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Integration of donor mesenchymal stem cell-derived neuron-like cells into host neural network after rat spinal cord transection

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ABSTRACT

Functional deficits following spinal cord injury (SCI) primarily attribute to loss of neural connectivity. We therefore tested if novel tissue engineering approaches could enable neural network repair that facilitates functional recovery after spinal cord transection (SCT). Rat bone marrow-derived mesenchymal stem cells (MSCs), genetically engineered to overexpress TrkC, receptor of neurotrophin-3 (NT-3), were pre-differentiated into cells carrying neuronal features via co-culture with NT-3 overproducing Schwann cells in 3-dimensional gelatin sponge (GS) scaffold for 14 days *in vitro*. Intra-GS formation of MSC assemblies emulating neural network (MSC-GS) were verified morphologically via electron microscopy (EM) and functionally by whole-cell patch clamp recording of spontaneous post-synaptic currents. The differentiated MSCs still partially maintained prototypic property with the expression of some mesodermal cytokines. MSC-GS or GS was then grafted acutely into a 2 mm-wide transection gap in the T9-T10 spinal cord segments of adult rats. Eight weeks later, hindlimb function of the MSC-GS-treated SCT rats was significantly improved relative to controls receiving the GS or lesion only as indicated by BBB score. The MSC-GS transplantation also significantly recovered cortical motor evoked potential (CMEP). Histologically, MSC-derived neuron-like cells maintained their synapse-like structures *in vivo*; they additionally formed similar connections with host neurites (i.e., mostly serotonergic fibers plus a few corticospinal axons; validated by double-labeled immuno-EM). Moreover, motor cortex electrical stimulation triggered c-fos expression in the grafted and lumbar spinal cord cells of the treated rats only. Our data suggest that MSC-derived neuron-like cells resulting from NT-3-TrkC-induced differentiation can partially integrate into transected spinal cord and this strategy should be further investigated for reconstructing disrupted neural circuits.

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1. Introduction

Severe spinal cord injury results in complete loss of central motor control of function below the injury site. The pathophysiology of spinal cord injury is complex and, therefore, to achieve functional recovery [1] several challenging objectives, such as decreasing glial and neuronal cell death, reducing scarring and

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cavitation, blocking inhibitory molecules in the lesion area and stimulating functional axonal regeneration [2–3], should be taken into consideration. Several studies using repair strategies have indicated that damaged axons can regenerate through the lesion site if given an appropriate environment [4–7], but regeneration of axons is limited by the intrinsic disabilities of adult central nervous system (CNS) neurons [8], and by a hostile post-injury environment which mainly derived from chronic inflammation and accumulation of inhibitory factors [9–12]. It is insufficient for regenerating axons to overcome physical barriers such as scars or cavities, as well as the inhibitory molecules [13]; hence descending corticospinal tract (CST) axons can hardly reestablish signal connection with intact lower motor neurons, which eventually would result in motor function disability [13,14]. However, based on the premise that CST axons could sprout into the intact side of spinal cord with an incomplete spinal cord injury (SCI) rat and made contacts with the propriospinal neurons (PSNs), and that such configurations were maintained over the lesion, and finally output signals to lumbar motor neurons indirectly [15,16], it is promising to rearrange the cortical hindlimb motor map by harnessing the spared PSNs or other interneurons as a relay. On the other hand, for complete or more severe SCI, since most of the effector neurons or interneurons within the injured area are totally destroyed, tissue engineering approach would be desirable to bridge a larger lesion. The core concept of tissue engineering and its success hinge on nature of transplanted cells, bioactive molecules and scaffolds, and their interactions. With optical combination of the three elements mentioned, tissue engineering approach is expected to bridge the cavities, to rectify the microenvironment of the injured area and to probably serve as relay ‘cell stations’ like the PSNs.

To achieve this goal, the *ex vivo* engineered cell-scaffold constructs should be transplanted to the site of injury. Neuronal replacement by grafted cells would compensate lost endogenous neurons [17–20]. In the present study, we focused on mesenchymal stem cells (MSCs) derived from the bone marrow. MSCs have the advantage of their easy acquisition, and relatively rapid expansion *in vitro*, together with their neuroprotection and modulating post-injury microenvironment ability, making them ideal candidates for CNS repair [21]. Transdifferentiation of MSCs into neurons is still controversial although many *in vitro* studies [22–26] and transplantation studies [27–29] showed neuronal properties of MSCs derived cells under different inducing environments. One major concern is that the induced cells failed to integrate into the host neuronal network. Nevertheless, several electrophysiological studies did show electrical activities of MSCs derived neurons [26,30], suggesting the potential of transdifferentiation. Using Schwann cells (SCs) to induce neural differentiation of MSCs, we had demonstrated varied results in 2-dimensional (2D) culture [31] or 3-dimensional (3D) poly (lactide-co-glycolide) (PLGA) multiple-channel conduits culture [32], indicating potential neuronal inductive effect of 3D culture. Considering the host niches may exert profound influence on stem cells [33], we have used in the present study an alternative 3D gelatin sponge (GS) scaffold with good cytocompatability and histocompatibility [34] to deliver cells. We have reported that besides action on neuronal differentiation of MSCs [31,32,35] or neural stem cells (NSCs) [36,37], neurotrophin-3 (NT-3) could protect neurons [38–40] and promote axon regeneration [36,39,41]; hence, NT-3 and its receptor TrkC as bioactive molecule were further investigated in this study. We aimed to construct tissue engineered synaptic connections originated from MSC-derived neuron-like cells, and to examine whether they could survive and integrate with host neural networks, and most importantly whether they would serve as relay neuron-like cells to restore hindlimb motor function after SCI.

2. Materials and methods

2.1. Isolation and culture of MSCs

MSCs were isolated as previously described [31] from green fluorescent protein (GFP) transgenic Sprague–Dawley rats (Osaka University, Osaka, Japan) which express ubiquitously GFP in all their tissues. Briefly, one-week-old rats were sacrificed and their femurs were flushed with and then cultured in the low glucose Dulbecco's modified Eagle's medium (L-DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, TBD Co, Tianjin, China) and 4 mM L-glutamine (Invitrogen) in an incubator with 5% CO_2 at 37 °C. When the adherent cells grew to 80% confluence, they were passaged (1:3) into different culture flasks. MSCs of passages 3–6 were employed for the study.

2.2. Purification of SCs

Acquisition of SCs was also performed as previously described [31]. Five-day-old Sprague–Dawley neonatal rats were killed by decapitation and cleaned by brief submersion in 70% ethanol. Sciatic nerves and brachial plexus were dissected, and placed in a sterile culture dish containing ice-cold D-Hank's solution without calcium chloride or magnesium chloride, where they were maintained until the nerves had been harvested from all of the rats. Epineurial membranes were removed under an anatomical microscope. Nerves were cut into small pieces (<2 mm) and digested with 0.16% collagenase (Sigma–Aldrich, St. Louis, MO) in 37 °C for 15 min. Dissociated tissue were plated on polylysine-coated culture dishes with some DMEM/F12 medium consisting of 10% FBS (TBD). After 30 min, an extra 2 ml of the above-mentioned culture medium was added to the culture dishes. Cells were maintained in a cell culture incubator at 37 °C, with a 5% CO_2 humidified atmosphere. The medium was changed every 2 days. The cells were subcultured when the culture dishes were 80% confluent, typically after 5–7 days in culture, depending on the initial SC population size, and the cell proliferation rate. Cells were purified by differential velocity adherent methods. As with our previous study, the purity of the SCs seeded to the scaffold material was 95–96% [31,32].

2.3. Adenovirus infection

The SCs and MSCs were engineered to over-secrete NT-3 and overexpress TrkC by recombinant adenovirus (Ad) carrying NT-3 gene (Ad-NT-3) and TrkC gene (Ad-TrkC), respectively. Following procedures described in our previous studies [31,32], SCs were transfected by Ad-NT-3 (100 multiplicity of infection [MOI]), and MSCs were transfected by Ad-TrkC (300 MOI) for 3 h. Then, the medium was replaced by DMEM/F12 with 10% FBS to allow growth for another 24 h.

2.4. Co-culture of MSCs and SCs in 3D gelatin sponge scaffolds

The construction of 3D gelatin sponge (GS) scaffolds was performed as previously described. Before cells were seeded to a GS scaffold, the scaffold was soaked with the culture medium for 10 min, and then excessive fluid was absorbed by a Waterman filter paper. A total of 1×10^5 cells in 10 μl culture medium were seeded to each GS scaffold. The cell number of MSCs versus that of SCs was 1:1. Altogether, 6 groups of cells were designed in this experiment *in vitro*. They were the MSCs (M), MSCs+SCs (M+S), MSCs+NT-3-SCs (M+SN), TrkC-MSCs (MT), TrkC-MSCs+SCs (MT+S) and TrkC-MSCs+NT-3-SCs (MT+SN) groups. In order to facilitate cell adhesion, the scaffolds with seeded cells were incubated in humidified atmosphere at 37 °C for 15 min in a 24-well plate (1 scaffold per well) before being submerged in 500 μl culture medium. The culture medium was changed every 2 days. At 14 days after culture, some of the scaffolds in each group were immersed in the 4% paraformaldehyde fixative in 0.1 M PBS (pH 7.4, 4 °C overnight) followed by cytoprotection in 0.1 M PB containing 30% sucrose for 1–2 days at 4 °C. The scaffolds were then embedded in an optimum cutting temperature (OCT) compound, cut serially in transverse sections at 30 μm thickness with a cryostat, and mounted on gelatin-coated slides. Prolonged culture period up to 28 days was adopted to test the overall cell survival condition after long-term culture using caspase-3 immunostaining.

2.5. 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 of MSC-derived neuron-like cells in the MT+SN group were performed at room temperature (22–24 °C). We tested altogether 78 cells with benign gigaseal of membrane for the induction of action potential and recording of spontaneous post-synaptic currents (sPSCs). Forty-three cells in the M group were tested as a control. Once sPSCs were recorded, cells were bathed with antagonists of neurotransmitter receptors as follows: PTX (picrotoxin 10 μM, Sigma) for GABA-A receptor, APV (2-amino-5-

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