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**Research Report**

# Intravenous administration of mesenchymal stem cells derived from bone marrow after contusive spinal cord injury improves functional outcome

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**ABSTRACT**

Transplantation of mesenchymal stem cells (MSCs) derived from bone marrow has been shown to improve functional outcome in spinal cord injury (SCI). Systemic delivery of MSCs results in therapeutic benefits in a number of experimental central nervous system disorders. In the present study we intravenously administered rat MSCs derived from bone marrow at various time points after induction of a severe contusive SCI in rat to study their therapeutic effects. MSCs were systemically delivered at varied time points (6 h to 28 days after SCI). The spinal cords were examined histologically 6 weeks after SCI. Stereological quantification was performed on the spinal cords to determine donor cell (MSCs transduced with the LacZ gene) density in the lesions. Light microscopic examination revealed that cavitation in the contused spinal cords was less in the MSC-treated rats. A limited number of cells derived from MSCs (LacZ<sup>+</sup>) in the injury site expressed neural or glial markers. Functional outcome measurements using the Basso–Beattie–Bresnahan (BBB) score were performed periodically up to 6 weeks post-SCI. Locomotor recovery improvement was greater in the MSC-treated groups than in sham controls with greatest improvement in the earlier post-contusion infusion times. The availability of autologous MSCs in large number and the potential for systemically delivering cells to target lesion areas without neurosurgical intervention suggests the potential utility of intravenous cell delivery as a prospective therapeutic approach in acute and subacute SCI.

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**1. Introduction**

Cell therapy is a promising experimental strategy to promote functional recovery after SCI. Local injection of Schwann cells

(Honmou et al., 1996; Martin et al., 1996), olfactory ensheathing cells (Raisman, 2007; Ramon-Cueto and Nieto-Sampedro, 1994; Sasaki et al., 2004, 2006), neural stem cells (Schultz, 2005; Tarasenko et al., 2007), bone marrow stromal cells

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(Chopp et al., 2000; Sasaki et al., 2009), and genetically-modified fibroblasts (Liu et al., 1999) into the spinal cord have been suggested to provide therapeutic benefits after SCI.

Mesenchymal stem cells (MSC) derived from bone marrow are multipotent stem cells present in adults, and in culture have the ability to differentiate into a variety of lineages including neurons and glia (Brazelton et al., 2000; Iihoshi et al., 2004; Kim et al., 2006; Sanchez-Ramos et al., 2000; Zhao et al., 2003). The systemic injection of mesenchymal stem cells (MSCs) prepared from adult bone marrow has therapeutic benefits after cerebral artery occlusion in rats (Honma et al., 2006; Horita et al., 2006; Iihoshi et al., 2004; Liu et al., 2006; Nomura et al., 2005; Omori et al., 2008; Onda et al., 2008; Toyama et al., 2009) and may have multiple therapeutic effects at various sites and times within the lesion as the cells respond to a particular pathological microenvironment. Cizkova et al. (2006) demonstrated improved functional outcome in a balloon compression rat SCI model, which induced relatively minor SCI, following intravenous infusion of human MSCs. Several hypotheses to account for MSC therapeutic benefits have been suggested, including neuroprotective effects from release or stimulation of growth factors and cytokines, the induction of neovascularization and axonal sprouting, and the replacement of damaged cells (Chopp et al., 2000; Hirouchi and Ukai, 2002; Kurozumi et al., 2004; Radtke et al., 2007).

In the present study, MSCs were intravenously delivered in a severe rat contusive SCI model at various times after injury to investigate whether systemic injections improve the therapeutic outcome after contusive SCI, and the relative

effectiveness of cell delivery at acute and subacute times. Histological examination, immunohistochemical and biochemical analysis, and behavioral studies were performed to evaluate the therapeutic potential of the MSCs. Our results indicate that systemic MSC delivery results in reduction in cavitation formation, increased BDNF levels at the injury site and improved functional outcome. Moreover, cell delivery at earlier time points was more effective than more delayed delivery. Thus, systemic delivery of MSCs in severe contusive spinal cord injury can reduce lesion size and enhance functional recovery following relatively acute delivery.

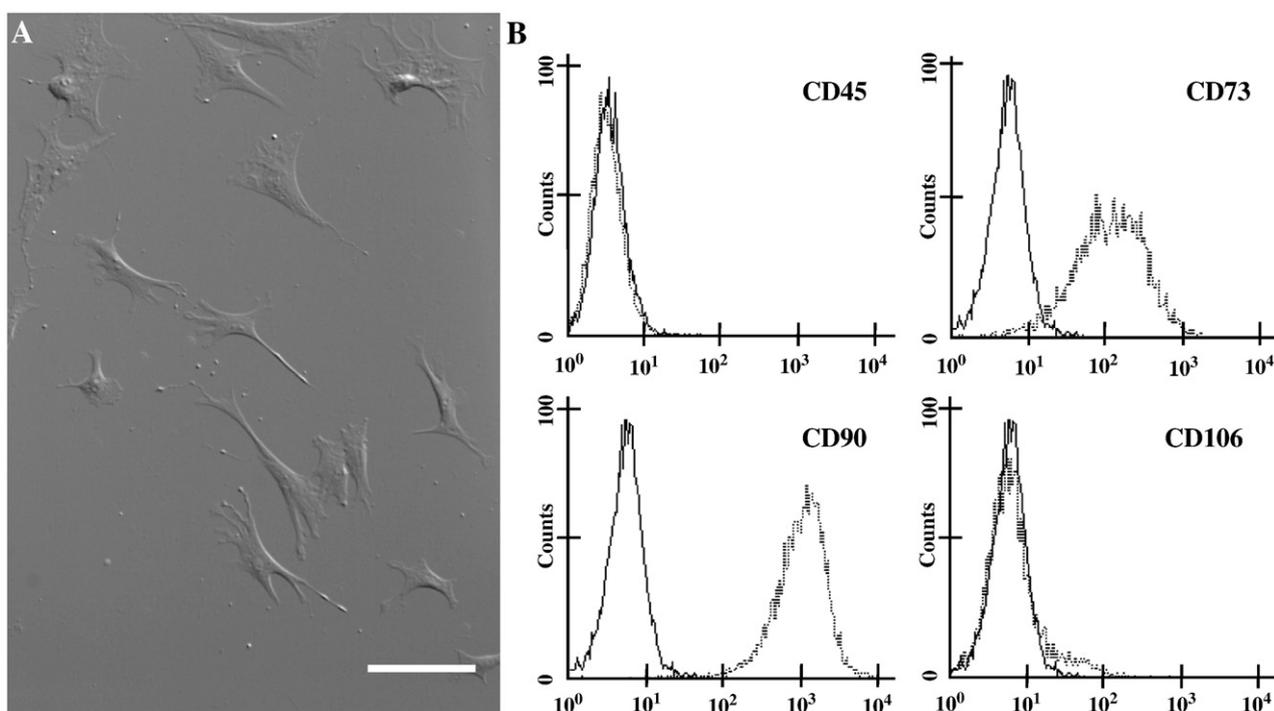
## 2. Results

### 2.1. Characteristics of rats MSCs

MSCs cultured as plastic adherent cells were maintained *in vitro*. The morphological features of the MSCs are shown in Fig. 1A. Characteristic flattened and spindle-shaped cells can be recognized. Flow cytometry analysis of the MSCs indicated a CD45 (-), CD73 (+), CD90 (+), and CD106 (-) cell surface phenotype (Fig. 1B), which is characteristic of MSCs and consistent with previous studies (Kim et al., 2006; Ukai et al., 2007).

### 2.2. Identification of donor cells *in vivo*

In a set of experiments immunohistochemical studies were carried out to identify LacZ-positive cells in the lesion in rats transplanted with LacZ-transfected MSCs ( $n=65$ ). LacZ-



**Fig. 1** – Phase-contrast photomicrograph of MSCs (A) in culture. Flow cytometric analysis of cultured MSCs (B) with CD45, CD73, CD90, and CD106 antibodies. Each panel indicates isotype-matched mouse IgG antibody control staining. Scale bar = 20  $\mu$ m.

transfected MSCs that had been intravenously administered were identified 5 days after transplantation. The LacZ-expressing MSCs were found primarily in and around the damaged site in the spinal cord (Fig. 2A–C). Although virtually no LacZ-positive cells were observed in the nondamaged areas of the spinal cord, a large number were observed in the contusion damaged spinal cord. The number of cells was greatest after early post-contusion injection times (MSC infusions at 6 h and 1 day) and attenuated with progressively delayed cell infusions. These results are summarized in Fig. 2D.

Immunohistochemical analysis demonstrated that some of the LacZ-positive donor cells (Fig. 3A-1 and B-1) expressed the neuronal marker NeuN (Fig. 3A-2, -3) or the astrocyte marker GFAP (Fig. 3B-2, -3). Merged images for LacZ and Neu and GFAP, respectively, are shown in Fig. 3A-3 and B-3. A relatively small number of the LacZ-positive donor cells expressed NeuN ( $10.0 \pm 0.8\%$ ,  $n=4$ ) or GFAP ( $9.6 \pm 3.6\%$ ,  $n=4$ ).

### 2.3. BDNF levels in vivo

BDNF levels in spinal cord tissues were measured by a sandwich ELISA 3 days after injection (medium or MSCs;  $n=7$  for each group) in the SCI rats. BDNF levels in the non-transplant (medium alone) rat brain were slightly increased compared to the normal spinal cord, but BDNF levels were

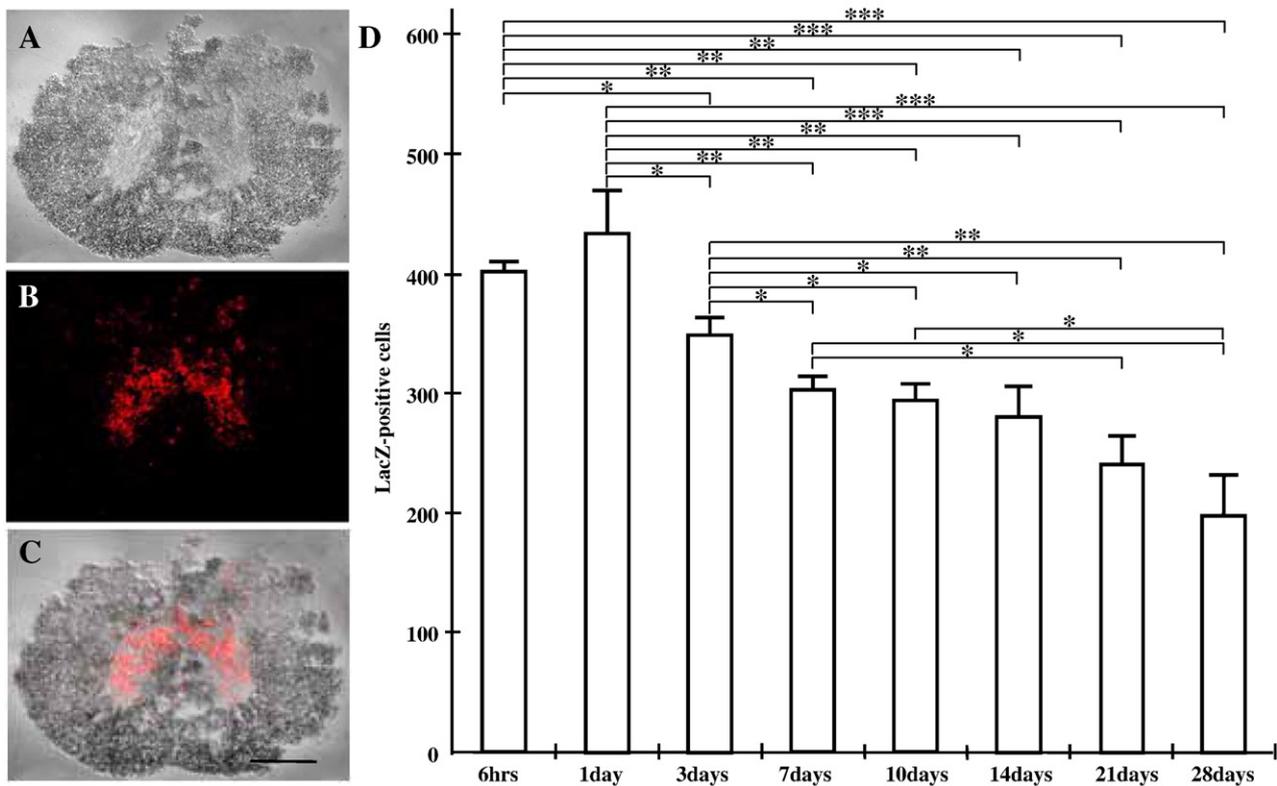
markedly increased in the spinal cord in the MSC-treated group (Fig. 4).

### 2.4. Histological determination of damaged lesion and lesion volume assessment

Spinal cords in all groups were stained with H-E 42 days after SCI (Fig. 5) ( $n=36$ ) and sample sections were obtained from the middle of the lesion. Necrotic cavity volumes in the MSC-treated groups (Groups 2–9) were significantly smaller than those in the sham control SCI group (medium alone). MSC infusions in the early treated groups showed less cavity formation. These results are summarized in Fig. 6.

### 2.5. Behavioral testing

All animals had near complete hind limb paraplegia immediately after SCI, and gradually recovered varying degrees of motor performance over the time course of 42 days ( $n=178$ ). The sham control group (Group 1; medium alone) reached Basso–Beattie–Bresnahan scores of 7–8 by 6 weeks. The rats of all the treated groups achieved higher scores than the sham control rats. Among the treated groups, the rats injected at earlier time points showed greater recovery. MSC infusions at 6 h, 1 and 3 days showed the greatest functional recovery. The



**Fig. 2** – Identification of donor cells in vivo. Intravenously administered LacZ-transfected MSCs accumulated in the SCI lesions. (A) Light photomicroscopic images of spinal cord. Confocal images (B) show the transplanted LacZ-transfected cells in panel A. A merged image for (A) and (B) is shown in C. A number of LacZ-positive cells in the lesions were calculated and these results are summarized in D. Scale bar = 500  $\mu\text{m}$ .

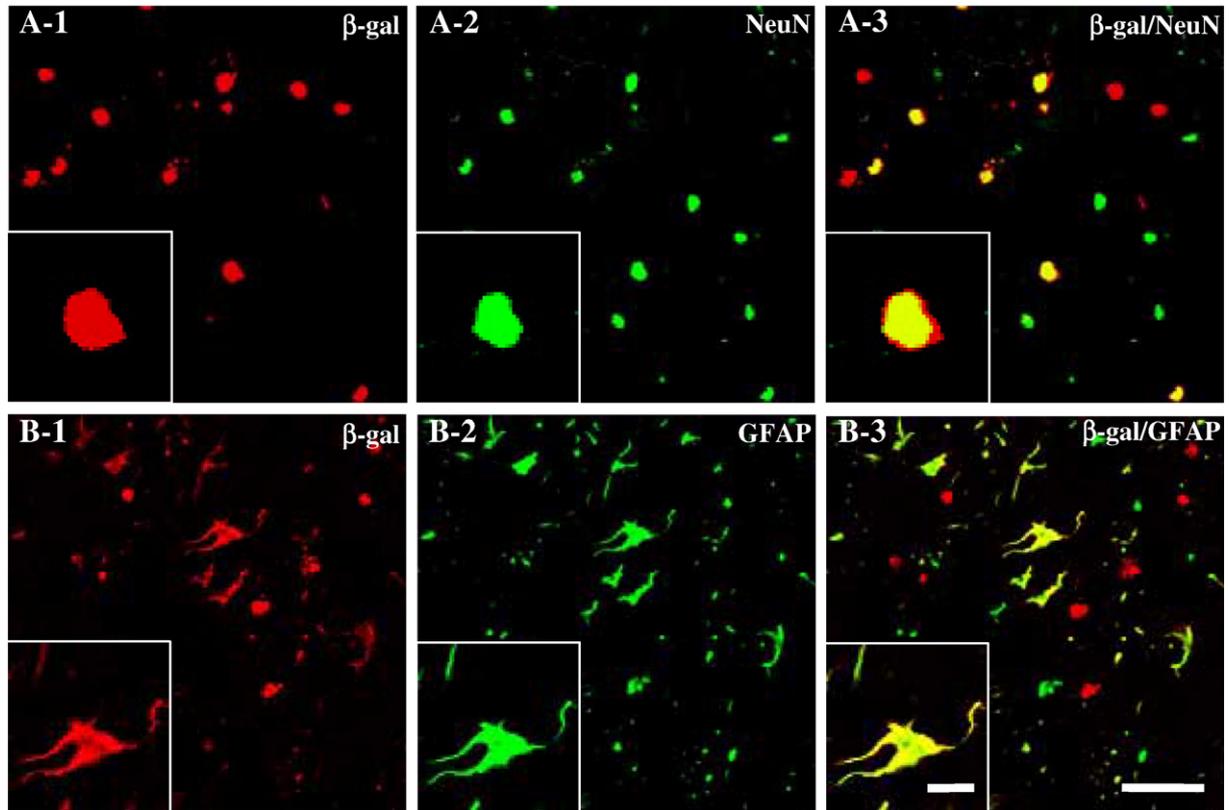


Fig. 3 – Confocal images show transplanted cells (LacZ in red; A-1 and B-1) immunostained with neural (NeuN in green; A-2) and astrocytic (GFAP in green; B-2) markers. Panels A-3 and B-3 confirm the co-labelling of LacZ/NeuN or LacZ/GFAP in the cells, respectively. Higher power confocal images are shown in insets. Scale bar=30  $\mu$ m and 10  $\mu$ m (inserts).

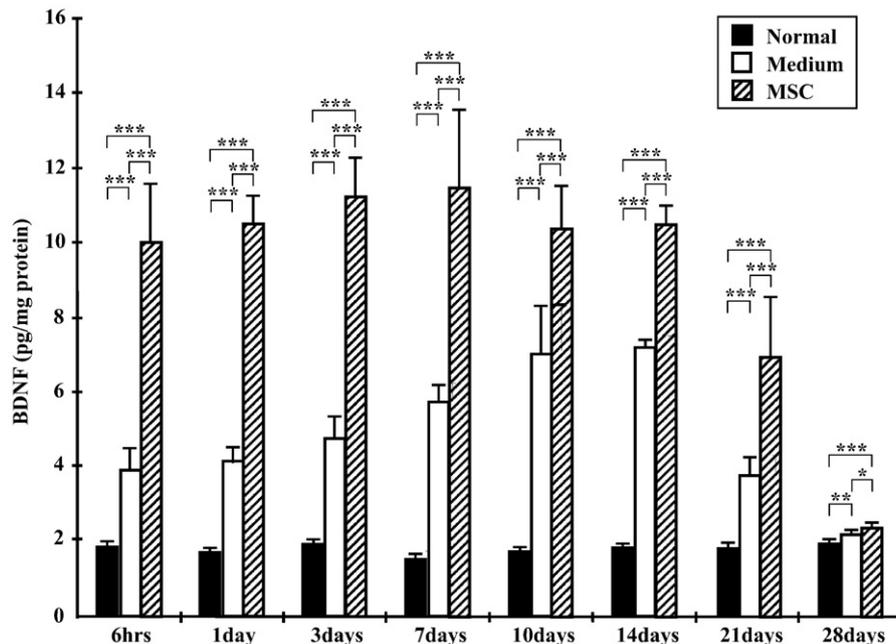
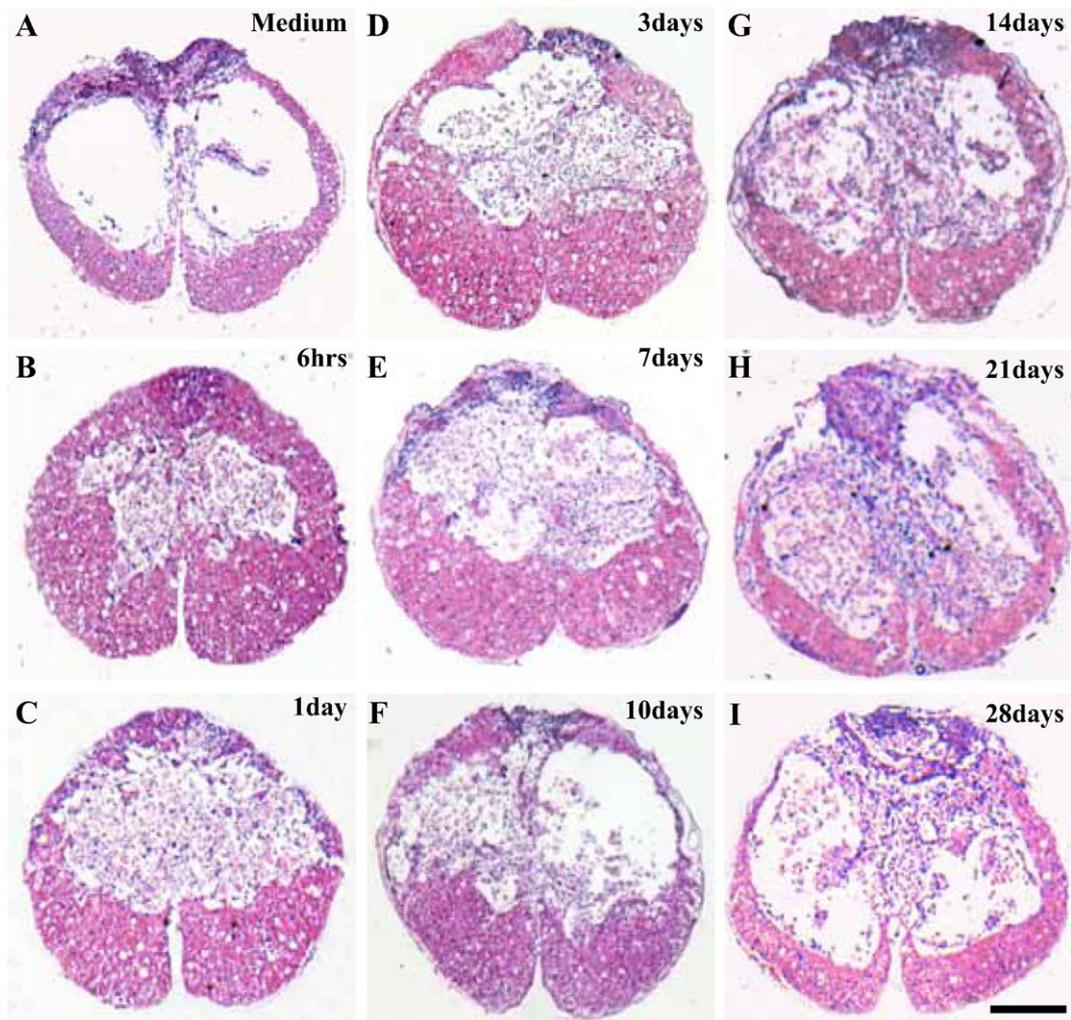


Fig. 4 – BDNF levels in the spinal cords were measured by a sandwich ELISA 3 days after injection. BDNF levels were significantly increased in the spinal cords in the MSC-treated group compared with the medium-injected group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Fig. 5 – Spinal cord slices stained with hematoxylin and eosin (H-E) to visualize the damaged lesions. The H-E-stained spinal cord slices from rats that were intravenously transplanted with MSCs at 6 h, 1, 3, 7, 10, 14, 21 and 28 days after SCI are shown in Panels B, C, D, E, F, G, H and I, respectively. A spinal cord slice from the nontreated SCI rats (medium alone) is shown for comparison (A). Scale bar=500  $\mu$ m.**

Basso–Beattie–Bresnahan scores for all nine groups at different time points are shown in Fig. 7.

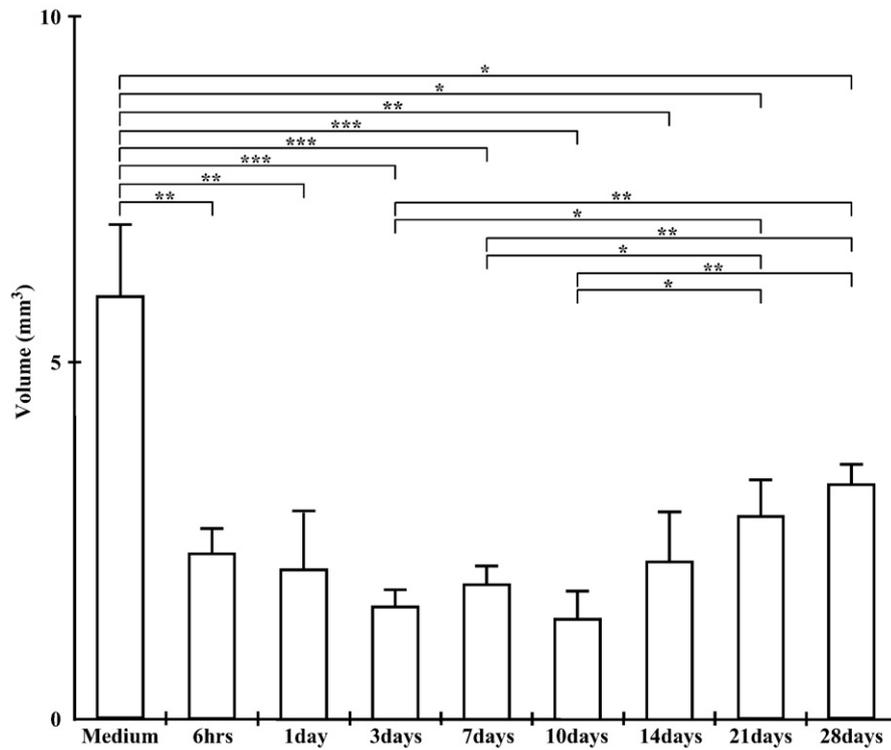
### 3. Discussion

In the present study, we demonstrate that intravenous administration of MSCs in a contusive SCI rats results in increased local BDNF levels, reduction in spinal cord lesion volume, and improved functional outcome. Immunohistochemical analysis of the tissue indicates that a relatively large number of LacZ labeled transplanted MSCs accumulated in the contusive injury site of spinal cord following systemic administration. Although MSC injections at early time points after injury were more effective than later time point injections, the later injections also showed beneficial functional effects. The precise “homings” mechanism of the infused cells to the lesion site is not known. One hypothesis is that disruption of the blood–spinal cord barrier by the trauma allows for selective cell entry from at the injury site. Previous

study suggests that the blood–spinal cord barrier may remain compromised for weeks after trauma (Popovich et al., 1996). This is consistent with our observation of therapeutic effects of MSCs delivered intravenously several weeks after SCI induction.

Several hypotheses to account for therapeutic effects of MSCs in CNS diseases including trauma and ischemia have been suggested. Replacement of damaged neurons has been suggested but is controversial and little data supports neurogenesis by transplanted cells as a major contributor to functional recovery. Current thinking is that neuroprotection (Chen et al., 2003; Honma et al., 2006; Horita et al., 2006; Iihoshi et al., 2004; Liu et al., 2006; Nomura et al., 2005), angiogenesis (Krupinski et al., 1994; Onda et al., 2008; Toyama et al., 2009; Ukai et al., 2007; Ward and Lamanna, 2004), and axonal sprouting (Liu et al., 2007; Sasaki et al., 2009) are the primary mechanisms.

The neurotrophic factor BDNF exhibits anti-apoptotic effects to promote cell survival after cerebral infarction (Hirouchi and Ukai, 2002; Koh et al., 1995). BDNF increases in



**Fig. 6 – Cavitation volumes at 42 days post SCI surgery are plotted for medium alone injection and for MSCs delivered at various times post-injury. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .**

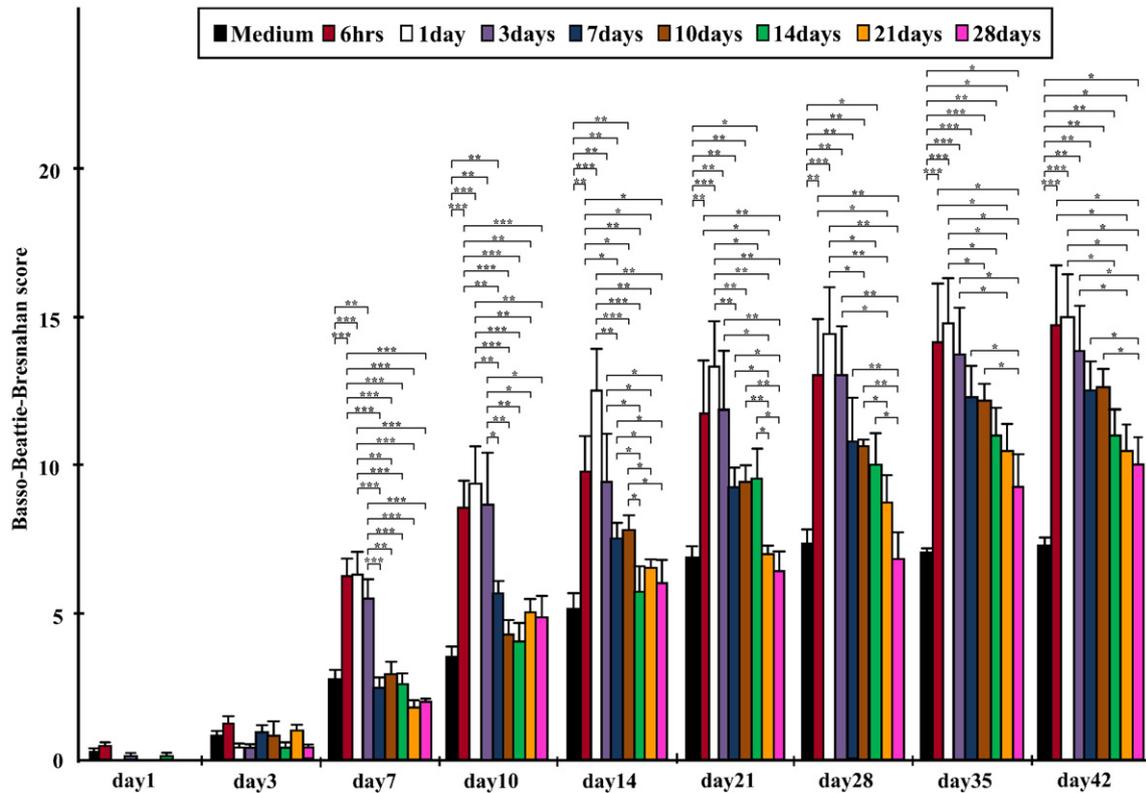
the ischemic brain following human MSC (hMSC) transplantation (Nomura et al., 2005). Transplantation of BDNF gene-modified hMSCs into a rat middle cerebral artery occlusion model results in better outcome as compared to nonmodified hMSCs (Kurozumi et al., 2004; Nomura et al., 2005). A transient non-injurious interval of spinal cord ischemia also leads to a significant increase in spinal BDNF expression, and protects spinal neurons against ischemia damage (Tokumine et al., 2003). BDNF delivery is protective to injured transected corticospinal tract neurons and can improve functional outcome after SCI (Jakeman et al., 1998; Kim and Jahng, 2004; Kwon et al., 2007; Namiki et al., 2000; Sasaki et al., 2009). Transplantation of olfactory ensheathing cells into dorsal hemisectioned spinal cord results in improved functional outcome and elevation in BDNF levels (Sasaki et al., 2006). It is not clear whether the transplanted cells account for the elevated levels of neurotrophin or if the cells have an inductive effect on host tissue. We recently reported that direct injection of human MSCs genetically modified to express BDNF was associated with increased axonal sprouting and improved functional outcome (Sasaki et al., 2009). In the present study, we demonstrate that BDNF increases in the SCI lesion following intravenous injection of MSCs. Thus, a neuroprotective mechanism through BDNF may be a contributing factor associated with the therapeutic benefits of MSC delivery for SCI.

One histological characteristic in the rat spinal cord after SCI is cavity formation, which is related to behavioral outcome (Basso et al., 1995, 2002). Our histological examination showed that early systemic administration of MSCs significantly reduced the cystic cavitation compared to the sham control

group, which is consistent with previous studies (Cizkova et al., 2006; Gu et al., 2009; Parr et al., 2008). Functional recovery was improved for all MSC-treated groups compared to the sham control group, but the greatest improvement in functional outcome was in the early treatment groups (6 h to 14 days). This functional improvement was maintained throughout the 42-day duration of the study indicating the stability of the functional improvement. Thus, the MSCs may have a neuroprotective effect whereby cavitation reduction leads to greater sparing of spinal cord tissue.

Increased sprouting of corticospinal tract and raphespinal serotonergic projections has been reported after direct injection of MSCs into a dorsal hemisection SCI model (Sasaki et al., 2009). An increased number of serotonergic fibers was observed in spinal gray matter including the ventral horn at and below the level of the lesion, indicating increased innervation in the terminal regions of a descending projection important for locomotion (Sasaki et al., 2009). While we did not investigate axonal sprouting in the present study, future studies should be carried out to investigate this potential mechanism.

Another possible mechanism is that the transplanted MSCs integrate into tissue and replace damaged cells, or improve neural regeneration (Chopp et al., 2000). MSCs have the capability to differentiate into cells with neural phenotypes (Lu et al., 2004; Neuhuber et al., 2004; Sanchez-Ramos et al., 2000; Woodbury et al., 2000) and transplanted MSCs can differentiate into neural lineage following SCI (Chopp et al., 2000; Lee et al., 2007). Our immunohistochemical data suggest that the transplanted LacZ-positive MSCs were predominantly located within and near areas of severe damage. Some of the



**Fig. 7 – Behavioral analysis of locomotor function.** Open-field locomotor scores, Basso–Beattie–Bresnahan score, for each groups tested at 1, 3, 7, 10, 14, 21, 28, 35, and 42 days after SCI. All animals showed a gradual improvement in hindlimb locomotor function during the 6-week recovery period. Statistical analysis indicated that the Basso–Beattie–Bresnahan scores in the MSC transplantation groups were significantly higher than those in the control group (medium alone). The greatest improvement was in rats injected with MSCs at 6 h, 1 and 3 days. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

LacZ-positive MSCs showed NeuN-positivity (about 10 %) or GFAP-positivity (about 9.6 %). They were minimally observed in the spinal cord areas away from the damaged area, suggesting that intravenously-injected MSCs were preferentially distributed to damaged areas of the spinal cord. The MSCs could also have an inductive effect on neurogenesis and angiogenesis which could contribute to tissue preservation and repair. Implantation of MSC-derived cells has been reported to elicit de novo neurogenesis and functional recovery in a nonhuman primate model of spinal cord injury (Deng et al., 2006).

Intravenous injection has advantages in that it is minimally invasive as compared to intra-spinal injection, larger number of cells can potentially reach a large area of SCI, and the risk of intra-spinal hemorrhage is minimal. The results of the present study demonstrate that intravenous infusion of MSCs in SCI rats resulted in an improved outcome, which is consistent with previous studies showing the therapeutic benefits of MSCs in experimental cerebral ischemic models (Honma et al., 2006; Horita et al., 2006; Liu et al., 2006; Nomura et al., 2005; Onda et al., 2008; Ukai et al., 2007; Zheng et al., 2010). Autologous human MSCs have been delivered to stroke patients in Phase 1 clinical studies (Bang et al., 2005; Honmou et al., 2008) and to date no adverse effects have been reported. As our understanding of basic mechanisms of therapeutic effects by MSCs in experimental studies, clinical studies on SCI

patients to determine not only safety but also efficacy will be important.

## 4. Experimental procedures

### 4.1. Preparation of mesenchymal stem cell prepared from rat bone marrow

The use of animals in this study was approved by the Animal Care and Use Committee of Sapporo Medical University and all procedures were carried out in accordance with institutional guidelines. Adult Sprague–Dawley rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg, i.p.). The femoral bone was taken out, and both edges of the femoral bone were cut. Bone marrow was aspirated with an 18-gauge needle, diluted to 25 ml with Dulbecco's modified Eagle's medium (DMEM) (SIGMA, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GibcoBRL, Grand Island, NY), 2 mM L-glutamine (SIGMA), 100 U/ml penicillin, 0.1 mg/ml streptomycin (SIGMA), was plated on 150 mm Tissue Culture Dish (IWAKI, Tokyo, Japan), and incubated in a humidified atmosphere of 5 % CO<sub>2</sub> at 37 °C for 3 days. MSCs, when selected by plastic adhesion, require the elimination of nonadherent cells by replacing the medium 48 h after cell seeding. When cultures almost reached confluence, the adherent cells were

detached with trypsin-EDTA solution (SIGMA) and subcultured at  $1 \times 10^4$  cells/ml. Some of cultured cells were rinsed in PBS for three times and fixed for 10 min with a fixative solution containing 4 % paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4, at room temperature. Phase-contrast photomicrographs were obtained using a Zeiss microscope.

#### 4.2. Phenotypic characterization of the MSCs

Flow cytometric analysis of primary MSCs were performed as previously described (Honma et al., 2006; Liu et al., 2006). Briefly, cell suspensions were washed twice with PBS containing 0.1% bovine serum albumin (BSA). For direct assays, aliquots of cells at a concentration of  $1 \times 10^6$  cells per milliliter were immunolabeled at 4 °C for 30 min with the following antibodies: FITC-conjugated CD45, PE-conjugated CD73, PE-conjugated CD90, and PE-conjugated CD106 (Becton Dickinson, Franklin Lakes, NJ, USA). As an isotype-matched control, mouse immunoglobulin G1-FITC (Becton Dickinson) or mouse immunoglobulin G1-PE (Becton Dickinson) was used. Labeled cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson) with the use of CellQuest software. Dead cells were gated out with forward- versus side-scatter window and propidium iodide staining.

#### 4.3. Adenoviral vectors

In some experiments, Adex1CALacZ adenovirus was used to transduce the LacZ gene into the MSCs. Details of the construction of the recombinant adenoviruses were described elsewhere (Kurozumi et al., 2004; Takahashi et al., 2003; Yamauchi et al., 2003). Adenovirus-mediated gene transfection was performed as previously described (Kurozumi et al., 2004; Takahashi et al., 2003). Briefly, the cells were seeded at a density of  $2 \times 10^6$  cells per 15 cm plate. MSCs were exposed to the infectious viral particles in 10 ml DMEM at 37 °C medium for 60 min; cells were infected with AxCALacZ-F/RGD at an MOI of  $3.0 \times 10^3$  opu/cell. The medium was then removed, and the cells washed once with DMEM and then recultured with normal medium for 24 h, after which transplantation was performed.

#### 4.4. Spinal cord injury model

Adult Sprague–Dawley rats weighing 250 to 300 g were anesthetized with an intraperitoneal (i.p.) injection of ketamine (75 mg/kg) and xylazine (10 mg/kg), and were subjected to contusion injury in the spinal cord (SCI) using the NYU weight drop impactor as described previously (Constantini and Young, 1994). Contusion injury was induced using a 10 g weight dropped at 55 mm onto Th9–10 spinal cord exposed by laminectomy.

#### 4.5. Transplantation procedures

Experiments consisted of nine groups. In group 1 (sham control), rats received medium alone injected i.v. after SCI. Rats were given MSCs ( $1.0 \times 10^6$ ) in 0.5 ml total fluid volume (DMEM) injected i.v. (through the femoral vein) 6 h after SCI

(Group 2), 1 day after (Group 3), 3 days after (Group 4), 7 days after (Group 5), 10 days after (Group 6), 14 days after (Group 7), 21 days after (Group 8), and 28 days after (Group 9), respectively. All rats were given daily injections with cyclosporine (10 mg/kg; i.p.) beginning 1 day before surgery.

#### 4.6. Immunohistochemical analysis

One week after transplantation, analysis of the transplanted cells in vivo was performed using laser scanning confocal microscopy. Spinal cord of the deeply anesthetized rats were removed, fixed in 4 % paraformaldehyde in phosphate-buffer, dehydrated with 30 % sucrose in 0.1 M PBS for overnight, and frozen in powdered dry ice. Transverse section was obtained at the level of spinal cord injury (Th9–10), and cryostat sections (10  $\mu$ m) were processed for immunohistochemistry. In order to identify the transplanted MSCs, immuno-labeling studies were performed with the use of the primary antibody to beta-galactosidase (monoclonal mouse anti-beta-galactosidase antibody, Promega, Madison, WI, USA). The primary antibody was visualized by using Alexa Fluor 594-labeled reagent (Zenon mouse IgG labeling kits, Invitrogen, Carlsbad, CA, USA). A 561-nm laser line from a Diode Pumping Solid State laser was used to excite the Alexa Fluor 594 (red). The numbers of LacZ-positive cells were counted in whole spinal cord slices at the middle of SCI lesions (four section per rat, and five separate rats for each group).

To identify the cell type derived from the transplanted cells, double-labeling studies were performed with the use of primary antibodies to neurons (monoclonal mouse anti-neuronal nuclei (NeuN) antibody, Millipore, Billerica, MA, USA) and astrocytes (monoclonal mouse anti-gial fibrillary acidic protein (anti-GFAP) antibody, Sigma, St. Louis, MO, USA). The primary antibodies were visualized by using Alexa Fluor 488-labeled reagent (Zenon mouse IgG labeling kits, Invitrogen, Carlsbad, CA, USA). To excite the Alexa 488 Fluor (green), a 488-nm laser line generated by an argon laser was used. Confocal images were obtained using a Zeiss laser scanning confocal microscope and Zeiss software.

#### 4.7. Detection of BDNF in vivo

Three days after injection (medium or MSCs), rats were deeply anesthetized with ketamine and xylazine. Spinal tissues were dissected on ice and were stored at  $-80$  °C until use. Subsequently, each tissue sample was suspended in an equal weight of homogenate buffer (3 ml; 137 mM NaCl, 20 mM Tris, 1% NP40, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 0.5 mM sodium vanadate) and homogenized with an ULTRAS homogenizer VP-5 S (TAITEC, Saitama, Japan). The homogenate was centrifuged (10,000 g) for 15 min at 4 °C, and the supernatant (2.7 ml) collected for analysis. BDNF ELISA kits (Promega, Madison, WI, USA) were used to quantify the concentration of BDNF in each of the samples.

#### 4.8. Hematoxylin and eosin (H–E) staining and estimation of damaged tissue volume

Forty-two days after SCI, rats were anaesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde

in PBS. Spinal cord were harvested and immersed in 4% paraformaldehyde in PBS for 2 days prior to paraffin embedding. Transverse sections (3  $\mu\text{m}$ ) were cut, and were counterstained with hematoxylin and eosin. For injured volume analysis, the cross-sectional area of injury in each 300  $\mu\text{m}$  spinal slices was examined with a dissection microscope and was measured using image analysis software, NIH image. The total lesion volume for each spinal cord was estimated by summation of the target area of SCI slices.

#### 4.9. Neurological outcome

The neurological outcome was studied over the time course of 6 weeks after SCI. Hindlimb motor function of the experimental rats were evaluated by Basso, Beattie, and Bresnahan locomotor rating scale (BBB) (Basso et al., 1995) 1, 3, 7, 10, 14, 21, 28, 35, and 42 days after SCI. The Basso–Beattie–Bresnahan score consists of combinations of hindlimb movements, trunk position and stability, stepping, coordination, paw placement, toe clearance, and tail position. In this study, the assessment of hind-limb motor function was strictly based on the objective criteria.

#### 4.10. Statistical analysis

Data are presented as mean values  $\pm$  SEM, and were statistically analyzed. Differences among groups were assessed by ANOVA with Scheffes *post hoc* test to identify individual group differences. Differences were deemed statistically significant at  $p < 0.05$ .

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