

Full Length Research Paper

Schwann cells promote neuronal differentiation of bone marrow stromal cells

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Bone marrow stromal cells (BMSCs), a type of multipotent stem cell, can differentiate into various types of cells. It has been suggested that the BMSCs have the capacity to differentiate into neurons under specific experimental conditions, using chemical factors. In this study, we showed that BMSCs can be induced to differentiate into neuron-like cells when they are co-cultured with Schwann cells by Brdu pulse label technology. It was found that a large scale of BMSCs showed a typical neuronal morphology and axon extended about 10 day after beginning of co-culture. These findings support the transdifferentiation of BMSCs, and the theoretical utility of these cells for the treatment of degenerative and acquired disorders of the nervous system and spinal cord injury.

Key word: Bone marrow stromal cells, Schwann cells, co-cultured, Brdu pulse label, axon, differentiation.

INTRODUCTION

Schwann cells are peripheral glial cells that ensheath axons to form myelin in the peripheral nervous system, following nerve injury. Schwann cells lose myelin, are activated and proliferate within the distal nerve segment to produce a variety of neurotrophic factors, cytokines and cell adhesion molecules thereby providing the pathway for regenerating axons. This process is collectively called Wallerian degeneration (Fawcett et al., 1990; Hall, 2001; Radtke and Vogt., 2009; Shields et al., 2010). Schwann cells also play a significant role in the endogenous repair of peripheral nervous system by reconstructing myelin which is indispensable for nerve function. They are also known to support reconstruction of the injured central nervous system (CNS) where successful axonal regeneration and functional reconstruction do not normally occur. A lot of experiments in the spinal cord and some other areas in the central nervous system have

shown that either the injection or transplantation of the polymer tube filled with cultured Schwann cells could promote axonal growth (Bunge, 2002, 2008; Hill et al., 2006; Plant et al., 1998; Vukovic et al., 2007). For these reasons, Schwann cells have long attention and are thus one of the most widely studied cell types for axonal regeneration both in the peripheral nervous system and CNS. Although Schwann cells have a strong ability to induce nerve regeneration, it is difficult to obtain a sufficient amount of Schwann cells for clinical use. Furthermore, technical difficulties exist in the harvesting and expansion of Schwann cells to obtain sufficient amount of cells for cell-based therapy within a reasonable time period. Accordingly, it is desirable to establish cells with Schwann cell characteristics from sources other than the peripheral nervous system (PNS) that are easy to access, capable of rapid expansion, amenable to survival and able to integrate into the host tissue to elicit axonal regeneration and contribute to remyelination.

Bone marrow stromal cells (BMSCs) are mesenchymal elements normally providing structural and functional support for hemopoiesis (Pittenger et al., 1999), and have the potential to differentiate into other kinds of cells such as osteoblasts, adipocytes and chondrocytes (Pittenger et al., 1999; Prockop, 1997). Taking into account these considerations, in this study, we co-cultured BMSCs with Schwann cells by Brdu pulse label technology and demonstrated that Schwann cells could induce and

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Abbreviations: BMSCs, Bone marrow stromal cells; MSCs, marrow stromal cells; PNS, peripheral nervous system; CNS, central nervous system; EDTA, ethylenediamine tetraacetic acid; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; SD, Sprague Dawley; FBS, fetal bovine serum; HS, horse serum.

promote *in vitro* biological differentiation of bone marrow stromal cells toward a neuron. These findings imply that the utility of cocultures of BMSCs and Schwann cells could be considered for the treatment of nervous system disorders in the future.

MATERIALS AND METHODS

Experimental animal and main reagent

Sprague Dawley (SD) rats (200 to 250 g) was provided by Laboratory Animal Center of Liaoning Medical College (China). Mouse Brdu antibody, Brdu and Type II Collagenase bought from Sigma (USA); Rabbit anti-mouse IgG antibody labeled by FITC was from ZhongShan Golden Bridge Biotechnology (China); Rabbit anti-mouse CD44, CD45 Rabbit anti-rat NSE and S-100 protein antibody were bought from Boster (China); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and horse serum (HS) were purchased from Sigma, Gibco (USA).

Culture and identification of MSCs

All animal experiments described in this report were approved by the animal care and experimentation committee of Laboratory Animal Center of China Medical University.

The rats were anesthetized with 1% pentobarbital (40 mg/kg body weight). The bones were dissected clean of attached muscles and the marrow was expelled from the marrow cavity using 1 ml syringe with complete culture medium (DMEM + 9%FBS + 9%HS). Bone marrow was pelleted by centrifugation at 1000 rpm/min for 5 min. Subsequently, 2×10^6 /ml marrow cells were seeded into a 25 cm² tissue culture flask filled with DMEM supplemented. The stromal cells were isolated by their adherence to plastic tissue flask. MSCs were cultured in a 100% humidified incubator with 5% CO₂ at 37°C. The medium was changed every 3 to 4 days. After the primary cultures reached confluency, they were rinsed three times with phosphate-buffered saline (PBS) and dissociated with 0.25% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA) for 5 min at 37°C and subcultured at a density of 8×10^3 cells/cm². The marrow stromal cells (MSCs) must be labeled 1 h with 10 μmol/L of Brdu before co-culture. The MSCs were selected and made 2×10^5 /ml seed into a six well plate containing coverslips. After 24 h, we detected the expression of CD44 and CD45 in MSCs by immunocytochemistry. MSCs coverslips were fixed with 10% formaldehyde for 30 min at room temperature, and rinsed with phosphate buffered saline solution three times for 5 min each, and permeated with 0.1% TritonX-100 for 20 min at room temperature. Then coverslips were washed with PBS 3 times for 5 min each, blocked 30 min with 1% bovine serum albumin at room temperature. The plate was incubated overnight with a primary antibody (Rabbit anti-mouse CD44 and CD45 antibody, 1:100). They were rinsed three times with PBS. Secondary antibody (Rabbit anti-mouse IgG antibody labeled by FITC) was used to incubate the plate for 1 h away from light. For negative controls, PBS was substituted for primary antibody. The coverslips were mounted and images were captured by Zeiss microscope.

Culture and identification of Schwann cells

To culture of Schwann cells, sciatic nerves of adult rats were obtained from adult SD rats under aseptic conditions and placed into Hank's balanced salt solution supplemented. The epineurium and connective tissue were removed under microscope, and the sciatic nerves were cut into 2 to 3 mm fragments, and dissociated

with dispase (1.25 U/ml) and collagenase (0.05%) for 6 to 8 h at 37°C. The cells from sciatic nerve of rats by mix enzyme digestive were gently shook 30 to 60min at 37°C, following, terminated digestion with complete culture medium and centrifuged at 1000 r/min for 5 min. Then, the cells seeded at 8×10^5 /ml into 25 cm² culture flasks in DMEM supplemented with 15% fetal bovine serum, 2 mmol/L glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. The medium was changed every 3 to 4 days. The cells were digested with 0.25% tyrosin and 1 mmol/L EDTA when cells grew to 80% confluence. The Schwann cells were selected and 2×10^5 /ml seed were made into a six well plate containing coverslips. After 24 h, we determined the expression of S-100 protein in Schwann cells by immunocytochemistry. The concrete steps corresponded to the earlier MSCs. The images were captured by Zeiss microscope.

Co-culture MSCs and Schwann cells

The 1×10^5 /ml MSCs cells labeled with Brdu and 1×10^5 /ml Schwann cells were co-cultured in 1 ml DMEM culture medium without serum been added into the six-well culture plate. The medium was changed every 3 to 4 days. Morphological variance was analyzed by invert microscope according to the expression of NSE and Brdu after 10 days' co-culture.

Observation of morphological changes of MSC

In order to detect differentiation of MSCs, series of experiments were conducted. First, morphological variance of marrow stromal cells was analyzed by invert microscope 10 day after beginning of co-culture. Subsequently, the length of axonal extension was measured by random selection of 10 neuron-like cells with invert microscope and the length of axon was described as result (mean ± standard deviation).

Evaluation of differentiation of MSCs by immunostaining

Immunostaining experiment was implemented to ascertain the percentage of differentiation of MSCs. The detailed manipulation protocol was as follows: The co-cultured MSCs and Schwann cells were fixed with 3.7% formaldehyde for 30 min at room temperature, and washed with PBS, then permeated for 10 min with 0.1% TritonX-100 and blocked for 30 min with 1% bovine serum albumin (BSA). The cells were then incubated overnight at 4°C with primary antibody (Rabbit anti-rat NSE and mouse anti-Brdu antibody, 1:100). The coverslips were incubated with secondary antibody (goat anti-rabbit IgG-FITC; rabbit anti-mouse IgG-TRITC 1:200) at 37°C for 1 h. Morphology of BMSCs was observed by Zeiss microscopy. Then co-culture group and MSCs group randomly selected 10 fields in 200× magnification and the differentiation rate was analyzed.

Statistical analysis

Statistical analyses were performed by using GraphPad PRISM® software and SPSS11.5 software.

RESULT

Cell culture and detection

The first step of our study was to culture BMSCs obtained

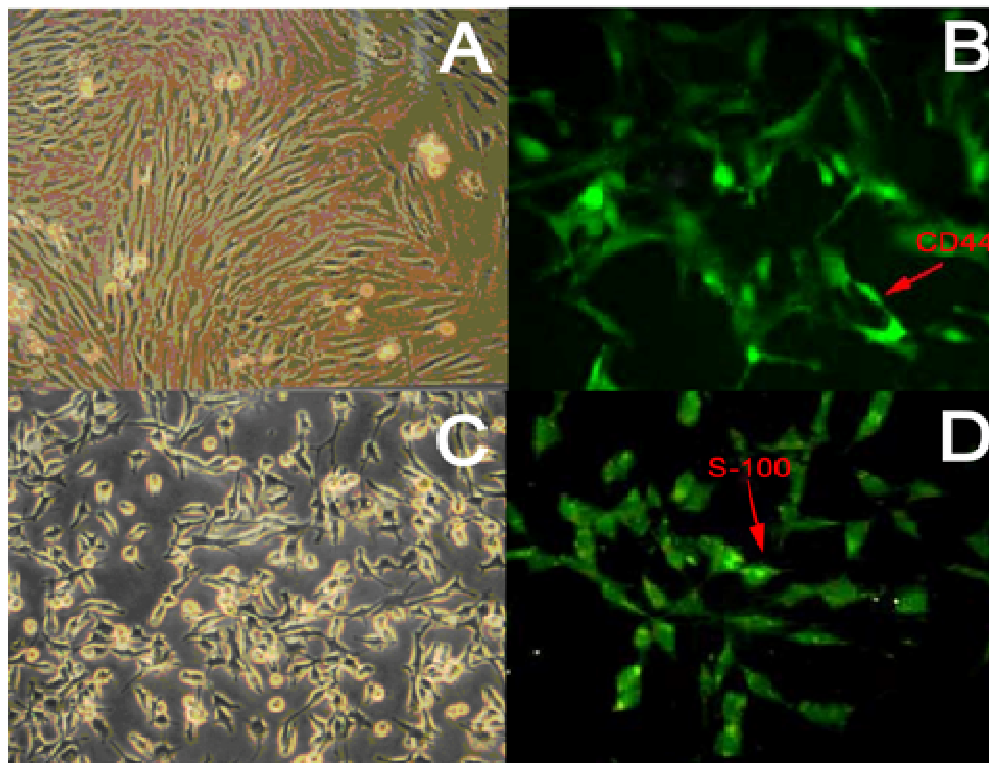


Figure 1. Bone marrow stromal cells and Schwann cells culture and detection. A: Bone marrow stromal cells were observed by the inverted microscope (100X). B: The expression of CD45 in bone marrow stromal cells were observed by the inverted microscope (200X). C: Schwann cells were observed by the inverted microscope (100X). D: the expression of S-100 in Schwann cells were observed by the inverted microscope (200X)

from adult Sprague Dawley (SD) rats. These cells were expanded *in vitro* for at least three passages, remaining undifferentiated (Figure 1A) and showing positivity for CD44 (Figure 1B), which seemed to develop better when they were cultured at low densities. They were negative for surface markers associated with hematopoietic cells, such as CD45, and these were very refringent during cell division. These results showed that we successfully obtained and cultured BMSCs from SD rats.

Schwann cells were successfully expanded and used for subsequent analyses. Single cells of spindle morphology were observed 2 to 3 days after initial seeding (Figure 1C). Schwann cells could express S-100 protein (Figure 1D), which imply that our method of culture Schwann cells was feasible.

Morphological changes of MSC after Schwann cells induction

It was observed that bone marrow stromal cells and Schwann cell could co-exist and the Schwann cells located at a radiation position along the MSCs 10 day after co-cultured (Figure 2A). The MSCs was in a ball shape, radiated and arranged like starfish (Figure 2A) 10 day

after co-cultured. However, the MSCs group, did not present these characteristic 10 day after cultured (Figure 2B). The slim cellular protuberance (Figures 2A and B) was neuronal axon because neuronal axonal cell only was slim in morphology, moreover, this result was confirmed by NSE immunofluorescence (Figure 3). The length of axonal cell were measured in this study, the result showed that axonal length of co-culture group was longer than MSCs culture group (Table 1). These results implied that axonal cell could be prolonged by Schwann cells induction.

Evaluation of differentiation of MSCs after Schwann cells induction

In order to confirm differentiation rate of MSCs after Schwann cells induction, NSE and Brdu immunofluorescence were conducted. As shown in Figure 3, the neuron-like cells, which came from differentiation of MSCs, were stained green by NSE, and the nuclei retained red by Brdu. Original MSCs were stained red by Brdu. Differentiation rate was calculated, and the result showed that differentiation rate of co-culture group was significantly higher than solely MSCs group (Table 2).

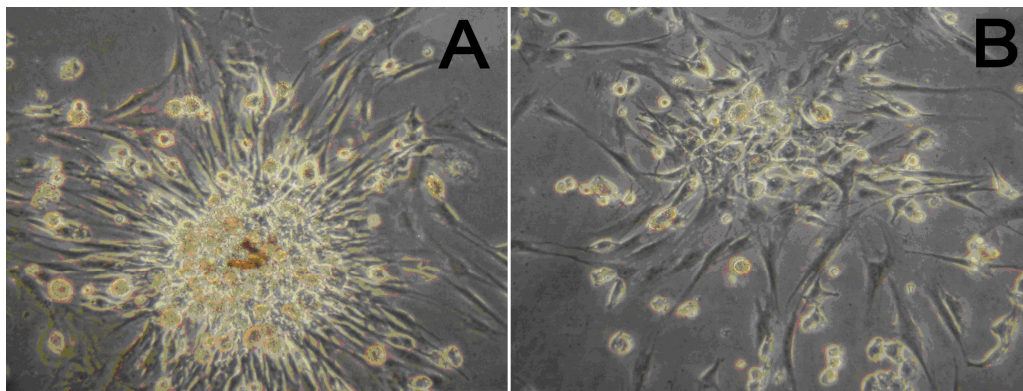


Figure 2. Morphological changes of MSC after Schwann cells induction. A: Morphological changes of MSC about 10 day after co-cultured were observed by the inverted microscope (100X). B: Morphological changes of MSC 10 day after alone cultured were observed by the inverted microscope (100X).

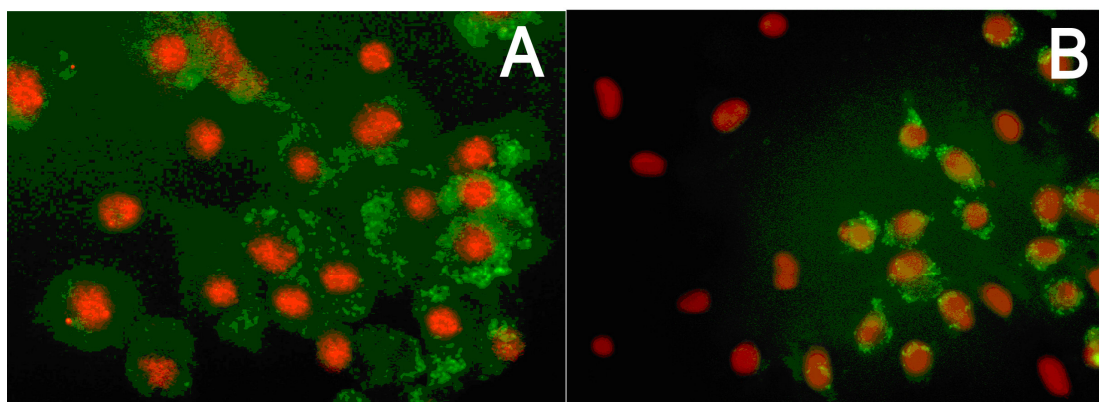


Figure 3. The expression of Brdu and NSE in bone marrow stromal cells. A: The expression of Brdu and NSE in bone marrow stromal cells after Schwann cells induction. B: The expression of Brdu and NSE in bone marrow stromal cells without Schwann cells induction.

Table 1. The length of axon about 10 day after MSC co-cultured group and alone cultured group. Different letters represent the significant difference at $p < 0.01$.

	Co-culture group	MSCs group
$\bar{x} \pm S (\mu\text{m})$	12.18 ± 0.92^a	10.32 ± 0.89^b

These results demonstrated that Schwann cells could promote neuronal differentiation of bone marrow stromal cells.

DISCUSSION

This study have shown neuronal differentiation of bone marrow stromal cells (BMSCs) when they are co-cultured with Schwann cells and Schwann cells promote neuronal

differentiation of bone marrow stromal cells. It is a well-known property of Schwann cells in regenerative processes of the nervous system (Bunge, 1991; Dezawa et al., 2001; Shields et al., 2005; Zurita et al., 2005; Zurita et al., 2001) Our results may be due to the release of neurotrophic factors by Schwann cells, but the frequent observation in co-cultures of Schwann cells contacting with BMSCs function, suggests that intercellular contacts could play certain role in the process of bone marrow stromal cells transdifferentiation. This study showed that the culture of BMSCs in the presence of Schwann cells is a simple biological method to obtain neuron-like cells from BMSCs. This finding suggests that neuronal differentiation of BMSCs can be obtained *in vivo* by the presence of environmental neurotrophic factors.

Bone marrow stromal cells (BMSCs) provide potential possibilities for clinical application, since they can be efficiently expanded *in vitro* to achieve a therapeutic dose. Firstly, BMSCs are easily accessible through aspi-

Table 2. Differentiation rate of co-culture group and MSCs group. Different letters represent the significant difference at $p < 0.01$.

Data	Co-culture group			MSCs group		
	NSE	BrdU	N/B (%)	NSE	BrdU	N/B (%)
1	46	52	88.5	38	58	65.5
2	45	53	85.9	36	56	64.3
3	50	62	80.7	34	53	64.2
4	49	58	84.5	36	59	61.0
5	48	56	85.7	28	57	49.1
6	43	56	76.8	32	55	58.2
7	46	57	80.7	35	59	59.3
8	44	57	77.2	36	60	60.0
9	40	55	72.7	26	57	45.6
10	42	58	72.4	37	59	62.7
$\bar{x} \pm S$			80.51±5.65 ^a			58.99±6.62 ^b

ration of the bone marrow, and can be easily expanded in large scale for auto-transplantation. Secondly, BMSCs can be obtained without serious ethical problems, which is a great advantage for clinical use. In addition, bone marrow stromal cells are referred to as mesenchymal stem cells because they are capable of differentiating into multiple mesodermal tissues, including bone (Beresford et al., 1992) cartilage (Lennon et al., 1995), fat (Beresford et al., 1992) and muscle (Wakitani et al., 1995). Moreover, differentiation into neuron-like cells expressing neuronal markers has been reported (Deng et al., 2001; Woodbury et al., 2000). Schwann cell is a special neurogliaocyte in peripheral nervous system. It is well known that Schwann cell can produce a variety of neurotrophic factors, cytokines and cell adhesion molecules, thereby providing the pathway for regenerating axons (Ishikawa et al., 2009; Kamada et al., 2005). Therefore, in the present study, neuronal differentiation of BMSCs when they are co-cultured with Schwann cells was investigated, the results showed that Schwann cells promote neuronal differentiation of bone marrow stromal cells, which were compatible with some previous reports (Mercedes et al., 2005), however, we concisely counted differentiation rate by Brdu pulse label technology, therefore, our study not only makes a certain theoretical sense, but also has practical and extensive prospect.

The present studies have demonstrated that Schwann cells promote neuronal differentiation of bone marrow stromal cells and bone marrow stromal cells induced from Schwann cells exert trophic effects and provide a strong foothold for regenerating axons (Dezawa et al., 2001; Ishikawa et al., 2009; Kamada et al., 2005; Mimura et al., 2004; Shimizu et al., 2007; Someya et al., 2008) Therefore, induced BMSCs cells by Schwann cells is a valuable candidate source for cell therapy in peripheral nerve injury. We hope that our study will contribute to bring one of the solutions to the cell therapy designed for the regeneration of peripheral nervous system and central

nervous system.

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