Biomaterials 33 (2012) 1363–1375

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

The role of microstructured and interconnected pore channels in a collagen-based nerve guide on axonal regeneration in peripheral nerves

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ARTICLE INFO

Article history:
Received 19 October 2011
Accepted 24 October 2011
Available online 13 November 2011

Keywords:
Nerve regeneration
Schwann cells
Scaffold
Neurotmesis
Sciatic nerve
Bands of Büngner

ABSTRACT

The use of bioengineered nerve guides as alternatives for autologous nerve transplantation (ANT) is a promising strategy for the repair of peripheral nerve defects. In the present investigation, we present a collagen-based micro-structured nerve guide (Perimaix) for the repair of 2 cm rat sciatic nerve defects. Perimaix is an open-porous biodegradable nerve guide containing continuous, longitudinally orientated channels for orientated nerve growth. The effects of these nerve guides on axon regeneration by six weeks after implantation have been compared with those of ANT. Investigation of the regenerated sciatic nerve indicated that Perimaix strongly supported directed axon regeneration. When seeded with cultivated rat Schwann cells (SC), the Perimaix nerve guide was found to be almost as supportive of axon regeneration as ANT. The use of SC from transgenic green-fluorescent-protein (GFP) rats allowed us to detect the viability of donor SC at 1 week and 6 weeks after transplantation. The GFP-positive SC were aligned in a columnar fashion within the longitudinally orientated micro-channels. This cellular arrangement was not only observed prior to implantation, but also at one week and 6 weeks after implantation. It may be concluded that Perimaix nerve guides hold great promise for the repair of peripheral nerve defects.

1. Introduction

The peripheral nervous system has an intrinsic ability to regenerate following injury. When the continuity of the endoneurium is maintained, regenerating axons are guided from the proximal to the distal nerve stump through preserved connective tissue elements. Schwann cell (SC) proliferation within the endoneurial tubes of the distal nerve generates the Bands of Büngner [1]. These aligned Schwann cell tubes guide the regenerating axons to their appropriate distal target organs and support functional recovery [2]. However, when peripheral nerve injury (PNI) leads to a gap, including discontinuity of the perineurium and epineurium, nerve regeneration is severely hampered. Only few, if any, axons reach the distal nerve stump. In such cases, surgical intervention is required to promote axon regeneration.

Direct nerve suture repair (nerve coaptation) is the preferred clinical treatment for short nerve defects. However, in the case of large nerve defects (where tensionless nerve repair is not possible) the interposition of a bridge between the proximal and distal nerve stumps is required. An autologous donor nerve, usually a sensory nerve (e.g. sural nerve [3]), is chosen to bridge such gaps. Despite its gold standard status, autologous nerve transplantation (ANT) is not

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doi:10.1016/j.biomaterials.2011.10.069

ideal for a number of reasons, including specific- (e.g. loss of sensory function) and general- (wound infection, haematoma, scarring, wound pain etc.) co-morbidities at the donor site. Furthermore, the availability of donor nerves is limited and unwanted complications may develop, including neuropathic pain [4]. Interestingly, only 40–50% of patients regain useful function when treated with autologous nerves [5]. Therefore, a wide range of materials, both biological and synthetic, have been investigated as alternatives to ANT in the repair of peripheral nerve defects [3].

The therapeutic effect of a bridging material in the repair of PNI can be influenced by its ability to physically and biochemically mimic aspects of the lost peripheral nerve. Such considerations have led to a tissue repair concept containing at least two essential components, as summarized by Lundborg [6]. Firstly, nerve guides should ideally contain an orientated matrix or scaffold. This can act as replacement for the endoneurial tubes, providing guidance and mechanical support for directed SC migration and axonal growth. Secondly, nerve guides should contain growth factors. These can either be produced by cells (e.g. by cultured- or migrating, endogenous SC) or be incorporated into the matrix for stimulation of effective and orientated axonal growth.

Although a large number of investigations have used hollow conduits as bridging devices, a growing body of interest has emerged in the development of micro- or nanostructured scaffolds [7]. A similar principle has also been devised for the repair of traumatically injured central nervous system. For example, a multi-component polymer scaffold seeded with neural stem cells has been designed to assist in the repair of traumatically injured spinal cord [8]. We have recently described the *in vitro* characteristics of a collagen-based micro-structured scaffold (Perimaix) containing continuous, longitudinally orientated and interconnected pores [9]. The structure of the scaffold has been designed to resemble the geometric dimensions of a peripheral nerve and 3D tissue culture experiments revealed it to be an excellent substrate for orientated axon outgrowth [10,11]. In earlier studies, two forms of the scaffold (termed Perimaix-17% and Perimaix-33%), differing in physical and molecular properties, were seeded with highly enriched populations of SC. The SC survived and formed aligned columns which followed the longitudinal orientation of the scaffold's internal microstructure, resembling Bands of Büngner. In the present investigation, the properties of Perimaix-17% (PM-17) and Perimaix-33% (PM-33) have been assessed in an *in-vivo* model of PNI. The model uses the epineurium of the lesioned rat sciatic nerve to secure and maintain the position of the implanted scaffold for the duration of the experiment. The hypothesis tested was that Perimaix nerve guides could promote directed axonal growth across 2 cm long nerve defects.

2. Materials and methods

All experiments were conducted in accordance with national- and EU regulations regarding animal care. Experimental animals were housed in a temperature- and humidity-controlled environment with a cycle of 12 h (h) light and 12 h darkness, and allowed free access to food and water. Every attempt was made to minimize the number of animals used, as well as any pain and discomfort that they may feel. Female inbred Lewis rats (12 weeks old, Charles River, Germany) and female transgenic rats (Lewis rat background) expressing green fluorescent protein (GFP) were used in the study. The transgenic GFP rats were provided by Prof. E. Kobayashi, (Jichi Medical University, Japan) and the husbandry and genotyping were performed by Prof. R. Tolba (Institute for Laboratory Animal Science, RWTH Aachen University). GFP transgenic rats were used at the age of 10–12 weeks for obtaining GFP-expressing Schwann cells (GFP/SC) [12].

2.1. Isolation of Schwann cells

SC were isolated and expanded *in vitro* as described earlier [13]. Briefly, both sciatic nerves were removed from terminally anaesthetized rats. The nerves were chopped into small pieces (1–2 mm) and were placed in Petri dishes in standard medium consisting of Dulbecco's modified essential medium (DMEM; Invitrogen™, Karlsruhe, Germany) supplemented with 10% foetal calf serum (FCS; PAA™, Pasching, Austria) and 1% Penicillin/Streptomycin (10.000 U/mL of penicillin, 10 mg/mL Streptomycin; PAA™, Pasching, Austria). After 7 days of incubation at 37 °C, the sciatic nerve fragments were enzymatically digested by incubation in collagenase followed by trypsin-EDTA (0.25%). Dissociated cells were plated onto poly-L-lysine/laminin-coated culture flasks (pll/lam; both Sigma–Aldrich™, Munich, Germany) and maintained in growth medium (DMEM containing 10% FCS, 40 μg/ml transferrin, 41.69 μg/ml μg bFGF, 41.698 μg/ml heregulin, 472.5 ng/mlforskolin, 10 μg/ml insulin 0.1% gentamicin, 1% glutamax).

2.2. Purification of SC

To obtain highly enriched SC, cell purification was performed using the Magnetic Assisted Cell Sorting (MACS®) system (Miltenyi Biotec, Bergisch Gladbach, Germany). Flasks with unpurified cells (i.e. SC and fibroblasts) were washed with phosphate buffered saline (PBS) and incubated with trypsin (0.05%) for 5 min. The cells were collected in growth medium, centrifuged at 300×*g* for 5 min at 4 °C, and washed with PBS supplemented with 2 mM EDTA (PE). For SC selection, the cell suspension was incubated with 2 μL of undiluted polyclonal rabbit anti-mouse low affinity nerve growth factor-receptor antibody (p75/LNGFr Chemicon International Ltd, Hampshire, United Kingdom) in PE containing 0.5% BSA (PEB) for 10 min at 7 °C. At the end of the incubation period, the cells were centrifuged, washed and incubated with 20 μL microbead-linked rat anti-mouse IgG1 (diluted 1:500; Miltenyi Biotec, Bergisch Gladbach, Germany) in 80 μL PEB for 15 min at 7 °C. After two rinsing steps with PE, an MS-column (Miltenyi Biotec, Bergisch Gladbach, Germany) was placed in the MiniMACS® magnet (Miltenyi Biotec, Bergisch Gladbach, Germany) and flushed with PEB. A maximum of 10^7 cells were resuspended in 500 μL PEB and applied to the MS column followed by 3 rinses with 500 μL PEB to wash out unbound cells (i.e. p75/LNGFr-negative cells, mainly fibroblasts). After removal from the magnet, the column was flushed with 1 mL PE, which allowed the collection of the p75/LNGFr-positive, Schwann cell fraction. The high purity (>95%) of the p75/LNGFr-positive Schwann cell fraction was confirmed by vimentin and S100β double immunofluorescence (Supplementary Figure 1).

2.3. Preparation of nerve guides

Perimaix nerve guides were generated by Matricel GmbH (Herzogenrath, Germany) using a patented uni-directional freezing process [10] (see Supplementary video 1 in [9]). Purified porcine collagen, characterized by low levels of non-collagenous and non-elastin markers, was used as a starting material for the nerve guide (Table 1). The main geometrical requirements for the novel nerve guide were: (1) a cylindrical form with a diameter of 1 mm and a length greater than 2 cm, (2) densely packed, longitudinally orientated microchannels capable of allowing SC adhesion, proliferation and migration as well as axonal growth.

The degree of cross-linking of the nerve guides was related to a decrease in free amine group content and an increase in denaturation temperature. The free amine group content of the samples was determined spectrophotometrically after reaction of the primary amine groups with 2,4,6-trinitrobenzenesulphonic acid (TNBS) as described previously [9]. The free amine group content was expressed as the number of free amine groups present per 1000 amino acids (n/1000 n). The percentage of reacted amine groups was calculated by setting the amine group content of non-crosslinked sample to 100%. The denaturation temperature of the nerve guides was determined by differential scanning calorimetry (DSC) using a TA Instrument Q100. In an empty hermetic pan, approximately 1 mg of scaffold was weighed, followed by the addition of 11 mg phosphate buffered saline solution. The sample was allowed to rehydrate overnight at room temperature before it was scanned at 5 °C/min in the range of 15–95 °C. Excess acid present from the directional solidification process was removed to prevent unwanted pH shifts. The peak temperature was taken as the denaturation temperature.

Fully hydrated samples of the cross-linked, sterilized, and orientated nerve guides were placed between the upper and lower plates (25mm diameter) of a controlled stress rheometer (Rheometrics Scientific, Piscataway, NJ). The oscillation frequency was varied from 0.01 to 20 Hz, and the strain was kept within the linear visco-elastic region for the measurements. The measurements were

Table 1
Characterization of cross-linking of the Perimaix nerve guides. Please note the decrease in denaturation temperature after gamma sterilization, which is a well-known phenomenon for collagen-based biomaterials [11]. In this previous study, the decrease in denaturation temperature resulted in a slightly faster degradation rate. (All measurements were performed $n = 3$, data is presented as mean + standard error of the mean).

Code	Non sterile samples		Sterile samples
	% of free amine groups reacted	Denaturation temperature (°C)	Denaturation temperature (°C)
Perimaix-17%	17	71.1 + 0.3	55.0 + 0.4
Perimaix-33%	33	76.7 + 0.2	60.5 + 0.4