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Neural stem cells promote nerve regeneration through IL12-induced Schwann cell differentiation

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ABSTRACT

Regeneration of injured peripheral nerves is a slow, complicated process that could be improved by implantation of neural stem cells (NSCs) or nerve conduit. Implantation of NSCs along with conduits promotes the regeneration of damaged nerve, likely because (i) conduit supports and guides axonal growth from one nerve stump to the other, while preventing fibrous tissue ingrowth and retaining neurotrophic factors; and (ii) implanted NSCs differentiate into Schwann cells and maintain a growth factor enriched microenvironment, which promotes nerve regeneration. In this study, we identified IL12p80 (homodimer of IL12p40) in the cell extracts of implanted nerve conduit combined with NSCs by using protein antibody array and Western blotting. Levels of IL12p80 in these conduits are 1.6-fold higher than those in conduits without NSCs. In the sciatic nerve injury mouse model, implantation of NSCs combined with nerve conduit and IL12p80 improves motor recovery and increases the diameter up to 4.5-fold, at the medial site of the regenerated nerve. In vitro study further revealed that IL12p80 stimulates the Schwann cell differentiation of mouse NSCs through the phosphorylation of signal transducer and activator of transcription 3 (Stat3). These results suggest that IL12p80 can trigger Schwann cell differentiation of mouse NSCs through Stat3 phosphorylation and enhance the functional recovery and the diameter of regenerated nerves in a mouse sciatic nerve injury model.

1. Introduction

Peripheral nerve injury causes degeneration of nerve fiber and degradation of surrounding tissues, leading to reduced motor and sensory activities. The regeneration of injured peripheral nerve is a multiplex process in which Schwann cells play an important role (Ren et al., 2012). After injury, resident Schwann cells transdifferentiate into proliferating repair cells, secrete neurotrophins and recruit macrophage to clear debris (Fairbairn et al., 2015; Navarro et al., 2007; Scheib and Hoke, 2013). The method of peripheral nerve repair involves using suture; this, however, is difficult in nerve-gap reconstruction, when axons must traverse two coaptation sites bridged by nerve grafts. Autologous nerve grafts represent the gold standard for transplantation, but sources are limited. In these situations, alternative devices such as conduits must be considered (Fairbairn et al., 2015; Kehoe et al., 2012). Nerve conduits provide mechanical support and direct axonal sprouting between the injured nerve stumps. Conduits have been shown to retain neurotrophic factors secreted from or recruited by the damaged cells and prevent ingrowth of fibrous tissue at the injury site (Kehoe et al., 2012). However, these implanted conduits do not contain Schwann cells and must be repopulated with endogenous Schwann cells migrating from residual nerve stump (Fairbairn et al., 2015). Recent studies reveal that implantation of neural stem cells (NSCs) in conduits promotes regeneration of injured peripheral nerves may depend on the ability of implanted NSCs to differentiate into Schwann cells, to secrete neurotrophic factors per se, to create a microenvironment to enrich neurotrophic factors from milieu, and to assist in myelination (Ren et al., 2012; Shi et al., 2009; Zhang et al., 2008). However, the nature of cytokines or growth factors that are involved in this process is not clear. The molecular mechanism for the Schwann cell differentiation of the implanted NSCs into newly regenerated axons is also not well established.

In our previous studies, we have confirmed that micropatterned poly (L-lactic acid) (PLA) conduit seeded with NSCs benefited axon regeneration. We hypothesized that aligned NSCs either secreted or recruited neurotrophic factors for nerve repair and may have participated in the nerve repair at a later stage (Hsu et al., 2009a). Therefore, we aimed to identify factors that were involved in NSCs-mediated nerve regeneration and functional recovery. We found that the levels of IL12p80 (the bioactive homodimer form of IL12p40) (Heinzel et al., 1997) in

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conduits with NSCs were shown to be 1.6-fold higher than those in conduits without NSCs. Implantation of NSCs and IL12p80 within the nerve conduit improved motor function recovery, increased nerve conduction activity, and enhanced nerve regeneration. We showed that IL12p80 induced the Schwann cell differentiation of mouse NSCs in vitro through phosphorylation of signal transducer and activator of transcription 3 (Stat3). These results provide a possible application of IL12p80 in developing sciatic nerve injury treatment.

2. Materials and methods

2.1. NSCs isolation and culture

Mouse neural stem cells (NSCs) isolation and culture was performed as described in our previous publication (Hsu et al., 2009b; Lee et al., 2009). Briefly, KT98 cells were derived from F1B-TAg transgenic mouse brain and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco)/F12 (Gibco) (1:1) containing 10% fetal bovine serum (FBS, Hyclone), 1% penicillin/streptomycin (Gibco). For NSCs isolation, KT98 cells were transfected with F1B-GFP plasmid, which showed an ability to select neural stem cells. KT98/F1B-GFP cells were cultured in KT98 cell culture medium supplemented with 500 μg/ml G418 (Merck Millipore). The GFP-positive KT98/F1B-GFP (KT98/F1B-GFP$^+$) cells were sorted using FACSAria cell sorter (BD Bioscience) and cultured in neurosphere formation medium: DMEM/F12 containing 1× B27 (Gibco), 20 ng/ml EGF (PeproTech Inc.), 20 ng/ml FGF2 (PeproTech Inc.), 2 μg/ml heparin (Sigma-Aldrich), and 500 μg/ml G418 for 7 days, which induced KT98/F1B-GFP$^+$ neurosphere formation. Then KT98/F1B-GFP$^+$ neurosphere-derived single cells were used in subsequent animal experiments and cell differentiation assays. All cells were cultured at 37 °C with 5% CO_2.

2.2. Animal surgery: sciatic nerve injury and conduit implantation

In this study, all animal experimental procedures followed ethical guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Health Research Institutes (Protocol No. NHRI-IACUC-101067A). FVB mice (8–10 weeks old) were used for animal experiments and were maintained in the National Health Research Institutes Animal Center. Before surgery, mouse was anesthetized by 5% isoflurane (Halocarbon) air inhalation; anesthetization was maintained by 2% isoflurane air inhalation during surgery. In sciatic nerve injury surgery, a 3 mm mouse sciatic nerve segment in the left leg was excised with microscissors. For the surgical implantation of nerve conduits, the micropatterned poly(L-lactic acid) (PLA) conduit was used, as previously described. In brief, the micropatterned conduit was beneficial to cell proliferation, gene expression and directing alignment of NSCs (Hsu et al., 2009a).

The 5-mm conduit with or without NSCs and/or mouse IL12p80 was implanted into the sciatic nerve injury site to connect the 3-mm nerve injury gap. Proximal and distal nerve stumps of the sciatic nerve injury site were sutured into the conduit with 1-mm residual nerve using 6-0 nylon microsutures. Therefore, axons existed in the central region of conduit were considered newly regenerated nerve. Mice that received the same surgical protocols, but did not undergo sciatic nerve injury were defined as the Sham control group (n = 3) and no implantation mice (Neg. control group) were defined as mice with sciatic nerve damage but that were not implanted with either conduit or conduit with NSCs.

The surgical implantation groups included the Conduit only group (n = 8), Conduit + NSC group (n = 8), Conduit + NSC + mIL12 group (n = 8), and no implantation (Neg. control) group (n = 3). In the Conduit only group, conduits were filled with 5 μl matrigel (BD Bioscience)/phosphate buffer saline (PBS) mixture (1:1). For the Conduit + NSC group (n = 8), conduits were filled with 5 μl matrigel/PBS mixture (1:1) with 1×10^6 NSCs. In the Conduit + NSC + mIL12 group (n = 8), conduits were filled with 5 μl matrigel/PBS mixture (1:1) with 1×10^6 NSCs and 100 ng mouse IL12p80 (BioLegend).

2.3. Functional assessments: walking track analysis and Rotarod test

Sciatic functional index (SFI) is a calculated score of data from Walking track analysis that combines gait analysis and the temporal and spatial relationship of one footprint to another during walking. Walking track analysis was performed using the Treadmill/TreadScan system (CleverSys) and presented as sciatic functional index (SFI) every week after surgery. The formula for SFI is as follows:

$$SFI = -38.3 \times \left(\frac{EPL-NPL}{NPL}\right) + 109.5 \times \left(\frac{ETS-NTS}{NTS}\right) + 13.3\left(\frac{EIT-NIT}{NIT}\right) - 8.8$$

SFI calculation was according to normal (N) and experimental (E) feet, where PL indicated the length of the footprint (the longitudinal distance between the tip of the longest toe and the heel), TS indicated the total toe spread (the cross-sectional distance between the first and fifth toes) and IT indicated the intermediate toe spread (the cross-sectional distance between the second and the fourth toes). The SFI is on a scale from −100 to 0, where −100 refers to a complete loss of function and 0 corresponds to the normal walking function. Adult FVB mice were used to obtain the normal walking video (total 1500 frames were collected in a complete walking period of one mouse) for TreadScan software calibration (10–12 outlines of each step were sufficient to train the software for identification of the paw position). After calibration, we used the well-established program to exclude abnormal walking status and irregular toe spread during an entire walking period of sciatic nerve injury mice.

The Rotarod test was executed using an RT series Rotarod Treadmill (SINGA) at the fourth and eighth week after surgery. In the Healthy Control group, mice were defined as without nerve dissection and muscle injury (n = 8 at the fourth week and n = 8 at the eighth week). Before formal data collection, each mouse ran on the rotating rod for three times each at 10 rpm, 12 rpm, and 15 rpm as a pre-test course. For data collection, mice ran on the rotating rod six times at 20 rpm. The maximal recording time was 120 s.

2.4. Protein array and IL12p80 identification

Protein samples were extracted from the implanted conduits with or without NSCs using 1× RIPA buffer (10× RIPA Lysis Buffer, EMD Millipore, diluted with deionized water) containing 1× protease inhibitor cocktail (Roche) at fourth week after surgery. A 100 μg protein sample was used in the mouse angiogenesis protein antibody array (RayBiotech) analysis, which followed the manufacturer's protocol. Protein levels were detected with chemiluminescence methods. To confirm IL12p80 expression level, protein samples were suspended in 1× non-reducing sample buffer [0.25% bromophenol blue, 50% glycerol, 10% sodium dodecyl sulfate (SDS)] and performed Western blotting as following description with rabbit polyclonal antibody against interleukin 12 p40 (IL12p40, 1:1000; GeneTex GTX88847).

2.5. Neural differentiation assay

Single cells were dissociated from the neurospheres using 1× HyQTase (Hyclone). 2×10^3 cells were seeded onto Poly-D-Lysine (PDL, BD Bioscience)-coated chamber slides (Nunc, Naperville, IL, USA) for following immunofluorescence staining. For Western blotting, 5×10^5 cells were seeded onto PDL-coated Coring 100 mm tissue culture dish. The differentiation medium was DMEM/F12 containing 2% FBS (as control) supplemented with or without inducing factors. In the CNTF + T3group, differentiation condition was modified from our