Experimental Neurology 292 (2017) 92–101

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Tissue engineering with peripheral blood-derived mesenchymal stem cells promotes the regeneration of injured peripheral nerves

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ARTICLE INFO

Article history:
Received 3 November 2016
Received in revised form 8 February 2017
Accepted 6 March 2017
Available online 7 March 2017

Keywords:
Peripheral blood-derived mesenchymal cells
Schwann cells
Differentiation
Peripheral nerve injury
Nerve regeneration
Tissue engineering

ABSTRACT

Peripheral nerve injury repair can be enhanced by Schwann cell (SC) transplantation, but clinical applications are limited by the lack of a cell source. Thus, alternative systems for generating SCs are desired. Herein, we found the peripheral blood-derived mesenchymal stem cells (PBMSCs) could be induced into SC like cells with expressing SC-specific markers (S100, P75NTR and CNPase) and functional factors (NGF, NT-3, c-Fos, and Krox20). When the induced PBMSCs (iPBMSCs) were transplanted into crushed rat sciatic nerves, they functioned as SCs by wrapping the injured axons and expressing myelin specific marker of MBP. Furthermore, iPBMSCs seeded in an artificial nerve conduit to bridge a 10-mm defect in a sciatic nerve achieved significant nerve regeneration outcomes, including axonal regeneration and remyelination, nerve conduction recovery, and restoration of motor function, and attenuated myoatrophy and neuromuscular junction degeneration in the target muscle. Overall, the data from this study indicated that PBMSCs can transdifferentiate towards SC-like cells and have potential as grafting cells for nerve tissue engineering.

1. Introduction

Schwann cells (SCs) play pivotal roles in developmental, neurodegenerative, and regenerative processes in the peripheral nervous system (Bunge, 1994; Kim et al., 2013; Al-Zer and Kalbouneh. 2015). SCs are the most promising seed cells for peripheral nerve tissue engineering because they can synthesize and secrete various neurotrophic factors and extracellular matrix to guide and promote axonal growth, as well as remyelinate regenerated axons or demyelinated axons (Wiberg and Terenghi, 2003; Lopez-Verrilli and Court, 2012). The limitations of the clinical application of SCs are attributed to the traditional SC isolation methods, which cause damage at the donor site, and to inefficient cell production (Wakao et al., 2010; Sowa et al., 2016). Recently, generating SCs by differentiation from stem cells was proposed as a potential strategy (Thoma et al., 2014).

Due to their ability to differentiate into multiple cell lineages, mesenchymal stem cells (MSCs) have attracted global attention in recent decades. MSCs can be isolated from various tissues (Huss, 2000; Karaminekoo et al., 2016; Mak et al., 2016), and those derived from bone marrow (Wang et al., 2013), adipose (Gao et al., 2015), amniotic fluid (Zhu et al., 2015), nasal respiratory mucosa (Zhang et al., 2015), dental pulp (Martens et al., 2014) and umbilical cord (Peng et al., 2011) have been documented to selectively transdifferentiate into SCs. However, the induction of peripheral blood-derived MSCs (PBMSCs) into SCs has not been reported.

Compared with the above-mentioned tissue sources, harvesting MSCs from peripheral blood is less invasive and easier. Therefore, it is much more acceptable to obtain autologous MSCs from peripheral blood, which could avoid the conventional limitations on stem cells such as immune rejection and ethical problems (Koerner et al., 2006; Zheng et al., 2015). Thus, PBMSCs have received increasing attention in recent years. Our group previously reported that PBMSCs can be induced into osteoblasts to enhance bone reconstruction (Wu et al., 2015).

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http://dx.doi.org/10.1016/j.expneurol.2017.03.005
0014-4886/

In the present study, we aimed to explore whether PBMSCs can be induced to SCs and to determine the potential of induced PBMSCs (iPBMSCs) to repair injured peripheral nerves.

2. Materials and methods

2.1. Ethics statement

All the animal experiments, including surgery and tissue collection, were carried out with the approval of the Southern Medical University Animal Care and Use Committee in accordance with the guidelines for the ethical treatment of animals. All efforts were made to minimize animal suffering.

2.2. Isolation of peripheral blood mesenchymal stem cells (PBMSCs)

Fresh blood was taken from four-week-old green fluorescence protein (GFP) transgenic Sprague-Dawley (SD) rats ("green rat CZ-004" SD TgN (act-EGFP) OsbCZ-004) and immediately diluted with an equal volume of heparinized saline. The diluted mixture was gently layered onto Lymphocyte Separation Medium (TBD Science) and then horizontally centrifuged for 25 min at 1600*g*. The mononuclear cells were extracted, washed twice with Hank's Balanced Salt Solution (Gibco), and cultured at 10^6 cells/cm^2 in DMEM/F12 medium (Gibco) supplemented with 20% foetal bovine serum (FBS, Gibco). The medium was replaced every three days, and non-adherent cells were discarded. After the primary culture reached approximately 80% confluence, cells were passaged regularly and cultured in DMEM/F12 medium with 10% FBS. Cells at passage5 (P5) were used for the experiments. These cells were identified by flow cytometric analysis of positive and negative markers of MSCs, such as CD29d, CD44, CD45, CD90, CD105, and CD146, and their multilineage differentiation potential was assessed by inducing differentiation into osteoblasts, chondroblasts, adipocytes, neurons and SCs, which was described in our previous publication (Wu et al., 2015).

2.3. Transdifferentiating PBMSCs towards SCs

The P5 PBMSCs were induced into SCs by the following three-step protocol. First, the cells were treated with 1 mM β-mercaptoethanol (β-ME, Amresco). After 24 h, the medium was replaced with α-MEM (Gibco) containing 350 ng/mL all-trans-retinoic acid (ATRA; Sigma) and 10% FBS; the cells were cultured in this medium for 3 days. Then, in the third step, the cells were transferred to α-MEM medium containing 12.5 μM forskolin (Sigma), 5 ng/mL platelet-derived growth factor-AA (PDGF; Gibco), 10 ng/mL basic fibroblast growth factor (bFGF, Gibco), 10 ng/mL recombinant human heregulin-beta1 (Peprotech) and 10% FBS. The third step lasted for 10 days, and the inductive medium was replaced every 3 days. Finally, the cells were identified by immunocytochemistry, quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting analyses.

To identify the immunophenotypes of the transdifferentiated cells, an aliquot of the completely induced cells was collected and seeded onto coverslips in a 24-well plate (1×10^4 cells/well). After the cells were cultured in inductive medium for an additional24 h, they were fixed in 4% paraformaldehyde for immunocytochemistry of specific markers of Schwann cells, including S100, P75NTR and CNPase. Briefly, the cultures were permeabilized with 0.5% Triton X-100 for 30 min and blocked in 5% deep sea fish gelatin (Sigma) in 0.01 mol/L PBS for 1 h. Then, the cells were incubated overnight at 4 °C with primary antibodies against rabbit anti-CNPase (1:200; Bioworld), mouse anti-P75NTR (1:100; Molecular, Upstate) or mouse anti-S100 (1:100; Millipore) and then incubated with Alexa568-conjugated donkey anti-mouse or anti-rabbit secondary antibodies (1:400, Molecular Probe) at room temperature for 2 h. Finally, the cell nuclei were labelled with DAPI.

To further assess whether the induced cells expressed functional SC factors, the mRNA levels of *Ngf*, *Nt-3*, *c-fos* and *Krox20* were detected by qRT-PCR. Briefly, total RNA was extracted from induced or un-induced PBMSCs using an RNA extraction kit (Dong Sheng, China). Total RNA was subjected to reverse transcription (RT) with a Revert Aid First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed in triplicate with an ABI Step One Plus System (Applied Biosystems) and fluorescence-labelled specific primers (Fermentas). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an endogenous control (primer sequences and product sizes are listed in Table 1). All the analyses were repeated in three independent experiments. The results were analysed with SOS2.1 software (Applied Biosystems).

To confirm the results of above immunocytochemistry and RT-PCR, we further performed western blot to examine the protein levels of S100, P75NTR and CNPase as well as NGF, NT-3, c-Fos and Krox20. Briefly, PBMSCs and iPBMSCs were homogenized in RIPA buffer (Sigma) containing 1% protease inhibitor cocktail (Cell Signaling). Extracts were separated in Laemli sample buffer on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad). Blots were blocked in 5% bovine serum albumin (BSA) for 1 h and incubated with primary antibodies against CNPase (1:500), S100 (1:500), P75NTR (1:700; Molecular, Upstate), NGF (1:500; Abcam), NT-3 (1:500; Santa Cruz), c-Fos (1:500; Santa Cruz), or Krox20 (1:200; Santa Cruz) overnight at 4 °C. All antibodies were detected with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (1:1000; Promega) for 2 h at room temperature. Immunoreactive proteins were detected and imaged by ECL (Millipore) using Lumazone system (Roper). The results for each protein were confirmed in three sets of experiments. Immunoblotting for β-actin (1:1000, Beijing Ray Antibody Biotech) was performed as a loading control.

2.4. Tracing the fate of induced PBMSCs (iPBMSCs) in an injured peripheral nerve

Adult wild type female SD rats (n = 6, weight 200 g–250 g, purchased from the animal centre of Southern Medical University) were anesthetized with12 mg/mL tribromoethanol (90 mg/kg body weight). Using an operating microscope, the sciatic nerves were exposed at the mid-thigh and crushed with a microscopic needle holder, which resulted in a 1-mm area of damage with unabridged epineuria. Subsequently, 1.5×10^6 iPBMSCs suspended in 3 μL of medium were microinjected into the injured site. After surgery, antibiotics were routinely administered for postoperative health care and cyclosporine (Huadong Pharmaceutical, China, 100 mg/L) was administered in the drinking water throughout the surviving period to prevent grafted cell rejection (Lu et al., 2010). Four weeks post the surgery, the sciatic nerves were collected and fixed with 4% paraformaldehyde (PFA) after cardiac perfusion. The fixed sciatic nerves were cut into longitudinal or transverse sections using a cryostat (Leica) for immunohistochemistry analysis with antibodies against neurofilament (NF; 1:400; Sigma) and myelin basic protein (MBP; 1:200; Calbiochem). Images were captured with confocal microscope (Olympus F1-1000 or Zeiss LSM 800).

Table 1
Quantitative real-time PCR primer sequences.

Gene	Sequence (5′–3′)	
	Forward	Reserve
Ngf	TAAGACCACAGCCACGGACATCA	ATGTTCACCTCGCCCAGCACT
Ntf3	CCCAAAGCAGAGGCACCCA	TGCAATCATCGGCTGGAATTCT
c-fos	AAATCAAAAGCAACCGCATGGA	CAGGCCTGGCTCACATGCTACT
Krox20	CTGCGAGCCCTTCCCTTTGA	CCCAAGCCATTAAAGTGCCACA
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTT