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Long-lasting significant functional improvement in chronic severe spinal cord injury following scar resection and polyethylene glycol implantation

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ABSTRACT

We identified a suitable biomatrix that improved axon regeneration and functional outcome after partial (moderate) and complete (severe) chronic spinal cord injury (SCI) in rat. Five weeks after dorsal thoracic hemisection injury the lesion scar was resected *via* aspiration and the resulting cavity was filled with different biopolymers such as Matrigel™, alginate-hydrogel and polyethylene glycol 600 (PEG) all of which have not previously been used as sole graft-materials in chronic SCI. Immunohistological staining revealed marked differences between these compounds regarding axon regeneration, invasion/elongation of astrocytes, fibroblasts, endothelial and Schwann cells, revascularization, and collagen deposition. According to axon regeneration-supporting effects, the biopolymers could be ranked in the order PEG >> alginate-hydrogel > Matrigel™. Even after complete chronic transection, the PEG-bridge allowed long-distance axon regeneration through the grafted area and for, at least, 1 cm beyond the lesion/graft border. As revealed by electron microscopy, bundles of regenerating axons within the matrix area received myelin ensheathment from Schwann cells. The beneficial effects of PEG-implantation into the resection-cavity were accompanied by long-lasting significant locomotor improvement over a period of 8 months. Following complete spinal re-transection at the rostral border of the PEG-graft the locomotor recovery was aborted, suggesting a functional role of regenerated axons in the initial locomotor improvement. In conclusion, scar resection and subsequent implantation of PEG into the generated cavity leads to tissue recovery, axon regeneration, myelination and functional improvement that have not been achieved before in severe chronic SCI.

Introduction

Spinal cord injury (SCI) generally results in life-long severe impairments for the patients. Present research in the field of SCI largely targets acute SCI. However, the majority of SCI patients are those with chronic lesions who may benefit insufficiently from therapeutic treatments designed for acute application. Compared to treatments of acute experimental SCI, the efficacy of therapies promoting axonal regeneration seems impaired in chronic models (Houle and Tessler, 2003). Accumulation of growth-inhibitory molecules associated with central myelin (Buchli and Schwab, 2005; Cafferty and Strittmatter, 2006; Filbin, 2003) or the lesion scar (Fawcett, 2006; Klapka and Muller, 2006; Silver and Miller, 2004) could be responsible for regeneration failure in both acute and chronic SCI. Using iron chelators to inhibit collagen-biosynthesis we have previously demonstrated the beneficial effects of transient suppression of fibrous scarring in an acute SCI model (Klapka et al., 2005; Schiwy et al., 2009). However, since iron chelators suppress the formation of fibrotic scarring but do not degrade an existing scar, this treatment is not transferable to chronic SCI where a mature lesion scar, with a plethora of axon growth-inhibitory molecules attached (Bundesen et al., 2003; Davies et al., 2004; Niclou et al., 2006), has already formed. Careful surgical resection of the scar and filling-in of scaffolding matrices into the resulting cavity could be a possible option for a regeneration-supporting therapy in chronic SCI.

Here we describe a chronic (5 weeks) SCI and scar resection model in rat, and different viscous matrix materials in regard to their ability to support axon regeneration. As shown previously, the scar is fully developed at the injury site after this time while the biosynthesis of most

Abbreviations: HX, hemisection; TX, complete transection; RX, scar resection; ALG, alginate-hydrogel; MG, Matrigel™; PEG, polyethylene glycol; mBBB, modified BBB open field test; wpl, week post lesion; wpr, week post resection; dpReTX, days post axonal re-transection; rT, rostral tracing; Ax, axon, As, astrocyte; Bv, blood vessel; Fi, fibroblastic cell; Sc, Schwann cell.

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scar-associated CSPGs has subsided (Tang et al., 2003) and spontaneous behavioral recovery has reached its plateau (Ung et al., 2007). For cavity-filling after scar resection we chose three biopolymers: (i) Matrigel™ (MG), (ii) alginate-hydrogel (ALG) and (iii) polyethylene glycol 600 (PEG).

These materials were chosen because of their reported beneficial effects in the treatment of acute SCI: MG is a gel obtained from the Engelbreth–Holm–Swarm sarcoma and has been described as a suitable gel matrix for tissue regeneration (Cassell et al., 2001) and axon growth (Tonge et al., 1997). ALG is a polysaccharide distributed widely in the cell walls of brown algae and has previously been applied as a cell carrier substance in SCI research to promote axonal regeneration and elongation, both *in vitro* and *in vivo* (Kataoka et al., 2004; Novikov et al., 2002, 2006). Application of PEG successfully yielded repair of crushed as well as transected spinal cord axons leading to immediate recovery of axonal conduction in an acute injury model (Borgens et al., 2002; Shi and Borgens, 2000). It should be noted that both terms "biomatrix" and "biopolymer" have been used to describe these materials because there is no generally accepted definition for either term. MG and ALG are often called as either biomatrices or biopolymers. By the broad definition of a biomatrix as a biological or biochemical matrix PEG can also be termed a biomatrix, but because of the lack of a cross-linking reaction we prefer to use the term "biopolymer" for PEG. We found the latter term more suitable than "biomatrix", because PEG is considered a technical biopolymer showing biodegradability and compatibility with biological tissue.

The present investigation is, to our knowledge, the first that directly compared MG, ALG and PEG with respect to their suitability as regeneration-supporting matrix materials in animal models of chronic moderate and complete SCI. We have investigated and compared spinal cord tissue responses to these biopolymers with respect to axon regeneration, cell invasion, vascularization and functional locomotor outcome. We could identify and select PEG as a highly suitable biopolymer-matrix which supports glial cell invasion, neovascularization as well as regeneration of myelinated axons into and beyond the lesion zone leading to a significant degree of improvement of locomotor behavior that has not been matched or outbalanced by any other treatment of complete chronic SCI.

Materials & methods

Surgery procedures

During all surgical procedures animals were placed on a heating pad to maintain body temperature. The timelines in Supplementary Fig. 1 provide an overview of the experimental procedures.

Spinal cord injury

Dorsal hemisection lesion model (HX, Supplementary Fig. 1A): Lesioning was performed according to a modification of a previously published protocol (Schiwy et al., 2009). In brief, adult female Wistar rats (HanTac:WH; Taconic) weighing 200–230 g were anesthetized with isoflurane (Forene, Abbott; 2–3% in O_2 and N_2O at a ratio of 1:2). Following laminectomy at thoracic level Th8/9 and opening of the *dura mater* via a longitudinal cut, a dorsal hemisection injury was performed with a Scouten wire knife (Bilaney). After suture of the *dura mater* the lesion area was covered with a piece of Elvax copolymer (ethylene vinyl acetate; a gift from Erbslöh GmbH) or with a small piece of a thermoplastic polymer (Nescofilm®, Roth). Finally, the overlying muscles and skin were sutured in layers, and the animals were housed with food and water *ad libitum*. Post-operative care included prophylactic daily oral Baytril™ (Bayer Health Care) administration for one week. Animals received daily manual bladder expression when necessary. They were inspected for signs of infection, dehydration or automutilation with appropriate veterinary assistance as needed. Institutional guidelines for animal safety and comfort were adhered to, and all surgical interventions and pre- and post-surgical animal care were provided in compliance with the German Animal Protection law (State Office, Environmental and Consumer Protection of North Rhine-Westphalia, LANUV NRW).

Complete transection model (TX, Supplementary Fig. 1B): Following laminectomy the *dura* at Th8/9 was opened with a traverse cut and the spinal cord was slightly lifted and separated from the dura with a spinal cord hook (Fine Science Tools). The spinal cord was completely transected by a traverse cut with a pair of micro-scissors which resulted in the retraction of the transected cord leaving a small gap between the two segments. Two small spatulas were used to carefully pull the segments apart to ensure complete transection. After suture of the *dura mater* the lesion area was covered with a small piece of Nescofilm®, and overlying tissues were sutured in layers. Housing and post-operative care were performed as mentioned above.

Spinal cord re-transection (ReTX) at 8 months post-resection (mpr): One week prior to sacrifice, the spinal cord was completely re-transected at the rostral border of the graft following laminectomy at Th7 in a small group of PEG-treated animals ($n = 3$). TX only-controls did not undergo this procedure because they did not show any noteworthy spontaneous recovery as revealed by their generally low mBBB scores. After one week post ReTX the animals underwent mBBB open field testing. The one week post ReTX time point was chosen for locomotor testing in order to exclude the possibility that an observed disappearance of the locomotor recovery upon ReTX is merely a result of spinal shock.

Scar resection and matrix insertion

Five weeks after the initial SCI, animals were randomly assigned into the respective treatment groups. Animals of the RX-groups were re-anesthetized and the lesion area was re-opened. The Nescofilm® cover was removed and the sutures were cut open to expose the lesioned spinal cord. Two incisions, one above and one below the spinal cord lesion were made at a distance of 4 mm apart from each other with a pair of micro-scissors. Spinal cord tissue was removed via aspiration with a small pipette connected to a vacuum pump (Supplementary Fig. 2).

The region between the two incisions was aspirated until the central canal became visible (hemisection) or until all scar tissue (recognizable by its stiff texture) was removed, respectively. The tested matrix materials were injected with a 10 μl Hamilton syringe (#701 LT) equipped with a blunt canula using a stereotactic device. Aliquots of Matrigel™ (BD Biosciences) were thawed on ice for liquefaction. For Matrigel (MG) injection, cooled instruments were used in order to prevent premature gel formation. For alginate-hydrogel (ALG) preparation, a 2% (w/v) solution of alginate (Pronova UP LVM; NovaMatrix) in 150 mM sodium chloride (NaCl) was filtered with a sterile syringe filter. The alginate was set by exposure to 0.1 M calcium chloride ($CaCl_2$) resulting in a viscous hydrogel. For chelator-containing ALG solution, first a solution of 40 mM iron chelator [2,2′-bipyridine]-5,5′-dicarboxylic acid (BPY-DCA) and 150 mM NaCl in H_2O was prepared. Via addition of 2% (w/v) alginate powder and subsequent filtration, an injectable ALG was created. PEG (Merck) was heated to 37 °C prior to implantation to allow liquefaction. Application of low molecular weight PEG such as PEG 600 is safe as it is almost completely absorbed and excreted in urine (European Food Safety Authority, 2006). Systemic administration of PEG has previously been described to successfully yield repair of crushed as well as transected spinal cord axons leading to immediate recovery of axonal conduction in an acute injury model (Borgens et al., 2002; Shi and Borgens, 2000). Prior to this study, we tested PEGs of different molecular weights (PEG 400, PEG 600, PEG 1000, and an aqueous solution of PEG 2000). In addition to chemical cues, mechanical stimuli could also influence neuronal growth. PEG 600 proved to be the most suitable PEG for the intended purpose, whereas the viscosity of the