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The integration of NSC-derived and host neural networks after rat spinal cord transection

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ABSTRACT

Rebuilding structures that can bridge the injury gap and enable signal connection remains a challenging issue in spinal cord injury. We sought to determine if genetically enhanced expression of TrkC in neural stem cells (NSCs) and neurotrophin-3 in Schwann cells (SCs) co-cultured in a gelatin sponge scaffold could constitute a neural network, and whether it could act as a relay to rebuilt signal connection after spinal cord transection. Indeed, many NSCs in the scaffold assumed neuronal features including formation of synapses. By whole-cell patch clamp, the synapses associated with NSC-derived neurons were excitable. Grafting of the scaffold with differentiating NSCs + SCs into rats with a segment of the spinal cord removed had resulted in a significant functional recovery of the paralyzed hind-limbs. Remarkably, the NSC-derived neurons formed new synaptic contacts suggesting that the scaffold can form a relay for conduction of signals through the injury gap of spinal cord.

1. Introduction

Severe spinal cord injury (SCI) is often accompanied by irreversible spinal tissue damage, limited endogenous repair, and permanent loss of motor, sensory, and autonomic functions [1,2]. A variety of cellular and fetal tissue grafting tactics have been used, with reports showing different degrees of tissue repair and behavioral improvements after spinal cord injury [3,4]. However, to date there is still lack of a tangible approach to effectively restore synaptic connectivity after spinal cord transection.

Neural stem cells (NSCs) encompass precursors for neurons, astrocytes and oligodendrocytes. In view of this, they render therapeutic potential for neurological diseases, such as SCI [5–7]. Therefore, effort should be made to increase the survival and neuronal differentiation of NSCs in cases where cell replacement is desirable. However, it has remained to be fully investigated whether NSCs are able to form functional synaptic contacts and neuronal network for effectively functioning connections in the injured spinal cord.

Schwann cells (SCs) is one of the most studied cell types for spinal cord repair. They have been shown not only to myelinate (remyelinate) axons after transplantation into the injured spinal cord, but also form a permissive substrate for regenerating axons [8]. SCs secrete neurotrophic factors (NTFs), which have positive effects on the survival and neuronal differentiation of NSCs and mesenchymal stem cells (MSCs) [8,9]. Consequently, a combined strategy has been developed taking advantage of SCs along with bioengineering products, adhesion molecules or NTFs to promote functional axonal regeneration through the lesion site as well as to enhance plasticity and recovery of spinal cord injuries [8,10].

NTFs are vital promoting factors for neural regeneration. Neurotrophin-3 (NT-3) is one of the best-characterized neurotrophic factors that is important in survival and differentiation of neuron, and synaptogenesis [6–8]. The action of NT-3 is mediated by its preferential binding to the receptor TrkC. Previous work in our laboratory has demonstrated that NSCs were promoted to differentiate into neuron-like cells and form functional synapses

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in vitro when transfected with adenovirus carrying NT-3 (Ad-NT-3) gene and TrkC (Ad-TrkC) gene [11].

It has been reported that three-dimensional (3D) poly lactide-co-glycolide (PLGA) scaffold could permit genetically modified NSCs to differentiate toward neurons which establish connections and exhibit synaptic activities *in vitro* [11]. However, when the PLGA scaffold was transplanted into injured spinal cord, acid product (acid microenvironment) of PLGA degradation could prevent the regeneration of corticospinal tract (CST) axons [12]. Gelatin sponge (GS) as engineered biomaterials can provide a platform to deliver therapeutic cells and/or neurotrophic molecules, to supply guidance for regenerating axons, and to prevent the infiltration of scar tissue and cyst formation in the SCI animal models [13]. In the present study, we attempted to co-culture NT-3 gene modified SCs and TrkC gene modified NSCs in 3-dimensional (3D) gelatin sponge scaffolds, to explore whether these cells could form a neural network with functional synapses, and to find out whether this neural network could be used to recapitulate a biologically permissive microenvironment for neural restoration at the lesion gap following spinal cord transection.

2. Materials and methods

2.1. NSCs and SCs preparation and identification

NSCs were isolated as previously described [14], from green fluorescent protein (GFP) transgenic Sprague–Dawley (SD) rats (Osaka University, Osaka, Japan). Briefly, rats (1–3 days old) were anesthetized. The whole hippocampus was dissected and dissociated. Basal medium, including DMEM/F12 (1:1), B27 supplement and basic fibroblast growth factor (bFGF). To obtain SCs, SD neonate rats (5–6 days old) were decapitated and sterilized. Sciatic nerves and brachial plexus were dissected. The epineurium and connective tissue were removed under a dissecting microscope. All nerves were cut into small pieces (<2 mm) and dissociated with 0.16% collagenase (Sigma). Culture medium contain DMEM/F12, 10% FBS, 2 μmol/l forskolin (Sigma) and 20 μg/ml bovine pituitary extract (Sigma). The cells were passaged when 90% confluence was reached and were purified by differential adhesion and differential digestion techniques [7].

2.2. NSCs and SCs transfection and seeding in three-dimensional gelatin sponge scaffolds

Recombinant adenoviral (Ad) vectors (Ad-TrkC and Ad-NT-3) were produced as described in our previous study [14]. Neurospheres were infected with Ad-TrkC. SCs were infected with Ad-NT-3. Five experimental groups were established: the NSCs, SCs + NSCs, SCs + TrkC-NSCs (or SCs + T-NSCs), NT-3-SCs + NSCs (or N-SCs + NSCs) and NT-3-SCs + TrkC-NSCs (or N-SCs + T-NSCs) groups. The transfection efficiency with these viral vectors has been published multiple times in the past [6,7,14]. Three-dimensional (3D) gelatin sponge scaffolds were prepared as previously described [13]. A total of 5×10^5 cells (NSCs and SCs were mixed in 1:1 ratio) in 10 μl culture medium were seeded to each scaffold. The scaffolds were incubated for 14 days.

2.3. Western blot

After 14 days in culture, 3 scaffolds from each group were dissolve intracellular and extracellular proteins, equal amounts of the protein suspension were loaded on a 10% polyacrylamide gel, separated by gel electrophoresis, and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with rabbit anti-PSD95 antibody, rabbit anti-ChAT antibody and rabbit anti-GFP antibody at 4 °C overnight, followed by incubation with anti-rabbit HRP-conjugated IgG. The bands were detected with an enhanced chemiluminescence (ECL) Western blot substrate kit.

2.4. Whole-cell patch clamp

The whole-cell configuration was used to record the electrical activities with an HEKA EPC amplifier 10 (HEKA Inc., Germany), and Patchmaster software (HEKA Inc., Germany). Data were filtered at 1 kHz and sampled at 5 kHz. The external solution contains 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (320 mOsm, pH set to 7.3 with Tris-base). The patch electrodes had a resistance of 3–5 MW, when filled with pipette solution, containing 140 mM CsCl, 2 mM $MgCl_2$, 4 mM EGTA, 0.4 mM $CaCl_2$, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM GTP. The pH was adjusted to 7.2 with Tris-base, and the osmolarity was adjusted to 280–300 mOsm with sucrose. Electrophysiological recordings were performed at room temperature (22 °C–24 °C). Spontaneous postsynaptic currents (sPSCs) were counted and analyzed using Fitmaster (HEKA Inc., Germany).

2.5. Spinal cord transection and transplantation

Three days before surgery, animals were given cyclosporine A subcutaneous injection in belly of rats. Adult female SD rats (220–250 g, supplied by the Experimental Animal Center of Sun Yat-sen University) were anesthetized. Following laminectomy at the T9 vertebral level, a 2-mm cord segment including visible spinal roots was completely removed at the T10 spinal cord level. After hemostasis was achieved, NSC-derived neural networks (the N-SCs + T-NSCs group) or gelatin sponge scaffolds (the control group) were used to fill up the gap. Cyclosporine A was administrated once every day for two months. All experimental protocols and animal handling procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University, and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.6. Assessment of locomotor performance

Hindlimb function of the rats was assessed weekly after surgery, using the Basso, Beattie and Bresnahan (BBB) open-field locomotor test [15] and inclined-grid climbing test as described by Ramon-Cueto et al. [16]. The former quantitatively evaluates voluntary movement and body weight support. The latter assesses accuracy of foot placement and coordination qualitatively which differentiate local reflex activity from voluntary movement. Two independent investigators blind of the different experimental treatments determined the BBB scores.

2.7. Electrophysiological analysis

At the end of the experiment, evoked potentials (EP) were recorded as described previously to assess functional status of motor and sensory axonal conduction. Basically, following general anesthesia and exposure of the sciatic nerves and sensorimotor cortex (SMC), the electrodes (BL-420E Data Acquisition Analysis System for Life Science, Taimeng, Chengdou, China) were connected to the sciatic nerve and SMC. The cortical motor evoked potential (CMEP) were calibrated first, and then recorded as per our standardized protocols [6].

2.8. Immunofluorescence staining

Specific proteins were determined using immunofluorescence staining as described in our previous publication [6]. Briefly, sections of 25 μm thickness were cut using a cryostat. The sections were incubated with primary antibodies mixed in 0.3% Triton X-100 overnight at 4 °C, followed by incubation with secondary antibodies. The slides were then examined under a fluorescence microscope. A summary of antibodies used is provided in Table 1.

2.9. Ultrastructural observations

For scanning electron microscope (SEM), scaffolds with cells were firstly washed 3 times with PBS, fixed in 2.5% glutaraldehyde for 90 min, dehydrated with a series of graded ethanol, and then freeze dried for 2 days. The dried samples were coated with gold and examined under a scanning electron microscope (Philips XL30 FEG).

For transmission electron microscope (TEM), scaffolds were fixed with 2.5% glutaraldehyde at 4 °C for 1 h and osmicated with 1% osmic acid for 1 h. Scaffolds were dehydrated through graded ethanol and embedded in Epon overnight, followed by polymerization at 60 °C for 48 h. Semithin sections were cut on a Leica RM2065 microtome and mounted on glass slides. They were then stained with toluidine blue (5%, in a borax solution) and mounted using neutral balsam. Ultrathin sections were cut and examined under an electron microscope (Philips CM 10, Eindhoven, Holland).

For immunoelectron microscopy (IEM), rats were transcardially perfused with 0.1 mol/L of sodium phosphate buffer containing 187.5 units/100 ml of heparin, followed by perfusion with 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% saturated picric acid. Dissected spinal cords were postfixed overnight at 4 °C in fresh fixative and subsequently cut into 50 μm sagittal sections on a vibratome. To improve the penetration of antibodies, vibratome sections were transferred into cryprotectant solution containing 25% sucrose and 10% glycerol in 0.1 M PBS overnight at 4 °C, followed by a quick freeze-thaw in liquid nitrogen three times. After washing with PBS, the sections were treated for 1 h with 20% goat serum (Tris buffer, pH 7.4) to block nonspecific binding of the antibody. Sections were first incubated with primary antibodies in 2% normal goat serum solution at 4 °C for 24 h, then incubated with secondary antibodies overnight at 4 °C, and postfixed in 1% glutaraldehyde for 10 min. The sections were detected by SABC-DAB Kit and silver enhanced with HQ silver Kit (NanoProbe 2012, Yaphank, NY), osmicated, dehydrated, and embedded in Epon. Epon blocks were sectioned and examined under the electron microscope (Philips CM 10, Eindhoven, Holland).