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Human mesenchymal stem cell transplantation promotes functional recovery following acute spinal cord injury in rats

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Abstract. Many attempts have been made in animals to produce cellular regeneration in the spinal cord using a variety of transplanted cell types. The present study was to investigate whether transplantation of human mesenchymal stem cells (hMSCs) into the spinal cord after contusion injury promotes a functional outcome. Spinal cord injury (SCI) was induced using an NYU impactor and hMSCs were transplanted 1 week after SCI. Behavioral testing was performed weekly for 2 months. Somatosensory (SSEPs) and motor evoked potentials (MEPs) were recorded to determine functional recovery. Hindlimb performance was modestly improved in the transplanted group based on BBB scaling and pain tests. SSEP latencies in the transplanted group were significantly shorter than in the media-treated group. Pathologically, LacZ and hTau positive cells were located at the injury and adjacent sites. The data indicate improvement in functional outcome in animals treated with hMSC transplantation compared to media-treated animals.

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Key words: spinal cord injury, human mesenchymal stem cell, transplantation, behavioral test, electrophysiology

INTRODUCTION

Functional deficits following spinal cord injury (SCI) result from damage to axons, loss of neurons and glia, and demyelination. Recent studies indicate that the adult mammalian central nervous system is responsive to certain molecules that regulate neuronal recovery and axonal growth after injury, including neurotrophic factors (Tuszynski and Kordower 1999), substrate molecules (Davies et al. 1999), and neutralizers to inhibitory molecules associated with myelin and the extracellular matrix (Morgenstern et al. 2002, Qiu et al. 2002, Schwab and Bartholdi, 1996). Several therapeutic strategies have been developed to manipulate these molecules in an attempt to promote axonal growth and replace lost neurons after SCI.

Cell transplantation to repair SCI is an active area of research with the goal of reducing functional deficit (Falci et al. 1997, Lee et al. 2005, McDonald et al. 1999). The ideal "transplantable cells" should be easily accessible, rapidly expandable in culture, immunologically inert, capable of long-term survival and integration in the host tissue, and amenable to stable transfection and expression of exogenous genes (Azizi et al. 1998). Mesenchymal stem cells (MSCs) are a source of stem-like and progenitor cells. Following transplantation into host tissue, MSCs show the capacity to differentiate into neurons (Li et al. 2001, Lu et al. 2000, 2001, Mahmood et al. 2001) and astrocytes (Azizi et al. 1998, Kopen et al. 1999). MSCs have been tested as therapeutic agents for the repair of brain injury (Li et al. 2001). Recent studies have reported that MSCs promote partial functional recovery after grafting to SCI sites (Chopp et al. 2000, Hofstetter et al. 2002), although mechanisms underlying this recovery have not been defined. Grafted MSCs survived in spinal cord tissue, forming cell bridges within the traumatic centromedullary cavity. In this tissue, cells expressing neuronal and astroglial markers were seen, together with a marked ependymal proliferation, showing nestin-positivity (Zurita and Vaquero 2004). Nonetheless, applications of MSCs to nerve regeneration are still relatively recent and could develop into clinical studies in the near future. The goal of this study, therefore, was to investigate whether the transplantation of human MSCs into the spinal cord after contusion injury promotes a functional outcome, as measured by behavioral tests, electrophysiological tests, and immunostaining.

METHODS

Subjects

Male Sprague-Dawley rats (Daehan Biolink, Chungbuk, Korea) weighing 300–350 g at the time of surgery, were used. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine.

Surgery

Spinal cord contusion surgeries were performed under pentobarbital (50–70 mg/kg) anesthesia and prophylactic administration of atropine sulfate (0.8 mg/kg). The T9 cord was contused with a New York University (NYU) weight-drop device in which a 10 g weight impact rod was dropped from a height of 25 mm to produce a moderately contused SCI model. Prophylactic gentamycin sulfate (1 mg/kg) was regularly administered for a week.

Cell culture

MSCs were cultured from the iliac crest of six normal individuals aged 10–15 years old and undergoing bone marrow harvests for future allogeneic transplantation. Permission to use human MSC was granted by Ajou University Health Science Center's Ethical Committee. The experiments were conducted with the understanding and written consent of each subject. Approximately 5 ml of bone marrow aspirates were centrifuged through a density gradient (Histopaque; 1.077 g/ml; Sigma, Saint Louis, Missouri, USA) for 30 min at 400 × g. Cells were plated in 100-mm dishes (1–2 × 10^7 nucleated cells per dish) containing DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, CA, USA). After 6-hour incubation, non-adherent cells were removed and adherent cells were washed once with fresh media. Cells were then continuously cultured for 1–4 weeks with fresh medium with and without 10 ng/ml basic fibroblast growth factor (bFGF, Dong-A Pharmaceutical Co., Yongin, South Korea) every 2 days. Cells were used in the experiments after three or four passages. A retroviral vector encoding β-galactosidase was prepared by transfecting a lacZ expressing retroviral plasmid, pMSCV-puro/lacZ (Clontech, Palo Alto, CA, USA) into a packaging cell

line, PA317. Virus was obtained from the medium, diluted with growth medium containing polybrene (Sigma, St. Louis, MO) to a final concentration of 8 μg/ml, and added to hMSCs (MOI = 100) that were plated at approximately 3 000 cells/cm^2 and maintained in growth medium overnight. For repetitive transduction, cells were subjected to the same procedure twice as described above on the 2nd and 3rd day.

Cell transplantation

For transplantation, rats were assigned, without bias, to the media-treated group or the hMSC transplantation group. Seven days after injury, rats were anesthetized with halothane (1:2, N_2O:O_2). Using a capillary glass tube, 5 μl cultured hMSCs (1×10^5/μl) were transplanted to the epicenter of the injury. The media-treated group received 5 μl of culture medium injected to the epicenter of the injury. In order to suppress the immune system cyclosporine A (1 mg/100 g, i.p.) was injected daily from 2 days prior to the transplant until the completion of the experiment.

Behavioral tests

Behavioral tests were performed to measure the functional recovery of the hindlimbs. Twenty-nine rats were tested by BBB scaling (Basso et al. 1996). Briefly, rats were gently adapted to the open field. Once a rat walked continuously in the open field, two examiners evaluated locomotor activities using the BBB locomotor rating scale. Postoperative (p.o.) open field testing occurred at least once a week from Day 1 p.o. to 9 weeks p.o. for all animals.

Pain behavioral signs representing mechanical allodynia were monitored with the rats placed on a metal mesh floor under a transparent acrylic box. Rats were allowed to adapt for about 15 min or until explorative behavior ceased. Responses to mechanical stimuli were tested with a calibrated von Frey filament. The 50% withdrawal threshold was determined using the up-down method (Chaplan et al. 1994). A series of eleven von Frey filaments with approximately equal logarithmic incremental (0.19) von Frey values (3.38, 3.56, 3.75, 3.93, 4.12, 4.31, 4.49, 4.68, 4.86, 5.05, and 5.24) were used to determine the threshold stiffness required for 50% paw withdrawal. Since von Frey values are logarithmically related to gram (g) values (VF = log (10 000 × g)), these chosen von Frey numbers are equivalent to 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10, and 12 in gram value, respectively. Starting with filament 4.31, von Frey filaments were applied perpendicularly to the ventral surface of the paw. Interpolation of the 50% threshold was carried out according to the method of Dixon (Dixon 1980).

Electrophysiological tests

For the electrophysiology experiments, rats were anesthetized with pentobarbital sodium (Entovar®, 40 mg/kg) and atropine sulfate (0.8 mg/kg) was injected to reduce tracheal secretions. In the SSEP recording, a special electrode (NE-120, Rhodes Medical Instruments, Inc., distributed by David Kopf Instruments, Tujunga, CA, USA) was used for recording in the sensorimotor cortex (bregma –2 mm, lateral 2 mm). A bipolar platinum wire electrode placed in the contralateral sciatic nerve was used as a stimulating electrode. For MEP recording, the recording electrode was inserted into the gray matter of the L1 spinal cord and the contralateral sensorimotor cortex (bregma –2 mm, lateral 2 mm) was stimulated. The pointed tip of the electrode was used as active and the rounded part of the electrode was used as a reference electrode. A single square pulse (0.1 ms duration) of electrical stimulus was delivered by a stimulus isolator (A365D or A365, World Precision Instruments, Sarasota, FL, USA), which was driven by a pulse generator (Pulsemaster A300, World Precision Instruments, Inc.). The analog signals of the evoked potentials were amplified (×10 000), filtered (bandpass 300–1 000 Hz), and fed to an IBM-compatible PC through an AD/DA converter (CED, Cambridge, UK) to be averaged using Spike 2 software. Each SSEP consisted of an average of 100–300 single sweep epochs. The threshold of electrical stimulation was first determined prior to the experiments. The effect of electrical stimulation on SSEPs and MEPs was analyzed in terms of latency and amplitude.

Immunohistochemistry

To evaluate the cellular characteristics of transplanted cells *in vivo*, an immunohistochemical analysis was performed. Nine weeks after the induction of SCI, rats were perfused with PBS and 4% paraformaldehyde (PFA). The spinal cords were removed and fixed with 4% PFA for 4 hours followed by 30% sucrose in PBS overnight. A serial longitudinal section of the spinal cord was obtained using a cryostat (12 μm thick:

Microm/HM500V, Walldorf, Germany). In order to identify the cell populations and characterize the cellular response at the injury/graft sites, selected sections were immunolabeled with one or more antibodies. Goat anti-β-galactosidase (1:500, Biogenesis, Poole, UK), anti-Tau (1:50, Chemicon, Temecular, CA, USA) and human mitochondria (1:100, Chemicon) antibodies were used to identify the survival of grafted human MSCs. To test if transplanted hMSCs were differentiated into neuronal cells, anti-Tau (human specific) and anti-glial fibrillary acidic protein (anti-GFAP, 1:500, BD, San Jose, CA, USA) were employed for neuron and astrocyte identification. All primary antibodies were diluted in 0.1M PBS, containing 0.1% goat serum and 0.3% Triton X-100. Sections were incubated at 4°C overnight in a humid chamber. After several rinses in PBS, biotinylated anti-mouse IgG or IgM (1:20, Vector, Burlingame, CA, USA) secondary antibodies were applied for one and a half hours. Fluorescent conjugated Streptavidin complex (1:250, Vector) was incubated and visualized using FITC or Texas red. Sections were mounted on glass slides with fluorescent mounting medium (Vectorshield, Vector) and observed under a fluorescence microscope (BX51, Olympus, Tokyo, Japan) or confocal microscope (LSM 510, Zeiss, Gottingen, Germany). Appropriate controls were included to confirm that the primary antibodies produced specific staining in tissue, including omission of either primary or secondary antibodies.

Statistical analysis

Data were expressed as mean ± standard error. An independent *t*-test was used to determine statistical differences between the hMSC-transplanted and media-treated groups for behavioral results. One-way ANOVA followed by Dunnett's *post-hoc* multiple comparison tests was used to evaluate the electrophysiological study results to compare the media-treated group with the hMSC-transplanted group.

RESULTS

Measurement of functional recovery

After SCI, the animals seldom moved their hindlimbs. Fig. 1 shows changes in locomotor deficits after SCI. Prior to transplantation, BBB tests were per-

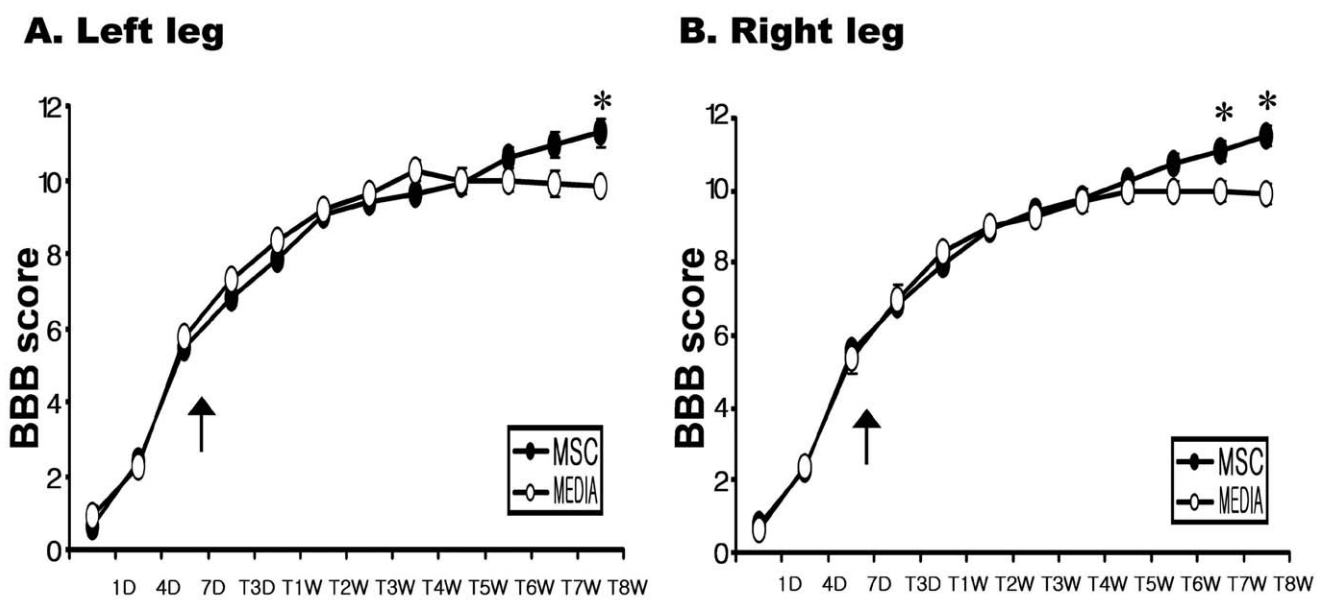

Fig. 1. Analysis of locomotor recovery as measured by BBB scores. (A) Left leg; (B) Right leg. X-axis indicates postoperative time in day or week (T means times after transplantation.) BBB scores decreased after injury and then slowly and gradually increased. The hMSC-transplanted rats showed significantly improved hindlimb performance from 8 weeks after transplantation in the left leg and 7 weeks after transplantation in the right leg compared to the media-treated rats. (Arrow indicates time of transplantation, *P<0.05, independent *t*-test).

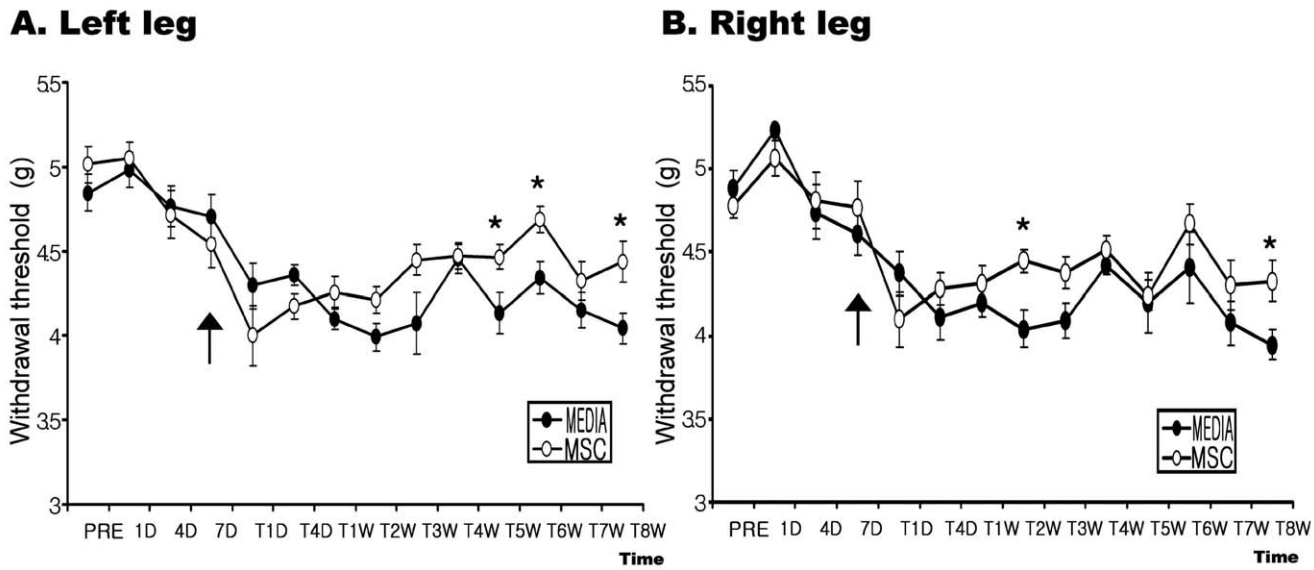

Fig. 2. Fifty percent withdrawal threshold to graded von Frey stimulation applied to the hindpaw. (A) Left leg; (B) Right leg. X-axis indicates postoperative time in day or week (T means times after transplantation.) After contusion spinal cord injury, mechanical hyperalgesia developed in both hind paws in media-treated (closed circle) and hMSC-transplanted (open circle) groups, as evidenced by a dramatic decrease in the PWT as compared with the pre-contusion value. This decrease in the PWT of hMSC-transplanted rats was significantly attenuated in both hind paws when compared with that of media-treated rats (Arrow indicates time of transplantation, *P<0.05, independent t-test).

formed in all animals at 1, 4, and 7 days after SCI. Animals with low scores and equally malfunctional hindlimbs were selected for the experiment. The media-treated group (n=11) scored 0 in both legs at 1 day post injury then gradually increased to a final score of 9.82 ± 0.23 in the left leg and 9.91 ± 0.28 in the right leg by 9 weeks after the injury. The hMSC-transplanted group (n=18) had significantly improved hindlimb performance from 8 weeks after transplantation in the left leg and 7 weeks after transplantation in the right leg compared to the media-treated group (t_{14}=4.170, P<0.05). The mean BBB score of the hMSC- transplanted group was 11.3 ± 0.37 (left) and 11.5 ± 0.28 (right) at 8 weeks after transplantation.

A behavioral test for mechanical allodynia was applied after SCI. A 50% withdrawal threshold to von Frey filament application to the plantar surface of the hindlimb was determined. Fig. 2 shows the results after spinal cord contusion. Before SCI, rats were seldom responsive to even the highest strength. There was a significant difference in the magnitude of withdrawal threshold reduction and the incidence of allodynia between the hMSC-transplanted and media-treated groups at 5 weeks after transplantation. In the former, withdrawal threshold reduction was more conspicuous than in the latter, indicating that the media-treated group produced a more severe mechanical allodynia (t_{15}=2.422, P<0.05). Paw withdrawal to von Frey stimulation was sometimes accompanied by a supraspinal reaction such as licking the paw, escaping from the stimulus, and attacking the von Frey filament.

Recovery of neural conduction

The SSEPs of the sensorimotor cortex in response to the stimulation of sciatic nerve were measured. Upon stimulation of the sciatic nerve, the latencies of SSEPs were classified as initial, N1- and P1-peak latencies. Figure 3A shows the representative wave forms of SSEPs stimulated with 6mA intensity, which is the standard point for data analysis. The initial, N1- and P1-peak latencies in animals from the hMSC-transplanted group (n=10) were significantly shorter than those from the media-treated group (n=8, Fig. 4A). The ANOVA of standard point of the SSEPs from the electrophysiological test yielded $F_{2,28}$=5.954 (P<0.05) in initial latency, $F_{2,28}$=13.743 (P<0.05) in N1 latency, and P1 latency was $F_{2,28}$=26.634 (P<0.05) in P1 latency and Dunnett's *post-hoc* multiple comparison test showed that there is significant differences between the hMSC-transplanted

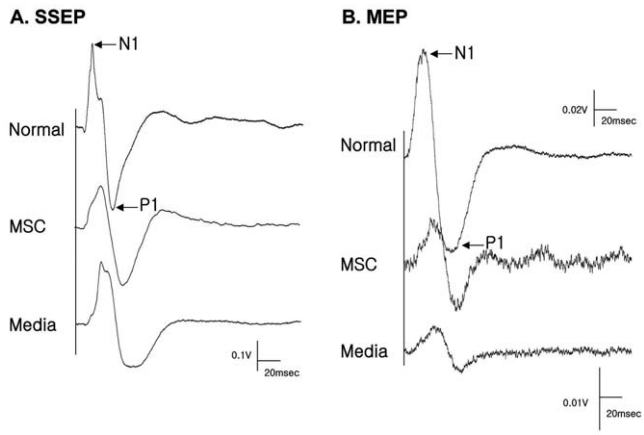

Fig. 3. Representative somatosensory (SSEPs) and motor evoked potentials (MEPs) recorded in normal, media-treated, and hMSC-transplanted animals. The latencies and amplitudes of SSEPs (A) and MEPs (B) were lengthened and reduced after spinal injury, respectively. (N1) peak of first negative deflection; (P1) peak of first positive deflection.

group and media-treated groups (P<0.05). The amplitude of both experimental groups, media-treated and hMSC-transplanted, was significantly lower than normal animals (n=7) without injury ($F_{2,28}$=42.787, P<0.05) but there was no significant differences between two experimental groups revealed by Dunnett's *post-hoc* multiple comparison test (P>0.05; data not shown).

The MEPs in the L1 spinal cord were measured using a bipolar disk electrode after the stimulation upon hindlimb area of the sensorimotor cortex. The waveform was similar to the SSEP pattern with negative-positive-negative deflection. Animals with spinal injury showed longer MEP latency with reduced amplitude. Fig. 3B depicts the representative waveforms of MEPs of normal (n=7) without injury, media-treated (n=7), and hMSC-transplanted (n=10) animals. In MEPs, the latency of the initial, N1-, and P1-peak of the media-treated and hMSC-transplanted group was significantly longer than the normal group (Dunnett's *post-hoc* multiple comparison test, P<0.05, Fig. 4B). ANOVA of the obtained each standard MEP peak point yielded $F_{2,18}$=9.490 (P<0.05) in initial latency, $F_{2,18}$=11.275 (P<0.05) in N1 latency, and $F_{2,18}$=11.386 (P<0.05) in P1 latency. There was no difference between the media-treated and hMSC-transplanted groups (Dunnett's *post-hoc* multiple comparison test, P>0.05).

Immunohistochemical study to support survival, migration, and differentiation patterns of transplanted cells

Exogenous MSC cell survival was determined by human mitochondria staining. Immunoreactivity of

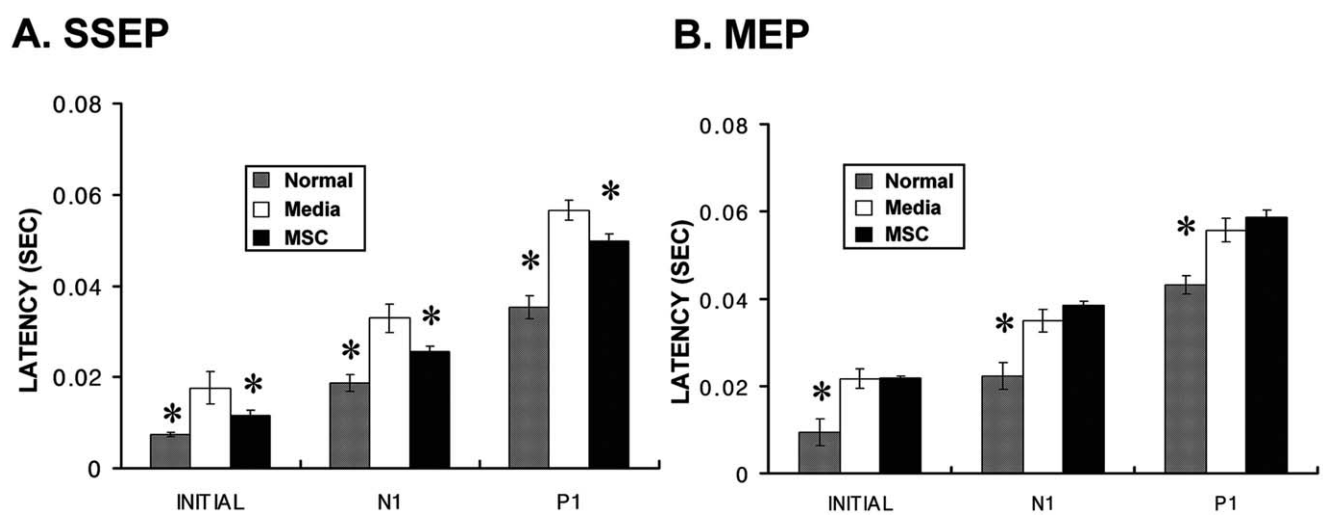

Fig. 4. Comparisons of the latencies in SSEPs and MEPs after media and hMSC transplantation. Data are expressed as mean ± SE. In SSEPs (A), the latencies in hMSC-transplanted rats were shortened compared to the media-treated group. In MEPs (B), the latencies in the media and hMSC-transplanted groups were lengthened compared to the normal control group (*P<0.05, Dunnett's *post-hoc* multiple comparison test). (Initial) Latency of initial part in evoked potentials; (N1) latency of first negative peak (upward deflection); (P1) latency of first positive peak (downward deflection).

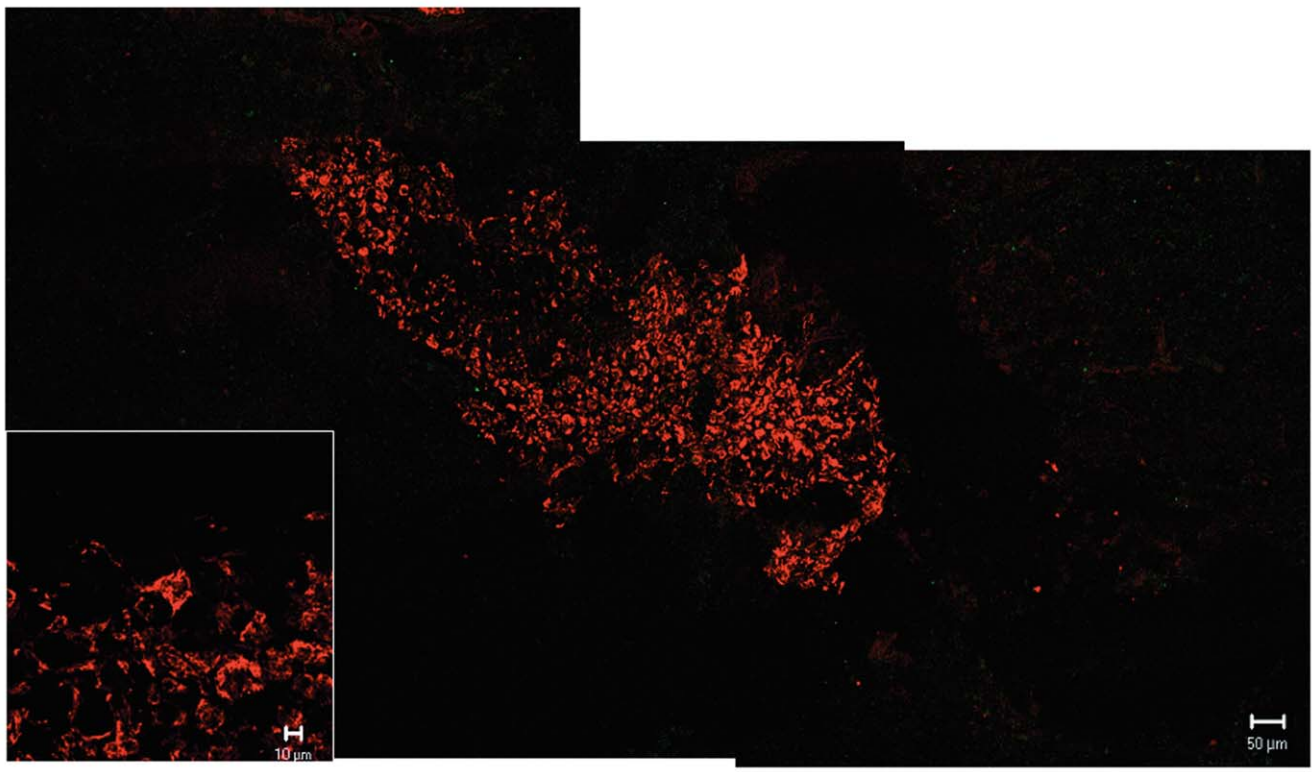

Fig. 5. Photomicrographs that demonstrate graft of hMSC after transplantation. Immunoreactivity of human specific mitochondria antibody was observed by the confocal microscope in injured site 3 weeks after transplantation, indicating that hMSC transplantations have successfully been grafted into the lesion site. An asterisk indicates the location of the injury site. The inset shows a magnified photograph of immunoreactive cells to human specific mitochondria antibody.

human specific mitochondria antibody was observed by the confocal microscope in the injured site 3 weeks after transplantation which indicates that hMSC transplantations have successfully been grafted into the lesion site (Fig. 5). Fig. 6 shows double staining of ß-galactosidase and GFAP (A, B, and C) or β-galactosidase and Tau (D, E, and F). β-galactosidase and GFAP positive cells or β-galactosidase and Tau positive cells were located around the injury site, indicating that some of the exogenous cells differentiated into astrocytes or neurons, although the number of differentiated cells was low.

DISCUSSION

In the present study, we observed that transplantation of human MSCs into the spinal cord after contusion injury promotes a functional outcome as measured by behavioral tests, electrophysiological tests, and immunostaining. The moderate contusion model applied in this study has been widely used for cell transplantation and closely parallels the type of spinal injury that most often occurs in SCI patients (Basso et al. 1996, Constantini and Young 1994, Gruner 1992, Lee et al. 2004). Lindsey et al. (2004) observed sensory changes following spinal contusion using the NYU impactor and reported that rats with moderate injuries showed the most consistent hindpaw withdrawal threshold decrease to von Frey hair stimulation among all three different injury groups. Hindpaw withdrawal thresholds to von Frey stimulation decreased after spinal contusion, indicating that mechanical allodynia developed below the level of the injury (Yoon et al. 2004). In our study, the hMSC-transplanted group demonstrated significantly improved functional outcome at 7 weeks after transplantation in the BBB tests, which is in agreement with Chopp and coauthors (2000). In addition, the rats in the media-treated group had a higher incidence of allodynia and a greater decrease in withdrawal threshold compared with MSC-transplanted group. Hyperalgesia in the hindlimbs declined at 1 week and significantly decreased

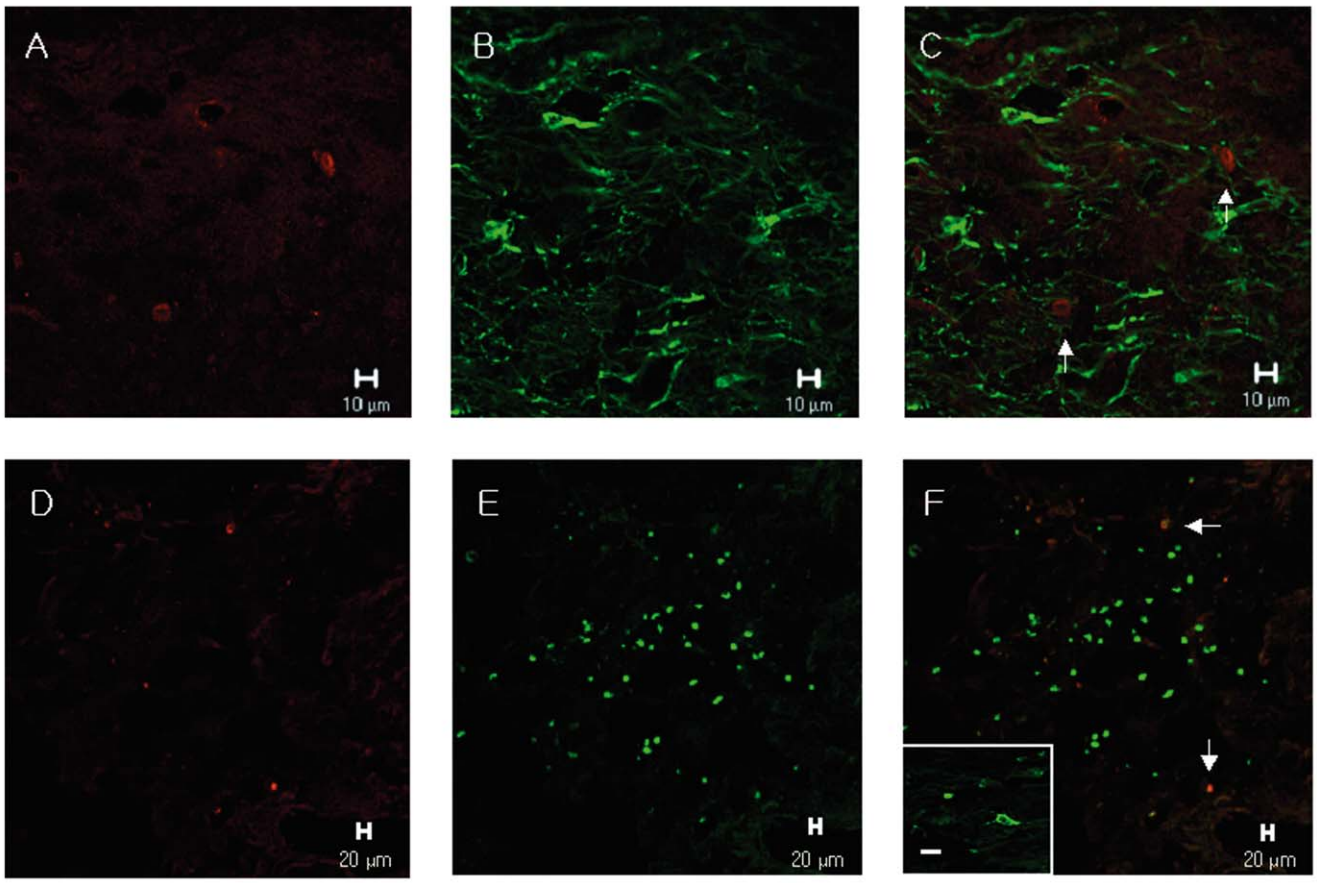

Fig. 6. Double staining with β-galactosidase and human specific anti-Tau or GFAP. Transplanted hMSC cells (red; A, D) are present among the GFAP-positive cells (green; B) or some Tau positive cells (green; E). (C) and (F) indicate the overlaid cells in each antibody. These suggest that the transplanted hMSC cells can differentiate into astrocytes or neurons. Arrows indicate the transplanted hMSC cells. The inset in (F) shows a magnified photograph of immunoreactive cells to human specific anti-Tau (scale bar is 10 μm).

at 5 weeks after MSC transplantation. Previous reports suggest that MSCs can promote functional recovery when grafted into contusion injury models (Chopp et al. 2000, Himes et al. 2006, Hofstetter et al. 2002) and progressive functional motor recovery was also achieved one year after the administration of MSC in chronic paraplegia (Zurita and Vaquero 2006). But there were few reports demonstrating the conduction recovery even though many showed the behavioral improvement using the BBB test. Our electrophysiological data demonstrate that the MSC-transplanted group had a shorter SSEP latency, which was very much in agreement with our pain test results.

Functional recovery includes sensory as well as motor components. Behavioral tests in our study showed that somatic sensation as well as locomotor performance was improved after hMSC transplantation. Electrophysiological results showed that SSEPs were recovered but MEPs were not. Improved SSEPs can explain the recovery of somatic sensation. However, the recovery of locomotion can not be explained directly by electrophysiological data because any improvement was not observed in MEPs after transplantation. At present, we speculate that improved somatic sensation including pain relief may contribute to the recovery of locomotion. For example, recovered processing of sensory information arising muscles, joints, or cutaneous sensory receptors may be beneficial to the coordinated performance of locomotion.

MSCs have several advantages as candidates for neurotransplantation. In particular, they can be readily obtained from bone marrow and expanded in culture. MSCs do not express growth factors when cultured *in vitro* yet MSC grafts contain significant quantities

of NGF, BDNF, and NT-3 *in vivo*, possibly produced by transplanted cells migrating to form grafts, like Schwann cells (Blesch and Tuszynski 2003, Jones et al. 2003, Lu et al. 2001). MSCs migrate into host tissue for short distances from the lesion site (Lu et al. 2005). Our results are agreement with Lu and others (2005). We found lacZ-positive cells in and around the injury area. Neuroprotective effects of cytokines such as growth factors may account for MSC-mediated functional recovery in contusion models since results of our study indicate that MSCs grafted *in vivo*. MSCs do not survive as other cells when transplanted into injured spinal cord in outbreed strains. Neither human MSC nor MSC from SD rats survived more than a few weeks (Himes et al. 2006, Neuhuber et al. 2005, Wu et al. 2003), using immunosuppression treatment. There was poor long-term survival of hMSC when grafted into the rat spinal cord even under condition of immunosuppression. We also could not detect the human specific mitochondria-positive cells 3 weeks after transplantation. It would demonstrate that even though the grafted cells survived for at least a short time, perhaps this short-term survival was sufficient for long-term benefits to the injured rats. So it could influence the functional recovery from SCI.

Human 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 NeuN-positive cells following SCI (Chopp et al. 2000). We also observed that transplanted MSCs were Tau- and GFAP-positive. Even though the number of differentiated cells is low, these cells may contribute to functional recovery either by preserving existing circuits or by helping to establish new ones. Therefore, MSCs may be a very useful source to reconstitute a cellular matrix in the lesion site as one of the functional repair approaches to SCI.

CONCLUSION

In this experiment we demonstrated that the transplantation of hMSC into the injured spinal cord in rats improves functional recovery in motor and sensory tests. The electrophysiological result especially showed improved conduction velocity in the sensory components of the spinal cord. Some of the transplanted hMSCs differentiated into neural cells in the spinal cord. This study suggests that transplantation of MSCs can be beneficial among the approaches to functional recovery.

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