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Distal segment extracts of the degenerated rat sciatic nerve induce bone marrow stromal cells to express Schwann cell markers *in vitro*

Hui Wang[a,*], Hongyu Zhang[b], Mo Liu[a], Ningyu Wang[a,**]

[a] *Department of Otolaryngology Head & Neck Surgery, Beijing Chaoyang Hospital, Key Laboratory of Otolaryngology Head and Neck Surgery, Capital Medical University, Beijing, PR, China*

[b] *Clinic of Beijing Branch, China Southern Airlines Co., Ltd., PR, China*

HIGHLIGHTS

- Injured nerve microenvironments induce MSCs into functional Schwann-like cells.
- Distal nerve segments play important roles in MSC induction to Schwann-like cells.
- Distal segments may be crucial for MSC differentiation to myelinating Schwann cells.
- The results elucidate mechanisms by which MSCs function in peripheral nerve repair.

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ABSTRACT

Bone marrow stromal cells (MSCs) have the ability to support nerve regeneration when transplanted into lesion sites, but the mechanism is unclear. We hypothesized that specific factors in the lesioned microenvironment induce the differentiation of transplanted MSCs into functional Schwann-like cells. To test this hypothesis and determine the origin of such factors, we investigated the effects of different extracts from degenerated rat sciatic nerves on MSCs *in vitro*. After 3 days of degeneration, extracts from the distal segment (Ds) and proximal segment (Ps) of the rat sciatic nerve were used in experiments. After 1 day of treatment, the morphology of MSCs cultured with Ds extracts were spindle shaped, and the cells interconnected with each other, followed by gradual loss of typical morphology during culture. After 7 days of treatment, western blotting and RT-PCR analyses indicated that the cells cultured with Ds extracts had significantly higher expression of glial fibrillary acidic protein (GFAP), Sox10, Oct6, and early growth response 2(Egr2) than that of cells cultured with Ps extracts and the untreated cells. Our study suggests that, in the microenvironments of nerve lesions, specific factors induce MSCs to differentiate into functional Schwann-like cells, which may originate from the Ds of the degenerated nerve. These results may help to elucidate the mechanisms by which MSCs function in peripheral nerve repair.

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1. Introduction

Bone marrow stromal cells (MSCs) are multipotent stem cells that localize in the stromal compartment of bone marrow. Recently, MSCs have attracted a great deal of research interest because of their potential use in cell-based therapies for various disorders including neural injury and degeneration. A number of studies have provided accumulating evidence showing that MSCs can be differentiated into Schwann-like cells *in vivo* or *in vitro*, and improve the functional outcomes of peripheral nerve repair [4,5,9,11,19]. These studies indicate that MSC is a powerful tool in nerve regenerative medicine.

Although methods to induce the differentiation of MSCs into Schwann-like cells *in vitro* have been reported in many studies [1,6,10,28], the mechanisms by which MSCs differentiate into Schwann cells *in vivo* and their functions are still uncertain. The most acceptable explanation is that MSCs differentiate into Schwann-like cells to produce large amounts of various cytokines and growth factors that may promote neural cell survival and neuritogenesis, or take part in myelin formation directly [7,16,20,22,26]. Because implanted MSCs generate neural phenotypes specific to the lesion site [4,17,18,23,24], another transdifferentiation mechanism of MSCs is believed to be due to spontaneous fusion of MSCs with host cells rather than actual transdifferentiation [27].

Regardless of the specific mechanism, MSC support of nerve regeneration only occurs when the transplanted MSCs contact or

* Corresponding author at: 8 Gongti Nanlu, Chaoyang Distract, Beijing 100020, China. Tel.: +86 0 13683243872.
** co-corresponding author.
E-mail addresses: whtel@163.com (H. Wang), wny@sohu.com (N. Wang).

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are in close proximity to the lesion sites. Therefore, we hypothesized that specific factors in lesioned microenvironments promote fusion or induction of the transplanted MSCs into functional Schwann-like cells. To test this hypothesis, we investigated the effects of extracts from degenerated sciatic nerves on MSCs *in vitro*.

2. Materials and methods

All animal procedures were conducted aseptically in accordance with the animal ethics guidelines of the Chinese National Health and Medical Research Council, and were approved by the Animal Experimentation Ethics Committee of Capital Medical College.

2.1. Cell harvesting and culture

Eight-week-old Wistar rats weighing 230–270 g were obtained from the Experiment Animal Center of Beijing, China. MSCs were purified from bone marrow according to a previously described technique [10], and cultured in α-modified Eagle's medium (α-MEM). Cells were passaged every 5–7 days, and the third passage cells were used in experiments.

2.2. Characterization of cultured MSCs

The third passage MSCs were fixed with 4% paraformaldehyde, incubated for 1 h with monoclonal antibodies against rat CD90, CD45, and CD34 (All purchased from Caltag), or control mouse IgG, which were conjugated to fluorescein isothiocyanate. Single cell suspensions were washed three times in PBS, counted, and adjusted to the appropriate concentrations. A total of 1×10^6 cells were then analyzed by FACSCalibur flow cytometer (BD).

2.3. Harvesting of degenerated sciatic nerve extracts

Thirty healthy male Wistar rats were used to prepare the extracts. All surgeries were performed under deep anesthesia by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). Both sides of the sciatic nerve were carefully exposed and transected at the middle of the thigh, and then the cut ends of the proximal and distal nerves were ligated and left for degeneration.

Degenerated sciatic nerves were harvested at 3 days after surgery and divided into two groups: proximal segment (Ps) and distal segment (Ds). Harvested nerves were frozen immediately after surgery and pulverized using a mortar and pestle while immersed in liquid nitrogen. All subsequent procedures were performed at 4 °C or in an ice water bath. The pulverized tissue was suspended in α-MEM at approximately 2 g wet tissue weight per 10 ml α-MEM. The suspension was then manually homogenized in a glass homogenizer, and then centrifuged at $31{,}000 \times g$ for 5 min. The lipid layer was removed with a spatula and the supernatant was decanted. The final supernatant was filtered through an Acrodisc® 0.2 micron syringe (Pall Corp) and stored at −70 °C until use.

2.4. MSC culture with nerve extracts

Degenerated nerve extracts were added to the culture medium of the third passage MSCs at a ratio of 1:8 (extract:medium). To mimic *in vivo* cell transplantation, the extract was added before the cells adhered. The culture medium was changed at day 3, and the cells were harvested at day 7 for RT-PCR and western blot analyses. MSCs passaged at the same time, but cultured with α-MEM were used as controls. All groups were repeated with 4 independent experiments.

2.5. Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Total RNA (1 μg) was used as a template in a 20 μL cDNA synthesis reaction. cDNA was synthesized using the Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. PCR was performed as recommended by the manufacturer using a Power SYBR Green PCR Master Mix (Applied Biosystems). The reaction contained 10 μL SYBR green reaction mix, 1 μM primers and 2 μL cDNA in a total volume of 20 μL. PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was performed for all reactions to ensure homogeneity of the amplicon. The PCR product size for each primer set was confirmed by gel electrophoresis. Potential contamination was monitored using no template controls as references. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to normalize the transcript levels of target genes. The primer sequences for S100, glial fibrillary acidic protein (GFAP), nerve growth factor receptor (NGFR; P75), Sox10, Oct6 and Egr2 are show in Table 1. All results were repeated with three independent experiments.

2.6. Western blot analysis

Cells were washed in PBS and lysed in RIPA buffer (Roche). The protein concentration was determined using BCA protein assay reagent (Pierce Biotechnology). Total protein samples (10 μg) were denatured at 100 °C for 5 min. After transferring the proteins onto polyvinylidene fluoride-membranes (Bio-Rad), the membranes were blocked in 5% bovine serum albumin/PBS for 1 h, and then the membranes were probed with specific primary antibodies at 4 °C overnight. After three washes with TBST (TBS with 0.1% Tween-20), the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). Membranes were washed and treated with an ECL chemiluminescent substrate (Amersham) and developed by exposure to Kodak X-OMAT light-sensitive film. β-Actin served as an internal control. The relative gray values were analyzed using Image-Pro Plus 5.1. All western blots were repeated three times.

2.7. Statistical analysis

Results are presented as the means ± standard error. The significance of differences was determined using one-way ANOVA by SPSS software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of rat MSCs

Bone marrow contains a heterogeneous population of cells. When MSCs were grown at a low density, the cells exhibited a triangular shape, but the morphology changed to a fibroblast-like morphology at confluence. Flow cytometry was used to determine the phenotypes of rat MSCs. The results indicated that rat MSCs were immunopositive for CD90 (99.74%), although some CD34- and CD45-positive cells were present (0.16% and 0.8%, respectively), indicating that the isolated rat MSCs were not contaminated with cells of hematopoietic or endothelial origins (Fig. 1).

3.2. Effects of degenerated sciatic nerve extracts on rat MSC morphology

After 1 day of culture with extracts from the Ds, MSCs showed a spindle shape and interconnected with each other, followed by gradual loss of the spindle morphology during culture. Compared

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