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Cell-seeded alginate hydrogel scaffolds promote directed linear axonal regeneration in the injured rat spinal cord

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ABSTRACT

Despite recent progress in enhancing axonal growth in the injured spinal cord, the guidance of regenerating axons across an extended lesion site remains a major challenge. To determine whether regenerating axons can be guided in rostrocaudal direction, we implanted 2 mm long alginate-based anisotropic capillary hydrogels seeded with bone marrow stromal cells (BMSCs) expressing brain-derived neurotrophic factor (BDNF) or green fluorescent protein (GFP) as control into a C5 hemisection lesion of the rat spinal cord. Four weeks post-lesion, numerous BMSCs survived inside the scaffold channels, accompanied by macrophages, Schwann cells and blood vessels. Quantification of axons growing into channels demonstrated 3–4 times more axons in hydrogels seeded with BMSCs expressing BDNF (BMSC–BDNF) compared to control cells. The number of anterogradely traced axons extending through the entire length of the scaffold was also significantly higher in scaffolds with BMSC–BDNF. Increasing the channel diameters from 41 μm to 64 μm did not lead to significant differences in the number of regenerating axons. Lesions filled with BMSC–BDNF without hydrogels exhibited a random axon orientation, whereas axons were oriented parallel to the hydrogel channel walls. Thus, alginate-based scaffolds with an anisotropic capillary structure are able to physically guide regenerating axons.

Statement of Significance

After injury, regenerating axons have to extend across the lesion site in the injured spinal cord to reestablish lost neuronal connections. While cell grafting and growth factor delivery can promote growth of injured axons, without proper guidance, axons rarely extend across the lesion site. Here, we show that alginate biomaterials with linear channels that are filled with cells expressing the growth-promoting neurotrophin BDNF promote linear axon extension throughout the channels after transplantation to the injured rat spinal cord. Animals that received the same cells but no alginate guidance structure did not show linear axonal growth and axons did not cross the lesion site. Thus, alginate-based scaffolds with a capillary structure are able to physically guide regenerating axons.

1. Introduction

Throughout the world, between 250,000 and 500,000 people per year suffer a new spinal cord injury (SCI), mainly through traumatic causes [1]. Patients are affected by permanent and substantial disabilities, depending on the level and severity of the injury. Like in other parts of the mammalian central nervous system (CNS), the capacity for spontaneous regeneration in the spinal cord is very limited [2]. Mechanisms that contribute to the lack of recovery include the limited activation of axon growth-promoting genetic programs in CNS neurons [3,4], the inhibitory environment in and around a spinal cord lesion [5–7] and the absence of appropriate axon growth stimuli [8,9]. In addition, many molecules and guidance cues that are present during development are lost in the adult nervous system or have altered functions [10].

One means to enhance axonal growth and to provide a substrate at a site of SCI is the transplantation of cells such as neural stem cells [11,12], olfactory ensheathing cells (OEC) [13,14], Schwann cells [15,16], or BMSCs [17,18]. Overexpression of neurotrophic factors by genetically modified cells can further enhance the growth of regenerating axons into a spinal cord lesion [9,19]. In

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particular, BDNF has been shown to promote axon growth from several neuronal populations [20–23].

However, strategies that use cell suspension grafts alone or in combination with neurotrophins lack a 3-dimensional organization in the lesion site. Thus, even axons that are appropriately stimulated might not be directed towards appropriate targets [10]. Indeed, axons grow mostly in random orientation and only few axons are able to reach the distal part of a lesion, preventing the reconnection of disrupted axon pathways with their appropriate distal target neurons. Therefore, a structured scaffold that not only directs the regeneration of axons but also allows for the integration of growth promoting cell populations might be superior for long-distance axon regeneration in a directed fashion and axonal bridging over large lesions.

Several different types of hydrogel scaffolds of synthetic or natural origin have been developed to promote axonal regeneration. However, many of them have no oriented structure and therefore cannot support the directed growth of axons [24–26]. Scaffolds that partially provide a directed matrix to guide axonal regeneration are either composed of aligned fibers [27,28], contain a laminar structure [29] or channels [30,31].

We and others have previously investigated anisotropic capillary alginate hydrogels for axonal guidance [30,32]. Alginate is a natural heteromeric polysaccharide consisting of α-L-guluronate and β-D-mannuronate produced by brown algae and the two bacterial genera *Pseudomonas* and *Azotobacter*. It is frequently used in the food industry, for wound management or the entrapment of cells [33]. By directed diffusion of divalent cations, an alginate hydrogel with anisotropic channel structure can be produced [34]. Diameter and density of the capillaries formed by this process are dependent on the type of ions used, their concentration, the pH of the solution, the addition of other non-gel-forming electrolytes and the concentration of the alginate solution [35]. Previous in vitro studies have suggested that the capillary diameter influences neurite outgrowth of dorsal root ganglia explanted on capillary alginate hydrogels [32]. A higher capillary diameter resulted in more axon growth but these axons displayed less orientation. Alginate gels have also been used in spinal cord injury models, e.g. as an injectable gel in a hemisection lesion [36] or in preliminary experiments with small capillary hydrogels in a dorsal column lesion model [30], partially characterizing the biocompatibility of the material.

The current study aimed to determine whether alginate-based scaffolds with an anisotropic capillary structure are able to guide regenerating axons after spinal cord injury. Combining scaffolds of different channel diameter with a release of BDNF from incorporated cells, we find that this combination is superior for directed axonal growth compared to a suspension graft of the same cells without scaffold. In addition, scaffolds with BMSCs secreting BDNF are superior to scaffolds with control GFP-expressing BMSCs.

2. Materials and methods

2.1. Fabrication of alginate-based anisotropic capillary hydrogels

Alginate-based anisotropic capillary hydrogels were fabricated as previously described [30,32]. Briefly, a solution of 20 g/l (2% w/w) sodium alginate (Pronova UP LVG, Novamatrix) and solutions of 1 M $Sr(NO_3)_2$ and $Zn(NO_3)_2$ were prepared in purified water (Milli-Q®, Millipore) and subsequently filtered through a 0.2 μm pore size filter (Nalgene vacuum filtration system, VWR International).

Anodized cylindrical aluminum molds (5.5 cm in diameter and 4 cm in height; Schuett-Biotec) were used for gelation. 65 g of sodium alginate solution were poured into the molds and carefully covered with 20 ml of electrolyte solution using pump spray bottles. The solution was allowed to stand for at least 36 h at room temperature (RT) until gel formation was completed. Gels were removed from the molds and immersed in purified water to remove excessive electrolytes (four times every 4 h). The top, capillary-free layer of the gel was removed [30].

To stabilize the gel structure, gel slices were dehydrated with acetone [32] and immersed in a solution of 0.1 M hexamethylene diisocyanate (HDI; Sigma) in dry acetone for 4 h at RT under slight stirring. Gel slices were washed in dry acetone for 5 min and put between two filter papers for 10 min to remove excessive HDI and acetone from the capillary lumens. Afterwards the gels were immersed in purified water at 70 °C until CO_2 development – resulting from HDI polymerization – stopped. To remove the divalent cations (Sr^{2+} or Zn^{2+}) complexed to the carboxylate groups of the alginate, the gels were immersed in 0.1 M HCl five times for at least 2 h at RT and then washed in purified water until a neutral pH was reached. Gels were cut with a vibratome (VT1000S, Leica) into cuboids of $2 \times 2 \times 1.3$ mm side length with the capillaries lying parallel to the 2 mm long edge. The gel pieces were sterilized and stored in 70% ethanol until further use. Before transplantation, hydrogel scaffolds were washed in Dulbecco's phosphate buffered saline (DPBS, Life Technologies) 3 times for 3 h and overnight at RT. Pore dimension and density were analyzed by light microscopy. Scaffolds with imperfect channel structure were excluded and not used for in vivo studies.

2.2. Preparation of adult BMSCs

BMSCs were isolated from adult Fischer 344 rats (Charles River). Experiments were carried out in accordance with national guidelines for animal care and in accordance with the European Union Directive (2010/63/EU). Animals were anesthetized intraperitoneally (5 ml/kg) with a mixture of ketamine (125 mg/kg, Bremer Pharma), xylazine (6.35 mg/kg, Ecuphar), and acepromazine (1.25 mg/kg, Ceva) and decapitated. Both tibias and femurs of the hind limbs were dissected, adherent muscle was removed and the bone was cut off on both sides just below the joint. The bone marrow was extracted by centrifugation at 3000 g for 2 min. The pellet was resuspended with 2 ml DPBS per animal. Cells were washed with 10 ml DPBS and resuspended. A total of 10^6 cells were seeded in a T75 flask (Greiner-Bio one) with 20 ml alpha-medium (Merck) supplemented with 20% fetal bovine serum (Merck), 100 units/ml penicillin and streptomycin (Life Technologies) and 2 mM L-glutamine (Life Technologies). Three days later, cells were washed with DPBS to remove cellular debris and non-adherent cells. Media was changed three times a week. The adherent BMSCs were detached with 0.25% trypsin solution once the culture reached near-confluence and split in several culture flasks. Cells were passaged up to 6 times and frozen in liquid nitrogen for storage.

2.3. Lentiviral vector production and infection of BMSCs

The lentiviral vectors used to transfect BMSCs were produced in 293-T cells by $CaCl_2$ transfection using a third generation lentiviral system as described [37]. For the expression of the neurotrophin BDNF and the GFP, a lentiviral construct expressing BDNF and GFP from a CMV-β-actin hybrid promoter was used [38]. BMSCs were infected with concentrated virus to express BDNF and GFP via an internal ribosome entry site (BMSC–BDNF) or GFP alone (BMSC–GFP). Transfected cells were purified to 90% by fluorescence-activated cell sorting (FACS) for the marker GFP. ELISA analysis of cell culture supernatants showed that BMSC–BDNF expressed 170 ng BDNF/10^6 cells/24 h. Cells were frozen in liquid nitrogen for storage. Before grafting to the spinal cord, cells