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Transplantation of neurospheres derived from bone marrow stromal cells promotes neurological recovery in rats with spinal cord injury

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Abstract Previous studies have revealed that cell therapy using bone marrow stromal cells (BMSCs) could promote motor functional recovery in animals with spinal cord injury (SCI). We describe here the development of cell biology technique and the experimental study of regeneration in SCI. The aim of this study was to investigate the potential for neurological recovery by transplantation neurospheres (NS) derived from BMSCs into thoracic SCI. Adult Fisher rats were used: 45 were subjected to complete thoracic SCI performed by the balloon compression method. BMSCs were cultured in vitro to obtain NS. Seven days after thoracic SCI, groups of 15 rats each received transplants of BMSCs-NS (group A), BMSCs (group B), or injection of medium only (group C) into the SCI lesion. Rats from each group were evaluated and compared longitudinally for motor function recovery. The spinal cords (SC) of injured rats were harvested at day 21 or day 42 and prepared for histological analysis. Five weeks after transplantation, many neuronal or axonal sproutings were observed and replaced by host cells in the SCI lesion of group A. Also, transplanted BMSCs-NS expressed neuronal lineage markers. Transplanted rats could walk with weight bearing and showed recovered motor evoked potentials (MEPs).

Key words Spinal cord injury · Cell therapy · Bone marrow stromal cell · Neurosphere · Motor evoked potential · Cell sprouting

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Introduction

Severe spinal cord injury (SCI) usually results in long-lasting deficits involving partial or complete paralysis and loss of sensation below the level of the injury. Cell transplantation to repair SCI is an active area of research with the goal of recovering the functional deficit.[1] The central nervous system (CNS) has long been regarded as incapable of regeneration, and hence the recent discovery of stem cell populations in the CNS has generated intense interest.[1,2] Neural stem cells (NSCs) are capable of undergoing expansion and differentiating into neurons, astrocytes, and oligodendrocytes in vitro and in vivo. However, NSC sources are deep inside the brain, and this inaccessibility severely limits their clinical utility. Identification of alternate sources of neural cells is therefore highly desirable. Multipotent stem cells, which have been detected in multiple tissues in adult mammals, participate in normal replacement and repair[3–5] while undergoing self-renewal. BMSCs contain a small fraction of stem cells that can differentiate into neurons or glial cells.[1,3–12] Transplantation of such stem cells has the potential to promote functional recovery after SCI.[10,12–15] BMSCs have potential clinical use as autografts whereas embryonic stem (ES) cells or stem cells from fetuses can be used as allografts.[15] Previous reports have shown that transplantation of only BMSCs after SCI was insufficient for high-order functional recovery because few BMSCs differentiate into neuronal cells.[9,12,13] We have previously reported an original cell line that collects neural stem cell-like masses from BMSCs.[16] This new cell biology technique has the potential to resolve problems with recent treatment for SCI using NSCs or BMSCs. The transplantation of BMSC-neurospheres (BMSCs-NS) in rats with SCI was investigated here, and a good level of neurological recovery was demonstrated. The present cell biology technique provides a new approach for SCI therapy involving autograft cell transplantation of neural stem cells.[16]

Materials and methods

BMSCs culture

BMSCs obtained from thigh bones of green fluorescent protein (GFP) rats[17] (mean age, 8 weeks; hemizygous GFP transgenic Wistar rats provided by Health Science Research Resources Bank, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM); Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin.[16] The green fluorescent protein (GFP) is a protein composed of 238 amino acid residues (26.9kDa) that exhibits bright green fluorescence when exposed to blue light. In cell and molecular biology, the GFP gene is frequently used as a reporter of expression. Almost all tissues of the GFP transgenic rat, including BMSCs, are green under excitation light. The cells were incubated in dishes at 37ºC in 5% CO_2 for 5 days, and nonadherent cells were removed by replacing the medium. After reaching confluence at approximately 2–3 weeks, the cells were harvested with 0.05% w/v trypsin and 0.02% w/v ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS; pH 7.4) for 5 min at 37ºC, replated, cultured again for 1 week, and finally harvested. Cells used in this study were harvested after three to five passages.

BMSCs-NS induction

Subconfluent cultures of BMSCs were changed to differentiation media that was a modification of that used by Suzuki et al.[16] Twenty-four hours before neuronal induction, the DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin was replaced with preinduction media consisting of DMEM/10% FBS/1 mM β-mercaptoethanol. To initiate neuronal differentiation, the preinduction media was removed and cells transferred to neuronal induction media composed of DMEM/1% FBS and containing 1% dimethyl sulfoxide (DMSO) and 200 μM butylated hydroxyanisole. After 12–72 h, many adherent cells detached from the dishes. Some of these floating cells changed into neural-like cells and were positive for nestin, a marker of neural progenitors. After counting and testing their viability, cells were cultured in medium containing Neurobasal A (Gibco-BRL), 100 U/ml penicillin G, 100 U/ml streptomycin, 2 mM L-glutamine, supplemented with B-27 (Gibco-BRL) and basic fibroblast growth factor (bFGF; 40 ng/ml; Kaken Pharmaceutical). This medium was a modification of that used by Svendsen et al.[18] Cells were maintained in this medium, and the bFGF was replaced each day. Spheroids (clusters of floating cells) became apparent after 7–15 days.

Spinal cord injury (SCI) model and transplantation

The balloon compression model using a 2 Fr. Fogarty catheter (M&I Medical Sales, Miami, FL, USA) was used for complete SCI in rats.[19,20] Studies were performed in 48 adult female Fisher rats weighing approximately 170 g (8 weeks of age) at the beginning of the experiment. Under anesthesia with intramuscular ketamine (64 mg/kg) and xylazine (4.5 mg/kg), partial laminectomy of the T9 vertebra was performed on 45 rats. A 2 Fr. Fogarty catheter was placed into the epidural space and the balloon tip inserted at the T10 level. The balloon was inflated with 70 μl saline for 3 seconds under the T9–T10 lamina. All rats showed complete paraplegia (BBB score of 0) after balloon compression.[20,21] Partial laminectomy was performed in 3 rats as a sham operation group. Transplantation was performed 7 days after SCI using a Hamilton syringe. Each rat was transplanted with 50 μl gel or medium containing cells into the injured spinal cord.[10] A total of 10 rats received 2×10^7 BMSCs-NS within 0.1% collagen gel for preparation before transplantation (group A). Another 10 rats received noninduced 2×10^7 BMSCs in 0.1% collagen gel (group B). Finally, 10 rats were injected with medium only (group C). Animal experiments were carried out in accordance with the Guidelines for Animal Experiments at Yamaguchi University.

Behavioral and electrophysiological evaluations

Animals were carefully maintained in an air-conditioned room at 22ºC with appropriate humidity and had free access to food and water. Behavioral evaluation was performed using the open-field BBB scoring system.[21] Scores from 0 (complete paralysis) to 21 (normal gait) were recorded every day for 6 weeks after SCI. Scoring was performed by an investigator blinded to the treatment status. Significant differences between BBB scores were examined using the repeated measures analysis of variance (ANOVA) and Bonferroni post hoc analysis for multiple group comparisons to determine the statistical significance of the results. All values are given as mean ± SEM. Values of $P < 0.01$ were considered as statistically significant.

Motor evoked potentials (MEPs) following transcranial electric train stimulation were recorded from bilateral gastrocnemius muscles in rats anesthetized with ketamine.[22,23] Groups A–C and naïve rats were examined longitudinally every week before and after SCI. For electrical stimulation, bipolar needle electrodes were fixed onto the skull surface. The anodal (+) electrode was inserted percutaneously into the scalp and the cathodal (–) electrode was placed in the mouth.[23] The train was two times; the intensity of the stimulus was about 15 mA of 0.2-ms duration. The interstimulus interval (ISI) was at 2 ms. After the last MEP recording, all rats were checked for disappearance of the MEP wave following transection of the sciatic nerve.

Histological evaluations

Injured rat SCs were harvested at day 21 and day 42 after SCI. Animals were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg) and transcardially perfused with saline for 5 min followed by 4% para-

formaldehyde (PFA) in 0.01 M PBS. SCs were removed and postfixed in the same fixative overnight at 4ºC for frozen sectioning. They were then rapidly frozen using liquid nitrogen, and tissue sections 6 µm thick were cut longitudinally for hematoxylin and eosin (H&E) staining or immunohistochemical study. The source and dilution of primary antibodies used were as follows: mouse IgG anti-β-tubulin III (Tuj1), monoclonal (1:500; ChemiconR, Temecula, CA, USA), rabbit IgG anti-glial fibrillary protein (anti-GFAP), polyclonal (1:500; ChemiconR), mouse IgG anti-ED1, monoclonal (1:200; ChemiconR), rabbit IgG anti-GAP43, polyclonal (1:800; ChemiconR), and anti-GFP Alexa 488-conjugated (1:200; Molecular Probes, Eugene, OR, USA). For fluorescence staining, secondary antibodies were Texas red or fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or anti-mouse antibodies (1:100; DAKO, Denmark).[16] Staining without secondary antibodies was investigated in each experiment. Images were obtained with the confocal laser-scanning microscope (GB-200; Olympus, Tokyo, Japan).[16] For measurement of cavity volume, five rats in each group at 6 weeks after SCI were used. Sagittal cryostat sections were stained with H&E and examined with a light microscope.[24,25] The area of cavity within the SC was measured with the image processing and analysis program NIH Image R 1.61 on the section of the largest cavity formation in each SC. Values of $P < 0.05$ were considered statistically significant by Bonferroni post hoc analysis for multiple group comparisons.

Results

Neurological recovery

After induction, neurosphere formation from BMSCs was observed similar to a previous report from our laboratory.[16] Group A rats (transplantation of BMSCs-NS) were treated with these neurospheres. The BBB locomotor scores were assessed at 3, 10, 14, 21, 28, 35, and 42 days after SCI in all ten rats from the three groups and in the sham operation group. All rats had a score of 21 before SCI. Rats that had the sham laminectomy operation had a constant score of 21. Three days after SCI, all rats had a score of 0. The BBB score for group C showed almost no recovery and remained less than 3 for 42 days (Fig. 1). The scores for groups A and B showed gradual recovery 1 week after transplantation and remarkable recovery 2 weeks after transplantation (Fig. 1). The rate of improvement for group A was significantly greater than the rate for group B ($P < 0.005$) and C ($P < 0.0001$; two-way repeated measures of ANOVA). For absolute score attained, group A showed significant improvement compared with group B ($P < 0.001$) and C ($P < 0.0001$) for all time points from 21 days after SCI (ANOVA, Bonferroni post hoc analysis) (see Fig. 1).

The average mean of the final recovery scores in group A was 7.8 ± 1.42, indicating weight support with hindlimbs. In group B it was 5.7 ± 0.90, indicating active movement of three joints of the hindlimbs. The recovery score in group C was 1.4 ± 0.29, indicating slight movement of one or two joints of the lower limbs (see Fig. 1).[21] The highest scores in group A were 14, 13, and 12, indicating occasional coordination for weight support enabling steps with forelimbs and hindlimbs. The lowest scores for two rats in group A indicated lack of neurological recovery. Rats with good neurological recovery in group B could occasionally step with weight support; however, six rats could only step forward without weight support. MEPs were recorded at 3, 14, 21, 28, and 42 days after SCI in each of the ten rats of all three groups and in two naïve rats. MEP signals could be recorded in the two naïve rats at all times. After SCI, the MEPs for all the rats in groups A–C disappeared (Fig. 2), and the animals could no longer walk. MEPs could not be detected in all group C rats at any time. MEPs were detected 5 weeks after transplantation in a few group B rats, but the latency was more than doubled (20 ms), and amplitude was significantly decreased (Fig. 2). Two weeks after transplantation, no clear MEPs were recorded in group A rats. However 5 weeks after transplantation, MEPs similar to those of normal rats were recorded in two animals from group A (Fig. 2; cases 1 and 2). A MEP wave pattern with decreased amplitude was recorded in two rats (Fig. 2; case 3). These electrophysiological results correlated well with the behavioral results from each rats.

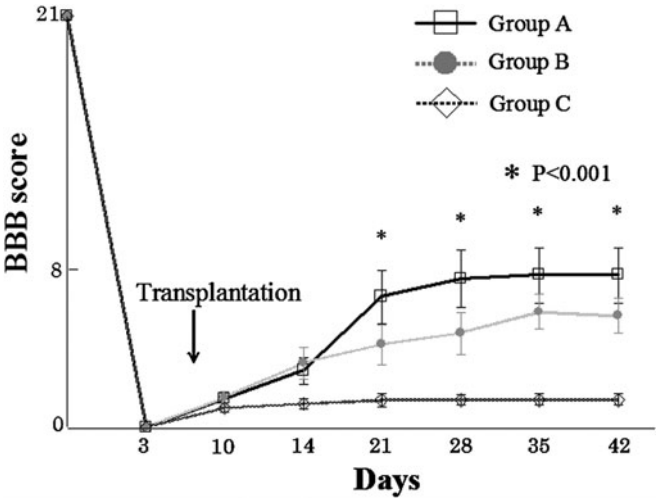

Fig. 1. BBB scores for the three groups. For absolute score attained, group A showed significant improvement compared with group B ($P < 0.001$) and C (*$P < 0.0001$) for all time points from 21 days after spinal cord injury (SCI). *Bars* ± SEM

Histology analysis

Five rats in each group were killed for H&E staining 42 days after SCI. H&E staining of parasagittal SC sections (50, 100, and 150 µm lateral from center) was followed by analysis by light microscopy in the SCI region of groups A–C in cavity size. Cavity size was 8.13 ± 0.927 mm^2 in group A, 11.36 ± 2.958 mm^2 in group B, and 19.35 ± 3.2 mm^2 in group C. Animals showed significant differences in cavity size between group A and group C ($P < 0.05$) (Fig. 5). The cavity size in group B tended to be smaller than that in group C ($P = 0.155$).

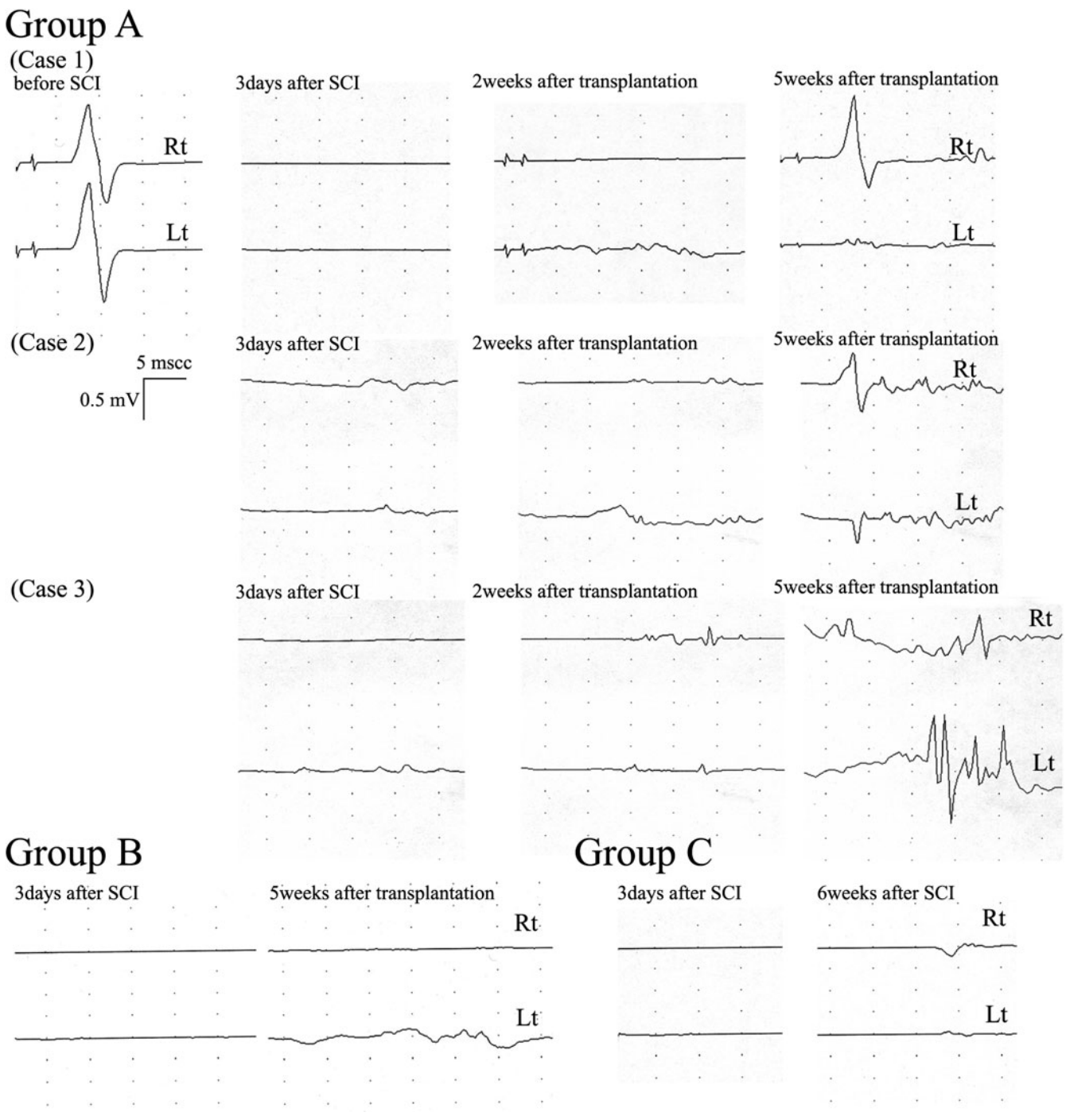

Fig. 2. Motor evoked potentials (MEPs) for the three groups. Five weeks after transplantation, MEPs similar to normal rats were recorded in two animals from group A (*case 1* and *case 2*). An MEP wave pattern with decreased amplitude was recorded in two rats (*case 3*). *SCI*, spinal cord injury

Immunohistochemistry with antibodies to Tuj1 (a neuron marker), GFAP (an astrocyte marker), and GFP (a donor cell) produced the following observations. Fourteen days after transplantation in group A, double-positive cells with GFP (green, donor cells) and each antibody stain (red, indicating neurons and astrocytes) were observed in the SCI region, and these cells migrated into the normal SC (Fig. 4A,B). In group B, GFP-positive cells were observed in the SC, but double-positive cells with neuronal markers were not observed (Fig. 3). Numerous GFP-positive donor cells were observed in SCs of group A but not group B 14 days after transplantation. However, 42 days after SCI, very few GFP-positive cells were observed in the SCs of group A. Two weeks after transplantation, the survival rate of transplanted cells was 0.0066% ± 0.0027% in group A and 0.0012% ± 0.0003% in group B. Five weeks after

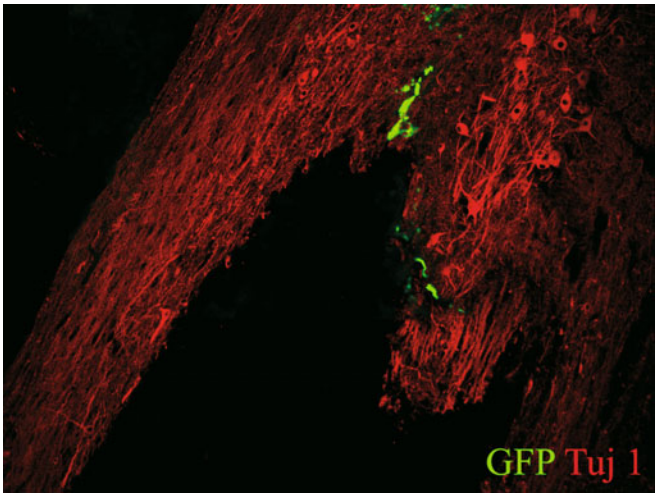

Fig. 3. Two weeks after transplantation, immunohistochemistry of transplanted bone marrow stromal cells (BMSCs) in group B within the spinal cord (SC) lesion. The host SC tissue was stained for Tuj1 (*red-labeled* cells and processes, indicating neuron and axon). Green fluorescent protein (GFP)-positive cells (*green-labeled* cells) were transplanted BMSCs. BMSCs were identified as green fluorescent cell assemblies of various sizes in the spinal cord 2 weeks after transplantation. Cavity volumes in group B appeared smaller than SC in group C because the host Tuj1-positive cells were preserved around the surviving BMSCs

transplantation, BMSCs were no longer identified in group B; however, a small number of BMSCs-NS cells (230 ± 128) survived in group A. Surviving cells expressed Tuj1 (neuron marker: 10.2% ± 5.4%) and GFAP (astrocyte marker: 54.3% ± 16.1%). Increased host GFAP-, Tuj1-, and ED1-positive cells were observed around the smaller cavity region of groups A and B relative to group C (Fig. 6). ED1-

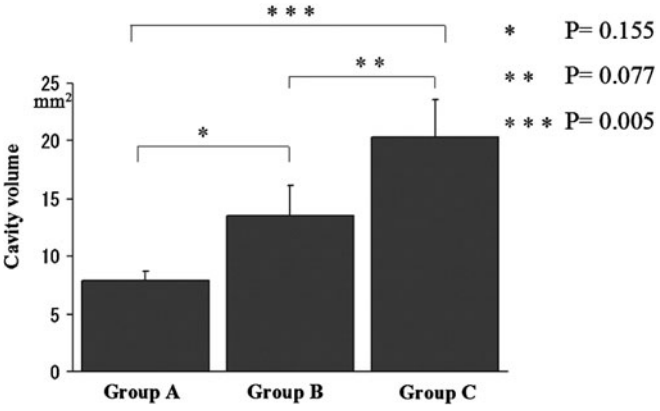

Fig. 5. Measurement of cavity volume. Six weeks after SCI, area of cavity within the spinal cord (SC) was measured and the section of the largest cavity formation in each SC was analyzed. Cavity size was 8.13 ± 0.927 mm^2 (group A), 11.36 ± 2.958 mm^2 (group B), and 19.35 ± 3.2 mm^2 (group C). Analysis of cavity size showed significant differences between group A and C ($P < 0.05$). *Bars* ± SEM

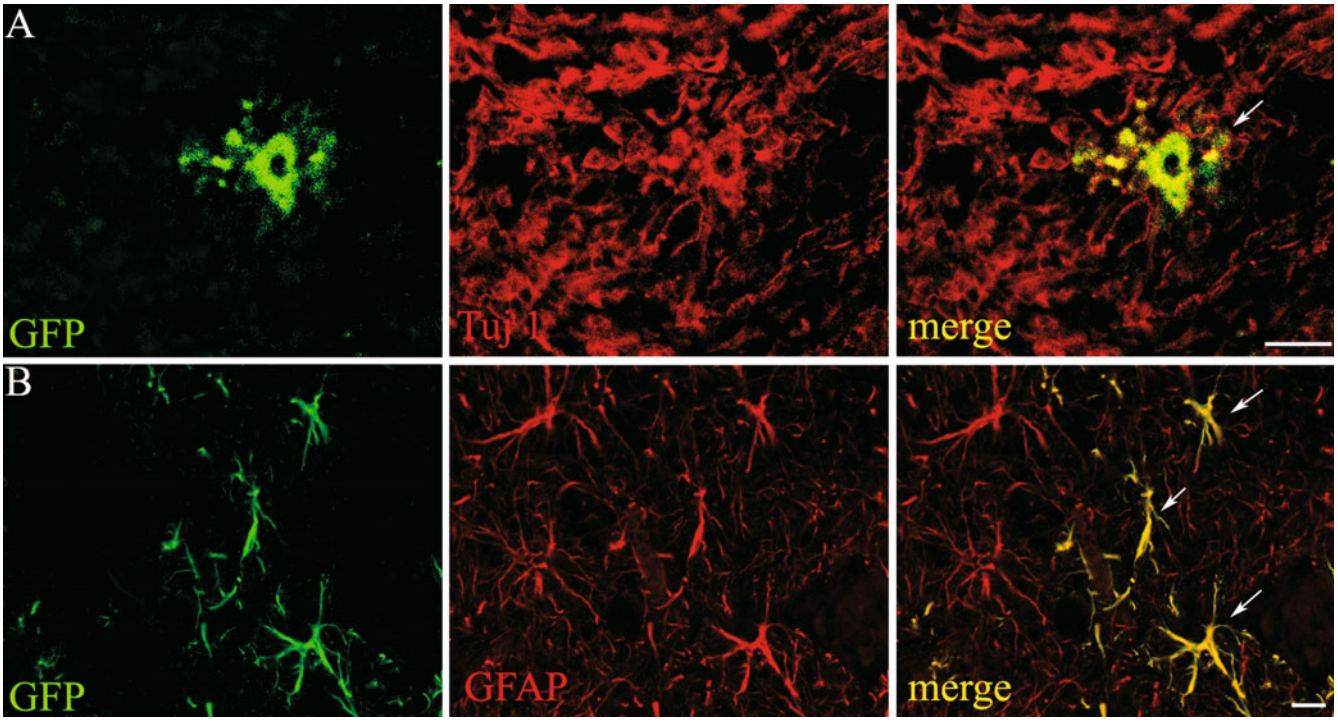

Fig. 4. Expression of specific lineage markers in BMSCs-NS 2 weeks after transplantation indicated by *GFP* (*green-labeled* cells, indicating transplanted and surviving BMSCs-NS) (**A**, **B**), *Tuj1* (indicating neurons), glial fibrillary protein (*GFAP*) (indicating astrocytes), and their combination (*merge*) in the spinal cord in group A (*arrows*). Tuj1 was labeled with a *red* marker in the *upper middle* panel. GFAP was also labeled with a *red* marker in the *lower middle* panel. Transplanted BMSCs-NS expressed GFP and Tuj1 or GFAP. Tuj1- and GFP-positive cells indicated that transplanted BMSCs-NS cells survived and functioned as neurons in the spinal cord. GFAP- and GFP-positive cells indicated that these cells performed as astrocytes. *Bars* 50 μm

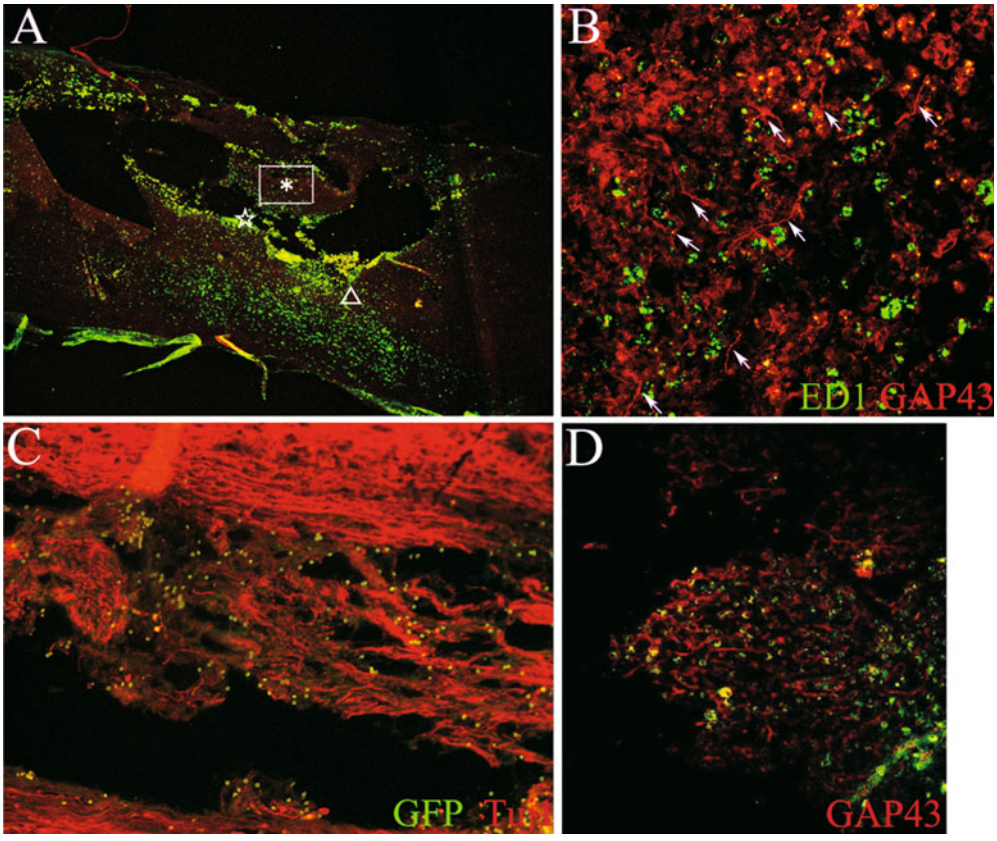

Fig. 6. Reaction of host SC at 5 weeks after BMSCs-NS transplantation. **A** Around the smaller cavity region in group A, increased host ED1 (*green-labeled* cells, indicating macrophage or microglia)-positive cells and fibroblasts were mainly observed (*triangle*). **B** In the region (shown by *boxed* * in **A**) of these increased cellular scaffolds, GAP43 (*red-labeled* fibers, indicating regenerated new axons; *arrows*) were mainly observed among ED1-positive cells and fibroblasts. **C** Besides the region (*) of the cavity, host Tuj1-positive cells and processes (indicating neurons and axons) were observed among some surviving BMSCs-NS cells (*green-labeled* cells). **D** Border area of injured and normal spinal cord; GAP43-positive fibers were increased and crossed the obstacle area

positive cells (indicating macrophages or microglia) and fibroblast cells were more numerous in the cavity region of group A than in group B (Fig. 6A). Host Tuj1-positive cells and processes (indicating neurons and axons) were observed among these cells. In addition, GAP43-positive cells, indicating regenerated new axons or preserved host axons, were observed among ED1-positive cells and fibrous tissues (Fig. 6B). Tuj1-positive fibers (indicating neurons and axons) were observed around the reduced cavity (Fig. 6C). GAP43-positive fibers were increased around the injured area and crossed the obstacle area (Fig. 6D).

Discussion

We demonstrated in the present study that transplantation of both the BMSCs and BMSCs-NS into SCs after compression injury promoted the neurological recovery of hindlimb motor function. Electrophysiological evaluation suggested clear regeneration of the long tract by cell transplantation in some animals. It would be preferable to transplant cells during acute-phase SCI; however, here the BMSCs and BMSCs-NS were transplanted 7 days after SCI because at least 1 week is needed to cultivate these cells for clinical use. Transplanted BMSCs and BMSCs-NS survived in the lesion of host SCs for 2 weeks after transplantation. Transplanted BMSCs-NS expressed some neuronal lineage markers, whereas BMSCs maintained their original phenotype. Few cells survived for 5 weeks after transplantation; however, the SCI regions were repopulated with host neuronal or inflammatory cells. In the BMSCs-NS group in particular, the transplanted cells mobilized not only neuronal cells but also the first processes of regeneration with inflammatory cells (macrophages or microglia) in the damaged region. We believe these reactions may be the first step in regeneration of the host SC, because in other tissues it has been shown that degenerated myelin or neurons must first be eliminated before regeneration can occur.[26,27]

The precise mechanism by which transplantation of NSCs or BMSCs promotes functional recovery after SCI is still unclear. It has been suggested that BMSCs-NS transplantation promotes regenerative potential within the host SCI. Transplanted BMSCs expressed no neuronal markers in the spinal cord. However, BMSC transplantation had an effect on the injured spinal cord. We think that the transplanted BMSCs survived for only a short time in the spinal cord and mainly function as a cellular scaffold in the acute phase. In addition, the present results suggest that BMSCs-NS survive in host SCs from the acute to subacute phase as neuronal phenotypes and play roles as temporal components of neural tissues until regeneration of the host SCs has begun. We think that these differences may be the reason for changes of functional recovery rate in groups A and B. The CNS has long been regarded as incapable of regeneration, but the recent discoveries of SCI research

have revealed that the CNS environment is globally permissive of regeneration.[28–31] Also, failure of regeneration may be the result of locally unfavorable conditions in the lesion site, based on findings that marked regeneration similar to normal projections can occur spontaneously.[28] This phenomenon is observed in the clear-cut SCI model, but it was suggested that regeneration occurred in SCs of some BMSCs-NS transplanted rats.[28]

Untreated SCIs have the potential to regenerate; however, cavity formation, neuronal necrosis, and increased apoptosis were finally observed.[16,32] Therefore, we believe it is important in the acute to subacute phase that neuronal replacement to promote and assist SC regeneration occurs to start the regeneration brought about by host cells. Subtle differences in the SC microenvironment provided by cellular scaffolding in these phases may have allowed good functional recovery to occur.[31,33] Because of the relatively short survival of transplanted cells, the BMSCs-NS were unlikely to have disturbed the initial process of SC regeneration.

Transplantation of NSCs for SCI was recently reported to induce recovery, but this procedure might present problems when applied for clinical use.[34–36] If many NSCs survive in SCs for a long time, transplanted cells will interfere with regeneration of host SCs because we cannot control abnormal proliferation of NSCs in the present technology.[12,15,36]

It has been proposed that BMSC transplantation for SCI promotes neurological recovery or decreases neuronal apoptosis.[7,10,13,34–39] However, the mechanism of action for SCI is still unclear. Previous studies suggest the main reasons for neuronal recovery were secretion of soluble growth factors or cell fusions.[37–39] BMSC transplantation in the present study caused rats with SCI to undergo good neurological recovery. It was well known that BMSCs secrete neurotrophic factors,[38] and it has been reported that nestin-positive BMSCs favor the astroglial lineage in neural progenitors and stem cells by releasing active bone morphogenetic protein (BMP)-4.[39]

Depending on the approach used to treat SCI, BMSCs-NS may be very useful in this procedure because BMSCs-NS have the advantages of both NSCs and BMSCs while excluding their separate disadvantages.

Conclusions

In conclusion, the efficacy and safety of BMSCs-NS implantation were demonstrated in a rat model of SCI. It was shown that engrafted cells acquired neural and glial phenotypes. This differentiation led to new axonal sprouting associated with improvements in SC function. Cellular treatment with BMSCs-NS is clinically relevant in patients with SCI. BMSCs-NS can be readily harvested from adult bone marrow, expanded in vitro, and readministered to the patient, thus avoiding immunosuppressive therapy. For these reasons, SCI treatment using BMSCs-NS might provide an alternative to NSCs derived from fetuses or embryonic stem cells.

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