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Biodegradable poly-β-hydroxybutyrate scaffold seeded with Schwann cells to promote spinal cord repair

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Abstract

Cavity formation is an important obstacle impeding regeneration after spinal cord injury and bridging strategies are essential to provide physical substrate allowing axons to grow across the lesion site. In this study we evaluated effects of biodegradable tubular conduit made from poly-β-hydroxybutyrate (PHB) scaffold with predominantly unidirectional fiber orientation and supplemented with cultured adult Schwann cells on axonal regeneration after cervical spinal cord injury in adult rats. After transplantation into the injured spinal cord, plain PHB conduit was well-integrated into posttraumatic cavity and induced modest astroglial reaction. Regenerating axons were found mainly outside the PHB with only single fibers crossing the host–graft interface. No host Schwann cells migrated into the graft. In contrast, when suspension of adult Schwann cells was added to the PHB during transplantation, neurofilament-positive axons filled the conduit and became associated with the implanted cells. Although rubrospinal fibers did not enter the PHB, numerous raphaespinal and CGRP-positive axons were found within the conduit. Modification of PHB surface with fibronectin, laminin or collagen significantly increased Schwann cell attachment and proliferation *in vitro*. However, transplantation of PHB conduit pre-coated with fibronectin and seeded with Schwann cells did not alter axonal growth response. The results demonstrate that a PHB scaffold promotes attachment, proliferation and survival of adult Schwann cells and supports marked axonal regeneration within the graft.

Keywords: Animal model; Spinal cord injury; Nerve tissue engineering; Neural prosthesis; Transplantation

1. Introduction

Spinal cord injury in humans results in complete failure of the severed nerve tracts to regenerate across the lesion site. The pathophysiology of spinal cord injury is multifactorial and, therefore, to achieve functional recovery several challenging objectives must be met, such as minimizing glial and neuronal cell death, reducing scarring and cavitation, blocking inhibitory molecules in the trauma zone and stimulating functional axonal regeneration [1–3].

In recent years, promising effects on axonal regeneration have been reported using transplantation of Schwann cells [4], olfactory ensheathing cells [5] and bone marrow stromal cells [6]. These cells are responsible for the supportive environment as they produce extracellular matrix molecules, integrins and trophic factors. However, in many cases neural repair requires bridging grafts to provide a physical substrate for directed axonal growth across the lesion gap and to allow axons to re-enter the host tissue in the appropriate location.

Although nerve grafting is the gold standard for neural repair, the limited access to autologous donor material and the problems with allograft rejection, have prompted a search for immunologically inert artificial materials [7]. At present, biomaterials appear to be more advantageous for spinal cord repair because of their structural and chemical versatility and accessibility. During the last few years, various biodegradable and non-biodegradable polymers have been tested in

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different experimental models of spinal cord injury [8]. The results of these studies demonstrate that bioengineering technology utilizing cellular transplantation strategies with Schwann cells, olfactory ensheathing cells and stem sells can promote repair of the injured spinal cord [2].

Previous reports from our laboratory demonstrate that poly-β-hydroxybutyrate (PHB) sheet with unidirectional fiber orientation could be used as a wrap-around implant to guide axonal growth after peripheral nerve injury [9]. In addition, we have developed biodegradable conduit for spinal cord repair which is based on strands of PHB fibers coated with alginate hydrogel and supplemented with cultured Schwann cells [10]. The conduit is well-integrated into the lesioned spinal cord, provides neuroprotection for injured rubrospinal neurons but supports only limited axonal ingrowth. Moreover, regeneration appears to be obstructed by the slowly degrading alginate hydrogel. We also recently report that alginate hydrogel could transform suspended Schwann cells into atypical cells with spherical shape and inhibit their metabolic activity [11]. Alginate hydrogel also inhibits neurite outgrowth *in vitro*, although this effect could be attenuated by addition of fibronectin and Schwann cells.

In the present investigation, we tested the hypothesis that cultured adult Schwann cells could be directly attached to the structurally guiding PHB scaffold and that this conduit will support Schwann cell survival and promote axonal regeneration of long spinal tracts after cervical spinal cord injury in adult rats. To enhance Schwann cell attachment and proliferation on PHB, we also tested various components of extracellular matrix molecules added to the material.

2. Materials and methods

2.1. Experimental animals

The experiments were performed on adult (10–12 weeks, $n = 35$) female Sprague-Dawley rats (Møllegaard Breeding Centre, Denmark). The animal care and experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 86-23, revised 1985) and were also approved by the Northern Swedish Committee for Ethics in Animal Experiments. All surgical procedures were performed under general anesthesia using a mixture of ketamine (Ketalar®, Parke-Davis; 100 mg/kg i.v.) and xylazine (Rompun®, Bayer; 10 mg/kg i.v.).

2.2. Schwann cell culture

Schwann cells were obtained from sciatic nerves of adult female Sprague-Dawley rats ($n = 5$) as described previously [12]. Nerves were cut into small pieces and placed in culture dishes in DMEM/10% heat-inactivated fetal calf serum, FCS (PAA Laboratories GmbH, Zinz, Austria) and kept at 37 °C, 95% humidity and 5% CO_2. After 2 weeks, the pieces were transferred into sterile universal container where they were enzymatically dissociated and then replated on 25 cm^2 poly-D-lysine-coated (PDL) tissue culture flasks (Sigma–Aldrich Sweden AB, Stockholm, Sweden) in DMEM/10% FCS supplemented with recombinant human glial growth factor 2 (rhGGF2; 40 ng/ml; Acorda Therapeutics, Inc., Hawthorne, NY) and 10 μM forskolin (Invitrogen AB, Täby, Sweden). Once the Schwann cells became confluent, the purification procedure was carried out. Immunomagnetic depletion of fibroblasts was carried out by using MACS goat anti-mouse IgG MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to manufacturer recommendations. The cells were resuspended and incubated in PBS/0.5% BSA/2 mM EDTA supplemented with Thy 1.1 mouse anti-rat serum (1:1000; MAB 1406, Chemicon) for 10 min followed by a washing step. After addition of goat anti-mouse IgG MicroBeads the cells were incubated for 15 min at 4 °C and then passed through a MiniMACS magnetic separation unit. Immediately after purification Schwann cells were subcultured on PDL-coated Lab-Tek® slides (Nalge Nunc International, Rochester, NY) at a density of 1×10^3 cells/well for 24 h. The purity of cells was assessed by using immunostaining for S100 protein (see below). After purification Schwann cells were replated on 75 cm^2 poly-D-lysine-coated (PDL) tissue culture flasks, expanded and prepared for transplantation at passage P4. The resulting purity of Schwann cells before transplantation was about 98%.

2.3. Culture of Schwann cells on PHB

For *in vitro* experiments, sterilized poly-β-hydroxybutyrate (PHB) sheets of approximately 150 kDa molecular weight and unidirectional fiber orientation (Astra Tech, Mölndal, Sweden) were cut into 8 × 8 mm pieces and then incubated for 24 h at 37 °C in the following solutions: (a) plain DMEM ($n = 9$), (b) fibronectin in DMEM (50 μg/ml; $n = 9$; Sigma–Aldrich Sweden AB), (c) laminin in DMEM (20 μg/ml; $n = 9$; Sigma–Aldrich Sweden AB) and (d) collagen IV in DMEM (2.56 μg/ml; $n = 9$; Sigma–Aldrich Sweden AB). After incubation, PHB sheets were briefly washed in DMEM and transferred into 8 chambers Lab-Tek® slides. The Schwann cells were detached with trypsin/EDTA, concentrated to 1×10^5 cell/ml in growth medium and then seeded on PHB at a concentration of 25×10^3 cells/well. The cell density was assessed after 1 day, 7 days and 14 days in culture. The growth medium was changed every 48 h. To evaluate Schwann cells attachment and proliferation, the cultures were immunostained for S100 protein and counterstained with DAPI (see below). Labeled cells were counted in 25 randomly selected areas of PHB sheet at ×250 final magnification using 400 × 400 μm frame.

2.4. Spinal cord injury and PHB transplantation

After cervical laminectomy, spinal cord was hemisected between C3 and C4 spinal segments and the trauma zone was expanded in the rostro-caudal direction to create a 2–3 mm long cavity. Immediately after completing the injury and haemostasis, PHB conduit was implanted into the cavity.

In experimental group 1 ($n = 6$), tubular conduits (14 mm long, 1.6 mm inner diameter) were constructed from a sheet of PHB with longitudinally oriented fibers as described previously [13]. To support Schwann cell transplants, loose unidirectional PHB fibers of a fixed weight were threaded through the lumen of a PHB conduit [14]. The conduits were stored in DMEM at 4 °C for maximally 6 h and were trimmed before implantation to fit the injury site.

In experimental group 2 ($n = 19$), Schwann cells were suspended in growth medium at a concentration of 80×10^6 cells/ml [10,13,14] and 5 μl of cell suspension was slowly injected into PHB conduit. To track grafted Schwann cells *in vivo*, the growth medium was supplemented with 10 μM 5-bromo-2-deoxyuridine (BrdU; Sigma–Aldrich Sweden AB) at 48 h before transplantation.

After obtaining early *in vitro* and *in vivo* results (see Section 3), we added a third experimental group ($n = 5$) to test if improved attachment and proliferation of Schwann cells on PHB material could be transformed into enhanced axonal regeneration within the conduit. PHB sheets (8 × 8 mm) were incubated in DMEM–fibronectin (50 μg/ml) for 24 h at 37 °C and then seeded with 5×10^4 Schwann cells. The latter cell density resulted in about 70% confluence after 24 h on PDL-coated 8 chambers Lab-Tek® slides. After 24 h in culture, PHB sheets were removed from Lab-Tek® slides, gently folded in to produce 2–3 mm long tubular conduits [9] and transplanted into spinal cord cavity. To minimize injury to seeded Schwann cells during conduit preparation, no loose PHB fibers were added. Dura mater, muscles and skin were closed in layers and the rats were given saline (4 ml s.c.) and benzylpenicillin (Boehringer Ingelheim; 60 mg i.m.).

2.5. Anterograde tracing of rubrospinal axons

At 8–9 weeks postoperatively, the rats ($n = 8$) from experimental groups 2 and 3 were mounted in a stereotaxic frame and a 2 mm hole was drilled in the