Biomaterials 35 (2014) 1450–1461

Contents lists available at ScienceDirect

Biomaterials

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

A synthetic oxygen carrier-olfactory ensheathing cell composition system for the promotion of sciatic nerve regeneration

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ARTICLE INFO

Article history:
Received 12 September 2013
Accepted 27 October 2013
Available online 15 November 2013

Keywords:
Olfactory ensheathing cell
Fluorocarbon
Oxygenation
Peripheral nerve
Nerve regeneration
Functional recovery

ABSTRACT

The treatment of lengthy peripheral nerve defects is challenging in the field of the regenerative medicine. Thus far, many nerve scaffolds with seeded cells have been developed, which hold great potential to replace nerve autograft in bridging lengthy nerve defects by providing guiding and bioactive cues. However, low oxygen status has been found within nerve scaffolds after their implantation *in vivo*, which has been shown to result in death or loss of function of supportive cells, and significantly limit nerve regeneration and functional recovery after nerve injury. In the present study, perfluorotributylamine (PFTBA) was introduced into a collagen-chitosan conduit within which olfactory ensheathing cells (OECs) were seeded to increase oxygen supply to OECs, as well as regenerating axons. The "PFTBA-OECs" enriched scaffolds were then used to bridge a 15-mm-long sciatic nerve defect in rats. Both nerve regeneration and functional recovery were examined at pre-defined time points after surgery. We found that the number of GFP-labeled OECs was significantly higher in the "PFTBA-OECs" scaffold than that in the single OECs scaffold. In addition, PFTBA was found to enhance the beneficial effect of OECs-enriched scaffold on axonal regeneration and functional recovery. All these findings indicate that the "PFTBA-OECs" enriched scaffolds are capable of promoting nerve regeneration and functional recovery, which might be attributable, at least in part, to their beneficial effect on the survival of OECs after their implantation *in vivo*.

1. Introduction

Peripheral nervous injury (PNI) affects 1 in 1000 individuals each year [1]. In current therapies, nerve regeneration after injury is still a challenge. For small gap injuries (1–2 mm), the natural regrowth of peripheral nerves makes direct end-to-end nerve suture possible. However, for large nerve defects, a graft is generally required to provide a bridge for regenerating axons. At present, autologous nerve graft has been widely used for bridging lengthy nerve defects [2]. However, autograft transplantation is limited by donor nerve availability and postoperative complications of donor sites [3–5]. Thus far, many artificial nerve scaffolds have been developed to bridge lengthy nerve defect [6,7], and has been shown to partially restore nerve regeneration by providing guiding cues for regenerating axons. To further promote axonal regeneration, many supportive cells have been introduced to nerve scaffolds to establish a beneficial local microenvironment for regenerating axons at the site of nerve defect [8–11], which holds great potential to replace nerve autograft in repairing nerve defect.

Olfactory ensheathing cells (OECs) have attracted considerable attention because of their exceptional ability to promote nerve regeneration in the central nervous system (CNS) [12–15]. In addition, recent studies have also demonstrated that OECs are capable of enhancing functional recovery of peripheral nerve injury after their transplantation into the injured site [16–18]. Thus, OECs have been considered as potential supportive cells for tissue engineering-based therapeutic strategies after nerve injury. However, low oxygen supply was found within nerve scaffolds after their implantation *in vivo*, which has been shown to result in death or loss of function of supportive cells, and significantly limits nerve regeneration and functional recovery after nerve injury repair [19–

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http://dx.doi.org/10.1016/j.biomaterials.2013.10.071

21]. Therefore, the strategy which is capable of overcoming the hypoxic status within nerve scaffolds holds great potential to improve the efficacy of cell-based nerve scaffolds in promoting nerve regeneration and functional recovery.

For overcoming the hypoxic condition within nerve scaffolds, extensive efforts have been devoted to promote vascularization within nerve scaffold by different methods. However, it generally takes days to weeks for new blood capillary to form and grow *in vivo*, during which the survival of supportive cells dramatically decreased due to low oxygen level. Therefore, adequate supply of oxygen over the initial two weeks might offer a promising, as well as challenging avenue to improve the performance of OECs in the treatment of lengthy nerve defect.

The perfluorocarbons (PFCs), such as PFTBA, offer a promising way to increase the level of oxygen within tissue-engineered grafts. The PFCs have been widely used as a successful blood substitutes due to their high solubility of oxygen, commercial availability, chemical and biological inertness, and ease of sterilization [22,23]. In addition, PFCs emulsions have a linear relationship between oxygen partial pressure and oxygen concentration [24,25]. Several studies have shown the beneficial effect of PFCs on different types of cells under hypoxic condition [26,27]. It has been reported that incorporating PFCs in a human HepG2 hepatomas-calcium alginate hydrogels increased cell metabolic activity and cell survival [28]. In addition, PFCs have also been shown to promote survival of mesenchymal stem cells and bone formation *in vivo* [29]. All these findings **make** PFTBA an attractive supplementation to nerve scaffold to overcome the detrimental effect of hypoxic condition on supportive cells *in vivo*, which holds the potential to improve nerve regeneration and functional recovery after nerve injury repair. Such a hypothesis has not been investigated thus far.

In this study, we prepared a “PFTBA-OECs” enriched fibrin hydrogel, which was injected into a collagen-chitosan nerve scaffold uniformly. Then the nerve conduits were used to bridge a 15-mm-long sciatic nerve defect in rats. The viability and distribution of OECs within scaffolds after surgery were examined by GFP labeling. The effect of “PFTBA-OECs” conduits on axonal regeneration and functional recovery was evaluated by both morphological analysis and functional assessments.

2. Materials and methods

2.1. Fabrication of the chitosan-collagen conduits and microstructure observation

The chitosan-collagen conduit was prepared following the procedures described previously [9]. In brief, the type I collagen (2.8 wt.%; Sigma, St. Louis, MO) and chitosan (0.7 wt.%; Sigma, St. Louis, MO) were dissolved in a solution of 0.05 M acetic acid (PH 3.2) at 4 °C. The mixture was centrifuged, and the suspension was vacuumized and injected into a self-designed mold. The mold was lyophilized for 48 h. Then the conduit was removed from the mold and cut into cylinders (15 mm in length, 1.5 mm in inner diameter, 2.0 mm in outer diameter). In addition, the conduits were cross-linked with a solution of genipin (1 wt.%, Challenge Bioproducts, Taichung, Taiwan) for 48 h, rinsed three times with distilled water, dehydrated for 30 min with 95% of ethanol, and air dried for 1 week. Finally, the conduits were sterilized with an exposure to 20 kGy ^{60}Co radiation before surgery (Fig. 1A).

The microstructure of conduits was examined under a scanning electron microscope (SEM; S-3400N; HITACHI, Tokyo, Japan) at an accelerating voltage of 5 kV. The conduits were washed three times with distilled water, and then dehydrated in serial ethanol solutions followed by a brief vacuum drying. Thereafter, the dry samples were sputter-coated with gold at 40 mA. The mean diameter of interconnected micropores on the wall was 20.68 ± 4.61 μm (range, 22.32–55.16 μm) (Fig. 1B–D).

2.2. Cell culture and purification

The method for isolation of OECs we adopted was modified from classical Nash's method [30], which was based on differentiation adhesion. Besides differential adhesion, chemical sorting has also been used, which has been found to increase the purity of OECs. Using this method, the purification of OECs ranged from 93.2% to 95% in previous studies [14,31]. Olfactory ensheathing cells (OECs) were collected from the olfactory bulb of 4- to 8-week-old Sprague-Dawley rats in the present study. Briefly, rats were deeply anesthetized with 1% pentobarbital and decapitated. Olfactory bulbs were removed and dissected free of meninges. The caudal one-third of the bulb was removed and discarded as much white matter as possible to isolate the outer nerve layer. Then the tissue was finely minced with a pair of scalpel blades (#10) on plastic culture dishes, and incubated for 25 min in collagenase A (0.75 mg/ml; Roche, Indianapolis, IN), collagenase D (0.75 mg/ml; Roche), and papain (12 U/ml; Worthington, Lakewood, NJ) in calcium-free complete saline solution with a trace of cysteine for 25 min at 37 °C on a rotary shaker in a CO_2 incubator. The tissue suspension was then centrifuged for 7 min at 300 × *g*, the supernatant was discarded, and tissue was resuspended in 2 ml of DMEM (Invitrogen, Carlsbad, CA) with 10% FCS using gentle mechanical triturating with fire-polished pasture pipettes with successively reduced diameters. The volume of media was immediately increased to 20 ml, and undissociated pieces of tissue were allowed to settle for 2 min before transferring the cell suspension to another culture tube and centrifuging as before. Cells were washed twice, resuspended, and preplated for 1 h in a culture flask at 37 °C in aCO_2 incubator. Nonadherent cells were gently washed off with DMEM, and cells were centrifuged and resuspended three times in DMEM before counting and concentrating cells to3.0 × 10^4 cells/μl just before transplantation. Then the double Immunofluorescence assay was processed to evaluate the purification of OECs, and the purity of cell cultures was determined with P75NGFR (ab62122; Abcam Inc., UK) and GFAP (ab49874; Abcam Inc., UK) antibody, DAPI (Invitrogen, USA). The images were acquired from a fluorescence microscope (DM6000; Leica, Germany). The cell purity is represented with the average of the ratio of NGFRp75 and GFAP double positive cell number to DAPI positive cell number. The purity of OECs was over 95% in the present study, and the result was shown in Fig. 1E–H.

OECs were genetically engineered in our laboratory to constantly express green fluorescent protein (GFP) reporter gene by using retrovirus-based vectors [32,33]. In this paper we call these cells GFP-expressing OECs. These cells were cultured in the same conditions as the OECs.

2.3. Procedure of OECs seeding into the chitosan-collagen conduit

The mixture conduits, which were used in the present experiment, were composed of chitosan-collagen conduits and GFP-expressing OECs in fibrin gel with or without PFTBA. The cell-fibrin gel mixture was constructed as previous method [29] and prepared immediately prior to implantation. In brief, the fibrinogen (Tisseel kit; Baxter AG, Vienna, Austria) was diluted with sterile saline to reach an 80 mg/ml concentration. The GFP-expressing OECs were trypsinized and counted using the Trypan blue exclusion method. Then aliquots of cells (0.5 × 10^6 GFP-expressing OECs) were suspended in 10 μl fibrinogen solution. To reach coagulation, the cell-fibrinogen mixture solution was mixed with the solution containing 10 μl thrombin (5 IU/ml) − PFTBA (10 wt.%; Sigma). Then the final cell-gel mixture was injected into the conduits prior to surgery, in one of the control groups, the cell-gel mixture without PFTBA was also injected into the conduits.

2.4. Animals and surgical procedures

All protocols involving the use of animals followed the ethical guidelines of the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1985), and were approved by Institutional Ethical Committee of the Forth Military Medical University.

Three hundred and eighty three male adult Sprague-Dawley rats, weighing approximately 200–220 g, were obtained from Laboratory Animal Centre of Fourth Military Medical University (FMMU) and were randomly divided into five groups as shown in Table 1. All animals were anesthetized with 1% sodium pentobarbital solution (40 mg/kg, i.p.). Under aseptic conditions, the left sciatic nerve was exposed using a muscle-splitting incision. A segment of sciatic nerve was removed, leaving a 15-mm-long defect after retraction of the nerve ends, and then sutured by 3 perineural 10/0 nylon. In the autograft group, the removed nerve segment was rotated 180° and re-implanted under 40× magnifications. In other four groups, the nerve defect was bridged with the nerve conduit sutured to both the proximal and distal nerve stumps. In details, in the CF group, the conduit was only filled with fibrin. In the CFO group, the conduit was filled with fibrin-OECs mixture. In the CFP group, the conduit was filled with fibrin-PFTBA mixture. In the CFOP group, the conduit was filled with fibrin, OECs and PFTBA (Fig. 1I). In all animals, the skin was closed with 6-0 stitches. After surgery, all animals were returned to their cages, and fed with food and water as libitum as usual.

2.5. Analysis of cell viability after surgery

At 3, 7, 14, 28 days after surgery, the rats (n = 6, at each time point of each group) were anesthetized prior to intracardial perfusion with 0.1 M phosphate buffer (Sigma) followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). Then the grafts (15.0 mm in length, 1.5 mm in inner diameter and 2.0 mm in outer diameter) were harvested, postfixed in buffered 4% paraformaldehyde for 4 h, cryoprotected in 30% sucrose overnight at 4 °C, and sectioned on a cryostat. Serial 25 μm thick longitudinal sections of the graft were mounted on slide (Superfrost*/Plus microscope slides, Fisher Scientific). Then the GFP-expressing OECs were examined under a fluorescent microscope (DM6000; Leica Germany). For quantitative analysis, we choose the midline section as well as the 5th and 10th 25 μm