Harvesting of bone marrow mesenchymal stem cells from live rats and the *in vitro* differentiation of bone marrow mesenchymal stem cells into neuron-like cells

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Abstract

In the bone marrow, there are certain populations of stem cell sources with the capacity to differentiate into several different types of cells. Ideally, cell transplants would be readily obtainable, easy to expand and bank, and capable of surviving for sufficient periods of time. Bone marrow mesenchymal stem cells (BM-MSCs) possess all of these characteristics. One of the most important benefits in using BM-MSCs is the possibility of autologous therapy. Recently, numerous studies have evaluated strategies that attempt to promote axonal regeneration in central nervous system (CNS) injuries. Among these strategies, cell transplantation is considered to be the most effective way. The differentiation of stem cells into different neural lineages (such as astrocytes and neural like cells) before transplantation has a critical role in achieving the best results in studies of CNS injury. In this study, BM-MSCs were isolated from bone marrow aspirates taken from the femurs of 103 live rats. The detection of BM-MSCs was performed with RT-PCR analysis, and they were then induced to differentiate into neuron-like cells in serum-withdrawal medium over a two week period using a multistep protocol. In addition to the morphological evaluation of differentiated cells, the process of neural differentiation was proven by immunocytochemical techniques using primary antibodies to Neuron Specific Enolase (NSE) to assess cell differentiation. PT-PCR analysis was performed for the evaluation of neural specific genes, which included NSE, MAP2, nestin, and ß-tubulin. Morphological evaluations detect neuron like cells with longitudinal processes. Using RT- PCR and immunocytochemistry assays, neuron specific genes and proteins following treatment of cells in serum-withdrawal induction medium was expressed. This study showed a simple and practical method for differentiating MSCs into neuron like cells, and feasibility of aspirate bone marrow from a live rat for autologous grafting.

Introduction

Recently, numerous studies have evaluated strategies that attempt to promote axonal regeneration in different kinds of central nervous system (CNS) injuries, which include the spinal cord and the brain. Among these strategies, cell transplantation is considered to be the most effective technique at present (Ohta *et al*., 2004). To date, several different kinds of cells have been transplanted into the spinal cord and brain to induce regeneration of cells, which include Schwann cells (Martin *et al*., 1996; Firouzi *et al*., 2006), cells of the embryonic spinal cord (Iwashita *et al*., 1994), olfactory ensheathing cells (Doucette, 1995), macrophages (Lazarov-Spiegler *et al*., 1996; Rapalino *et al*., 1998), choroid plexus ependymal cells (Ide *et al*., 2001; Kitada *et al*., 2001), neural stem cells (Ogawa *et al*., 2002), and bone marrow stromal cells (Ohta *et al*., 2004). Among many different kinds of cells, embryonic neural stem cells have been most enthusiastically studied (Kordower *et al*., 1995; McDonald *et al*., 1999). However, several difficulties,

including ethical issues and clinical complications, such as immune reactions and the formation of teratomas, make it impossible to use human fetal tissue as a practical and immediate source of therapeutic treatment (Bjorklund and Linvall, 2000; Constantini and Young, 1994; Krause *et al*., 2001).

In the bone marrow, there are certain populations of stem cell sources including hematopoietic stem cells, marrow stromal stem cells (Prockop, 1997) and multi-potent adult progenitor cells (Herzog *et al*., 2003; Muguruma *et al*., 2003). These multipotent adult progenitor cells, which comprise approximately 0.125% of the total number of marrow cells (Blanco *et al*., 2001), are multipotent stem cells with the capacity to differentiate into different cell types under specific experimental conditions (Hofstetter *et al*., 2002; Prockop, 1997; Wu *et al*., 2003). These cell types include osteoblasts, adipocytes, chondrocytes (Phinney *et al*., 1999), skeletal muscle fibers (Ferrari *et al*., 1998), cardiomyocytes (Orlic *et al*., 2001), hepatocytes (Petersen *et al*., 1999), neural lineages, including neurons and astrocytes (Sanchez-Ramos *et al*., 2000; Deng *et al*., 2001; Schwarz *et al*., 1999; Woodbury *et al*., 2000), and epithelial cells of the lung and intestinal tract (Krause *et al*., 2001). Ideally, cell transplants would be readily obtainable, easy to expand and bank, and capable of surviving for a sufficient period to facilitate appropriate axonal regeneration (Fischer *et al*., 2001). Bone marrow mesenchymal stem cells (BM-MSCs) have all of these characteristics.

Experimental studies have shown that BM-MSCs, when transplanted into the brain of adult rats, can reduce functional deficits after stroke (Chen *et al*., 2001) or traumatic injury (Chopp *et al*., 2002; Mahmood *et al*., 2004; Mahmood *et al*., 2002). Because of their ability to differentiate into a variety of cells, the ease of their isolation and expansion, and their potential use for clinical application, efforts have increased to better understand the biology of BM-MSCs. On the whole, undifferentiated BM-MSCs demonstrate glial characteristics at the site of the lesion and there is no evidence that the engrafted MSCs differentiate into neural cells (Ohta *et al*., 2004). So, induction of differentiation of MSCs to neuron like cells and then transplantation of such cells may contribute to better recovery of function in animal models of neurotrauma. Additionally, the ability to harvest stem cells successfully from live rats would enable the use of these cells for autologous transplantation studies and eliminating immune reactions.

Materials and Methods

One hundred and three male Fischer-344 Wistar rats were used in this experiment. The animals were between eight and 12 weeks of age and weighed between 300 and 350 g. All animals were housed separately in a large, well-lit laboratory that was controlled for temperature (21°C) and maintained with a daily photoperiod of 12 hr of light between 6:00 am and 6:00 pm. Each animal had *ad libitum* access to food and water and was fed on a complete and balanced standard laboratory diet. All experiments were performed in accordance with the European Animal Care Committee guidelines.

Isolation of BM-MSCs

BM-MSCs were isolated from bone marrow aspirates taken from the femur. Rats were anesthetized with intramuscular (im) ketamine (75 mg/kg) and xylazine (10 mg/kg). A small hole (1-1.5 mm) in the femur was created with burr micromotor drill following the skin incision (5 mm), and 0.5-1 ml of bone marrow was aspirated using a 2 ml syringe with a 21-gauge needle supplemented with 500-750 IU heparin. The samples were diluted in L-15 medium (2 ml) containing 3 ml Ficoll. The cells in the mononuclear layer were collected after centrifugation (2,000 rp, for 15 min) and were resuspended in 2 ml serum-free medium. After centrifugation (2,000 rpm, for 15 min), the cells were suspended in 1 ml Neural Progenitor Maintenance Media (NPMM).

Differentiation of BM-MSCs *in vitro*

BM-MSCs were induced to differentiate into neuron-like cells in serum-withdrawal medium over two weeks using a multistep protocol. BM-MSCs seeded at a density of 3000-4000 cells/cm^2 and were treated with the first step medium that contained DMEM/F12 (Gibco) supplemented with 1% insulin/transferrin/selenium (ITS) supplement (Gibco), 2% B27 supplement (Gibco), 10 ng/ml basic fibroblast growth factor (bFGF; Peprotech) and 5 µM retinoic acid (RA; Sigma). After one week, the medium was changed to a medium that contained ITS and B27 supplements with 1 µM dibutyryl cyclic AMP (dbcAMP; Sigma) and 100 µM ascorbic acid (AA; Sigma). Cells were incubated in this medium for four days. The third stage of treatment of the cells was performed in medium that contained ITS and B27 with 10 µM forskolin (Sigma), 0.1 µM isobutylmethylxanthine (IBMX; Sigma) and 100 µM AA for three days. All culture media were changed every two to three days. In addition to the morphological evaluation of differentiated cells, to prove that neural differentiation had occurred they were processed using immunocytochemistry techniques with primary antibodies to NSE. For immunocytochemical analyses, cells were fixed with 4% paraformaldehyde on the third day of differentiation and incubated for 1 hr in 4°C, permeabilized with Triton X-100 (0.2%) and processed for immunocytochemistry using primary antibodies to ß-Tubulin III at a dilution of 1:50 (mouse monoclonal antibody; Chemicon), and NFM at 1:500

(mouse monoclonal; Sigma). For fluorescence studies, fluorescein isothiocyanate (FITC)-conjugated (Sigma) anti-mouse secondary antibody (1:500) was applied. For the confirmation of cell differentiation, a reverse transcription polymerase chain reaction (RT-PCR) analysis was performed for the evaluation of neural specific genes that included neuron specific enolase (NSE), MAP2, nestin, and ß-tubulin (Fig. 6). For RT-PCR analysis, the total cellular RNA was extracted using TRI-reagent BD (Sigma). Synthesis of cDNA was carried out with M-MuLV reverse transcriptase (RT) and a random hexamer as the primer, in accordance with the instructions of the manufacturer (Fermentas). PCR amplification was performed using a standard procedure with Taq DNA Polymerase (Fermentas) with denaturation at 94°C for 15 s, annealing at 55°C or 60°C for 30 s according to the primers, and extension at 72°C for 45 s.

Results

Immunocytochemistry analysis

Differentiated stem cells expressed NSE, nestin, ß-tubulin, NFM and MAP-2 (Fig. 1, 2, 3, 4 and 5). This neuron-specific immunostaining confirmed the differentiation of BM-MSCs to neural lineages. The viability of the cells that were prepared for transplantation was in excess of 90%, as determined by trypan blue exclusion.

Total RNA isolation and RT-PCR analysis

The number of cycles varied between 30 and 40, depending on the abundance of the particular mRNA. The primers and product lengths are listed in Fig 3. Using RT-PCR, we defined the expression of several neural specific genes that included NSE, nestin, ß-tubulin, and MAP-2 three days after induction of differentiation (Fig. 6).

Discussion

Several experimental strategies to treat CNS are being investigated, and cell therapy may give the best results for improving clinical situation of a patient with paralysis (McKerracher, 2001). Among the different kinds of cells that have been used for this purpose, BM-MSCs seem to be one of the best candidates, because they are relatively easy to isolate, can be expanded rapidly *in vitro*, and differentiated into multiple cell types (Hofstetter *et al*., 2001). In addition, the differentiation of BM-MSCs into neuron-like cells, which express markers that are typical of mature neurons, has been reported (Woodbury *et al*., 2000, Deng *et al*., 2001). One of the most important benefits in the use of BM-MSCs is the possibility of autologous therapy, which avoids graft rejection, the risk of viral antigens, and the ethical concerns that are associated

Figure 1: In vitro differentiation of BM-MSCs into neuron-like cells three days after the induction of differentiation. Differentiated cells showed positive immunoreactivity for the NSE neuron-specific marker.

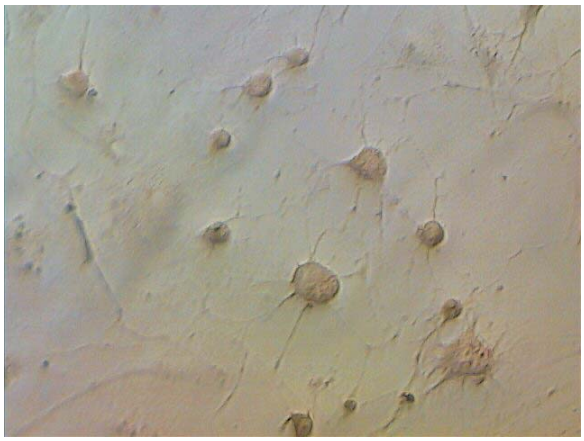


Figure 2: In vitro differentiation of BM-MSCs into neuron-like cells. Differentiated cells showed positive immunoreactivity for the neuron-specific marker, ß-tubulin III.

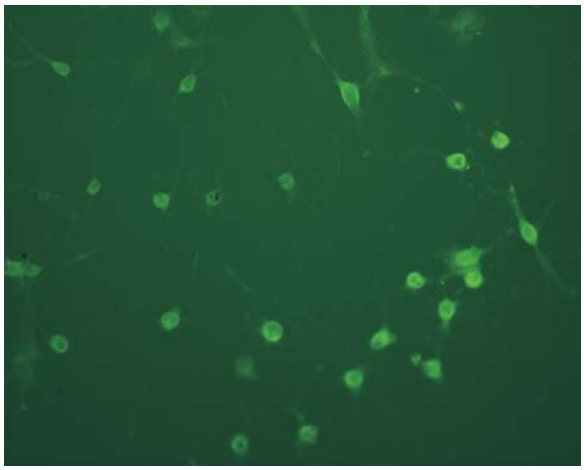


Figure 3: In vitro differentiation of BM-MSCs into neuron-like cells three days after the induction of differentiation. Differentiated cells showed positive immunoreactivity for MAP-2.

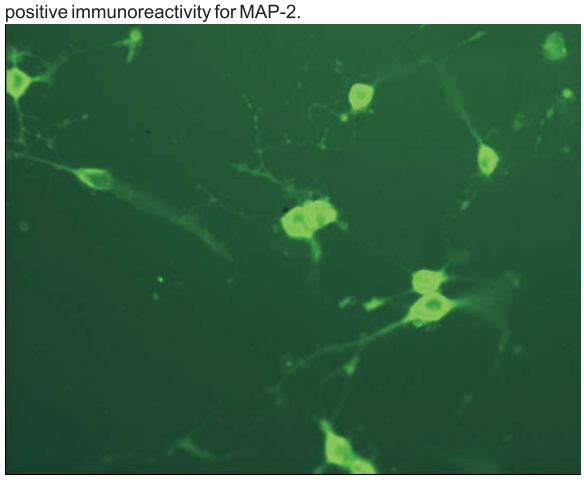


Figure 4: In vitro differentiation of BM-MSCs into neuron-like cells three days after the induction of differentiation. Differentiated cells showed positive immunoreactivity for nestin.

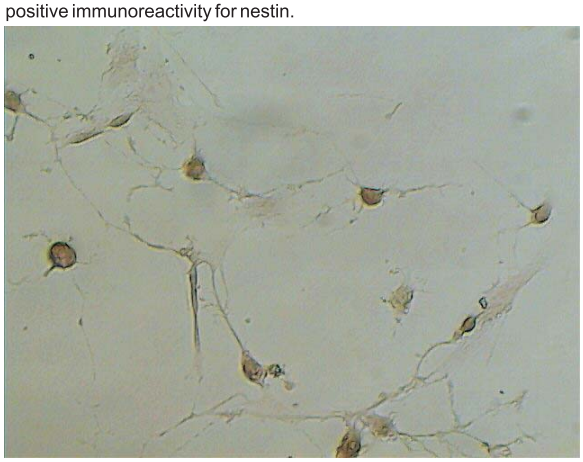
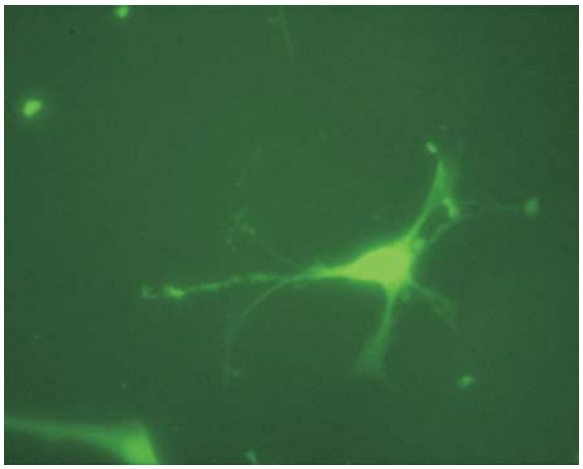


Figure 5: In vitro differentiation of BM-MSCs into neuron-like cells three days after induction of differentiation. Differentiated cells showed positive immunoreactivity for NFM.



with other sources of stem cells (Hofstetter *et al*., 2001). It is important to mention that most of the current cell therapy studies are based on the use of using allograft or xenograft cells. This study demonstrated the ease of harvesting bone marrow from live rats for this purpose.

Glial differentiation from the grafted neural restricted precursor cells and neural differentiation from the grafted glial restricted precursor cells have not been observed (Han *et al*., 2002, Svendsen *et al*., 1996). Therefore, neuronal replacement in the CNS is feasible but requires the grafting of predifferentiated stem cells or neuronal precursor cells (Han et al., 2002, Vescovi *et al*., 1999, Cao *et al*., 2002). Indeed, as the environment in the adult spinal cord restricts neural stem cell differentiation to a glial lineage, an important practical conclusion is that transplants into the CNS will require the predifferentiation of multipotential stem cells

Figure 6: Reverse transcription polymerase chain reaction (RT-PCR) analysis of neuron-specific markers (NSE, MAP2, ß-tubulin) and a neural progenitor marker (nestin). BM-MSCs that were treated by the neural induction protocol for three days expressed neuron-specific markers.

R-Nestin-F
TGGAACAGAGATTGGAAGGC
R-Nestin-R
CAGCAGAGTCCTGTATGTAGC

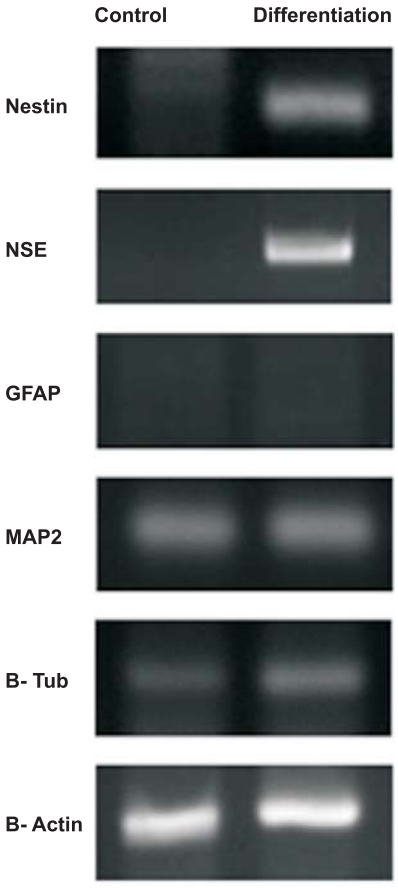
R-NSE-F
CGTTACTTAGGCAAAGGTGTC
R-NSE-R
CATCCAACTCCAGCATCAGG

GFAP-F
ACCATGCCACGTTTCTCCTTGTCTCG
GFAP-R
CCTTAATGACCTCACCATCCCGCATCT

R-Map2-F
ACCAACTCATCTCTCCTGTG
R-Map2-R
GGTTATTCCATCAGTGACTTTGT

ß-tubulin-F
TCACTGTGCCTGAACTTACC
ß-tubulin-R
GGAACATAGCCGTAAACTGC

ß-Actin-F
CATCCCCCAAAGATTCTAC
ß-Actin-R
CAAAGCCTTCATACATC



toward a neuronal lineage (Keirstead *et al*., 2001, Lee *et al*., 2000, Cao *et al*., 2001), or the use of lineage-restricted precursor cells (Han *et al*., 2000, Rao *et al*., 2000), to achieve control over the phenotype of the transplanted cells. Therefore, differentiation of MSCs into neuron-like or myelin-forming cells might be the key in achieving a greater degree of clinical improvement in CNS injuries (Chopp *et al*., 2000). Neural restricted precursor (NRP)-derived neurons have the potential of becoming relays for the repair and reconstruction of damaged circuits or the creation of novel neuronal connections. The present study examined neural differentiation of MSCs in serum-withdrawal neural induction medium. Thin cellular processes with branches were developed following the treatment of BM-MSCs in neurogenic culture medium over a period of two weeks, which was further confirmed by immunocytochemistry and RT-PCR assays. Undifferentiated cells did not express neuron-specific markers and did not stain positively for neuron-specific proteins. Nowadays, there are several methods to induce differentiation in BM-MSCs. However, the viability of cells after differentiation differs (Han *et al*., 2002). In this study, there was acceptable degree of viability (90%).

In conclusion, the expression of important genes that are associated with neuron-like cells in cells that are isolated from rat bone marrow, suggest that BM-MSCs and the neuron-like cells that are derived from these novel stem cells may be important candidates for the use of cell therapy for neurological diseases, such as spinal cord injuries, Parkinson's disease and multiple sclerosis. Further investigation will be required for the evaluation of the use of these cells as autograft sources in different neurological diseases.

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