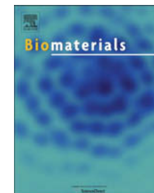
Biomaterials 30 (2009) 3711–3722

Contents lists available at ScienceDirect

Biomaterials

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



Synaptic transmission of neural stem cells seeded in 3-dimensional PLGA scaffolds

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ARTICLE INFO

Article history:
Received 10 March 2009
Accepted 19 March 2009
Available online 17 April 2009

Keywords:
Neural stem cell
Transplantation
PLGA
Synapse
NT-3
TrkC

ABSTRACT

To explore therapeutic potential of engineered neural tissue, we combined genetically modified neural stem cells (NSCs) and poly(lactic acid-co-glycolic acid) (PLGA) polymers to generate an artificial neural network *in vitro*. NSCs transfected with either NT-3 or its receptor *TrkC* gene were seeded into PLGA scaffold. The NSCs were widely distributed and viable in the scaffold after culturing for 14 days. Immunoreactivity against Map2 was detected in >70% of these grafted cells, suggesting a high rate of differentiation toward neurons. Immunostaining of synapsin-I and PSD95 revealed formation of synaptic structures, which was also observed under electron microscope. Furthermore, using FM1-43 dynamic imaging, synapses in these differentiated neurons were found to be excitable and capable of releasing synaptic vesicles. Taken together, our artificial PLGA construct permits NSCs to differentiate toward neurons, establish connections and exhibit synaptic activities. These findings provide a biological basis for future application or transplantation of this artificial construct in neural repair.

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1. Introduction

Spinal cord injury (SCI) is a highly prevalent medical problem, which may be resulted from motor vehicle accidents or other trauma [1]. For instance, there is an annual incidence of 40 SCIs per million populations in the USA (http://www.spinalcord.uab.edu/). Victims of SCI are often afflicted by severe neurological disabilities, yet there has been no effective treatment up to date. The pathophysiological processes in SCI are multifactorial, involving blood vessel rupture, ischemia, edema, metabolic derangement, and free radicals formation in acute phase, and followed by axonal degeneration/regeneration, loss of glial cells, demyelination/remyelination, and formation of cavities in the injured site [2]. These devitalized tissues have been proposed to be replaced by exogenous neural tissues to restore functions. Toward this end, effort has been made to transplant neural tissues to mitigate neurological disabilities in SCI [3].

Neural stem cell (NSC) are the cells that can continuously self-renew and differentiate into both neuronal and glial lineages, including neuron, astrocyte, and oligodendrocyte [4,5]. These features render therapeutic potential for neurological diseases [6], such as SCI [3,7–11]. However, effectiveness of NSC transplantation in the SCI is limited, particularly when descending/ascending pathways are separated by a gap. Grafted NSCs alone are often incapable of forming a neural network to bridge the gap in the injured spinal cord [7,10,11].

To circumvent this problem, engineered tissues, using poly(L-lactic acid) (PLLA) or poly(lactic acid-co-glycolic acid) (PLGA) polymers, have been investigated to be used as a structural frame of the bridge. Tissue compatible polymers have been used to carry exogenous cells (such as Schwann cells or NSCs) into SCI animal model and promote recovery of hindlimb motility [3,12–14]. This approach appears sustainable in long term. For instance, in hemisection SCI model of rat, implantation of PLGA scaffold with NSCs into the injured site resulted in a functional improvement for one year. The transplantation reduced tissue loss and glial scarring [3]. PLLA scaffold-Schwann cells construct also improved the hindlime motor recovery in completed transection model [13].

Both PLLA and PLGA appear biocompatible in the injured site of spinal cord [3,12–14], and can be easily manufactured. PLGA

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doi:10.1016/j.biomaterials.2009.03.046

scaffold is biodegradable and may prevent scarring and cyst formation in the SCI animal models [3,15]. While disassociated single-cells may have compromised viability [16], neurospheres of NSCs seeded into scaffolds of the polymers showed improved survival and differentiation [16,17]. However, the biological basis for the therapeutic effect of NSC–polymer construct is still unclear. Regeneration of axons only passes through the injury epicenter, but not through the injury core after the construct is transplanted into the SCI animal model [3]. Whether NSCs are able to form functional synapses and neuronal network in the construct, an indispensable pre-requisite for re-establishing effectively functioning connections in the injured spinal cord, is unknown.

Neurotrophins (NTs) are known to play important roles in neural survival, differentiation, and neurite outgrowth [18,19]. NSCs transfected with NT-3 yield a high percentage of differentiation toward neurons [20,21]. This percentage may be further increased when NSCs over-expressing NT-3 are mixed with NSCs expressing tyrosine receptor kinase C (*TrkC*), the NT-3 receptors [20]. Therefore, the present study engineers a PLGA neural construct containing grafted NSCs. The grafted NSCs transfected with either NT-3 or *TrkC* were mixed to optimize their differentiation toward neurons. This model is then allowed us to examine the formation of neural network in the artificial neural construct.

2. Materials and methods

2.1. Preparation of PLGA scaffolds

Macroporous PLGA scaffold was synthesized as described in our earlier work [22]. Briefly, PLGA with an 75:25 monomer ratio (D,L-lactide:glycolide) was synthesized by ring-opening polymerization using Sn $(Oct)_2$ as catalyzer and dodecenol as initiator. The average molecular weight of PLGA copolymer was 1.22×10^5 (M_n GPC). To obtain different pore sizes, the polymer concentration was increased from 2.5% to 20%. Sodium chloride as porogen was added into the polymer solution with PLGA/NaCl in a weight ratio of 1:9. PLGA scaffold formed variable sizes of pore from a few μm to 200 μm that were suitable for seeded neurospheres with diameters of 100–300 nm. PLGA rods with longitudinal parallel-channels were fabricated by an injection molding, combined with thermally induced phase separation. The lumens of the mold were pretreated with chlorotrimethylsilane, and placed into the freezer at −40 °C for at least one hour. Five% (w/v) PLGA solution in 1,4-dioxane was injected into the cold mold quickly with a syringe, and kept the injection pressure until the polymer solution at the injection port of syringe was completely frozen. The mold was placed in the −40 °C freezer for another two hours. The scaffolds were then lyophilized under 0.940 mbar at 0 °C for at least four days. The polymer scaffold was trimmed into a rod shape with two cm in length and five mm in diameter. The rods were sterilized by 70% alcohol for 10 min, rinsed with sterilized phosphate buffered solution (PBS, pH 7.4) for 30 min, and were stored in a desiccator. Upon seeding NSCs into the PLGA for culture, the PLGA rod was cut into two mm thickness slices in transverse for the seeding.

2.2. NSCs preparation and identification

NSCs were prepared as described previously [8]. Briefly, three-to-five-days-old Sprague–Dawley (SD) rat pups were anesthetized. Whole hippocampi were dissected and dissociated in D-Hanks' balanced salt solution (HBSS). After centrifuging at 1000 rpm for five minutes, the supernatant was removed. Pellet was resuspended in five ml basal medium including: DMEM/F12 (1:1) containing B27 supplement (20 μl/ml, Gibco, CA, USA) and bFGF (20 ng/ml, Invitrogen, CA, USA). The cells were plated onto 75-ml culture flasks. The medium was replaced every three days. Typically, the cells grew as suspending neurospheres and were passaged approximately once per week. To confirm the neurospheres were nestin-positive cells, cultures were fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature and rinsed in PBS, labeled with monoclonal anti-nestin (Table 1), a marker of neural precursor cell, followed by incubation with FITC-conjugated anti-mouse IgG (1:1000, Jackson Immunological Research, PA, USA). The slides were examined under fluorescence microscope.

2.3. NSCs transfection and seeding in PLGA scaffold

Recombinant adenoviral (Adv) vectors (Ad-NT-3 and Ad-*TrkC*) were produced as described in our previous study [20]. Ad-LacZ (gift from Dr. Huang WL) was used as control [10,11,20]. Neurospheres were infected with Ad-LacZ, Ad-NT-3 or Ad-*TrkC* at a multiplicity of infection (MOI) of 50. The cells were resuspended in five ml fresh basic medium and plated onto 25 ml-culture flasks for 48 h. Five experimental groups were established: NSCs only; LacZ–NSCs; *TrkC*–NSCs; NT-3-NSCs; NT-3/*TrkC*–NSCs (co-culture of NT-3-NSCs mixed with *TrkC*–NSCs). The transfection efficiency with these viral vectors has been published multiple times in the past [10,11,20]. Before seeding, we dissociated neurospheres mechanically and counted the number of cells on the cell board. We extracted 20 μl cell suspension and drop one drop on the board. Under light microscope, cells (A single neurosphere was regarded as one cell) were counted in the grids. Cells or neurospheres were also counted when they were on the right or upper border of the rectangular grid. The number of cells was calculated using a formula ((the total number of four quadrants of grids/ 100)$\times$400 $\times$ 10^4). For the NT-3/*TrkC* group, we first counted the cell number for both NT-3 and *TrkC* group, and then mixed them in 1:1 ratio. Difficulties were encountered initially to seed neurospheres into the scaffold. To circumvent this problem, 2.4×10^6 cells in 20 μl culture medium (including 1:1 DMEM/F12 and 10% fetal bovine serum) (TBD, Co, Tianjin, China) were placed on the top of PLGA slice. The seeding was facilitated by placing a Waterman filter paper (#1) underneath the slice to gently suck cells into the pores. The slices were incubated in 35 mm culture dish for 14 days. The culture medium was replaced every two days. In some experiments for the NT-3/*TrkC* group, 100 nM K252a (Calbiochem, Darmstadt, Germany), an inhibitor of neurotrophin-related tyrosine kinase, was added into the culture medium [21].

2.4. Live–dead staining

To evaluate the viability of the cells grafted into the PLGA, NSCs only, LacZ and NT-3/*TrkC* were seeded into PLGA slices with two mm thickness. After 14 days culture, the slices were rinsed with 0.1 M phosphate buffer (pH 7.4) for 30 min. The slices were incubated in two ml 0.1 M phosphate buffer containing 2 μM of calcein-AM and 4 μM of ethidium homodimer (EthD-III) (Viability/Cytotoxity Assay Kit for Live & Dead Animal Cells, Biotium, USA) for one hour at 37 °C. The slices were rinsed three times (20 min for each), and then fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. Transverse sections with 20 μm thickness were cut in succession using a cryostat. The 1st to the 5th sections were defined as peripheral sections and the 46th to the 50th as center section. The live-cells stained with calcein-AM show green color and the dead cells stained with EthD-III show red color under fluorescent microscope. Cell death rate was calculated by determining the percentage of EthD-1-positive cells over total cell number. For each experimental group, at least five fields of each section (including 4 corners and one centre) were imaged. The final cell death rate was derived from the average of three sets of experiments.

Table 1
Primary antibodies.

Antibodies	Source	Species	Type	Dilution	Reference
Nestin	Chemicon	Mouse	Monoclonal IgG	1:500	Jahr et al., 2003
Map2	Sigma	Mouse	Monoclonal IgG	1:500	Binder et al., 1986
GFAP	Sigma	Rabbit	Polyclonal IgG	1:500	Debus et al., 1983
MOSP	Chemicon	Mouse	Monoclonal IgG	1:1000	Mu et al., 1994
PSD95	Sigma	Mouse	Monoclonal IgG	1:500	Kornau et al., 1995
Synapsin-I	Sigma	Rabbit	Polyclonal IgG	1:1000	Stone et al., 1994
p-C-jun	Santa Cruz	Mouse	Monoclonal IgG	1:500	Yatsushige et al., 2005
β III tubulin	Sigma	Rabbit	Polyclonal IgG	1:500	Zhang et al., 2006
p-p38	Cell Signaling	Rabbit	Polyclonal IgG	1:1000	Ge et al., 2005
ChAT	Chemicon	Rabbit	Polyclonal IgG	1:500	Nunes-Tavares et al., 2000
5-HT	Sigma	Rabbit	Polyclonal IgG	1:500	Peressini et al., 1984
Glutamate	Boster	Rabbit	Polyclonal IgG	1:200	Ma et al., 2007
GABA	Boster	Rabbit	Polyclonal IgG	1:200	Li et al., 2005

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