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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
T. Okasora, JI. Jo, and Y. Tabata.	Augmented anti-tumor therapy through natural targetability of macrophages genetically engineered by NK4 plasmid DNA.	Gene Therapy	15	524-530	2008
M. Nakamura, JI. Jo, Y. Tabata, and O. Ishikawa.	Controlled delivery of T-box 21 siRNA ameliorates autoimmune alopecia (alopecia areata) in C3H/HeJ mouse model.	Am J Pathol.,	172(3)	650-658	2008
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REVIEW

Mari Dezawa

Systematic neuronal and muscle induction systems in bone marrow stromal cells: the potential for tissue reconstruction in neurodegenerative and muscle degenerative diseases

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Abstract Because bone marrow stromal cells (MSCs) are easily accessible from both healthy donors and patients and can be expanded on a therapeutic scale, they have attracted attention for cell-based therapy. Benefits of MSCs have been discussed mainly from two aspects: one is their tissue protective and immunomodulatory effects, and the other is their capability under specific manipulations to differentiate into various cell types. In this review, their differentiation into functional neural and muscle cell lineages is the focus, and their potential to the application for tissue reconstruction in neurodegenerative and muscle degenerative diseases is discussed.

Key words Mesenchymal cell · Transdifferentiation · Regenerative medicine · Cell therapy · Transplantation

Introduction

Bone marrow contains a category of nonhematopoietic multipotent cells that can be cultivated in vitro as plastic adherent cells. These cells are called bone marrow stromal cells, mesenchymal stem cells, or bone marrow stromal stem cells, and no uniform term is yet fixed.[1] In this review, these cells are called bone marrow stromal cells (MSCs). MSCs are mesenchymal elements normally providing structural and functional support for hemopoiesis. Because they exhibit diverse characters and consist of a heterogeneous population, their true nature is not fully known. The majority of MSC populations express mesenchymal markers such as CD29 (beta-1 integrin), CD90 (Thy-1), CD54 (ICAM-1), CD44 (H-CAM), CD71 (transferrin receptor), CD105 (SH2), SH3, Stro-1, and CD13, but a very small number of cells positive for hematopoietic surface markers CD34, CD3, and CD117 (c-kit) do exist.[2,3] Although they behave as do stem cells, their property as stem cells is not fully clarified.

These cells are easily accessible through aspiration of the patient's bone marrow and can easily be expanded on a large scale for autotransplantation. For example, 20–100 ml bone marrow aspirate yields 1×10^7 MSCs within several weeks that may provide a plentiful number of cells for cell therapy. In contrast to embryonic stem (ES) and tissue stem cells, we can collect MSCs without encountering serious ethical problems, and there is no need for us to use fertilized eggs or a fetus. Thus, MSCs are a strong and hopeful candidate for use in cell-based therapy.

Recently, MSCs have attracted attention mainly because of two reasons. One is that they contribute to the protection of host tissue after transplantation. Because MSCs originally support hematopoietic cells in the bone marrow, they produce various kinds of cytokines and trophic factors including neurotrophins, stem cell factors, and interleukins.[4] This nature is useful in tissue protection, controlling apoptosis and neovascularization. In fact, when naive MSCs are transplanted to neurotraumatic or neurodegeneration models such as spinal cord injury and stroke, they migrate into the damaged site, protect tissues, and contribute in part to functional recovery.[5–7] In addition, MSCs have recently been shown to transfer functional mitochondria to damaged host cells to rescue aerobic respiration.[8]

The other reason why MSCs came to the front is that they have an ability to differentiate into other kinds of cells that replenish lost cells in degenerated tissue. MSCs have been reported to differentiate into mesenchymal lineage cells such as osteocytes, chondrocytes, and adipocytes, and some of these systems are already being applied in clinical treatment.[1,9] Recently, however, the unorthodox plasticity of MSCs has been described, in that they have an ability to cross oligolineage boundaries which were previously thought to be uncrossable. Makino et al. showed that rhythmically contracting cardiomyocytes with cardiac muscle markers and electrophysiological characters could be induced from MSCs.[10] Furthermore, hepatocytes, insulin-producing cells, and airway epithelial cells are reported to

M. Dezawa (✉)
Department of Anatomy and Neurobiology, Kyoto University Graduate School of Medicine, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan
Tel. +81-75-753-4343; Fax +81-75-751-7286
e-mail: dezawa@anat2.med.kyoto-u.ac.jp

be inducible from MSCs.[11-13] Accordingly, the potential of MSCs to transdifferentiate from mesenchymal lineages to other lineages is now of interest. As to neurons, previous reports described that MSCs can differentiate into neuron-like morphology only by the administration of reducing agents and/or trophic factors.[14-16] However, some other reports expressed skepticism about these observations that simple treatment of MSCs only by reducing agents or factors do not fully induce their differentiation into functional neurons, and that these cells do not actually integrate into the host tissue to contribute to the functional recovery.[17-19] Although MSCs offer great potential for cell transplantation therapy, their practical application to human diseases is dependent on the ability to control their differentiation into certain functional cells with high efficiency and purity.

Recently, we have found a method to systematically induce Schwann cells, neurons, and skeletal muscle cells from human and rat MSCs at a therapeutic scale[20-22] (Fig. 1). This review focuses on the potential of MSCs to differentiate into neural and muscle cells and discusses the possibility of clinical application in neurodegenerative and muscle degenerative diseases.

Induction of cells with Schwann cell property from MSCs

Peripheral glial cells, Schwann cells, which constitute the peripheral nervous system (PNS), are myelin-forming cells and are known to support axonal regeneration after damage by providing various kinds of trophic factors and molecular footholds and to finally reconstruct myelin, which contributes to functional recovery.[23-25] Useful not only in the PNS, Schwann cells also support axonal regeneration and reconstruction of myelin in the central nervous system (CNS). For these reasons, they represent a good candidates for implantation to support regeneration of both PNS and CNS, including spinal cord injury.

Although Schwann cells are potentially useful cells, there is difficulty in clinical use to obtain a sufficient amount of cells. For their cultivation, another peripheral nerve must be newly damaged. Thus, it would be more desirable to establish cells of Schwann cell characteristic from sources that are easy to access and are capable of rapid expansion. We focused on MSCs and finally established a method to induce MSCs with Schwann cell properties.[20]

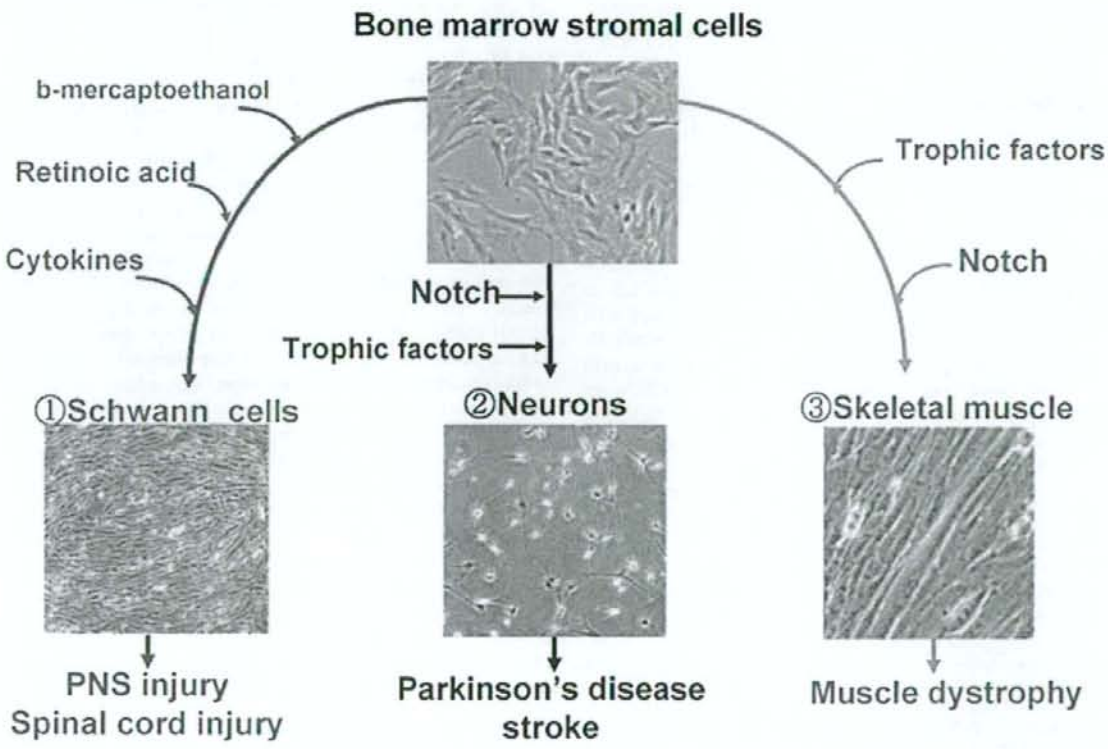

Fig. 1. Schematic diagram of Schwann cell, neuronal cells, and muscle cell induction from bone marrow stromal cells (MSCs) and their application for neuro- and muscle degeneration. *PNS*, peripheral nervous system

MSCs were treated with beta-mercaptoethanol (BME), followed by retinoic acid (RA), and finally administrated cytokines related to Schwann cell differentiation, namely forskolin (known to upregulate intracellular cAMP; FSK), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and neuregulin.[20] Treated cells were morphologically similar to Schwann cells and expressed p75, GFAP, S-100, O4, P0, and Krox20, all known as markers of Schwann cells.[20] Approximately 97% of the induced cells were positive for Schwann cell markers.

Recovery in spinal cord injury by the transplantation of induced Schwann cells

These MSC-derived Schwann cells (M-Schwann) are effective in promoting axonal regeneration and functional recovery in completely transected adult rat sciatic nerves and spinal cord.[26-28] In this section, the focus is on the potential of M-Schwann cells for contribution to spinal cord injury recovery.

When rat M-Schwanns were transplanted to the defective site of a completely transected model (the T7 spinal cord segment was completely removed), cells integrated well into the host spinal cord.[27] In contrast to a control group that had received only Matrigel (Collaborative Biomedical Products, Bedford, MA, USA), the number of neurofilament-positive nerve fibers was significantly larger in M-Schwann group. A large majority of these nerve fibers were revealed to be tyrosine hydroxylase (TH)-positive fibers, whereas some of the CGRP- and serotonin-positive fibers were also contained.[27] Hindlimb function recovered in the M-Schwann group from 4 weeks after transplantation, and a significant difference was recognized in BBB score up to 6 weeks after transplantation. The best recovery score in the M-Schwann group indicated weight-supporting plantar steps, but no forelimb–hind limb coordination. In contrast, the average recovery score in the control group was very low, showing only two joints of hindlimbs had extensive movement. Retransection of the grafts at their midpoint in the M-Schwann group was performed 6 weeks after transplantation, which completely abolished the recovered hindlimb function, and no significant recovery was observed even 4 weeks after retransection.[27] These results exclude the possibility that transplanted cells enhanced the activity of a locomotor pattern generator in the spinal cord, but rather emphasize axonal regeneration induced by transplanted M-Schwann cells contributed to functional recovery.

Induction of functional neuronal cells from MSCs

Recently we established a method to systematically and specifically induce neuronal cells from MSCs. Highly efficient and specific induction of postmitotic functional neuronal cells, without glial differentiation, can be achieved by gene transfer of Notch intracellular domain (NICD) followed by the administration of a certain combination of trophic factors.[21]

The Notch signaling pathway has been known to influence cell fate determination during development and to maintain a pool of uncommitted precursors to the terminal specification of cells.[29] In neural development, Notch is known to be one of the glial factors, and in fact, a series of studies have shown that when Notch signaling is activated, astrocytes and Schwann cells differentiate from neural stem cells (NSCs) and neural crest stem cells, respectively. Initially, we expected that MSCs would shift from mesenchymal to Schwann cell characteristics by Notch introduction when combined with administration of trophic factors related to neural development. After such treatment, however, it was very surprising to see a small population of neuron-like cells induced in the final product. My research team repeated the experiment, modified, and finally established a neuronal induction system from MSCs, as shown below.

The plasmid containing mouse Notch1 intracellular domain (NICD) cDNA was transfected into MSCs with lipofection followed by selection. After NICD introduction, MSCs acquired neuronal progenitor cell (NPC) property. In fact, they expressed markers related to neural stem cells and/or NPCs and demonstrated sphere formation in the free floating culture system. The morphology of NICD introduced cells does not differ from that of naive MSCs, but when they were expanded and then stimulated with trophic factors [bFGF, FSK, and ciliary neurotrophic factor (CNTF)] for several days, they changed their morphology drastically, extended neurite-like processes, and differentiated into postmitotic neuronal cells at an efficiency of approximately 96% (MSC-Ns). These cells were immunopositive to neuronal markers including MAP-2ab, neurofilament, and Tuj1, and action potential was recorded in some of MSC-Ns in a patch clamp experiment. The outstanding characteristics of MSC-Ns is that they are devoid of glial development in the final population. In fact, few cells positive to either GFAP (marker for astrocytes) and galactocerebroside and O4 (markers for oligodendrocytes) were detected in MSC-Ns. My research team then estimated whether MSC-Ns integrate into host brains to contribute to functional recovery in neurodegenerative disease models.

MSC-Ns contribute to functional recovery in stroke model

MSC-Ns were transplanted into the infarction area in the middle cerebral artery occlusion (MCAO) rat model. Seven days after the occlusion, a total of 50000 MSC-Ns was directly injected to the infracted area. The transplanted rat showed significant recovery in Beam balance (vestibulomotor function), Limb placing (sensorimotor function), and Morris water maze (cognitive function) test ($P < 0.01$). Histologically, green fluorescent protein (GFP)-labeled transplanted cells migrated from the injection site into the ischemic boundary area, expressed neuronal markers of neurofilaments, MAP-2ab, and beta3-tubulin, integrated into the hippocampus and cortex, and extended processes. Most of the transplanted cells are neuronal marker-positive cells; only a small number of cells (approximately 1%) were positive for GFAP. These results showed that induced neuronal cells are effective in the amelioration of the rat brain ischemic injury model.

Induction of dopamine-producing cells and their application for Parkinson's disease

For Parkinson's disease, transplantation of dopaminergic neurons is believed to be effective.[30] However, cells committed positive for TH, a marker for dopaminergic neurons, accounted for lower ratios (~3%) in MSC-Ns initially.[21] As glial cell line-derived neurotrophic factor (GDNF) is known to promote the generation and development of midbrain dopaminergic neurons,[31] GDNF was administered to MSC-Ns to increase the proportion of cells immunopositive for TH. This treatment was effective, and approximately 40% of MSC-Ns became TH positive (MSC-DNs). The dopamine release upon depolarization in vitro was measured by HPLC, showing that MSC-DNs actually released dopamine to the culture media in response to high K^+-depolarizing stimuli. These results indicate that functional dopamine-producing neuronal cells could effectively be induced from MSCs.[21]

Rat MSC-DNs were transplanted into the striatum of the Parkinson's disease model rat induced by 6-hydroxydopamine (6-OHDA). In these model rats, apomorphine injection induces abnormal rotational behavior, which is generally used as an indicator of Parkinson's disease symptoms in animal models. Rats grafted with MSC-DNs demonstrated substantial recovery from rotation behavior up to 10 weeks.[21] In addition to rotational behavior, nonpharmacological behavior tests, adjusting step, and paw-reaching tests demonstrated significant improvement in behavior in both experiments. In the immunohistochemistry of grafted striatum, GFP-positive transplanted cells expressed marker of neurofilaments, TH, and dopamine transporter (DAT), whereas few cells were positive to GFAP and O4, consistent with in vitro data. Animals grafted were followed up to 16 weeks, and no tumor formation was observed in the brain.

Human MSC-DNs were similarly transplanted into the striatum of Parkinson's model rats under the control of immunosuppressant FK 506, and rotational behavior was recorded at 4 weeks after cell transplantation. Grafting resulted in significant improvement in rotational behavior as well.[21]

The foregoing results demonstrated that functional mature neurons that have an ability to produce and release neurotransmitters were able to be induced from rodent and human MSCs.

Induction of skeletal muscle cells from MSCs

During the experiment of neural induction, the order of treatment was reversed to perform the control experiment (see Fig. 1). However, this event accidentally demonstrated the induction of skeletal muscle cells. The induction experiment was repeated, upgraded, and finally a new method to systematically and efficiently induce skeletal muscle lineage cells with high purity from large population of MSCs was established.[22]

Human and rat MSCs were first treated with trophic factors of bFGF, FSK, PDGF, and neuregulin, followed by transfection with a NICD expression plasmid by lipofection and selection, and allowed to recover to 100% confluency. At this stage, a large majority of MSCs developed to mononucleated myogenic cells expressing skeletal muscle cell-specific marker MyoD. Cells were then supplied with differentiation medium (2% horse serum or ITS insulin-transferrin-selenite-serum free medium, both of which are known to promote differentiation of myoblasts to myotubes).[32] After treatment, MSC-derived muscle lineage cells (MSC-Ms) were obtained. This final population contained three kinds of muscle-lineage cells: (1) postmitotic multinucleated myotubes expressing Myf6/MRF4 (a marker for mature skeletal muscle) and contractile proteins, (2) mononucleated myoblasts expressing MyoD, and (3) satellite-like cells positive for Pax7, a marker for muscle satellite cells.[33]

Application to muscle degeneration models

To estimate how workable these induced muscle lineage cells are in the repair of degenerated muscles, human MSC-Ms were transplanted into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment.[34] Cells were transplanted by local injection into degenerated muscles. Two weeks after transplantation, GFP-labeled transplanted cells incorporated into newly formed immature myofibers exhibited centrally located nuclei in treated animals. Four weeks after transplantation, GFP-positive myofibers exhibited mature characteristics with peripheral nuclei just beneath the plasma membrane. Functional differentiation of grafted human cells was also confirmed by the detection of human dystrophin in GFP-labeled myofibers.[22]

MSC-Ms contained satellite-like cells that developed into satellite cells in the host muscle. In general, muscle satellite cells are known to contribute to regenerating myofiber formation upon muscle damage.[35] Therefore, we tested whether transplanted satellite-like cells were able to contribute to muscle regeneration as satellite cells in vivo. Four weeks after transplantation of human MSC-Ms, cardiotoxin was readministered into the same muscles without additional transplantation. Two weeks after the second cardiotoxin treatment (6 weeks after initial transplantation), many regenerating GFP-positive myofibers with centrally located nuclei were observed. This result implies that, upon transplantation of MSC-Ms to muscles of patients, those retained as satellite cells should be able to continue to contribute to future muscle regeneration.[22]

Compared with the various muscle stem cell systems that have been reported, this system offers several important advantages. Because this induction system does not depend on a rare stem cell population but can utilize the general population of adherent MSCs, which can easily be isolated and expanded, functional skeletal muscle cells can be obtained within a reasonable time course on a therapeutic scale. In case of MSCs derived from inherited muscle dystrophy patients, genetic manipulation is possible after the isolation and expansion of MSCs. Moreover, transplantation of MSC-derived cells should encounter fewer ethical problems.

General conclusions

Although ES cells and tissue stem cells have great potential, MSCs also provide hopeful possibilities for clinical application, as they can be efficiently expanded in vitro to acquire a therapeutic scale. In addition, transplantation of MSC-derived cells should pose fewer ethical problems by preventing stem cell controversy because bone marrow transplantation has already been widely performed. As MSCs are easily obtained from patients or marrow banks, autologous transplantation of induced cells or transplantation of induced cells with the same HLA subtype from a healthy donor may minimize the risks of rejection (Fig. 2). Needless to say, bone marrow should at least be "normal and healthy" for transplantation. Particularly, in the case of autologous transplantation in muscular dystrophy, the replenishment of normal genes is necessary for usage of the patient's MSCs. In such a case, induction and transplantation of the same HLA subtype from a healthy donor may be a more realistic solution (Fig. 2).

Although we showed a high ratio and specific induction of Schwann cells, neurons, and skeletal muscle cells, the following problems need to be solved[20–22] Although there have been so far few reports referring to tumor formation after transplantation of untreated MSCs, further studies are needed to ensure the safety, tumor formation, and efficacy of manipulated MSCs over a long-term period using higher mammals such as primates. Second, as the potential of differentiation would differ by age, individual, race, and sex, each of these factors must be investigated in the future. Third, MSCs have been shown to be heterogeneous in terms of growth kinetics, morphology, phenotype, and plasticity. With the development of specific markers and detailed characterization of heterogeneous general adherent MSCs, their properties and plasticity can be studied and defined with more certainty. Fourth, the usage of fetus bovine serum in the induction system is problematic for infections and Bovine spongiform encephalopathy (BSE). Fortunately, I have already confirmed that human serum is more appropriate for the differentiation of human MSCs than fetus bovine serum (unpublished data); this system is able to provide a patient's MSC-derived cells using the patient's own serum.

Notch–Hes signaling is known to inhibit neuronal and myogenic differentiation in conventional development.[29] However, in our system, NICD introduction accelerated the induction of neuronal and skeletal muscle cells from MSCs. Although this results appear inconsistent with previous research, they do not refute the known role of Notch–Hes signals during development. In a previous report, JAK/STAT inhibitor administration and constitutive active STAT1/3 transfection showed that down regulation of STATs was tightly associated with NICD-mediated neuronal induction, whereas Hes, downstream of Notch, was not involved in the induction event.[21] Skeletal muscle induction was also revealed to be independent of Hes1/5. Thus, the results suggest the distinct cellular responses to Notch signals; for example, the repertoire of second messengers and active factors, may well be different between conventional neural stem cells and/or neural progenitor cells and MSCs. It might be possible that an unknown signaling pathway downstream of Notch is involved in these events, and thus further studies are needed to identify the factor involved in this phenomenon.

Because MSCs can be obtained from patients, it is possible to establish "auto cell transplantation therapy" using

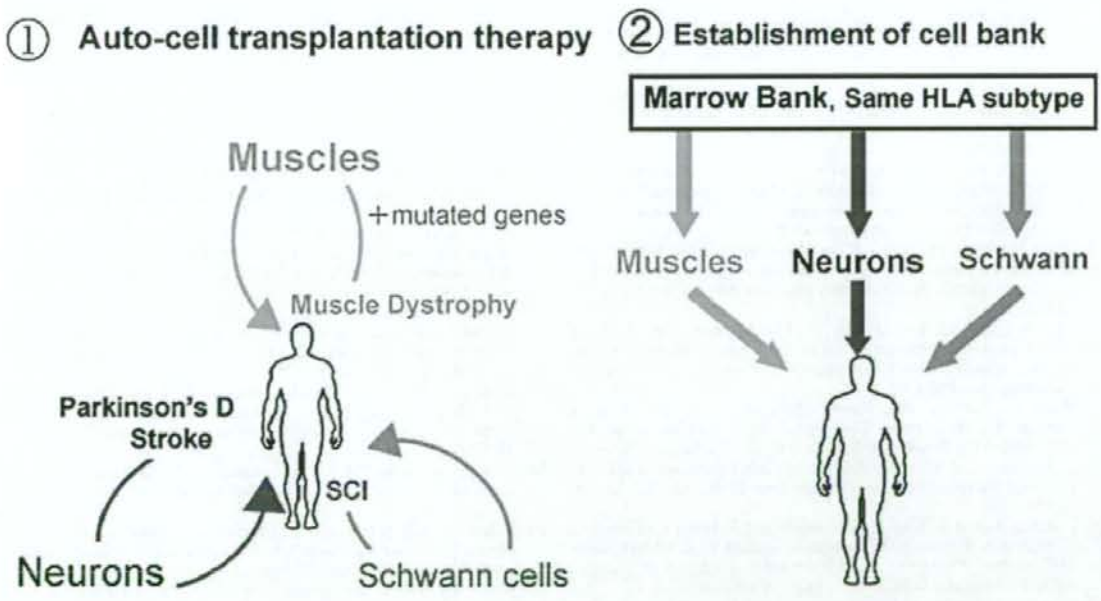

Fig. 2. Strategy for cell transplantation therapy in neurodegenerative and muscle degenerative diseases

MSCs. Fortunately, my research team already confirmed that human serum is more appropriate to the differentiation of human MSCs than fetus bovine serum (unpublished data). To realize this ideal, it is necessary to develop the regulatory system of differentiating MSCs into cells with a purpose. The method would be one of the possible ways to regulate MSC differentiation into functional Schwann cells, neurons, and skeletal muscle cells that will be applicable to neurodegenerative and muscle degenerative diseases.

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Reduction of cystic cavity, promotion of axonal regeneration and sparing, and functional recovery with transplanted bone marrow stromal cell–derived Schwann cells after contusion injury to the adult rat spinal cord

Laboratory investigation

Yukio Someya, M.D.,[1] Masao Koda, M.D., Ph.D.,[3] Mari Dezawa, M.D., Ph.D.,[4] Tomoko Kadota, M.D., Ph.D.,[2] Masayuki Hashimoto, M.D., Ph.D.,[3] Takahito Kamada, M.D., Ph.D.,[1] Yutaka Nishio, M.D., Ph.D.,[1] Ryo Kadota, M.D., Ph.D.,[1] Chikato Mannoji, M.D., Ph.D.,[1] Tomohiro Miyashita, M.D., Ph.D.,[1] Akihiko Okawa, M.D., Ph.D.,[1] Katsunori Yoshinaga, M.D., Ph.D.,[5] and Masashi Yamazaki, M.D., Ph.D.[1]

Departments of [1]Orthopaedic Surgery and [2]Bioenvironmental Medicine, Chiba University Graduate School of Medicine, Chiba; [3]Department of Orthopaedic Surgery, Prefectural Togane Hospital, Chiba; [4]Department of Anatomy and Neurobiology, Kyoto University Graduate School of Medicine, Kyoto; and [5]Chiba Rehabilitation Center, Chiba, Japan

Object. The authors previously reported that Schwann cells (SCs) could be derived from bone marrow stromal cells (BMSCs) in vitro and that they promoted axonal regeneration of completely transected rat spinal cords in vivo. The aim of the present study is to evaluate the efficacy of transplanted BMSC-derived SCs (BMSC-SCs) in a rat model of spinal cord contusion, which is relevant to clinical spinal cord injury.

Methods. Bone marrow stromal cells were cultured as plastic-adherent cells from the bone marrow of GFP-transgenic rats. The BMSC-SCs were derived from BMSCs in vitro with sequential treatment using beta-mercaptoethanol, all-*trans*-retinoic acid, forskolin, basic fibroblast growth factor, platelet derived–growth factor, and heregulin. Schwann cells were cultured from the sciatic nerve of neonatal, GFP-transgenic rats. Immunocytochemical analysis and the reverse transcriptase–polymerase chain reaction were performed to characterize the BMSC-SCs. For transplantation, contusions with the New York University impactor were delivered at T-9 in 10- to 11-week-old male Wistar rats. Four groups of rats received injections at the injury site 7 days postinjury: the first received BMSC-SCs and matrigel, a second received peripheral SCs and matrigel, a third group received BMSCs and matrigel, and a fourth group received matrigel alone. Histological and immunohistochemical studies, electron microscopy, and functional assessments were performed to evaluate the therapeutic effects of BMSC-SC transplantation.

Results. Immunohistochemical analysis and reverse transcriptase–polymerase chain reaction revealed that BMSC-SCs have characteristics similar to SCs not only in their morphological characteristics but also in their immunocytochemical phenotype and genotype. Histological examination revealed that the area of the cystic cavity was significantly reduced in the BMSC-SC and SC groups compared with the control rats. Immunohistochemical analysis showed that transplanted BMSCs, BMSC-SCs, and SCs all maintained their original phenotypes. The BMSC-SC and SC groups had a larger number of tyrosine hydroxilase–positive fibers than the control group, and the BMSC-SC group had more serotonin-positive fibers than the BMSC or control group. The BMSC-SC group showed significantly better hindlimb functional recovery than in the BMSC and control group. Electron microscopy revealed that transplanted BMSC-SCs existed in association with the host axons.

Conclusions. Based on their findings, the authors concluded that BMSC-SC transplantation reduces the size of the cystic cavity, promotes axonal regeneration and sparing, results in hindlimb functional recovery, and can be a useful tool for spinal cord injury as a substitute for SCs. *(DOI: 10.3171/SPI.2008.9.08135)*

Key Words • **bone marrow stromal cell** • **cell transplantation** • **hindlimb function** • **Schwann cell** • **spinal cord injury**

It has been widely believed that a lesioned adult mammalian spinal cord cannot regenerate. Recent advances in stem cell research have enabled us to repair injured spinal cords by means of various kinds of cell therapies, including the use of embryonic stem cells,[33] fetal neural stem cells,[36] and adult neural stem cells.[15] Major obstacles to the use of these cells in clinical trials

Abbreviations used in this paper: ANOVA = analysis of variance; BBB = Basso, Beattie, Bresnahan; BDA = biotinylated dextrin amine; BMSC = bone marrow stromal cell; BMSC-SC = BMSC-derived Schwann cell; FBS = fetal bovine serum; HSC = hematopoietic stem cell; MEM = minimum essential medium; PBS = phosphate-buffered saline; RT-PCR = reverse transcriptase–polymerase chain reaction; SC = Schwann cell; SCI = spinal cord injury; SEM = standard error of the mean.

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

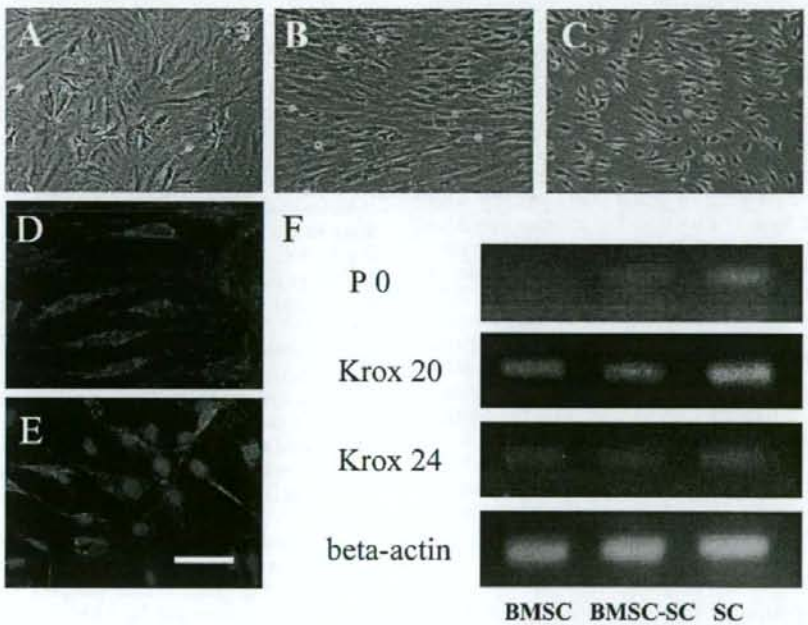


FIG. 1. Photomicrographs demonstrating the peripheral SC-like characteristics of BMSC-SCs. A: Phase-contrast microscopic image of BMSCs cultured from GFP-transgenic rats. B: Phase-contrast microscopic image of BMSC-SCs. The BMSC-SCs are morphologically and phenotypically similar to SCs. C: Phase-contrast 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.

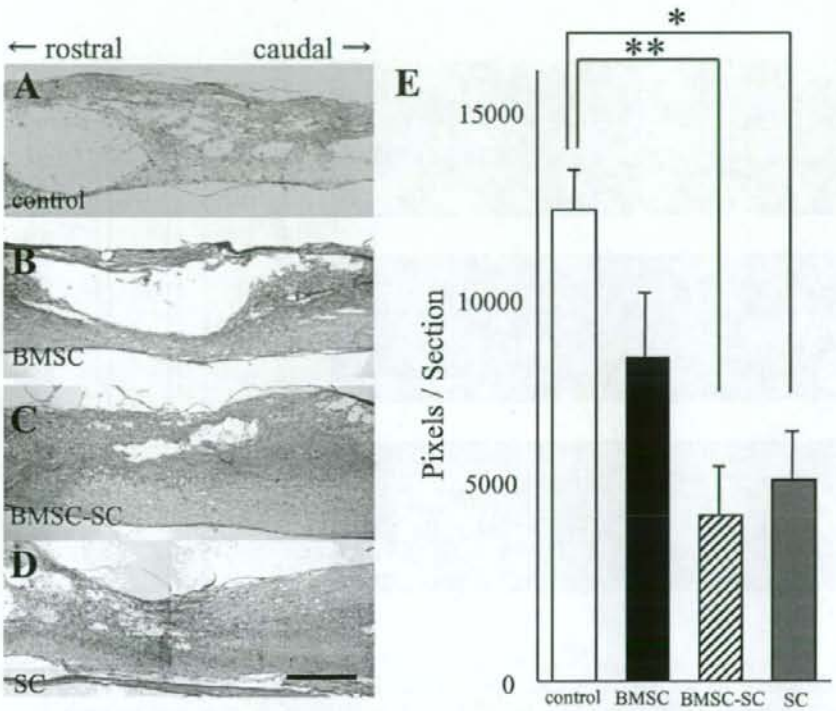


FIG. 2. A–D: Cresyl-violet–stained BMSC-SCs and SCs in preserved spinal cord tissue samples from all 4 groups. Representative image of cystic cavity in samples obtained in the control (A), BMSC (B), BMSC-SC (C), and SC (D) groups. The area of cystic cavity of BMSC-SC and SC groups were significantly 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;

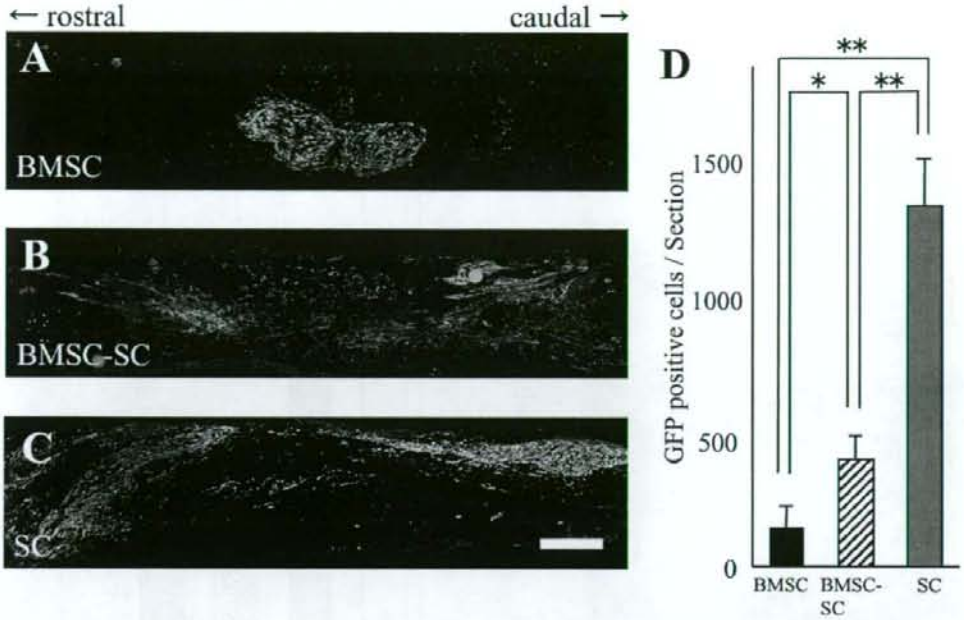


FIG. 3. Photomicrographs. Staining with GFP in the BMSC (A), BMSC-SC (B), and SC groups (C) 5 weeks after cell transplantation. Bar = 1 mm. D: Graph of the statistical analysis of number of GFP-positive transplanted cells. There were significantly more GFP-positive transplanted cells in the BMSC-SC group *(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-

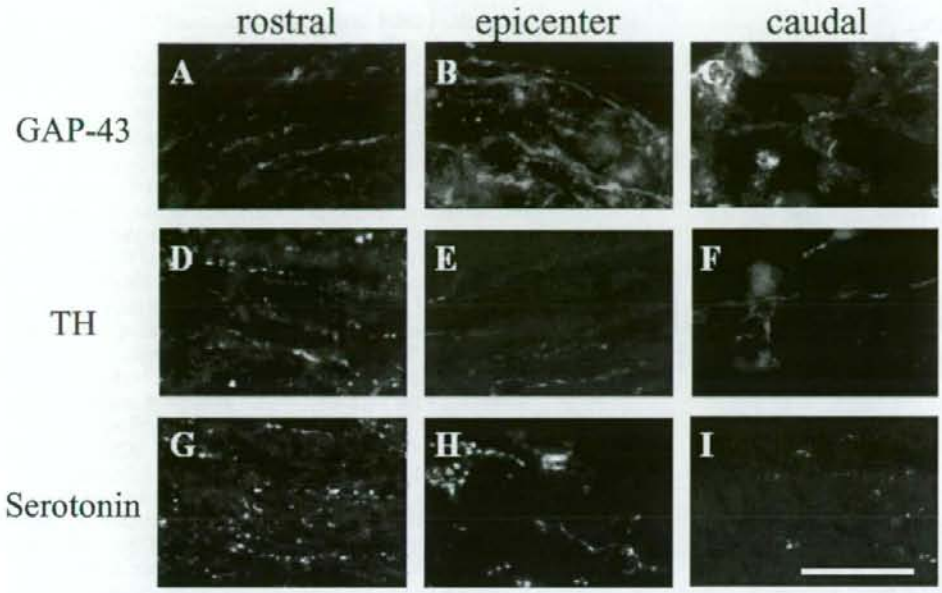


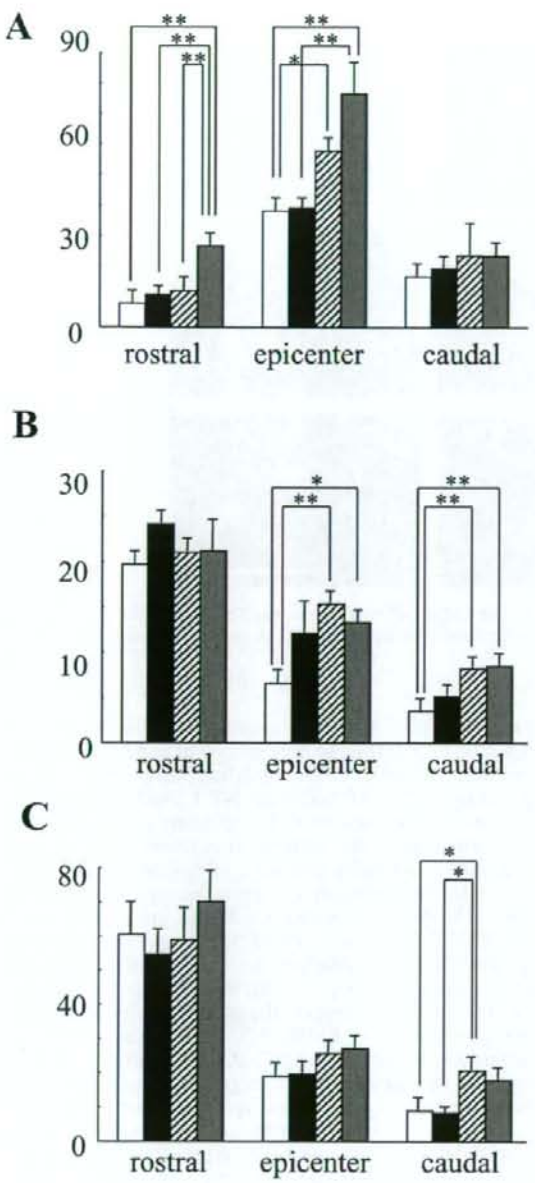
FIG. 4. Immunostaining for regenerated/spared nerve fibers after BMSC-SC transplantation. The Gap43-positive (A–C), Th-positive (D–F) and serotonin-positive (G–I) fibers were detected at the rostral (A, D, and G) epicenter (B, E, and H) and caudal (C, F, and I) levels. Bar = 50 μm.

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. *$p < 0.05$, **$p < 0.01$.

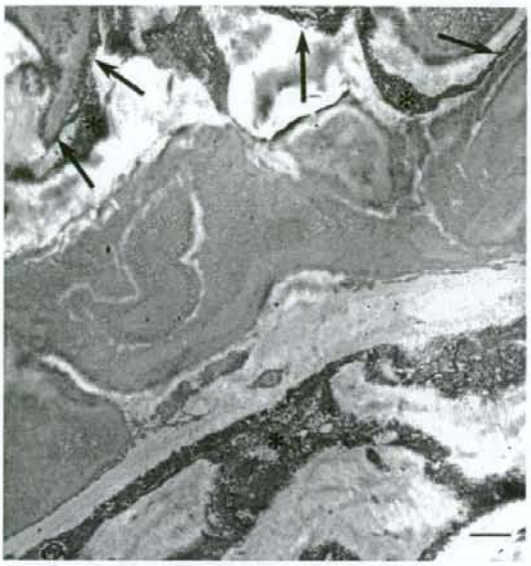


FIG. 6. Electron micrograph obtained in the BMSC-SC group. The GFP-positive transplanted cells *(asterisks)* had several thin processes, which were in immediate contact with host nerve tissues *(arrows)*. Bar = 2 μm.

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.

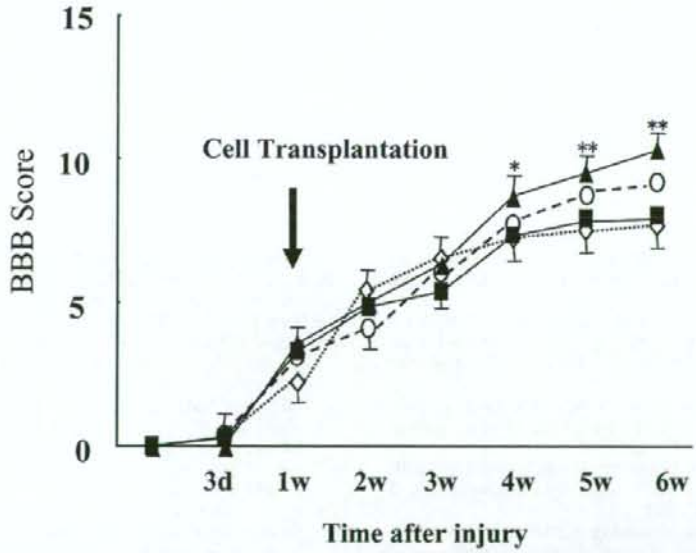


FIG. 7. Graph showing that rats with BMSC-SC transplantation showed better hindlimb functional recovery. The repeated measure ANOVA and post hoc test showed better functional recovery in BMSC-SC group *(triangle)* than in the control (*diamond* and *dotted line*) and BMSC group *(black square)*. There 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

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Address correspondence to: Masashi Yamazaki, M.D., Ph.D., Department of Orthopaedic Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. email: masashiy@faculty.chiba-u.jp.

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Review

Induction system of neural and muscle lineage cells from bone marrow stromal cells; a new strategy for tissue reconstruction in degenerative diseases

Masaaki Kitada and Mari Dezawa

Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

Summary. Since bone marrow stromal cells (MSCs) are easily accessible both from healthy donors and patients, and can be expanded on a therapeutic scale, they have attracted attention for cell-based therapy. MSCs contribute to the protection of host tissue after transplantation by Immune modulation and trophic effect. They also have an ability to differentiate into other cell kinds that will replenish lost cells in the degenerated tissue. This review discusses the potential of MSCs for tissue reconstruction in neuro- and muscle-degenerative diseases and their differentiation capacity into functional cells.

Key words: Mesenchymal stem cells, Muscle dystrphy, Parkinson's disease, Spinal cord injury, Stroke

Introduction

Bone marrow contains a category of nonhematopoietic cells that can be cultivated and expanded in vitro as plastic adherent cells. These cells normally provide structural and functional support for hematopoiesis, and are called bone marrow stromal cells, mesenchymal stem cells or bone marrow stromal stem cells, but an uniform term for these cells is not fixed yet. In this review, these cells are called bone marrow stromal cells (MSCs). Since they exhibit diverse characteristics and consist of heterogeneous population, their true nature is not fully understood. The majority of MSC population express mesenchymal markers, such as CD29 (beta1-integrin), CD90 (Thy-1), CD54 (ICAM-1), CD44 (H-CAM), CD71 (transferrin receptor), CD105 (SH2), SH3, Stro-1, and CD13, but a small number of cells are positive for hematopoietic surface markers, such as CD34, CD3, CD117 (c-kit) (Pittenger et al., 1999, 2000). They behave like stem cells, while their stem property is still subject for debate.

Offprint requests to: Mari Dezawa, Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, 2-1, Seiryo-machi, Sendai, Miyagi 980-8575, Japan. e-mail: mdezawa@m.tains.tohoku.ac.jp

They are easily accessible through the aspiration of the bone marrow, can be isolated from patients, and can be expanded in a large scale, both from healthy donors and patients. For example, 20-100 ml of bone marrow aspirate yields $1x10^7$ of MSCs within several weeks, which provides a plentiful number of cells.

Recently, MSCs have attracted attention mainly from two aspects. One is that they contribute to the protection of host tissue after transplantation, mainly by immune modulation and trophic effect. As MSCs originally support hematopoietic cells in the bone marrow, they produce various kinds of cytokines and trophic factors. This nature is beneficial to tissue protection, controlling apoptosis and neovascularization. In fact, when naive MSCs are transplanted to neuro-traumatic or -degeneration models, such as spinal cord injury, strokes and experimental autoimmune encephalomyelitis (EAE), or to myocardiac infarction, they migrate into the damaged site, protect tissues and partly contribute to the functional recovery (Chopp et al., 2000, 2008; Lu et al., 2001; Ohta et al., 2004, 2007; Ohnishi et al., 2007; Qu et al., 2007, 2008; Zhang et al,, 2005, 2006). The other reason is that they have an ability to differentiate into other cell kinds that will replenish lost cells in the degenerated tissue.

MSCs and immune system

MSCs have been suggested to be 'immune-privileged" because of their low expression of major histocompatibility complex (MHC) class I and no expression of class II (Uccelli et al., 2006). This characteristic of MHCs is expected to diminish the

reaction of graft rejection. There are several hopeful reports regarding the transplantation of MSCs (Liechty et al., 2000; Tse et al., 2003; Niemeyer et al., 2006; Fibbe et al., 2007; Wei et al,, 2008). Tse et al. reported that T-cells failed to detect MSCs (Tse et al., 2003), Liechty et al. showed that transplantation of human MSCs into sheep gave no specific rejection against the grafted cells (Liechty et al., 2000), and Wei et al. demonstrated that only the CD34-negative fraction derived from human bone marrow survived after grafting into rat intervertebral discs with high expression of Fas-ligand, which has been implemented in the reduction of allogenic rejection independent of apoptotic induction (Wei et al., 2008). However, there are some studies objecting to this idea. Grafted MSCs into allogeneic, MHC-mismatched mice resulted in considerable rejection (Eliopoulos et al., 2005; Nauta et al., 2006). Antigen presentation by MSCs under specific stimulation such as interferon-γ (IFN-γ) pretreatment was also reported (Chan et al., 2006). In our study, human MSCs implanted into the rat sciatic nerve tended to be rejected even with mild immunosuppression (Shimizu et al., 2007). Thus, more attention should be paid to evaluating the MSC's immune-privilege.

MSCs are known to modify their circumstances to suppress the immunoreaction. MSCs bring cell division arrest to T-cells (Glennie et al., 2005), B-cells (Corcione et al., 2006), natural killer (NK) cells (Spaggiari et al., 2006), and dendritic cells (DCs) (Ramasamy et al., 2007). The cell division arrest on T-cells by MSCs is caused by inhibition of cyclin D2 expression, thus cell cycle is arrested in the G0-G1 phase (Glennie et al., 2005). Crosstalk of MSCs and immune cells are also important for MSCs to exhibit the inhibition effect on proliferation of immune cells. T-cells and NK cells secrete IFN-γ (Krampera et al., 2006) to stimulate MSCs to produce indoleamine 2,3-dioxygenase (IDO), which inhibit proliferation of T-cells and NK cells (Krampera et al., 2006). Monocyte release IL-1ß so that MSCs are sensitized to secrete transforming growth factor-ß1 (TGF-ß1) (Groh et al., 2005). Neither system utilizes cell-cell contact mechanism, indicating that MSC-regulated inhibition of immune cell proliferation is dependent on cell-to-cell communication via soluble factors.

MSCs have been reported to affect other immunological reactions: secretion of cytokines and cytotoxicity of T-cells (Krampera et al., 2003; Rasmusson et al., 2003; Aggarwal and Pittenger, 2005; Zappia et al., 2005) and NK-cells (Spaggiari et al., 2006), maturation and antibody secretion of B-cells (Corcione et al., 2006), and maturation, antigen presentation, and activation of DCs (Ramasamy, Fazekasova et al., 2007). This mediation of immunoreactions by MSCs is considered to be regulated by secretion of molecules by MSCs, such as hepatocyte growth factor and TGF-ß1 (Di Nicola et al., 2002), IDO (Meisel et al., 2004), nitric oxide (Sato et al., 2007), and prostagrandin E2 (Aggarwal and Pittenger, 2005), demonstrated by in vitro studies. Further studies are needed to elucidate MSC's function on in vivo immunomodulation through specific molecules.

MSC's function on inhibition of immune reactions has been applied for treatment of autoimmune diseases, or diseases caused by immunological dysfunction. Graft-versus-host disease (GvHD) is one of the targets for MSC to play a critical role on immunomodulation (Le Blanc et al., 2004; Ringden et al., 2006). Co-infusion of MSCs, in addition to hematopoietic stem cells, both derived from sibling donor bone marrow, has been demonstrated to decrease the sporadic rate and severity of GvHD (Lazarus et al., 2005). This strategy is now under clinical trials, and the results from Phase I study proved the feasibility and safety of the grafting of cultured MSCs (Lazarus et al., 1995). The other study showed the similar effect on the treatment of GvHD using co-infusion technique, in which donor bone marrow was transplanted with host MSCs (Aksu et al., 2008). For other diseases, including rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis, MSCs have been applied in animal models, but the mechanism of improvement of pathological condition caused by MSC infusion is mostly unknown.

Tissue repair by MSCs implantation

Infused MSCs are reported to migrate to the vast majority of organs where these cells integrate and differentiate into tissue specific cells in the irradiated recipient (Devine et al., 2003). Also, MSCs are known to migrate into the injured site (Chen et al., 2001; Ohta et al., 2004), suggesting that they might have the property to recognize the environmental cues to make them chemotaxis to the injury tissues. Recent studies have shown the mechanisms of this chemotactic property of MSCs: MSCs have been shown to have receptors related to chemotaxis, such as chemokine (C-X-C motif) receptor 4 (CXCR4) (Sordi et al., 2005). CXCR4 plays a critical role in homing of MSCs to the bone marrow, and this phenomenon is mediated by interaction of CXCR4 and its ligand, stromal-derived factor-1 (SDF-1) (Ji et al., 2004). Recent study also showed that MSCs express functional formyl peptide receptor (FPR), which also mediates chemotactic signaling, and MSCs are chemotactically migrated under the ligand-binding assay with intracellular calcium increase, mitogen-activated protein kinases activation and Akt activation (Kim et al., 2007). Toll-like receptors (TLRs) are known to be expressed in MSCs, which regulate proliferation and differentiation of MSCs (Pevsner-Fischer et al., 2007). Besides, MSCs can pass through the basement membrane by secreting metalloproteases (MMPs), in which MSCs are activated by cytokines, including IL-1ß, TGF-ß1, and tumor necrosis factor-a (Ries et al., 2007).

MSCs have been shown to exhibit a protecting effect on injured tissues, especially in the animal models of some neurological diseases, such as spinal cord injury