Biomaterials xxx (2013) 1–11

Contents lists available at ScienceDirect

Biomaterials

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

Functional recoveries of sciatic nerve regeneration by combining chitosan-coated conduit and neurosphere cells induced from adipose-derived stem cells

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ARTICLE INFO

Article history:
Received 18 September 2013
Accepted 27 November 2013
Available online xxx

Keywords:
Chitosan
Sciatic nerve injury
Nerve conduit
Adipose-derived stem cell
Spheroid
Neurosphere

ABSTRACT

Suboptimal repair occurs in a peripheral nerve gap, which can be partially restored by bridging the gap with various biosynthetic conduits or cell-based therapy. In this study, we developed a combination of chitosan coating approach to induce neurosphere cells from human adipose-derived stem cells (ASCs) on chitosan-coated plate and then applied these cells to the interior of a chitosan-coated silicone tube to bridge a 10-mm gap in a rat sciatic nerve. Myelin sheath degeneration and glial scar formation were discovered in the nerve bridged by the silicone conduit. By using a single treatment of chitosan-coated conduit or neurosphere cell therapy, the nerve gap was partially recovered after 6 weeks of surgery. Substantial improvements in nerve regeneration were achieved by combining neurosphere cells and chitosan-coated conduit based on the increase of myelinated axons density and myelin thickness, gastrocnemius muscle weight and muscle fiber diameter, and step and stride lengths from gait analysis. High expressions of interleukin-1β and leukotriene B4 receptor 1 in the intra-neural scarring caused by using silicone conduits revealed that the inflammatory mechanism can be inhibited when the conduit is coated with chitosan. This study demonstrated that the chitosan-coated surface performs multiple functions that can be used to induce neurosphere cells from ASCs and to facilitate nerve regeneration in combination with a cells-assisted coated conduit.

1. Introduction

Despite advances in neuroscience and microsurgery, the process of repairing peripheral nerve injuries remains suboptimal, particularly when a nerve gap is present [1]. After the injury occurs, the distal stump undergoes axon demyelination and degradation, causing the atrophy of effector muscles and the impairment of normal functions, such as gait [2]. The most widely used technique for bridging the gap in an injured nerve is using an autologous nerve graft to guide the regenerating nerve [3]. However, the functional results of using a nerve graft are variable and the lack of a donor nerve also impedes the application of the nerve graft in a clinical setting [4]. Three types of conventional bridging material have been used in the past few decades. The first type is biological conduits such as blood vessels, skeletal muscles, and tendons, but the length of the conduit is limited because of problems related to tubular collapse, poor regeneration, scar tissue proliferation, and adhesion. The second type is nonbiologic synthetic conduits such as silicone tubes and nylon tubes. The results remain poor because that the cytotoxicity might cause a subsequent foreign body reaction and prolonged inflammation [5]. The third type is biodegradable ones including poly(lactide-*co*-glycolide), poly(phosphoester),

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0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.11.081

polyurethanes, chitosan, and decellularized biomatrices. The controllable degradation can be tailored by altering their molecular weight and composition. In addition, biofunctional modification including incorporation or surface-tethering of neurotrophic factors potentiates its clinical application [6].

In addition to conduits, using neural stem cell (NSC) therapy is another approach to facilitate peripheral nerve regeneration [7]. NSCs are primarily produced by isolating them from the fetal central nervous system or by deriving the NSCs from embryonic stem cells (ESCs), which raises critical ethical concerns and increases the risk of tumor formation [8]. The adipose-derived stem cells (ASCs) are sources of multipotent stem cells that can be differentiated into several distinct lineages [9]. The ASCs and bone marrow-derived stem cells (BMSCs) possess similar characteristics regarding multipotency and molecular signatures, and also share common genetic signals [10,11]. The advantages of using ASCs are the abundance of these cells, the ease of harvesting the cells by performing minimally-invasive procedures, and an autologous origin that requires no immunosuppression [12]. The ASCs can trans-differentiate into the neural lineage and form free-floating spheroid bodies in various neurotrophic media [13,14]. Similar genetic expression profiles are present in spheroid bodies derived from ASCs, BMSCs, and NSCs [15]. These spheroid bodies, called neurospheres, are capable of self-renewal and clonal isolation, and possess great potential for generating neuronal [16], glial [17], and oligodendrocyte [18] cells.

Numerous biomaterials have been proposed to guide peripheral nerve regeneration [6,19]. Among these biomaterials, chitosan tubes may facilitate and guide axon regeneration. Chitosan is a naturally occurring polysaccharide and is non-cytotoxic and highly biodegradable. Chitosan has been widely used in gene delivery [20,21], cell culture [22–24], and tissue engineering [25,26]. In addition, chitosan produces anti-inflammatory effects against neutrophil infiltration into organs, tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) levels in serum, as well as antioxidative properties [27]. Several studies have demonstrated that a chitosan conduit can act as a favorable scaffold biomaterial characterized by minimal cytotoxicity and high biodegradability to promote nerve regeneration when combined with Schwann cells [28], NSCs [29,30] or BMSCs [31–33]. Only a few studies has reported that chitosan was combined with ASCs to facilitate sciatic nerve regeneration [34,35]. In our previous studies, the neurogenic potential of human ASCs was induced and formed neurosphere on a chitosan-coated surface [36]. The current study designed several functional evaluations for illustrating the therapeutic outcomes of sciatic nerve regeneration to bridge a 10-mm gap by using conduits coated with or without chitosan, and by combining neurosphere cells derived from human ASCs. We also investigated the underlying mechanisms of therapeutic effects produced by a chitosan-coated conduit with or without performing the cell-based treatments.

2. Materials and methods

2.1. Preparation of the chitosan-coated plate and conduit

The preparation of cell culture plates and nerve conduits coated with chitosan (the degree of deacetylation was 85%; 417963, Sigma–Aldrich, St. Louis, MO, USA) was conducted in accordance with the process used in our previous study [36]. In summary, 1% *w*/*v* of chitosan powder was dissolved in 1 M of acetic acid, and the impurities were then removed using vacuum filtration (Nunc, Roskilde, Denmark). The chitosan solution was added to the tissue culture plates (Corning Inc., NY, USA), which were then dried at 60 °C for 24 h. After a thin film formed on a culture plate, the surface was neutralized by applying 1 N of aqueous NaOH for 30 min and then washed thoroughly using distilled water. The chitosan-coated plates were exposed to ultraviolet light overnight before the cells were seeded.

Similar coating methods were applied to the nerve conduits. A medical-grade silicone tube (1.5 mm inner diameter, 15 mm in length) (Versilic® 760110, Saint-Gobain, Courbevoie, France) was filled with the chitosan solution (1%). The silicone tube was placed upright, and the chitosan was evenly dried on the inner wall after the tube was incubated in an oven at 60 °C for 24 h. The coated tube was then neutralized and cut to 10 mm in length for nerve conduit. The conduits were exposed to ultraviolet light overnight before being implanted into an animal.

2.2. ASC culture and spheroid formation

Human ASCs were obtained from healthy donors with informed consent and with the approval of the Review Board of National Cheng Kung University (NCKU) Hospital. The ASC isolation protocols followed those of Dr. Zuk and Dr. Hedrick at the University of California, Los Angeles [9]. In summary, the raw lipoaspirates were washed to remove debris, and were treated with 0.075% collagenase (type I; Sigma–Aldrich) for 30 min at 37 °C. The collagenase was inactivated using an equal volume of Dulbecco's modified Eagle's medium (DMEM; Invitrogen Inc., Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), and the infranatant was centrifuged for 10 min at low speed. The pelleted stromal vascular fraction (SVF) was separated from the floating population of mature adipocytes. The SVF consisted of a heterogeneous cell population, and this was enriched in preadipocytes by using plastic adherence. All SVF cells were cultured and passaged in DMEM containing 10% FBS at least 3 times before use. The multipotency ability of isolated ASCs was assessed by performing osteogenesis, adipogenic, and chondrogenic induction, as in a previous study [36]. On Passage 3, ASC cells were seeded onto the chitosan-coated plate with 2×10^4 cells per cm^2 for 48 h to develop free-floating spheroids [36].

2.3. Sciatic nerve transection and treatments

In the process of peripheral nerve repair using conventional nerve tubes, a length limit or critical gap exists between the proximal and distal stumps of a cut nerve, above which regeneration does not occur. This distance is approximately 10 mm in the sciatic nerve of rats [37]. Therefore, we designed a rat sciatic nerve transection model containing a 10-mm gap. The experimental procedures used for this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at NCKU. Male Sprague-Dawley rats weighing 200–250 g (6–7 weeks old) were anesthetized using Zoletil (50 mg/kg intraperitoneally) (Virbac, Carros, France). An incision was made from the right sciatic notch to the distal thigh. The subcutaneous tissue was bluntly dissected under the skin to expose the biceps femoris muscle. The sciatic nerve was freed from the investing fascia and prepared for further manipulation (Fig. 1A, left panel). The animals were randomly assigned to the following 5 groups: the sham operation group (Sham) explored the sciatic nerve without any damage to the nerve tissue; the silicone group (S) used a silicone conduit to bridge the 10-mm gap (Fig. 1A, middle panel); and in the chitosan group (C), the gap was bridged using a chitosan-coated conduit. The neurospheres formed by seeding the ASCs on a chitosan-coated plate were dissociated into single cells and 1×10^5 cells were then applied to the silicone conduit (S + N) or chitosan-coated conduit (C + N). The wounds were closed by suturing the skin. After surgery, the animals were housed at 21 ± 0.5 °C in cages with free access to food and water. Six weeks after surgery, the rats were euthanized and the surgical sites were subsequently opened to harvest the nerve tissue (Fig. 1A, right panel).

2.4. Examination of myelin sheath

The nerve tissues were isolated and fixed overnight in 4% paraformaldehyde. In each animal, three 2-mm segments were cut in the proximal section of the anastomosis (proximal), the middle portion of the nerve gap (middle), and the distal section of the anastomosis (distal) (Fig. 2A). The nerve segments were fixed again overnight in 1% OsO_4 (Merck KGaA, Darmstadt, Germany). These three segments were separately dehydrated in a gradient of ethanol (EtOH) (50%, 75%, 85%, 95%, and 100%). During the dehydration process, gradient mixtures of EPON (Ladd Research Industries, Inc., Burlington, VT, USA) in 2-propanol were prepared (33%, 66%, and 100%). Tissues were treated with gradient EPON solutions and maintained in pure EPON overnight. Tissues were then embedded in EPON in an oven at 60 °C overnight. Semi-thin sections (0.5 μm) of the nerve explants were cut on a microtome (Reichert Jung 1130, Leica, Wetzlar, Germany). The myelin sheath was stained with toluidine blue (0.1%, Sigma–Aldrich). The images were captured using a digital camera (Sony, Japan) attached to a microscope (Eclipse TE300, Nikon, Japan) running ImagePro® software (Media Cybernetics, L.P., Silver Spring, MD, USA). The number of myelinated axons was counted using a high-power field objective lens (40×, Nikon, Japan) with 5 separate fields in each slice, 3 separate slices in each segment in random by three single-blinded students who were taught to identify the re-myelinated axons under the same criteria. The nerve regeneration was evaluated by examining the axon density and axon ratio from proximal to distal segments. To determine axon density, the number of myelinated axons per visual field was counted in the middle segments. The myelinated axons contained in the distal and proximal segments were counted, and the percentage of the distal myelinated axons to proximal myelinated axons ratio was then calculated.

To observe the ultrastructure of the myelin sheath, the nerve explants embedded in EPON were sectioned at 80 nm on a microtome (Leica). The sections were then placed on a copper mesh (Pleco, Clovis, CA, USA). The nuclear acid inside the sections was stained with 1% uranyl acetate (TAAB, Berkshire, UK), and the proteins were stained with 1% lead citrate (Ladd). After brief air-drying, the samples

Please cite this article in press as: Hsueh Y-Y, et al., Functional recoveries of sciatic nerve regeneration by combining chitosan-coated conduit and neurosphere cells induced from adipose-derived stem cells, Biomaterials (2013), http://dx.doi.org/10.1016/j.biomaterials.2013.11.081