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Relationship between Scaffold Channel Diameter and Number of Regenerating Axons in the Transected Rat Spinal Cord

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Abstract

Regeneration of endogenous axons through a Schwann cell (SC)-seeded scaffold implant has been demonstrated in the transected rat spinal cord. The formation of a cellular lining in the scaffold channel may limit the degree of axonal regeneration. Spinal cords of adult rats were transected and implanted with the SC-loaded polylactic co-glycolic acid (PLGA) scaffold implants containing seven parallel-aligned channels, either 450- μm ($n=19$) or 660- μm in diameter ($n=14$). Animals were sacrificed after 1, 2, and 3 months. Immunohistochemistry for neurofilament-expression was performed. The cross-sectional area of fibrous tissue and regenerative core was calculated. We found that the 450- μm scaffolds had significantly greater axon fibers per channel at the one month (186 ± 37) and three month (78 ± 11) endpoints than the 660- μm scaffolds (90 ± 19 and 40 ± 6 , respectively) ($P=0.0164$ & 0.0149 , respectively). The difference in the area of fibrous rim between the 450- μm and 660- μm channels was most pronounced at the one month endpoint, at $28,046 \mu\text{m}^2 \pm 6,551$ and $58,633 \mu\text{m}^2 \pm 7,063$, respectively ($P=0.0105$). Our study suggests that fabricating scaffolds with smaller diameter channels promotes greater regeneration over larger diameter channels. Axonal regeneration was reduced in the larger channels due to the generation of a large fibrous rim. Optimization of this scaffold environment establishes a platform for future studies of the effects of cell types, trophic factors or pharmacological agents on the regenerative capacity of the injured spinal cord.

Keywords

Biomedical Engineering; Tissue Development and Growth; Central Nervous System; Polymeric Scaffolds

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Introduction

Spinal cord injury (SCI) is a devastating condition with respect to the number of patients affected [1] and the profound cost to both individuals and society [2]. Experimental strategies successful in promoting axonal regeneration in animal models of SCI include modulation of neuronal intracellular signaling [3–7], antagonism of myelin inhibitory factors [8–10], and transplantation of cells [11–17]. Of the latter, Schwann cell (SC) suspensions have most consistently demonstrated to support axon regeneration in the central nervous system (CNS) [11,12,18–32]. Biodegradable polymer implants have been shown to stimulate axon regeneration in the peripheral nervous system (PNS) by providing structural stability, prolonged release of growth-promoting agents [33–39], and a reservoir for sustained therapeutic drug delivery [40]. This approach can be applied to the spinal cord [31,41–44], providing a platform for the study of factors which promote regeneration in a complete transection model.

Re-establishment of functional connections after SCI requires injured axons to grow through the graft, enter normal tissue, find target cells, and establish synapses to complete a functional circuit. SCs seeded in guidance channels have been shown to promote axonal regrowth into the distal spinal cord [18,25,31,45,46]. The use of biodegradable polymer implants has many advantages. They serve as conduits for the delivery of therapeutic cell types to the injured spinal cord, while also providing a structural support to facilitate axonal growth and regeneration. The future of graft technology depends on optimization of scaffold geometry and cross-sectional area (CSA) to increase the numbers and efficacy of ascending and descending axons traversing the graft. We propose that parallel-aligned channels within the biodegradable polymer graft allow for increased CSA as well as directed growth. The objectives of this research are to examine the effect of two different channel sizes (450- μm and 660- μm in diameter) on the number of regenerating axons within the scaffold and the size of the regenerative core and fibrous scar within each channel.

Materials and Methods

Scaffold fabrication

PLGA grafts were generated with an 85:15 ratio of lactide/glycolide as previously described [47]. Briefly, the grafts were made by injecting the liquid phase polymer into a teflon mold, followed by vacuum extraction of the solvent. The resulting construct was 3.0mm in diameter, with seven 2.0 mm-long parallel channels with consistent internal diameters of either 450- or 660- μm (Fig. 1). Seven channels can be very conveniently distributed within the circular scaffold, with six located peripherally and one centrally. The 660- μm diameter channels were the largest diameter of channel that could be enclosed in a 3mm diameter scaffold, while maintaining structural integrity. The 450- μm diameter channel yielded a total cross sectional area for regeneration that was half that of the 660- μm diameter channel. The detailed morphology of these PLGA scaffolds (including micro-computed tomography) has previously been published ([47]). Degradation was shown to occur gradually [47].

Preparation of purified populations of Schwann cells

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the Mayo Clinic. SCs were obtained from sciatic nerves of two to five-day-old Sprague-Dawley rats (Harlan, Indianapolis, IN) according to earlier described methods [48]. Large sciatic nerve explants were stripped of epineurium and connective tissue, cut into 1 mm³ pieces, and then digested enzymatically with 0.25% trypsin (Worthington, Lakewood, NJ) and 0.03% collagenase (Sigma-Aldrich, St. Louis, MO) in Hanks balanced salts solution (Invitrogen, Carlsbad, CA). After digestion, cells were pelleted in a Beckman TJ-6 centrifuge,

trypsin/collagenase mixture removed, and resuspended in 5 ml of DMEM/F12 (BioWhittaker, Walkersville, MD) containing 10% FBS (Invitrogen) and 100-units/ml penicillin/streptomycin (Invitrogen). Resuspended cells were then dissociated and maintained on a laminin (Sigma-Aldrich) coated dish and incubated at 37° C and 5% CO₂ for 3 days.

Seeding SCs into guidance channels

SCs were resuspended in chilled Matrigel (BD Biosciences, Bedford, MA) at a concentration of 1.2×10^8 cells/100 μ l. SCs were then inserted in each channel of the scaffold with a gel-loading pipette tip. Scaffolds with seeded SCs were incubated in the DMEM/F12 media with 10% FBS for 24 hours prior to surgical implantation.

Surgical procedure and animal care

Adult female Sprague Dawley rats (weighing 200–250g) (n=33) were anesthetized with an intraperitoneal injection of Ketamine (50–60 mg/kg) (Fort Dodge Animal Health, Fort Dodge, IA) and Xylazine (5 mg/kg) (Ben Venue Laboratories, Bedford, OH). Immediately prior to the surgery, Lacrilube Ophthalmic Ointment (Allergan Pharmaceuticals, Irvine, CA) was applied to the eyes to prevent drying. The animals were placed on a heating pad to maintain body temperature at $37 \pm 0.5^\circ$ C. All surgical procedures were performed under sterile conditions. A multilevel laminectomy was performed at T8–10 thoracic vertebrae and the underlying cord segments (T9–T12) were exposed. The dura was incised longitudinally and pulled laterally. A spinal cord transection was made at T9, followed by removal of a 2-mm segment. The 450- μ m (n=19) or 660- μ m (n=14) guidance scaffold was grafted into the lesion site in such a way that the cut ends of both spinal stumps were apposed to the SC-containing guidance scaffold. The dura overlying the exposed spinal cord was sutured closed. The spinal cord, with the implanted scaffold, was covered with muscles and fascia and then sutured closed. The skin was repositioned and closed with sutures.

After surgery, the animals received 0.1 ml of Gentamicin (50 mg/ml) (Schering-Plough, Union, NJ) intramuscularly for five days; Buprinex 0.5 mg/kg (Baxter Healthcare Corporation, IL, U.S.A) subcutaneously for 3–4 days to minimize pain; and 3–5 ml of Lactated Ringer's Solution (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA, U.S.A) subcutaneously as needed. Food and water were readily available. The bladders were manually expressed twice daily.

Perfusion of animals

One (n=7 for 450- μ m group and n=6 for 660- μ m group), two (n=6 for 450- μ m group and n=4 for 660- μ m group), or three (n=6 for 450- μ m group and n=4 for 660- μ m group) months post implantation, the rats were euthanized with an intraperitoneal injection of 0.4 ml pentobarbital sodium (40 mg/kg) (Fort Dodge Animal Health, Fort Dodge, IA). 120 ml PBS followed by 120 ml of 4% paraformaldehyde was infused through the aorta. The spinal column and cord were removed *en bloc* and post-fixed overnight in the same fixative at 4° C. Following post-fixation, the grafted area, including 5 mm of the rostral and caudal spinal cord, was dissected out.

Tissue processing for histology

The spinal cords were processed for paraffin embedding and cut transversely (8–10 μ m thick) on a Reichert-Jung Biocut microtome (Leica, Bannockburn, IL).

Immunostaining

Sections were selected at equal intervals from the rostral, mid-point and caudal portions of the scaffold from each group – the sections were thus $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$ along the length of the scaffolds.

Sections were then immunocytochemically stained. Sections were deparaffinised in xylene, rehydrated in graded ethanol and washed in distilled water. The sections were incubated in Proteinase K (2 µg/ml) diluted 1:10 with phosphate buffered saline (PBS) for 20 minutes at room temperature. They were then placed in 0.3% hydrogen peroxidase in methanol (30 min), rinsed in PBS for 5 minutes before they were covered with a protein block solution (InnoGenex, San Ramon, CA) for 20 minutes. The sections were incubated with the mouse monoclonal anti-neurofilament (NF) antibody against phosphorylated NF-H (Dako clone 2F11, Carpinteria, CA), diluted 1:150 overnight at 4° C, then rinsed in PBS (3×2 min), and incubated with the secondary Envision antibody (Dako) and horseradish peroxidase for 60 minutes.

Axon counting

The number of neurofilament-stained axons was counted at three levels through the scaffold. In each case, the scaffold and the adjacent spinal cord were serially sectioned from rostral to caudal. In each case, the sections representing the interface between the rostral spinal cord and scaffold and the interface between the scaffold and caudal spinal cord were identified. These numbers were then used to identify the rostral (1/4 of the way along the scaffold), midpoint (1/2 of the way along the scaffold), and caudal (3/4 of the way along the scaffold) levels in the scaffold. Axon profiles were readily identified in 10 µm-thick transverse sections of tissue due to their small russet-colored cylindrical appearance when the observer focused through the section. All axon counts were made by a single observer who was blinded to the different treatment groups.

Fibrous scar and regenerative core area measurements

Tissue cross-sections were imaged on a Zeiss Axioplan II microscope using an AxioCam digital camera. Image analysis was performed on each series of NF-stained sections using a digital image analyzing system (KS 400 Imaging System Release 3.0, Carl Zeiss Vision, Echting, Germany). Area measurements of total cross-sectional area (CSA), CSA of fibrous rim, and CSA of regenerating core were made by outlining these areas with the cursor. Actual areas were then calculated using the pixel calibration (both x and y) divided by the objective lens magnification. All histological analyses were assessed blindly with respect to treatment.

Statistical analysis

Data from all the channels at one level (i.e., rostral, mid-level or caudal) in one animal were pooled (as means) and the mean number of axons per channel were calculated for each animal. This mean was then used as a single independent observation for each animal. Statistical analysis was performed by comparing groups of observations between animals with 450-µm and 660-µm channel scaffolds. A two-tailed student t-Test was used to compare results of the number of axons; area of regenerative core and area of fibrous rim. Linear regression analysis was used to determine a relationship between number of axons and core regenerative area or fibrous rim area, independent of fabricated scaffold channel size.

Results

Effect of channel size on number of axon fibres

Immunohistochemical analysis for neurofilament expression was performed on transverse sections of the implanted scaffolds which had 450-µm (Fig. 2A) or 660-µm (Fig. 2B) diameter channels. Axonal growth was determined at three anatomic levels of the scaffold: rostral, midlevel, and caudal. The number of axon fibres per channel was averaged in the entire scaffold and compared at each endpoint (Fig. 3A) or the number of axon fibres per channel at each level was compared at the one month endpoint (Fig. 3B).

The number of axon fibres per channel was significantly higher at the one month (185.78 ± 36.78) and 3 month (77.67 ± 11.26) endpoints in the 450- μm channels compared to the 660- μm channels, which measured 90.11 ± 18.95 and 40.08 ± 6.49 respectively (Fig. 3A) ($P=0.0164$ and 0.0149 , respectively).

When the number of axons per channel at different levels of the scaffold was compared, the 450- μm channels showed significantly higher numbers present in the mid-point (109.43 ± 27.45) and caudal level (160.43 ± 51.82) of the scaffold compared to the 660- μm channels, which measured 49.85 ± 11.7 and 48.62 ± 8.51 , respectively (Fig. 3B) ($P=0.0308$ and $P=0.0101$, respectively).

Effect of channel size on cross sectional area of regenerative core and fibrous rim

A central aim in this study was to establish which channel size allowed for more axon growth by supporting a greater area of core regeneration with limited fibrous rim growth. The CSA of the regenerative core (circled in white in Fig. 2A & B) and fibrous rim (outer dense brown lining of the channel (Fig. 2A & B) was also measured at three anatomic levels of the scaffold: rostral, midlevel, and caudal. The regenerative core (Fig. 4A) and fibrous rim (Fig. 4B) area per channel was averaged in the entire scaffold and compared at each endpoint.

For the CSA of the regenerative core, no significant difference was found at any given time interval between the 450- μm and 660- μm groups at each endpoint (Fig. 4A). However, the CSA of the fibrous rim in the 450- μm group was shown to be significantly lower ($28,046.22 \pm 6,550.75 \mu\text{m}^2$) compared to the 660- μm group ($58,633 \pm 7062.9 \mu\text{m}^2$) at the 1 month endpoint (Fig. 4B) ($P=0.0105$).

The CSA of the regenerative core (Fig. 5A) and fibrous rim (Fig. 5B) at the rostral, middle and caudal levels in the 450- μm and 660- μm channels was also compared at the one month endpoint. No significant differences were noted here between the two groups at the different levels of the scaffold.

Relationship between area of regenerative core or fibrous rim to number of axon fibres

When all data from both experimental groups was combined, with the number of axon fibres plotted against the regenerative core area, there was a weak positive correlation between number of axon fibers and the CSA of the regenerative core of the scaffold available for regeneration ($R^2=0.2525$) (Fig. 6A).

In addition, when all data from both experimental groups was combined, with the number of axon fibres plotted against the area of fibrous rim, a minor negative correlation existed between the number of axon fibers and the area of fibrous reaction organized as a rim around the regenerative core of all channels of both sizes ($R^2= -0.1802$) (Fig. 6B).

Discussion

Schwann-cell-seeded PLGA scaffolds, with an 85:15 ratio of lactide/glycolide, implanted into transected rat thoracic spinal cord demonstrated axonal growth throughout the length of the graft over a three month period. We have previously demonstrated that these axons traverse the length of the channels and project up to 15 mm into the cord on the other side of the scaffold [47]. Guidance channels containing seven parallel-aligned channels, either 450- μm or 660- μm in diameter, maintained their structural stability over the course of the study. Optimal channel size for maximum axon regeneration remains unknown. The purpose of this experiment was to implant grafts with parallel-aligned channels of two different sizes, 450- μm and 660- μm , and observe their effects on axon growth, regenerative area and fibrous area. For technical reasons, it was not possible to determine if differences existed between the areas

of the spinal cord where the most regeneration took place (i.e., ventral, dorsal or lateral). While identification of the channels themselves was always possible, it was not clear how the channel corresponded to the original orientation of the scaffold. In part this was because of the polymer material. The scaffold channels tended to move during the processing of the sections on the slide. In addition, the micro-anatomy at the interface between the spinal cord and the scaffold became inconsistent and disorganised in a way that made it difficult to follow the formation of individual channels in transverse sections. At a depth of ~50µm into the channel, the consistent core and rim configuration became clear. We did however determine that the 450-µm diameter channels promote growth of significantly more axons than did 660-µm channels. In addition, we demonstrated no significant difference in the size of the regenerative core between the 450-µm and 660-µm channels. However, the area of fibrous rim was significantly larger in the 660-µm group, suggesting that the fibrous scar expanded to occupy more area inside the larger channels. As figure 2A demonstrates, the fibrous rim was even absent in some areas of the 450-µm channels. This growth of fibrous tissue may represent either an intrinsic CNS reaction or a fibrous reaction to the graft material. If the process was inherent to CNS regeneration, the ring of fibrous tissue would be homologous to perineurium (derived from fibroblasts), which is part of the normal microscopic anatomy of peripheral nerve [49,50]. However, if it is a fibrous reaction to the graft material, the rim could impede axon regeneration [51–55]. Our results here indicate that the latter is the case, since fewer axons per channel were evident in the 660-µm group, which demonstrated a more extensive fibrous rim when compared to the 450-µm group. We also more frequently observed macrophages in the fibrous rim of the 660-µm channels. However, this was not quantified.

In a previous study, den Dunnen and colleagues examined single-channeled biodegradable nerve guides of varying diameter and wall thickness in the PNS [56]. They concluded that increased wall thickness of the prosthesis (more polymer mass) caused more swelling during polymer degradation, and in combination with smaller diameter guides caused more nerve compression, thus, resulting in overall decreased peripheral nerve regeneration. In the CNS, however, we observed decreased quantity of regenerative axonal fibers with the larger 660-µm channels, which have less wall thickness, but more surface area lining the channel. Nerve compression and compromised scaffold stability were not observed.

The decrease in number of axon fibres per channel from the one month to the two month endpoint in both groups indicates that the scaffold environment is unable to support the regenerated axons over time, perhaps due to an inflammatory reaction, or the growing fibrous scar. Biodegradable polymers, similar in chemical composition to the ones used in the fabrication of the scaffold here, are widely used in orthopaedic, oral- and maxillofacial and other fields of surgery. Their use has been complicated by inflammatory reactions causing both swelling and pain at the site of implantation. The etiology of this inflammatory reaction is largely unknown, though it has been suggested that fibroblasts and macrophages adjacent to the polymer may play a role [57].

Another possibility for the loss of axon fibres from the one to two month interval is attrition of fibres that do not reach targets and establish functional synaptic connections. Using retrograde tracing, we have demonstrated that axons regenerating through the scaffolds originate from large neurons in the rostral spinal cord [58]. The majority come from segments immediately adjacent to the scaffold and up to 15mm into the caudal spinal cord. We have not however demonstrated that these axons establish functional synaptic connections. CNS and PNS neurons have unique requirements for growth and survival, and CNS neurons may have greater need for activity-dependent plasticity. If CNS neuronal growth depends on activity, and injured CNS neurons are less active, their capacity for regeneration would be impaired.

A third likely cause for the loss of axons may be growth of the fibrous scar. In our experiment, the 660- μm channels had a significantly increased amount of fibrous tissue at the one month interval compared to the 450- μm group, the same time period when we observe the largest difference in axon fibres between the two channel sizes. In addition, a slight negative correlation existed between the number of axon fibres and the area of fibrous rim around the regenerative core of all channels of both sizes.

In figure 3A, the number of axons per channel decreases with time in both channel diameters. The rate of decrease in the larger channels is slower but there are sequentially less axons at each time point. In the smaller channels, the number appears to stabilize at 2 months and then remains the same between 2 and 3 months. The reason for this difference is not clear.

Our study suggests that fabricating scaffolds with smaller diameter channels increases axonal regeneration over larger diameter channels. The versatility of this model allows it to be systematically altered to study the effects of different cell types, trophic factors or pharmacological agents on the regenerative capacity of the injured cord.

Conclusion

Channel dimensions within a scaffold implant can influence the formation of fibrous tissue around its circumference, which can impact the level of axonal regeneration taking place. Provision of an optimal environment for axonal regeneration will be essential for promoting functional recovery after SCI.

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Abbreviations

CNS	Central nervous system
CSA	Cross sectional area
NF	Neurofilament
PNS	Peripheral nervous system
SC	Schwann cell
SCI	Spinal cord injury

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Figure 1. Schematic representation of scaffolds for insertion into the transected spinal cord. Longitudinal (A) and transverse (B) views of 85:15 polylactic co-glycolic acid (PLGA) scaffolds containing 7 channels.

Figure 2.

Transverse section through a 450- μ m and 660- μ m channel. Light microscopy analysis of neurofilament stained axons (russet circular profiles) in the regenerative core (circled in white) in the 450- μ m (A) and 660- μ m (B) channels. Axons were identified as immuno-stained cylinders that could be visualised when focussing through the thickness of the section. The fibrous rim is composed of eosinophilic, lightly stained, concentrically oriented cells in the space between the the inner surface of the channels and the regenerative core. Profiles of regenerating axons were never seen within the fibrous rim. Clumps of darkly stained macrophages were seen within the fibrous rim (white arrow). Each channel typically contained a central capillary (*). Magnification: 400X (A&B). Scale bar: 50 μ m (A&B).

Figure 3.

Axon fibres per channel. A, Graphic representation of the average number of axon fibres per channel through the entire scaffold at 1, 2 and 3 months. Error bars represent standard error of the mean. * $P=0.0164$ and 0.0149 when comparing $450\text{-}\mu\text{m}$ and $660\text{-}\mu\text{m}$ channels at the 1 and 3 months, respectively. B, Graphic representation of the average number of axon fibres in the rostral, middle or caudal levels of the scaffold at 1 month. Error bars represent standard error of the mean. * $P=0.0308$ and 0.0101 when comparing $450\text{-}\mu\text{m}$ and $660\text{-}\mu\text{m}$ channels at the mid-point and caudal levels, respectively.

Figure 4.

Area of regenerative core and fibrous rim at 1, 2 and 3 months. A, Graphic representation of the average area of regenerative core (μm^2) through the entire scaffold at 1, 2 and 3 months. Error bars represent standard error of the mean. B, Graphic representation of the average area of fibrous rim (μm^2) through the entire scaffold at 1, 2 and 3 months. Error bars represent standard error of the mean. *P=0.0105 when comparing 450- μm and 660- μm channels at the 1 month endpoint.

Figure 5.

Area of regenerative core and fibrous rim in the rostral, middle or caudal levels of the scaffold channels. A, Graphic representation of the average area of regenerative core (μm^2) through the rostral, middle or caudal levels of the scaffold at 1 month. Error bars represent standard error of the mean. A, Graphic representation of the average area of fibrous rim (μm^2) through the rostral, middle or caudal levels of the scaffold at 1 month. Error bars represent standard error of the mean.

Figure 6.

Relationship between axon fibres and regenerative core or fibrous rim. A, Graphic representation of the relationship between axon fibres per channel and the regenerative core area. There is a positive correlation between the two ($R^2=0.2525$) ($n=303$). B, Graphic representation of the relationship between axon fibres per channel and the fibrous rim area. There is a negative correlation between the two ($R^2=-0.1802$) ($n=303$).