptosis. Giemsa staining showed nuclear condensation and fragmentation (Fig. 1A), both of which are the morphological characteristics of apoptotic cells. Immunocytochemistry showed that a number of Schwann cells were positive for activated caspase-3 and they also had condensed nuclei stained by nuclear staining with DAPI (Fig. 1B), showing these cells underwent apoptosis. In contrast, only a few Schwann cells in adhesive culture on PDL-coated dish showed condensation or fragmentation of cell nuclei and were positive for cleaved caspase-3 (not shown). These evidences showed that Schwann cells underwent anoikis after detachment.

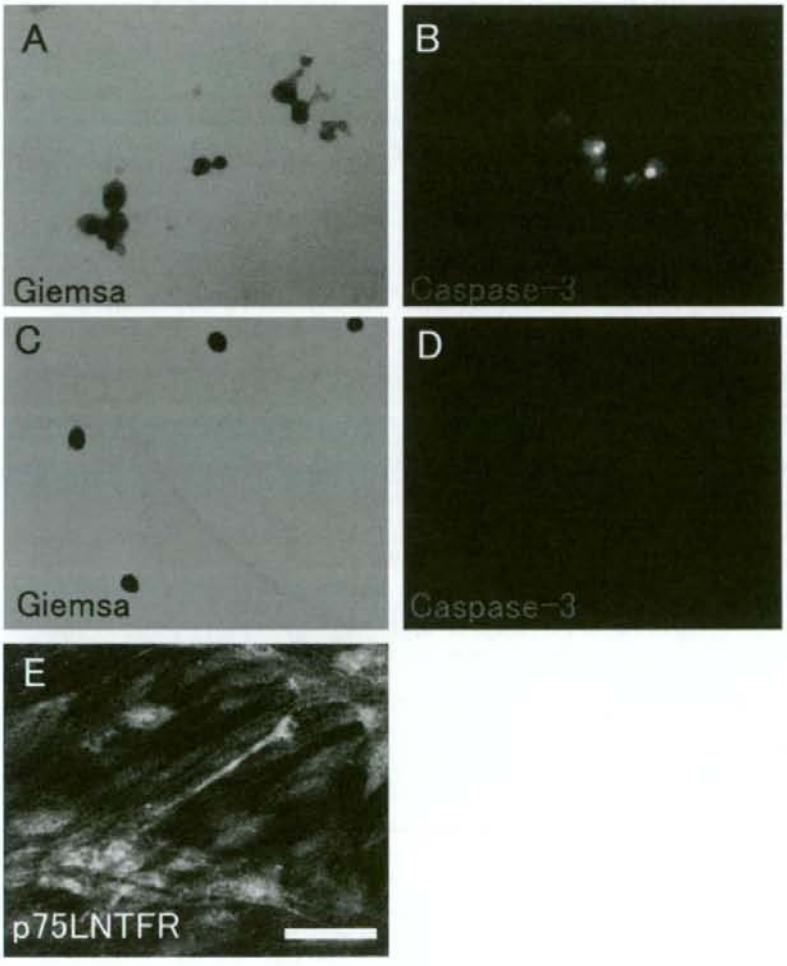

Fig. 1. Schwann cells underwent apoptosis after detachment. Twenty-four hours after detachment, apoptosis of Schwann cells was confirmed with Giemsa staining (A) and immunocytochemistry for activated caspase-3 (B). Giemsa staining revealed the condensation and fragmentation of cell nuclei, both of which are the characteristics of apoptotic cells (A). Immunocytochemistry revealed that caspase-3 was activated in Schwann cells after detachment (B). In contrast, only a few Schwann cells showed apoptosis on adhesive substrate PDL (C) and (D). Schwann cells cultured on PDL-coated dish were assessed with Giemsa staining (C) and immunocytochemistry for activated caspase-3 (D) immediately after the detachment by trypsin. (E) Purity of cultured Schwann was confirmed by immunocytochemistry for p75 low affinity nerve growth factor receptor (p75). The purity was approximately 95%. Bar = 50 μm.

Next, to elucidate which neurotrophic factor can suppress anoikis of Schwann cell, we added NGF, BDNF, NT-3 or GDNF to the culture medium. Without any neurotrophic factors, approximately 40% of Schwann cells died 24 h after detachment, whereas up to 10% of the Schwann cells died under adhesive culture condition on PDL-coated dish (Fig. 2A). Amongst the neurotrophic factors tested, only BDNF could suppress anoikis of Schwann cells (Fig. 2A). BDNF suppressed anoikis of Schwann cells by the dose-dependent manner (Fig. 2B). BDNF exerted its maximum anti-anoikis effect at the concentration of 100 ng/ml (Fig. 2B). Functional blocking of p75 receptor using anti-p75 antibody abolished the anti-anoikis effect of BDNF (Fig. 2C). In contrast, addition of K252a had no effect on anti-anoikis effect of BDNF. Moreover, competition of p75 receptor with excessive NGF also abolished the anti-anoikis effect of BDNF (Fig. 2D). Addition of K252a did not alter the competitive effect of NGF against BDNF-mediated anti-anoikis effect (Fig. 2D). These findings suggest that BDNF suppresses anoikis of Schwann cells via binding to the p75 receptor.

Immunohistochemistry for GFP revealed that BDNF promote survival of transplanted Schwann cells in injured spinal cord. Five weeks after transplantation, the number of GFP-positive transplanted Schwann cells was significantly larger in the BDNF-treated Schwann cell transplanted rats compared with that in control rats (Fig. 3). Locomotor assessment revealed that there was no significant difference between the BDNF and control groups in the recovery score (Fig. 4). The average recovery score in both groups were 10.0 in the control group and 11.6 in the BDNF group (Fig. 4).

Anoikis has been described in many types of cells, including thyroid cells, kidney epithelial cells [8], as well as in endothelial cells, fibroblasts [17], osteoblasts [16] and dopaminergic neurons [11]. In case of cell transplantation therapy, procedures for preparation of graft cells includes single cell isolation may induce anoikis. For example, pancreatic islet cells, hepatocytes [19] and dopaminergic neurons [11] undergo anoikis after single cell isolation. In the present study, we firstly showed that Schwann cells, which are widely known to promote axonal regeneration and functional recovery after transplantation into injured spinal cord, underwent anoikis-induced cell death after detachment. Although further exploration is needed, various types of cells may undergo anoikis during the graft preparation for transplantation into the injured spinal cord and may decline the survival of transplanted cells.

Addition of certain growth factors is one of the promised approaches to prevent anoikis. Hepatocyte growth factor (HGF) can suppress anoikis of MDCK epithelial cells [8], breast carcinoma cells [13]. Transforming growth factor-α inhibits anoikis of epithelial intestinal cell line [15], epidermal growth factor can suppress anoikis of keratinocytes [9] and insulin-like growth factor prevents anoikis of mouse fibroblast [17]. In the current study, we firstly showed that BDNF can effectively suppress anoikis of Schwann cells. Many growth factors activate pathways that confer resistance to anoikis. Some of the pathways activated by growth factors acting through their tyrosine kinase receptors converge on the signals activated by integrins, and these pathways include the PKB/Akt survival pathways. Additionally, in the context of anoikis, growth factors can also play a role through the pathway involving the extracellular-regulated kinase (ERK). In case of Schwann cells, they do not express tyrosine kinase receptors for BDNF (TrkB), but expresses p75 neurotrophin receptor which is known to promote death in some types of cells. On the other hand, it has been reported that p75 neurotrophin receptor can promote cell survival [1]. Whether p75 neurotrophin receptor promotes cell survival or cell death depends on cell type [4]. The present results showed that BDNF promotes cell

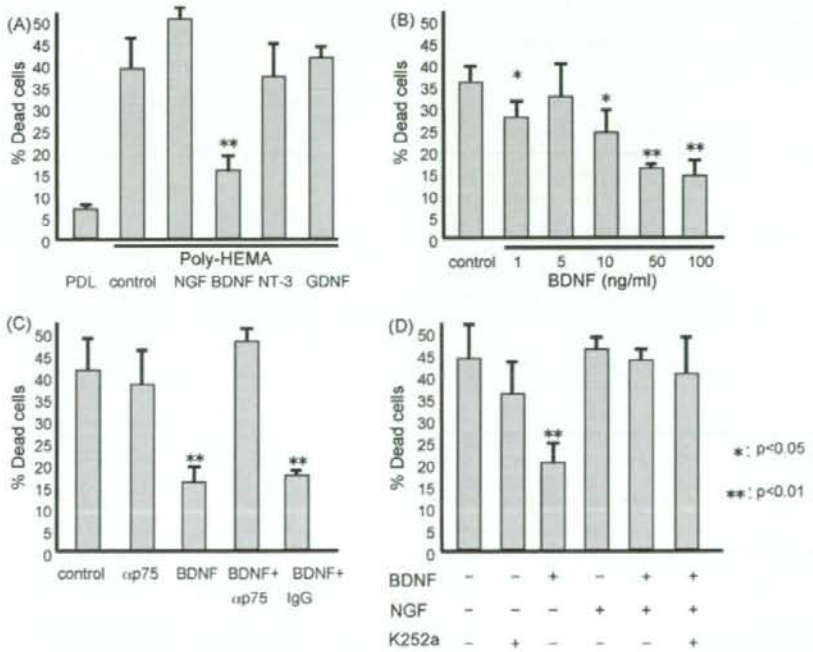

Fig. 2. Suppression of anoikis of Schwann cells by BDNF *in vitro*. BDNF suppressed anoikis of Schwann cells (A). BDNF suppressed anoikis of Schwann cells by the dose-dependent manner (B). Functional blocking of p75 receptor using anti-p75 antibody abolished the anti-anoikis effect of BDNF (C). Moreover, competition of p75 receptor with excessive NGF also abolished the anti-anoikis effect of BDNF (D). These findings suggest that BDNF suppresses anoikis of Schwann cells via binding to the p75 receptor.

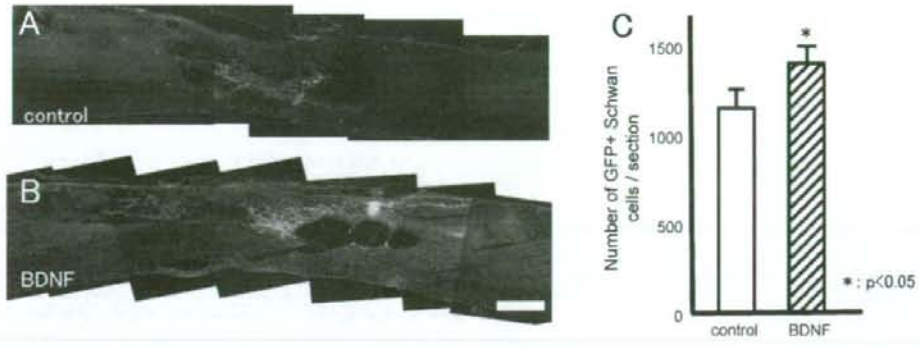

Fig. 3. BDNF treatment increased the number of survived Schwann cells after transplantation into the injured spinal cord. GFP transgenic rat-derived Schwann cells were transplanted into injured spinal cord with or without BDNF treatment. Immunohistochemistry for GFP revealed that the number of GFP-positive transplanted Schwann cells was significantly larger in the BDNF-treated Schwann cell transplanted rats compared with that in control rats.

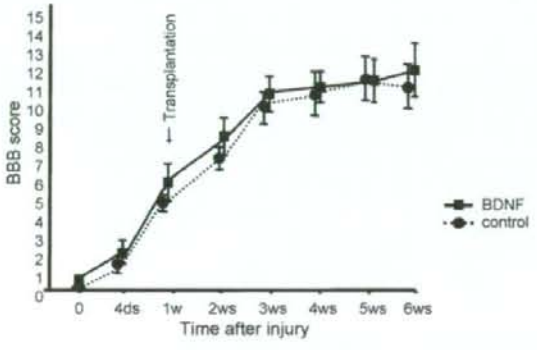

Fig. 4. Recovery of hind limb function. BDNF-treated (BDNF, closed square) or non-treated (control, closed circle and dotted line) Schwann cells were transplanted into lesioned site 1 week after spinal cord injury. There was no significant difference in functional recovery between the BDNF and control groups.

survival of Schwann cells after detachment via p75 neurotrophin receptor. NGF and NT-3, the other members of nerve growth factor family protein, have potential to bind to p75 receptor, however, they could not suppress the Schwann cell anoikis. Although the precise mechanism underlying this discrepancy between the nerve growth factor family proteins is still unclear, there is a possibility that the interaction of truncated TrkB and p75 has some role on the p75-induced suppression of Schwann cell anoikis. Further exploration is needed to elucidate precise mechanism of p75-mediated anti-anoikis effect.

In the present study, BDNF treatment cannot accelerate functional recovery in spite of significant increased number of survived Schwann cells. It is still unclear that appropriate cell number, location and time point for more effective functional recovery.

In conclusion, certain drug treatment during graft preparation can potentially promotes better cell survival after transplantation and can resolve one of the problems of cell transplantation therapy for SCI. Appropriate drug selection for each type of cell sources is needed to explore in future.

Acknowledgement

This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (17659460).

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Acta Neuropathol
DOI 10.1007/s00401-008-0476-x

ORIGINAL PAPER

Deletion of macrophage migration inhibitory factor attenuates neuronal death and promotes functional recovery after compression-induced spinal cord injury in mice

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Received: 13 August 2008 / Revised: 16 December 2008 / Accepted: 20 December 2008

Abstract Macrophage migration inhibitory factor (MIF) is a multipotential protein that acts as a proinflammatory cytokine, a pituitary hormone, and a cell proliferation and migration factor. The objective of this study was to elucidate the role of MIF in spinal cord injury (SCI) using female MIF knockout (KO) mice. Mouse spinal cord compression injury was produced by application of a static load (T8 level, 20 g, 5 min). We analyzed the motor function of the hind limbs and performed histological examinations. Hind-limb function recovered significantly in the KO mice starting from three weeks after injury. Cresyl-violet staining revealed that the number of surviving neurons in the KO mice was significantly larger than that of WT mice six weeks after injury. Immunohistochemical analysis revealed that the number of NeuN/caspase-3-active, double-positive, apoptotic neurons in the KO mice was significantly smaller than that of the WT mice 24 and 72 h after SCI. These results were related to in-vitro studies showing increased resistance of cerebellar granular neurons from MIF-KO animals to glutamate neurotoxicity. These results suggest that MIF existence hinders neuronal survival after SCI. Suppression of MIF may attenuate detrimental secondary molecular responses of the injured spinal cord.

Keywords Macrophage migration inhibitory factor · Spinal cord injury · Glutamate · Apoptosis · Knockout mouse

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Introduction

Macrophage migration inhibitory factor (MIF) is a T-cell-derived, soluble lymphokine [4]. MIF was originally found to inhibit the migration of macrophages and activate them at inflammatory loci. Furthermore, MIF functions as a hormone and immunomodulator, and as a proinflammatory cytokine, and has been identified in many organs (e.g., brain, kidney, and liver [11]).

In the normal central nervous system (CNS), MIF is found in astrocytes, ependymal cells, and epithelial cells of the choroid plexus [12]. It increases in cerebrospinal fluid soon after SCI in rats [7] and increases significantly in the cerebrospinal fluid of patients with multiple sclerosis and neuro-Behçet's disease [10]. Previously we reported in detail on the upregulation of MIF after SCI [9]. MIF mRNA is upregulated in microglia accumulating in the lesion epicenter three days after SCI and in astrocytes around the cystic cavity after one week. MIF is chemotactic for keratinocytes and facilitates wound healing by macrophages [1]. MIF production by peritoneal macrophages may contribute to paracrine and autocrine activation and to macrophage accumulation in the peritoneal cavity of women with endometriosis [2]. If macrophages in CNS play an important role in its regeneration, MIF depletion could hinder the recovery process after SCI because MIF

Published online: 06 January 2009

mRNA is upregulated in microglia accumulating around the lesion. Astrocytes compose glial scars after SCI to prevent inflammation outflow into circumferential normal tissue. MIF depletion could also negatively affect glial scar formation and functional recovery because MIF is upregulated after SCI in astrocytes [9].

The best way to elucidate MIF function in SCI is to use knockout (KO) mice for a comparison study with wild-type (WT) mice. We injured MIF KO and WT mouse spinal cords using a spinal cord compression apparatus and compared their locomotor recovery for six weeks. We detected a statistically significant difference between KO and WT mice in locomotor scale, immunohistological studies, and in-vitro glutamate assault of cerebellar granular neurons (CGN).

Materials and methods

Animals

In this study, we used female MIF-deficient mice (KO) and wild-type mice (WT), which were established by Honma [8]. Both strains were on a Balb/c background and were bred at Hokkaido University, Japan [13]. All animals were treated and cared for in accordance with the Chiba University School of Medicine Guidelines pertaining to the treatment of experimental animals.

Cell culture

Cerebellar granular neurons (CGN) were prepared from postnatal day-seven WT and KO mice. Fresh cerebella were dissected, and the tissue was dissociated with trypsin (2.5 mg/ml, Invitrogen, Carlsbad, CA, USA) and DNase I (0.3 mg/ml, Roche Applied Science, Indianapolis, IN, USA). Cells were plated on poly-L-lysine-coated chamber slides (Lab-Tek Chamber Slides Permanox, Nalge Nunc International, Rochester, NY, USA) at a density of 7.0×10^4 cells/cm^2 in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, penicillin–streptomycin (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, Invitrogen, Carlsbad, CA, USA), and 0.02 M HEPES. After 16 h in culture, the medium was replaced with DMEM 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.

Following seven-day culture, CGN were treated with glutamate (100 μM) for 6 h to induce cell death. Recombinant human MIF (R&D Systems, Minneapolis, MN, USA) was added to the culture medium of glutamate-treated CGN from KO mice in different concentrations (5–50 ng/ml) to assess the effect of MIF on neuronal death. Neuronal death was detected by double staining with propidium iodide and calcein using live/dead double staining kit (MBL, Nagoya, Japan). The numbers of dead cells were counted in two areas of 400× magnification microscope fields and averaged among four groups.

Surgery

Tissue samples were obtained from WT ($n = 24$) and KO ($n = 23$) mice. Under halothane anesthesia, laminectomy was performed at the T7–8 level, leaving the dura intact. The animals were then placed in a stereotaxic apparatus, and two adjustable forceps were applied to the spinous processes of both T6 and T9 to stabilize the spine. The dural tube was compressed with a steady load of 20 g for 5 min at the site of the T 7–8 laminectomy. The tip of the weight was a 1 × 2-mm rectangular plastic plate [5]. The mice were kept under a heating lamp until they regained consciousness. Bladder function was observed during the first few days after trauma for signs of urinary retention. The mice were kept in a temperature-controlled environment of 20°C and were exposed to alternate light and dark periods of 12 h. Food and water were given ad libitum. Manual bladder expression was performed twice a day until recovery of the bladder reflex.

Tissue preparation

Mice were sacrificed 24 h (WT: $n = 7$, KO: $n = 5$), 72 h (WT: $n = 6$, KO: $n = 7$), and 6 weeks (WT: $n = 11$, KO: $n = 11$) after SCI. Animals were perfused transcardially with ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) under deep pentobarbital anesthesia. Spinal-cord segments T 6–9 were excised and fixed overnight in the same solution. The tissue was then immersed for 48 h in 20% sucrose in PBS at 4°C, embedded in O.C.T. compound (Tissue-Tek; Sakura Finetech, Tokyo, Japan), and frozen on dry ice. We sliced 12 μm thick cross-sections and mounted every fifth slice on five sequential slides. Each slide had 20 slices. This methodology covered 6-mm spinal cord length around the epicenter of the lesion. The sections were subjected to histological (six weeks after injury), histochemical (72 h), and immunohistochemical (24 h, 72 h, and 42 days after injury) examination.

Histological studies

Immunohistochemistry was performed as previously described [9]. Briefly, the sections were rehydrated in 0.3% Triton X-100 in PBS for 1 h, washed with PBS for 5 min

three times, and blocked with blocking solution (0.05 M Tris–HCl (pH 7.6), 1% bovine serum albumin, Blockace (Yukijirusi, Sapporo, Japan), 0.15 M NaCl, and 0.1% Tween 20) for 1 h. The sections were then incubated overnight at 4°C with mouse antineuronal nuclei antibody (NeuN, 1:400 dilution; Chemicon, Temecula, CA, USA), as a marker for neurons, rabbit anti-caspase-3-active antibody (caspase-3-active, 1:800 dilution; Genzyme Techne, Cambridge, MA, USA), as a marker for apoptotic cells, and adenomatous polyposis coli (APC, 1:800 dilution, CalBiochem, San Diego, CA, USA), as a marker for oligodendrocytes. The slides were then washed with PBS three times for 5 min and incubated with secondary antibodies (goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594, both 1:800 dilution; Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. The slides were then washed with PBS three times and sealed with mounting medium (Dako Cytomation, Copenhagen, Denmark).

Lectin histochemistry was performed using tomato lectin (0.02 mg/ml; Sigma, St Louis, MO, USA) as a marker for microglia, followed by Alexa Fluor 488 streptavidin (1:800 dilution; Molecular Probes). The slides were then washed with PBS three times and mounted as described above.

Cell counts

We counted the NeuN/caspase-3-active or APC/caspase-3-active double-positive cells 24 and 72 h after injury. The double-positive cells indicated the presence of apoptotic cells that were induced by injury. We measured the slices macroscopically, and designated the two smallest slices as the lesion epicenter on each slide of an animal. Slices were selected from the slides and images were captured using a 20× objective lens field. Every cross-section was composed from four images. We printed the images and double-positive cells were counted. Averages were compared between the genotypes. We also counted the tomato-lectin-positive cells and compared these counts between WT and KO mice 72 h after injury as previously described. The positive cells indicated microglia that had gathered in the injured spinal cord.

We counted the cresyl violet-positive neurons in the ventral gray matter whose major diameters were larger than 30 μm as alpha motor neurons [18] and compared these between the WT and KO mice 42 days after injury, as previously described. We also counted surviving oligodendrocytes, which were stained with APC followed by Alexa Fluor 488-conjugated secondary antibody.

Behavioral test

Functional recovery of the hind limb was determined by measuring the hind-limb motor-function score as described previously [6]. Mice were allowed to move freely in an open field with a rough surface for 5 min at each time period tested. The hind-limb movements of mice were videotaped and scored by two independent observers who were unaware of the groups. Measurement of motor function was performed before surgery and 1–6 weeks (once a week) after SCI. The scale ranged from 0 to 13. In brief, a score of 0 indicated complete paralysis, 1–3 indicated movement of hind limbs without rhythmical stepping, 4–5 indicated rhythmical motion of hind limbs without weight-bearing ability, 6–7 indicated weight-bearing ability, 8–12 indicated walking ability with an increase in the hind-limb gait width, and 13 indicated full recovery.

Statistical analysis

Motor-function scores were subjected to repeated-measures ANOVA followed by a post hoc test using the Tukey–Kramer test. The motor-function score of each time point was statistically analyzed using Student's t test. The results of neuronal survival counts 42 days after SCI, apoptotic neuron counts, and tomato-lectin-positive microglia counts after 24 or 72 h were subjected to Mann–Whitney U tests. The numbers of dead cells from glutamate insult in vitro were subjected to one-way ANOVA followed by a post hoc test using the Tukey–Kramer test. Data are presented as mean values ± SE. Values of $p < 0.05$ were considered statistically significant.

Results

MIF deletion attenuated glutamate insult of CGN in vitro

After 6 h of glutamate insult, the number of dead cells was counted and compared between WT and KO (Fig. 1). The number of dead cells in CGN from KO mice (3 ± 0.6 in KO; Fig. 1b, d) was significantly fewer than that from WT (11 ± 1.9 in WT; Fig. 1a, d). We added recombinant human MIF to the culture medium of glutamate-treated CGN from KO mice in different concentrations (5–50 ng/ml) to assess the effect of MIF on neuronal death. Recombinant human MIF exacerbated the effect of glutamate insult on CGN in KO mice, and the number of dead cells was 5 ± 1.7 (5 ng/ml) and 4 ± 1.5 (50 ng/ml) (Fig. 1c, d). These results showed that MIF deletion attenuated the glutamate-induced insult of CGN.

MIF deletion facilitated recovery of hind-limb motor function after spinal cord compression

We assessed recovery of hind-limb function by measuring the hind-limb motor-function score, in which the maximum

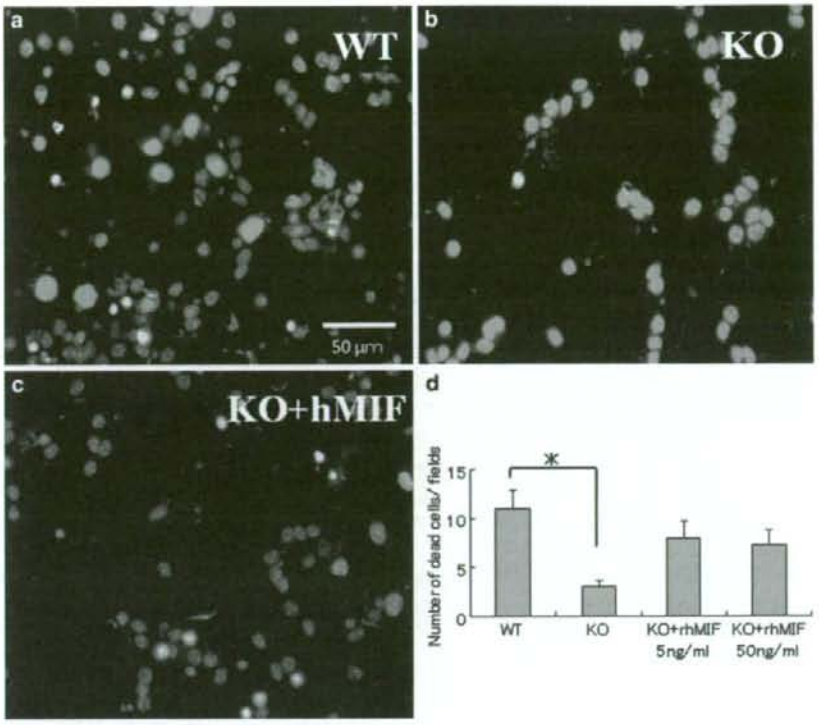

Fig. 1 Cerebellar granular neurons (CGN) from KO mice were more resistant to glutamate insults in vitro. Following 7 days of culture, primary cultured CGN were treated with glutamate (100 μM) for 6 h to induce cell death. Live cells (*green*) and dead cells (*orange*) were stained with live and dead cell staining kit. **a**, **b** More dead cells were stained in CGN from WT mice (**a**) than KO mice (**b**). **c** Recombinant human MIF (5 ng/ml) exacerbated neuron death in CGN from KO mice. **d** Each bar-graph shows the number of dead cells after glutamate insults to CGN from WT, KO, KO + MIF (5 ng/ml), and KO + MIF (50 ng/ml). The number of dead cells in CGN from KO mice was significantly fewer than in CGN from WT mice ($p < 0.05$). Although MIF addition resulted in increased neuronal death in CGN from KO mice, they did not reach a statistically significant difference. *$p < 0.05$. *Bar* 50 μm for **a**–**c**

hind-limb motor-function score was 13. All mice had a score of 11 before SCI, and the score was reduced to 0 immediately after compression. There was no significant difference in motor-function score between the WT and KO mice until three weeks after injury (Fig. 2). Three weeks after compression, significant recovery of hind-limb function was observed in KO mice compared with WT mice

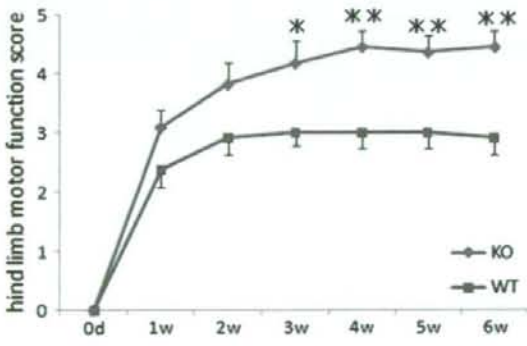

Fig. 2 Hind-limb locomotor score showed better recovery in KO mice after spinal cord compression injury. After spinal cord compression injury, hind-limb locomotor score for each genotype was recorded and compared. The scores of KO mice always surpassed those of WT mice and showed a statistical difference from 21 days after spinal cord injury ($n = 11$ in each genotype). *$p < 0.05$; **$p < 0.01$

(three weeks, $p < 0.05$; four weeks, $p < 0.01$, five weeks, $p < 0.01$; six weeks, $p < 0.001$). The time course of recovery of hind-limb function showed statistically significant differences between the genotypes in repeated-measures ANOVA ($p < 0.01$). The average recovery score in KO mice six weeks after transplantation was 4.45 ± 0.28 (range 2–6), and the corresponding score in WT mice was 2.91 ± 0.28 (range 2–5). A score of 4 indicated a gait characterized by stepping and forward-propulsive movements of one hind limb, but no weight bearing and external rotation of the hind limb. A score of 3.0, however, indicated an obvious movement of one or more joints in both limbs but no coordination, alternate stepping movement, or weight-bearing.

Histochemical assessment shows more neurons survived in KO than WT mice 42 days after SCI

Cresyl-violet-positive cells larger than 30 μm in major axis were classified as remaining motor neurons. Figure 3 shows representative slides of WT (a) and KO (b) spinal cords in the lesion epicenter. In high magnification view, some neurons were spared in the gray matter of the KO mice (d) compared with WT mice (c). Many more inflammatory cells were stained in the gray matter of WT (c) compared with KO (d) mice. The average number of

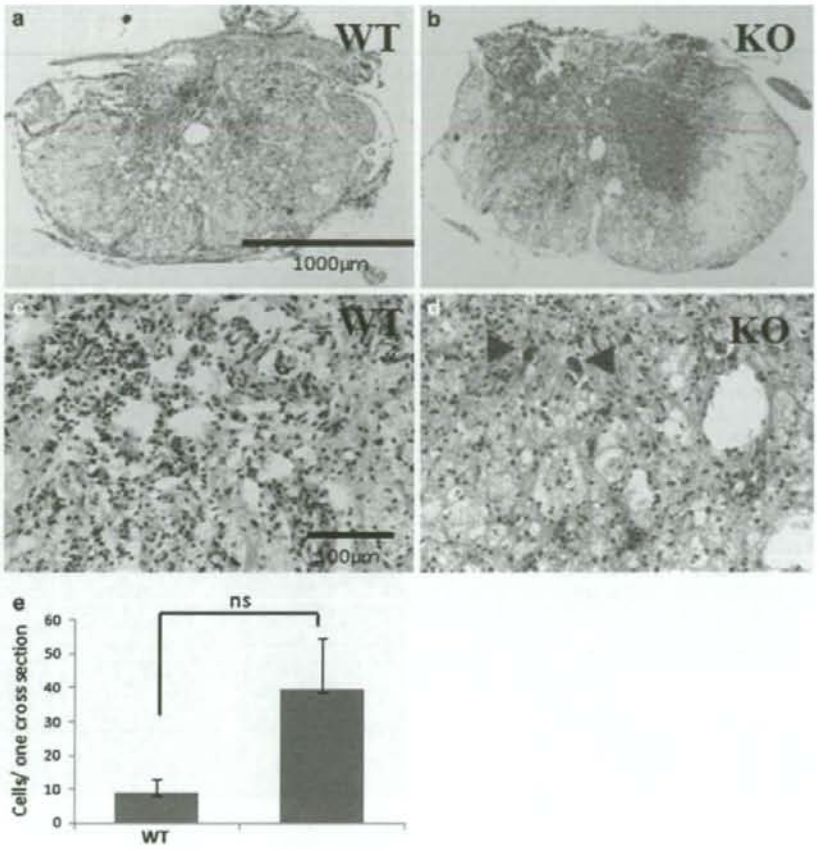

Fig. 3 More neurons survived in KO mouse spinal cord 42 days after spinal cord compression injury. Spinal cord cross-sections, 42 days after spinal cord injury, were stained with cresyl-violet. Motor neurons whose diameter was more than 30 μm were counted and compared. **a**, **b** Representative spinal cord cross-sections of the lesion epicenter 42 days after spinal cord compression injury (**a** = WT, **b** = KO). **c**, **d** High magnification view of **a** and **b**. Some neurons survived in KO mouse spinal cord in **d** (*arrow heads*). On the other hand, a few neurons were counted in WT mouse spinal cord, and more macrophages were gathered around the spaces where neurons were voided (**c**). **e** Despite a large difference in surviving neuron numbers, there was no statistical difference between the genotypes. *Bar* 1,000 μm for **a** and **b**, 100 μm for **c** and **d**

cresyl-violet-positive cells larger than 30 μm in KO mice 42 days after injury was 45.1 ± 15.0, whereas in WT mice it was 12.2 ± 3.9 (Fig. 3e). Although there was no statistically significant difference between genotypes ($p = 0.05$), the number of remaining neurons was indicative of a better recovery of hind-limb motor function in KO mice. On the other hand, there was no statistically significant difference in the number of APC-positive cells between WT and KO mice 42 days after injury (data not shown).

Immunohistochemical assessment a few days after injury showed fewer apoptotic neurons were counted in KO mice; a similar result was obtained for neuron counts after 42 days

As described earlier, more neurons survived in the lesion epicenter of KO mice than in that of WT mice after 42 days, and this result influenced better recovery in hind-limb motor function in KO mice 42 days after SCI. Moreover, more CGNs from KO mice were resistant to glutamate insults than those from WT mice. Based on the above results, we hypothesized that less acute neuronal death occurred in KO mice within a few days after SCI than in WT mice. Thus, we counted NeuN/caspase-3-active double-positive cells and compared between genotypes 24 and 72 h after SCI.

Figure 4 shows fluorescent immunohistochemistry of NeuN (a and b), caspase-3-active (c and d), and merged views (e and f) 24 h after SCI. The number of double-positive cells for NeuN/caspase-3-active in KO mice was significantly smaller than in WT mice 24 and 72 h after injury ($p < 0.05$ after 24 h; $p < 0.01$ after 72 h). The average number of double-positive cells for NeuN/caspase-3-active in KO was 18 ± 3.0 24 h after injury and 15.8 ± 2.1 72 h after injury, whereas that in WT mice was 27 ± 1.9 and 25.2 ± 0.6, respectively (Fig. 4g).

There was no significant difference in the number of microglia 72 h after injury between WT and KO mice ($p = 0.46$). The average number of positive cells for tomato lectin in KO mice 72 h after injury was 372 ± 60, whereas that in WT mice was 436 ± 48.