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Transplantation of bone marrow stromal cells for peripheral nerve repair

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Abstract

Cell transplantation using bone marrow stromal cells (BMSCs) to alleviate neurological deficits has recently become the focus of research in regenerative medicine. Evidence suggests that secretion of various growth-promoting substances likely plays an important role in functional recovery against neurological diseases. In an attempt to identify a possible mechanism underlying the regenerative potential of BMSCs, this study investigated the production and possible contribution of neurotrophic factors by transected sciatic nerve defect in a rat model with a 15 mm gap. Cultured BMSCs became morphologically homogeneous with fibroblast-like shape after *ex vivo* expansion. We provided several pieces of evidence for the beneficial effects of implanted fibroblast-like BMSCs on sciatic nerve regeneration. When compared to silicone tube control animals, this treatment led to (i) improved walking behavior as measured by footprint analysis, (ii) reduced loss of gastrocnemius muscle weight and EMG magnitude, and (iii) greater number of regenerating axons within the tube. Cultured fibroblast-like BMSCs constitutively expressed trophic factors and supporting substances, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), collagen, fibronectin, and laminin. The progression of the regenerative process after BMSC implantation was accompanied by elevated expression of neurotrophic factors at both early and later phases. These results taken together, in addition to documented Schwann cell-like differentiation, provide evidence indicating the strong association of neurotrophic factor production and the regenerative potential of implanted BMSCs.

Keywords: Bone marrow stromal cells; Neurotrophic factor; Regeneration; Sciatic nerve; Schwann cells; Transplantation

Introduction

The ability of regenerating neurites to penetrate through the structurally altered extracellular matrix, surrounding tissues, and infiltrating cells at the injured site to reach synaptic targets plays an important role in the regeneration of peripheral nerves. Treatment of peripheral nerve defects is primarily aimed at recreating continuity of the nerve to allow axonal re-growth into the nerve stump distal to the lesion. Therapeutic approaches for the reconstruction of the peripheral nerve defects include end-to-end suturing, fascicular suturing, nerve grafts, and nerve conduits. Evidence indicates that nerve grafting is essential to the reconstruction of long nerve defect. Recently, cell transplantation has become the focus of attention, especially that of Schwann cells, and reliable outcomes have been achieved in the regeneration of the sciatic nerve (Bryan et al., 1996). In addition, the implantation of neural stem cells, bone marrow stromal cells (BMSCs), or fibroblasts has been shown to exert a beneficial effect on peripheral nerve regeneration (Shen et al., 1999; Dezawa et al., 2001; Cuevas et al., 2002, 2004; Murakami et al., 2003; Heine et al., 2004; Mimura et al., 2004). Thus, cell transplantation has been proposed as a method of improving peripheral nerve regeneration.

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Multipotent stem cells, which participate in normal replacement and repair while undergoing self-renewal, have been detected in multiple tissues in adults. Stem cells are being investigated as replacement therapy for a variety of disorders and can be engineered to deliver appropriate support to the intrinsic cells in a diseased organ system (Park et al., 2002; Murakami et al., 2003; Heine et al., 2004). Several investigators have published reports on hematopoietic stem cells and mesenchymal stem cells derived from bone marrow. Bone marrow mesenchymal stem cells, which are also known as bone marrow stromal cells, have become one of the most interesting targets for the study of tissue regeneration because of their plasticity. BMSCs are multipotential cells that contribute to the regeneration of tissues such as bone, cartilage, fat, and muscle, and to the expression of many cytokines and cellular factors (Pittenger et al., 1999; Deans and Moseley, 2000; Dormady et al., 2001; Jackson et al., 2001; Bhagavati and Xu, 2004). Several studies have shown the capacity of BMSC-derived elements to localize in the murine central nervous system (CNS) and peripheral nervous system (PNS), to integrate in these tissues, and to assume the morphology of some resident cells (Eglitis and Mezey, 1997; Brazelton et al., 2000; Mezey et al., 2000; Sanchez-Ramos et al., 2000; Lu et al., 2001; Akiyama et al., 2002; Hudson et al., 2004). Given the pluripotency of BMSCs, the prospect of using them to elicit neuroprotection has been explored in spinal cord injury (Akiyama et al., 2002), traumatic brain injury (Lu et al., 2001), and peripheral nerve injury (Dezawa et al., 2001; Cuevas et al., 2002, 2004; Mimura et al., 2004). Cell replacement, trophic factor production, extracellular matrix molecule synthesis, guidance, remyelination, microenvironmental stabilization, and immune modulation have recently been proposed as beneficial mechanisms after cell implantation. Evidence suggests that BMSCs comprise a heterogeneous population of cells, including those with small rounded, large flattened and fibroblast-like morphology, with distinct plasticity and characterization (Sanchez-Ramos et al., 2000; Hudson et al., 2004). In the present study, our purpose was to elicit the functional recovery in peripheral nerve injury over a 15 mm gap defect by fibroblast-like BMSC implantation and to evaluate the kinetics of trophic factor expression during the regenerative process.

Materials and methods

Cell preparation

BMSCs were prepared from adult female Sprague–Dawley rats and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) as described previously (Cuevas et al., 2004). Briefly, after the dissociated cells were incubated for 24 h, the non-adherent cells were removed. The adherent cells were continuously cultured and then used for the experiments.

Differentiation studies

BMSCs with fibroblast-like morphology were cultured to confluence and changed to osteogenic medium (α-MEM supplemented with 10% FBS, 0.1 μM dexamethason, 10 mM β-glycerol phosphate, 50 μM ascorbate) and adipogenic medium (α-MEM supplemented with 10% FBS, 1 μM dexamethason, 5 μg/mL insulin, 0.5 mM isobutylmethylxanthine and 60 μM indomethacin) for 3 weeks. The differentiation potential for osteogenesis was assessed by the mineralization of calcium deposits by von Kossa histochemical staining. The production of intracellular lipid droplets was detected by Oil Red O staining for the evaluation of adipogenesis (Tsai et al., 2004).

Immunocytochemical analysis

Immunocytochemical detection was performed as previously reported (Chen et al., 2004a). Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X 100. The cells were blocked with 5% nonfat milk for 30 min and then incubated with primary antibody against fibronectin (Sigma Chemical, 1:500) overnight at 4 °C. After washing, the cells were incubated with horseradish peroxidase-conjugated secondary antibody (1:500) for 1 h at room temperature. The color was developed with 3,3′-diaminobenzidine (Sigma Chemical) and observed under a light microscope.

Surgery

The animal study protocol was approved by the Animal Experimental Committee of Taichung Veterans General Hospital. Forty adult male Sprague–Dawley rats (200–250 g), divided into BMSC-treated ($n=20$) and control ($n=20$) groups, were anesthetized i.p. with sodium pentobarbital (50 mg/kg) during all surgical procedures. After skin incision, the sciatic nerve was exposed using a muscle splitting incision. With the aid of an operation microscope, the right sciatic nerve was severed and removed (15 mm) near the obturator tendon in mid-thigh (Chen et al., 2001). A 20-mm silicone tube (1 mm inner diameter, 2 mm outer diameter) was interposed into this nerve gap (Chen et al., 2000). Both proximal and distal nerve stumps were anchored into the conduit with 9-0 nylon microsutures. Most transplantation studies were carried out by suspending cells with crucial supporting substances such as collagen, laminin, fibronectin, or Matrigel (Chen et al., 2000). To get further insights into the effect of implanted cells, we used relatively inert substance, gelatin, as a suspending matrix. Mixtures of 2% gelatin (control group) or BMSCs (1×10^6 cells/tube) suspended with 2% gelatin (BMSC group) were then injected into the lumen of the conduit, using a syringe. The wound was subsequently closed in layers using 4-0 Dexon sutures. The rats were given food and water *ad libitum*. Four animals in each group were sacrificed 3 days after surgery and the rest of the animals were sacrificed 10 weeks after surgery.

Functional assessment

Functional evaluation of sciatic nerve regeneration was expressed by the sciatic function index (SFI) (Mimura et al., 2004). A technical assistant who was blinded to treatment

allocation evaluated sciatic nerve function weekly after surgery. The rats' hind feet were dipped in ink and the rats were allowed to walk across a plastic tunnel so that the footprints could be recorded on paper loaded onto the bottom of the tunnel. The distance between the third toe and heel (PL), first and fifth toe (TS), and second and fourth toe (ITS) was measured on the experimental side (EPL, ETS, and EITS, respectively) and the contralateral normal side (NPL, NTS, and NITS, respectively). The SFI was calculated as follows: $SFI = -38.3 \times (EPL - NPL)/NPL + 109.5 \times (ETS - NTS)/NTS + 13.3 \times (EITS - NITS)/NITS - 8.8$. In general, the SFI oscillates around 0 for normal nerve function, whereas around −100 SFI represents total dysfunction.

Electrophysiological study

Electrophysiological recording was determined as reported by Mimura et al. (2004) with minor modification. Briefly, 10 weeks after surgery, the rats were anesthetized i.p. with sodium pentobarbital (50 mg/kg) and the sciatic nerves were exposed. Electric stimulation (duration of 0.1 ms, intensity of 2.3 mA) was applied to the proximal side of injured sites. The compound muscle action potential was recorded in the gastrocnemius with an active monopolar needle electrode 10 mm below the tibia tubercle and with a reference needle 20 mm from the active electrode. We calculated the area of the recorded muscle response (mV × ms) and amplitude, which can be considered to reflect the amount of activated fibers. During electrophysiological study, another 6 animals were added as normal animal control (normal, $n=3$) and non-treated injured animal (transected, $n=3$) to represent the status of normal and complete injury, respectively.

Histological examination

Ten weeks after implantation, the rats were anesthetized and sacrificed and the regenerated nerves within the tubes were harvested. The nerve grafts were fixed immediately in a cold buffered 3% glutaraldehyde solution. After fixation, these nerve tissues were post-fixed in 0.5% osmium tetroxide, dehydrated, and embedded in paraffin. The paraffin sections (4 μm) were then stained with toluidine blue (Chen et al., 2000). All nerve sections were observed under a light microscope, and photographs were taken using a digital camera. Images of the histological sections (central portion of the regenerated nerve) were digitized and subsequently analyzed using an image analysis system (Image-Pro). The number of axons was counted in randomly selected fields ($80 \times 60\ \mu m^2$) at a magnification of 400×. The axon numbers were then extrapolated by applying the area algorithm to estimate the total number of axons for each nerve.

Tissue preparation

At each indicated time, the animals were anesthetized and sacrificed. The gastrocnemius muscle in both legs was removed and weighted. Protein extracts within the silicone tubes were harvested with Laemmli SDS sample buffer (Chen et al., 2004a). The protein concentration in the supernatant was then determined by Bradford assay. Total RNAs within the silicone tubes or cells were extracted with a commercial RNA extraction kit (RareRNA).

Western blot

Protein extracts were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were first incubated with 5% nonfat milk in PBS for 1 h at room temperature to reduce nonspecific binding. Membranes were washed with PBS containing 0.1% Tween-20 (PBST), and then incubated for 1 h at room temperature with the indicated antibodies including neurofilament (1:2000, Chemicon), myelin basic protein (MBP) (1:2000, Chemicon), and β-actin (1:1000, Santa Cruz Biotechnology). After the membranes were washed again with PBST buffer, a 1:10,000 dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h. The blots were developed using ECL Western blotting reagents.

Reverse transcription polymerase chain reaction (RT-PCR)

The measurement of mRNA expression was assessed by RT-PCR as previously reported (Chen et al., 2004a). Briefly, the PCR reaction was performed under the following conditions: one cycle of 94 °C for 3 min, 28 cycles of (94 °C for 50 s, 58 °C for 40 s, and 72 °C for 45 s), and then 72 °C for 5 min. The primer sets used in this study were 5′-GCTCTTCTCTTCTTTGGTGCTGTCC and 5′-GGCGTCTGCCGCCCGCGCTTCG for myelin peripheral protein zero (P0); 5′-AGCAGCAGAGCTCCGAGTCTGGTCTGCTGT and 5′-GTGTCTCACTGTGTAGATGGCCGCTGCACT for peripheral myelin protein 22 (PMP22); 5′-CCAAGGACGCAGCTTTCTAT and 5′-CTCCGGTGAGTCCTGTTGAA for nerve growth factor (NGF); 5′-GATGACCATCCTTTTCCTTACTATGG and 5′-ACTATCTTCCCCTTTTAATGGTCAG for brain-derived neurotrophic factor (BDNF); 5′-GATGGCTTTCGCAGAGCAAACAC and 5′-GCTACATCTGCTTATCTTTGGC for ciliary neurotrophic factor (CNTF); 5′-GATGAAGTTATGGGATGTCGTG and 5′-GGTCAGATACATCCACACCG for glial cell line-derived neurotrophic factor (GDNF); and 5′-TCCTGTGGCATCCACGAAACT and 5′-GGAGCAATGATCTTGATCTTC for β-actin.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of NGF, BDNF, CNTF, and GDNF in the supernatants were measured with an enzyme immunoassay kit (R&D Systems), following the procedure provided by the manufacturer.

Extracellular matrix detection

The preparation and measurement of extracellular matrix components in cultured cells was performed as previously reported (Milner and Campbell, 2002). Briefly, BMSCs were

maintained with DMEM/10% FBS for 3 days. Before being harvested, cells were lysed in water for 30 min at 37 °C. The excess cell debris was removed by washing the substrate several times with PBS. The remaining extracellular matrix components were subjected to ELISA with antibodies against collagen I, collagen IV, fibronectin, and laminin (all obtained from Sigma Chemical).

Statistical analysis

Data are expressed as means ± standard error. A one-way analysis of variance (ANOVA) was performed for multiple comparisons, and if there was significant variation between treatment groups, the mean values were compared with respective control using Dunnett's test. A level of $p < 0.05$ was considered statistically significant.

Results

Characterization of cultured BMSCs

BMSCs obtained from the bilateral femurs and tibias of adult female rats comprised heterogeneous groups of cells after seeding and growing in culture plates. After initial plating, the adherent cells exhibited small rounded, spindle-shaped, or large flattened morphology (Fig. 1A). Most cells grew and exhibited fibroblast-like morphology in reaching confluence. Interestingly, the small rounded cells adhered to the surface of these cell layers (Fig. 1B). These small rounded cells disappeared after repeated passage, whereas the fibroblast-like cells became enriched. At passage 4, the fibroblast-like cells became morphologically homogeneous, with more than 95% purity (Fig. 2A). These cells were positive in fibronectin-immunoreactivity (Fig. 2B) and negative in nestin-immunoreactivity and Thy1.1-immunoreactivity (data not shown) and showed adipogenic (Fig. 2C) and osteogenic (Fig. 2D) potential, demonstrating mesodermal characteristics. Our characterization revealed the heterogeneity of the cell population in cultured BMSCs. Since the fibroblast-like population of BMSCs proliferated continuously in regular culture conditions and easily became enriched, it was used for further *in vivo* and *in vitro* studies.

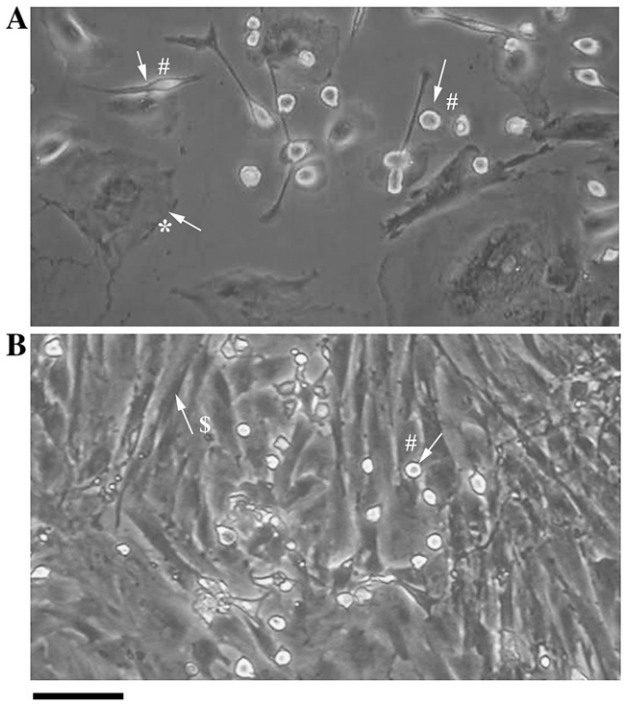

Fig. 1. Morphological characteristics of cultured BMSCs. Cultured BMSCs exhibited small rounded, large flattened and fibroblast-like morphology in the first passage of initial culture (A) and confluent culture (B). Representative cells with small rounded morphology, large flattened morphology, and fibroblast-like morphology are indicated by #, *, and $, respectively. Phase contrast, scale bar = 100 μm.

BMSC implantation promotes regeneration

To evaluate the efficacy of the fibroblast-like population of BMSCs on functional improvement in peripheral nerve injured rats, we first compared the SFI between the BMSC-treated group and the control group. Typical walking tracts obtained from normal, vehicle-treated (control), and BMSC-treated animals at 10 weeks after nerve transection are depicted in Fig. 3A. BMSC implantation improved functional recovery at each time point assessed in comparison with vehicle-treated transected sciatic nerve (Fig. 3B). In the electrophysiological study, the compound muscle action potential was recorded in experimental animals 10 weeks after surgery (Fig. 4A). The impaired conduction latency after injury was improved in the BMSC-implanted animals (Fig. 4B). The percentage of activated fibers was higher in the BMSC-implanted group than the control group, as evidenced by the increases of whole response area under the curve (Fig. 4C) and amplitude (Fig. 4D). After 10 weeks of nerve injury, the right gastrocnemius muscle degenerated and lost weight. When comparing the gastrocnemius muscle of the left intact side between different treated groups, we did not observe the occurrence in hypertrophy of the uninjured sides. Sciatic nerve injury-induced weight loss of the gastrocnemius muscle was ameliorated by BMSC transplantation (Fig. 5).

Macroscopic examination showed obvious regeneration of nerve tissues in 8 of the 16 tubes 10 weeks after surgery in the control group, whereas 13 of the 16 tubes exhibited such regeneration in the BMSC-treated group. Histological examination revealed that the regenerated nerve trunks selected from both groups displayed a similar ultrastructural organization (Fig. 6B and C). In most of the regenerated nerve sections from the two groups, the axonal diameter and maturity were still not complete in comparison with those in the normal sciatic nerve (Fig. 6A). Inspection of the transverse axial section in the central portion of the regenerated nerve sections in the BMSC-implanted group showed that the number of regenerated axons was significantly greater when compared with that of the control group (Fig. 6D). It should be noted that the increased number of axons might almost result from the regenerated nerves or likely axonal bifurcation. This issue was not completely answered in this study. Generally, the regeneration process requires the sequential expression of specific growth-associated and function-asso-

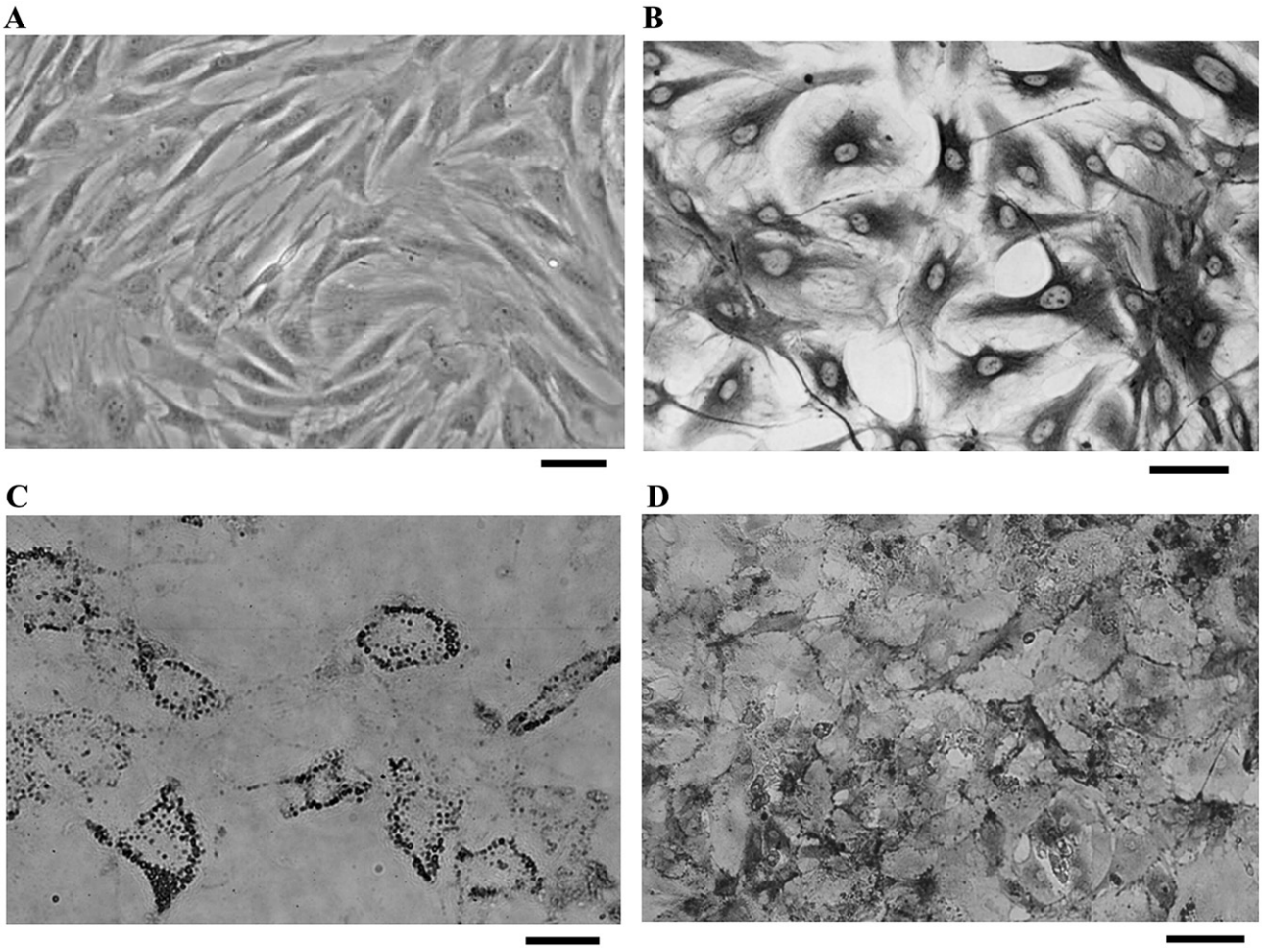

Fig. 2. Mesodermal differentiation of BMSCs. Cultured BMSCs exhibited large, fibroblast-like morphology (A, phase contrast) and fibronectin-immunoreactivity (B) at passage 4. BMSCs (passage 4) were subjected to differentiation induction for 3 weeks. After differentiation, the accumulation of intracellular lipid droplets was detected by Oil Red O staining (C) and the deposition of calcium was determined by von Kossa histochemical staining (D). Scale bar = 50 μm.

ciated genes in both sheath cells and neurons. Mammalian neurofilaments are the major components of the neuronal cytoskeleton and play an active role in axon outgrowth. Among them, low-molecular-weight neurofilament (NF-L) forms the core of the neurofilament (Liem and Hutchison, 1982). On the other hand, mature neurons require proper myelination. MBP, P0, and PMP22 are thought to be specific to Schwann cells essential for myelin formation (Pedraza et al., 1997). To further monitor the process of peripheral nerve regeneration, the expression of NF-L, P0, PMP22, and MBP was measured. Compatible with our histological examinations, results of Western blot showed elevated protein levels of both NF-L (1.9×) and MBP (1.7×) in the BMSC-implanted group (Fig. 7). By RT-PCR analysis, the same group expressed elevated levels of P0 and PMP22 (Fig. 9C). The findings suggest that BMSC implantation promoted the regeneration of the sciatic nerve, including axonal outgrowth and remyelination.

BMSCs produce supporting factors in vitro and in vivo

Functional benefits derived from cell transplantation include the ability to differentiate or integrate into local tissue and act as stem or progenitor cells, or to induce endogenous restorative activity by evoking the production of trophic and/or supporting substances (Shen et al., 1999; Brazelton et al., 2000; Sanchez-Ramos et al., 2000; Marcol et al., 2003; Chen et al., 2004b). Among them, neurotrophic factors and extracellular matrix proteins play an important role in the survival and proliferation of axons and Schwann cells and are regarded as axonal growth-promoting factors that stimulate axonal regeneration. Cultured fibroblast-like populations of BMSCs synthesized and secreted detectable neurotrophic factors ($p < 0.01$), including NGF, BDNF, GDNF, and CNTF (Figs. 8A and B) and produced extracellular matrix components ($p < 0.01$), including collagen I, collagen IV, fibronectin, and laminin (Fig. 8C). In contrast, there were no detectable levels of Schwann cell-specific and myelination-associated gene expression such as P0 and PMP22 (Fig. 8A). To further assess the neurotrophic factor-producing ability of BMSCs after transplantation, total RNAs were recovered from tubes and subjected to RT-PCR analysis for the measurement of the abovementioned genes. Intact sciatic nerves constitutively expressed P0, PMP22, NGF, BDNF, GDNF, and CNTF (Fig. 9A). After 3 days of transplantation, the expression of NGF, BDNF, GDNF, and CNTF but not P0 and

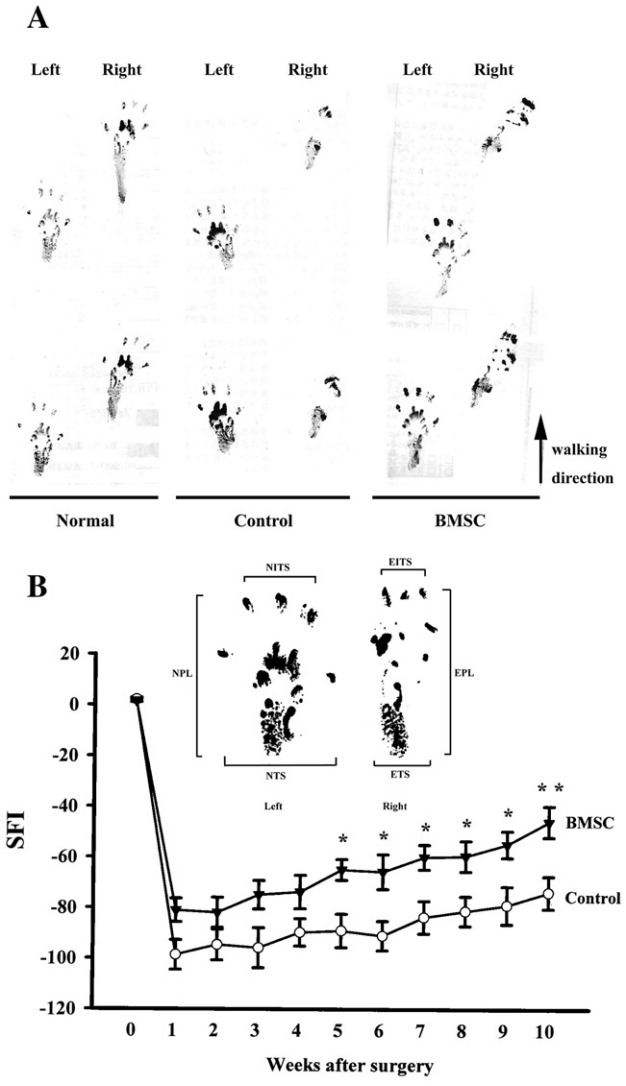

Fig. 3. Walking tract analysis. Typical tracks were obtained from normal, control, and BMSC-implanted animals 10 weeks after surgery (A). The calculated parameters are depicted in the upper panel of B as described in Materials and methods. SFI was determined in control ($n=8$) and BMSC-implanted ($n=13$) groups weekly after surgery (B). *$p<0.05$ and **$p<0.01$.

PMP22 was detected in tubes in the BMSC-treated group (Fig. 9B). However, their expressions were below detectable levels in the tubes in the control group (data not shown). At the end of the experimental period (10 weeks after surgery), we detected the expression of P0, PMP22, NGF, BDNF, GDNF, and CNTF in the regenerated tissues of both groups. In the BMSC-treated group, the expression levels of these genes were significantly elevated when compared with those of the control group (Fig. 9C). These findings suggest that a higher level of neurotrophic factor expression was accompanied by BMSC implantation within the regenerated tissues.

Discussion

Recent experimental studies demonstrate the beneficial effect of bone marrow aspirates in regeneration of damaged nerve tissues (Shen et al., 1999; Dezawa et al., 2001; Lu et al., 2001; Akiyama et al., 2002; Cuevas et al., 2002, 2004; Mimura et al., 2004). Bone marrow-derived cells can be aspirated directly from donors and comprise heterogeneous populations of cells with distinct plasticity. These cells express multipotential and differentiate into osteoblasts, cartilage, skeletal muscle cells, endothelium, cardiac muscle cells, hepatocytes, and neuroectoderm both *in vitro* and *in vivo* (Eglitis and Mezey, 1997; Petersen et al., 1999; Pittenger et al., 1999; Brazelton et al., 2000; Mezey et al., 2000; Sanchez-Ramos et al., 2000; Jackson et al., 2001; Bhagavati and Xu, 2004). In the present study, we successfully isolated BMSCs from adult female rats and found at least two populations of cell types within them. Small-rounded and spindle-shaped BMSCs exhibited nestin immunoreactivity but no fibronectin immunoreactivity and demonstrated neural potential (data not shown). However, the percentages of this type of cell rapidly decreased with repeated passage in regular serum culture. After culturing for *ex vivo* expansion, the remaining cells became morphologically homogeneous with fibroblast-like shape. These fibroblast-like BMSCs showed fibronectin immunoreactivity and no nestin immunoreactivity and demonstrated adipogenic and osteogenic potential. Sanchez-Ramos et al. (2000) and Hudson et al. (2004) also reported similar findings. In this study, we demonstrated the promotion in repairing 15-mm nerve defects by using cultured fibroblast-like BMSCs, as evidenced by (i) improved walking behavior as measured by footprint analysis, (ii) the reduction of gastrocnemius muscle atrophy as measured by muscle weight, (iii) reduced attenuation of EMG magnitude in the gastrocnemius muscle, and (iv) an increased number of axons with the tube. Results of this study indicate that fibroblast-like BMSCs have great potential to promote regeneration of peripheral nerve injury. The artificial graft made with cultured fibroblast-like BMSCs represents an alternative method for the reconstruction of a long distance defect in a peripheral nerve. However, there should be noted that our assays cannot afford the fully explanation in eliciting functional recovery in sciatic nerve. Nerve conduction measurement is a direct evidence for the study of nerve transmission. The EMG analysis presented simply addresses the degree of neuromuscular transmission at the neuromuscular junction. Rather than atrophy, contractile strength of the gastrocnemius muscle in response to proximal sciatic stimulation would have been a much better indicator of improved regeneration. In addition, morphometric analysis of individual nerve fiber diameter using fiber cross-sectional area and/or traced perimeter is usually used for the determination of myelination. The increased number of axons within the central portion of the regenerated nerve trunks might almost result from the regenerated nerves or likely axonal bifurcation. Currently, these issues were not completely answered in this study.

Studies have shown that transplantation of *in vitro*-differentiated BMSCs into the cut ends of sciatic nerves is capable of supporting nerve fiber regeneration (Dezawa et al., 2001; Mimura et al., 2004). In addition, the non-manipulated adult BMSCs also exert a beneficial effect against peripheral nerve injury (Cuevas et al., 2002, 2004). However, the detailed molecular mechanisms responsible for the therapeutic benefits

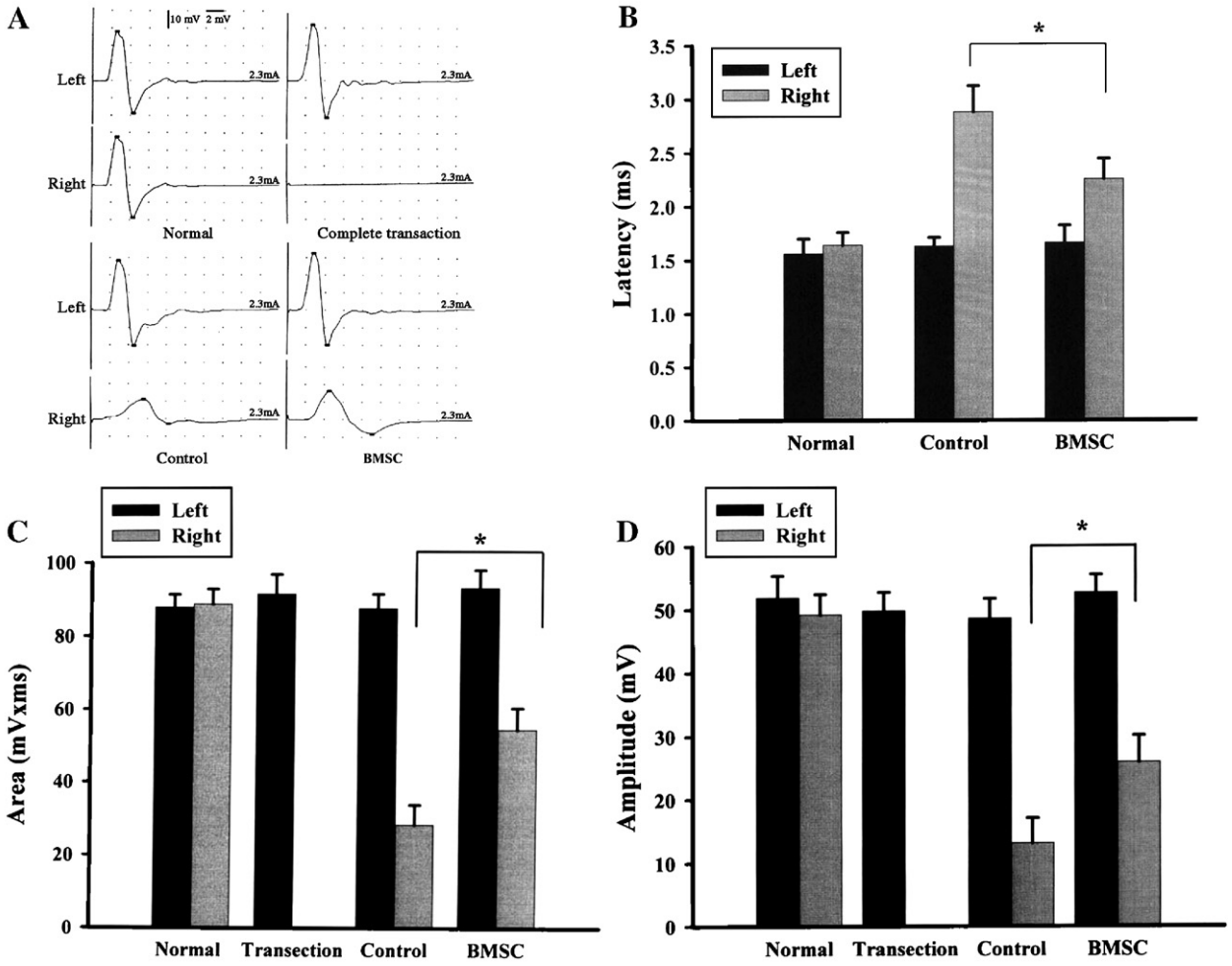

Fig. 4. Electromyographic (EMG) analysis. The representative EMG recording waves (intact left and injured right) were elicited in response to electrical stimulation of the sciatic nerve proximal to the lesion in normal ($n=3$), complete transection ($n=3$), control ($n=8$), and BMSC-implanted ($n=13$) animals 10 weeks after surgery (A). The time calibration bar is 2 ms and amplitude calibration bar is 10 mV. The stimulation intensity is 2.3 mA and the duration is 0.1 ms. The quantitative results of latency (B) and compound muscle action potential determined by the calculation of (i) mean area (mV × ms) under the whole response curve and (ii) EMG amplitude are depicted in panel C and panel D, respectively. *$p<0.05$.

of non-manipulated BMSC transplantation against peripheral nerve injury are largely unclear. PNS regeneration occurs mainly through a series of reactions produced by activated Schwann cells so that the axons of the proximal nerve stump grow through the distal stump in close contact with the Schwann cell bands. As a result of these integrated functions, nerve regeneration can be induced and the reinnervated Schwann cells can revert to an axon-associated phenotype (Agius and Cochard, 1998; Mimura et al., 2004). Therefore, the differentiation of implanted BMSCs into Schwann-like cells is one of several possibilities. In this study, the signals of BrdU were almost exhausted in the tubes of the BMSC-treated group 10 weeks after transplantation (data not shown) implying that we were unable to evaluate the fate of transplanted BMSCs. Cuevas et al. (2002) found that the improvement in a peripheral nerve defect was associated with the less than 5% of BMSCs that differentiated into Schwann cells. Most of the implanted BMSC population remained in an undifferentiated state. Therefore, there did not seem to be enough differentiation of BMSCs into Schwann-like cells to explain their beneficial effect against sciatic nerve injury.

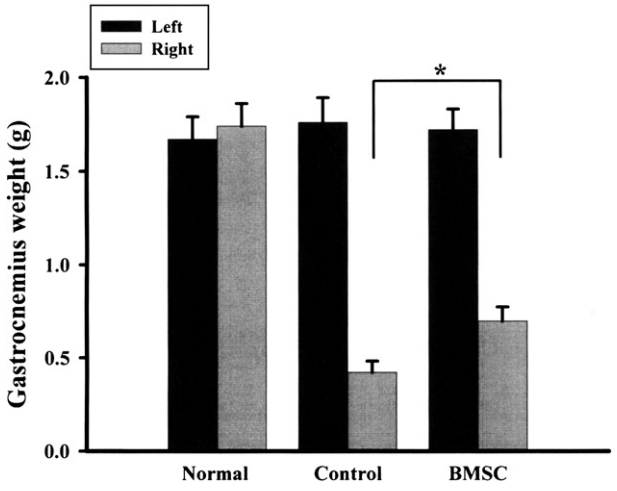

Fig. 5. Measurement of gastrocnemius muscle. The gastrocnemius muscle of both sides (intact left and the injured right) was removed and weighted in normal ($n=3$), control ($n=8$) and BMSC-implanted ($n=13$) animals 10 weeks after surgery. *$p<0.05$.

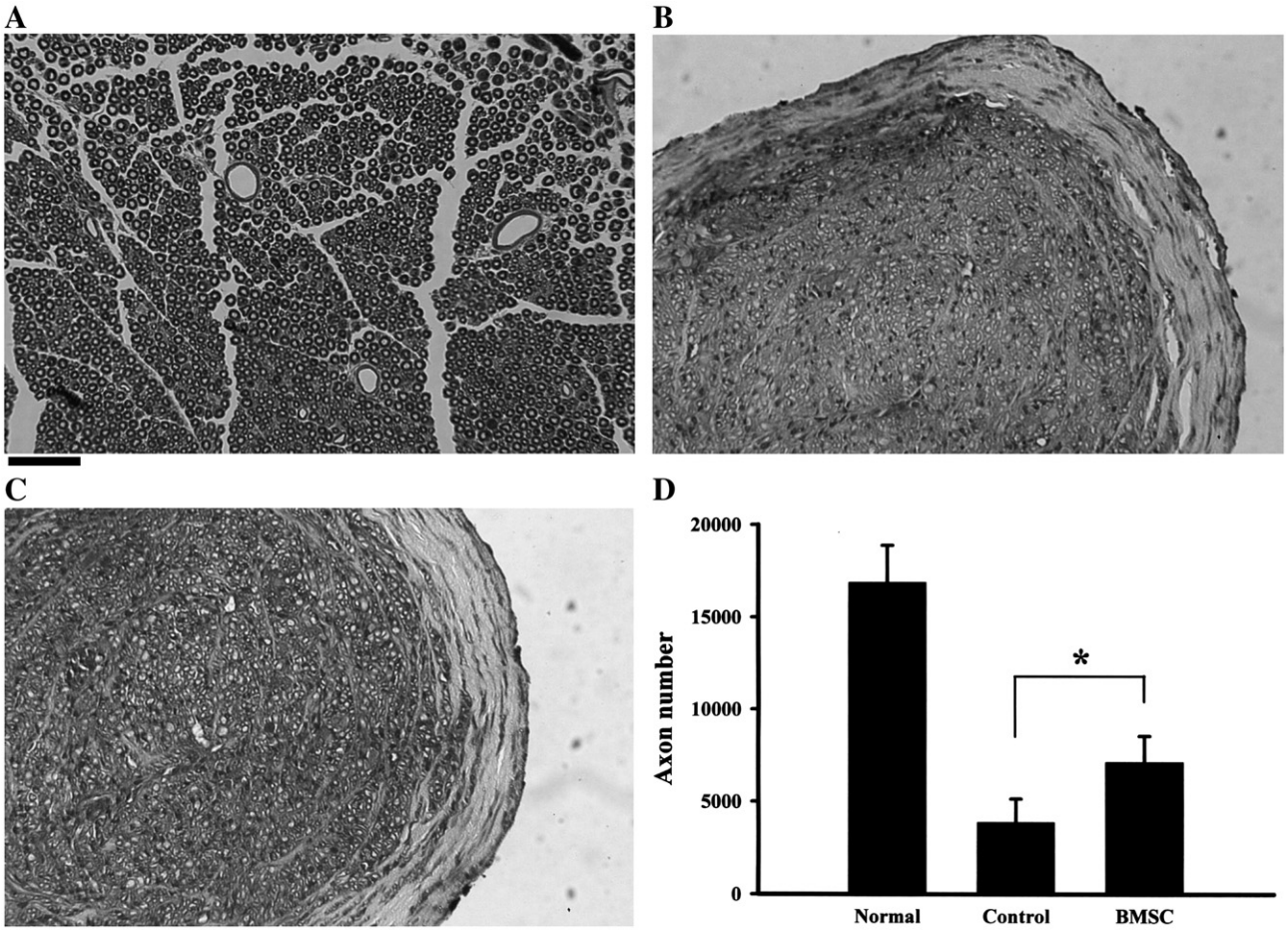

Fig. 6. Histological examination. Cross sections of normal sciatic nerve (A) and regenerated tissues (central portion within tubes) from control (B, $n=4$) and BMSC-implanted (C, $n=5$) animals 10 weeks after surgery were stained with toluidine blue. Bar graphs show the quantitative results of axonal counting (D). *$p<0.05$.

During peripheral nerve development and the regenerative process, trophic factors and supporting substances are essential molecules which play important roles (Chen et al., 2000, 2001; Lee et al., 2003; Marcol et al., 2003). Among them, neurotrophic factors and extracellular matrix proteins are regarded as growth-promoting factors that stimulate neurite outgrowth and Schwann cell proliferation and migration (Anton et al., 1994). Evidence indicates that the administration of neurotrophic factors and/or extracellular matrix proteins can promote early peripheral nerve regeneration, particularly for the regeneration of long nerve defect (Chen et al., 2000; Lee et al., 2003). Thus, in addition to Schwann-like cell differentiation, an alternative possibility for the beneficial effect is the production of trophic and supporting substances by implanted fibroblast-like BMSCs. In our study, cultured fibroblast-like BMSCs had the ability to synthesize and release neurotrophic factors such as NGF, BDNF, GDNF, and CNTF, as well as to produce extracellular matrix proteins such as collagen I, collagen IV, fibronectin, and laminin, *in vitro*. In comparison to the control group, the implanted BMSCs could express detectable levels of these neurotrophic factors in the early phase of the regenerative process. Similar to that of the *in vitro* gene expression profile, the presence of the Schwann cell-specific and myelin-associated P0 and PMP22 genes was not detected in the implanted BMSCs at this stage. In the later phase of the regenerative process (10 weeks after surgery), the regenerated tissues containing BMSC implantation were capable of synthesizing elevated levels of neurotrophic and myelin-associated factors. In the present study, we were unable to identify the specific cellular sources of the elevated neurotrophic factors derived from either implanted BMSCs

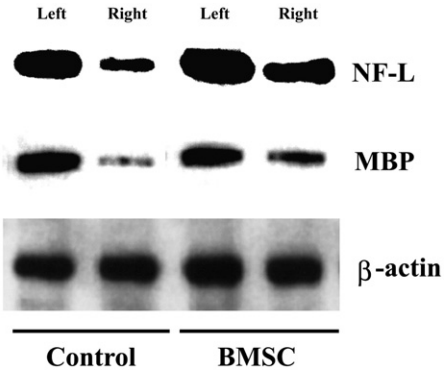

Fig. 7. Nerve fiber protein analysis. Cellular proteins were extracted from control ($n=4$) and BMSC-implanted ($n=8$) animals 10 weeks after surgery and subjected to Western blot with antibodies against neurofilament-L (NF-L), MBP, and β-actin. The left is the intact side and the right is the injured side.

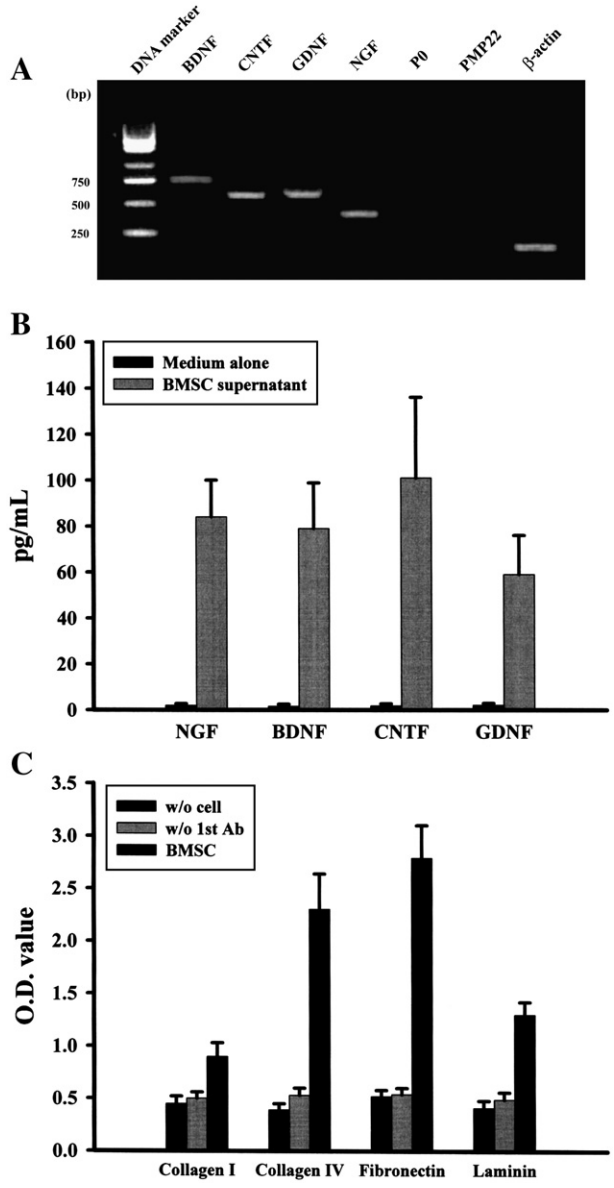

Fig. 8. Neurotrophin and extracellular matrix analysis of cultured BMSCs. Total cellular RNAs were extracted from confluent BMSCs (passage 4) and subjected to RT-PCR for the detection of BDNF, CNTF, GDNF, NGF, P0, PMP22, and β-actin. One of three independent experiments is shown (A). BMSCs (passage 4) were cultured for 3 days ($n=4$). The supernatants were collected and subjected to ELISA for the measurement of NGF, BDNF, CNTF, and GDNF (B) and the remaining cells were subjected to ELISA for the detection of collagen I, collagen IV, fibronectin, and laminin (C). In the analysis, those without cells (w/o cells) and without 1st antibody addition (w/o 1st Ab) were used as negative control; $n=4$.

or other regenerated tissues or both. However, our findings were in agreement with the correlation between higher neurotrophic factor production and BMSC implantation. Increasing evidence indicates that exogenous administration or enhanced expression of extracellular matrix proteins or neurotrophic factors stimulates peripheral regeneration (Chen et al., 2000, 2001; Lee et al., 2003; Marcol et al., 2003). In consideration of the well-known function of neurotrophic factors (Anton et al., 1994) and our *in vitro* characterization of BMSCs, those neurotrophic factors produced by cultured BMSCs might be possible protective candidates. Therefore, the trophic factor-producing ability might partly explain the beneficial effect of BMSC implantation against peripheral nerve injury. These factors could be expressed first by implanted BMSCs leading to the prevention of death of injured neurons and activation of resident Schwann cells and could also signal local tissues to release other growth-promoting substances favoring the regeneration.

The PNS retains a considerable capacity for regeneration, but this requires a microenvironment favorable to axonal outgrowth. A variety of conduits have been employed in attempts to bridge nerve gaps. The synthetic nerve conduit bridges the gap between the nerve stumps and directs and supports nerve regeneration. The conduits may be implanted empty, or they may be filled with growth factors, cells or

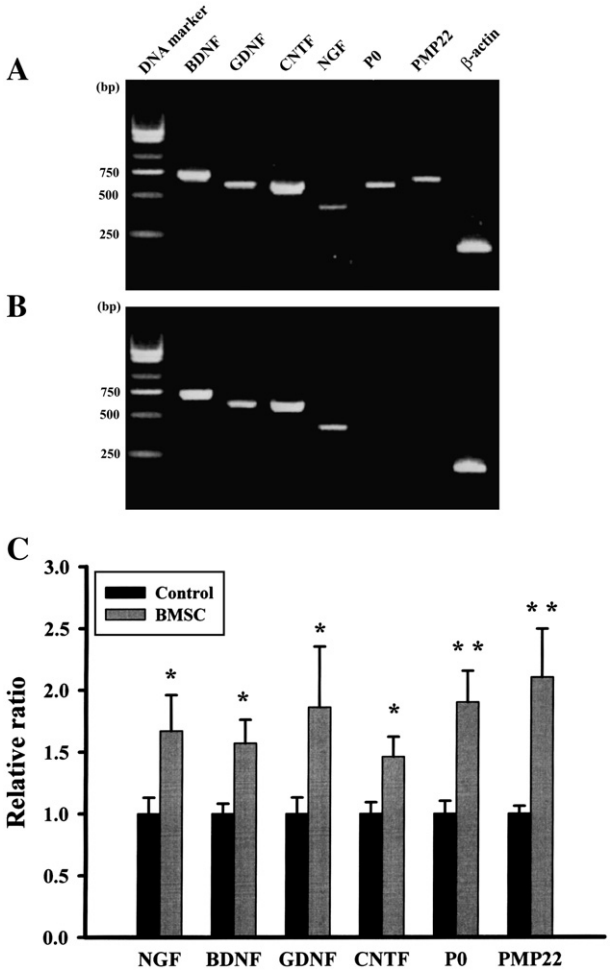

Fig. 9. RT-PCR analysis of gene expression in regenerated tissues. Total cellular RNAs were extracted from intact sciatic nerves (A) and BMSC-treated silicone tubes removed 3 days after implantation (B). After RT-PCR, the resultant DNA fragments were resolved by electrophoresis in a 1.5% agarose gel and stained with EtBr. One of four separate experiments is shown. Total RNAs were extracted from control ($n=4$) and BMSC-treated ($n=8$) tubes obtained 10 weeks after implantation and subjected to RT-PCR (C). The quantitation was performed by a computer image system. The ratio was calculated by the co-amplified β-actin internal control. The relative ratio was compared to each control (designated as 1). $*p<0.05$ and $**p<0.01$.

fibers. An artificial graft can meet many of the needs of regenerating nerves by concentrating trophic factors, reducing cellular invasion and scarring of the nerve, and providing directional guidance to prevent neuroma formation or excessive branching. Evidence suggests that the incorporation of Schwann cells into nerve conduits is the best choice (Bryan et al., 1996). In addition, a growing body of evidence implies that other cell types such as embryonic stem cells, neural stem cells, mesenchymal stem cells, olfactory ensheathing cells, or fibroblasts also promote nerve recovery. Although they differ in efficacy, their beneficial effects have been proposed for cell replacement, microenvironment stabilization, endogenous repair ability activation, or supporting substance production (Bryan et al., 1996; Shen et al., 1999; Cuevas et al., 2002, 2004; Murakami et al., 2003; Heine et al., 2004; Mimura et al., 2004). The results of this study suggest that the transplantation of fibroblast-like BMSCs may have a promoting effect on the regeneration of transected nerves. The beneficial effects of implanted BMSCs are strongly correlated with the production of trophic substances.

Acknowledgments

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