Poly(L-Lactide) Microfilaments Enhance Peripheral Nerve Regeneration Across Extended Nerve Lesions

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After injury, axonal regeneration occurs across short gaps in the peripheral nervous system, but regeneration across larger gaps remains a challenge. To improve regeneration across extended nerve defects, we have fabricated novel microfilaments with the capability for drug release to support cellular migration and guide axonal growth across a lesion. In this study, we examine the nerve repair parameters of non-loaded filaments. To examine the influence of packing density on nerve repair, wet-spun poly(L-Lactide) (PLLA) microfilaments were bundled at densities of 3.75, 7.5, 15, and 30% to bridge a 1.0-cm gap lesion in the rat sciatic nerve. After 10 weeks, nerve cable formation increased significantly in the filament bundled groups when compared to empty-tube controls. At lower packing densities, the number of myelinated axons was more than twice that of controls or the highest packing density. In a consecutive experiment, PLLA bundles with lower filament-packing density were examined for nerve repair across 1.4- and 1.8-cm gaps. After 10 weeks, the number of successful regenerated nerves receiving filaments was more than twice that of controls. In addition, nerve cable areas for control groups were significantly less than those observed for filament groups. Axonal growth across 1.4- and 1.8-cm gaps was more consistent for the filament groups than for controls. These initial results demonstrate that PLLA microfilaments enhance nerve repair and regeneration across large nerve defects, even in the absence of drug release. Ongoing studies are examining nerve regeneration using microfilaments designed to release neurotrophins or cyclic AMP.   © 2003 Wiley-Liss, Inc.

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Axonal regeneration can occur after peripheral nervous system (PNS) injury when the injury gap is short and the nerve ends can be anastomosed. When the gap is too long and anastomosis of the nerve ends is difficult, axonal regeneration and eventual innervation of the target organ is unlikely. Under these conditions, several repair materials have been proposed to increase the prospects of nerve regeneration, including autologous nerve grafts (Lundborg et al., 1982a; Hafiz et al., 1985; Evans et al., 1995), allografts (Dubuisson, 1997), xenografts, muscles (Norris et al., 1988; Gattuso et al., 1989; DeFranzo et al., 1994), and blood vessels (Chiu et al., 1982; Foidart-Dessalle et al., 1997). Although these materials enhance regeneration, there are numerous disadvantages, such as limited availability, a requisite additional surgical step, size mismatching (Rodriguez et al., 1999), a need for immunosuppression (allografts and xenografts) (Bain et al., 1988), and a loss of nerve function at the donor site (autologous grafts).

An alternative to using natural components is to employ entubulation techniques to bridge the nerve gap. Synthetic as well as natural materials have been shaped into tubes such that the two nerve stumps could be inserted into the ends of the tube, thereby allowing the nerve to regenerate across a well-defined gap. These tubes, typically referred to as nerve guides or nerve guidance channels, act to sequester chemotrophic and chemotactic molecules while reducing the formation of scar tissue around the reforming nerve. Tubular nerve guides have been constructed from a variety of materials, including silicone rubber (Lundborg et al., 1982b; Williams et al., 1983; Gibson and Daniloff, 1989; Danielsen et al., 1993; Dahlin et al., 1995), collagen (Archibald et al., 1991; Li et al, 1992; Chamberlain et al., 1998), and bioresorbable polymer tubes made of poly(L-lactide) acid (PLLA) (Seckel et al., 1984; da Silva et al., 1985), poly(glycolic acid; PGA) (Dellon and Mackinnon, 1988; Mackinnon and Dellon, 1990; Keeley et al., 1991), polyglactin (Molander et al., 1983), or blends of these components (den Dunnen et al., 1995, 1996, 1997; Aldini et al., 1996; Rodriguez et al., 1999). The latter group has the advantage of degrading

over time in vivo (Leenslag et al., 1987; Pistner et al., 1993), thus avoiding the need for a second surgery to remove scaffolding material. In most instances, nerve regeneration within hollow tubes is limited to approximately 1-cm gaps in the rat sciatic nerve (Lundborg et al., 1982b; Seckel et al., 1984).

To expand the regenerative potential of the entubulation method, tubes have been preloaded with fibrin (Williams and Varon, 1985; Williams, 1987), trophic factors (Rich et al., 1989), Schwann cells (Guenard et al., 1992; Levi et al., 1997), and extracellular matrix molecules, such as collagen (Wells et al., 1997; Labrador et al., 1998), fibronectin (Bailey et al., 1993), and laminin (Madison et al., 1985, 1988; Tong et al., 1994). It is thought that these materials provide a substrate that enhances migration of nonneuronal cells (e.g., fibroblasts, endothelial cells, and Schwann cells), which act to form a cell- and matrix-containing cable between the separated stumps (Labrador et al., 1998). The formation of this cellular cable provides the bridging material for axons to extend across the gap. Potentially, axonal regeneration could be further enhanced if the bridging structure was designed to direct and organize the longitudinal formation of the nerve cable. Lundborg et al. (1997) has demonstrated that bridging filaments made from resorbable suture greatly enhances axonal regeneration across 10- to 15-mm nerve gaps. We have examined the regenerative potential of novel resorbable synthetic microfilaments that can be fabricated for drug release. Release of neurotrophins, growth factor, or cyclic nucleotides could enhance further regeneration and segregate axonal growth to more appropriate target locations to increase functional reconnections, which is a consistent problem with this type of nerve repair.

To increase the guidance environment in the tube, we have fabricated bioresorbable PLLA microfilaments. The fabrication process produces a continuous filament or thread, $40-100$ µm in diameter that can be preloaded with up to 60% aqueous phase by volume. Proteins in the aqueous phase can be loaded into filaments for slow release as the filaments degrade. Previous studies in our laboratory have shown that these microfilaments can be coated with laminin and provide an improved substratum to organize Schwann cell migration and direct axonal growth in vitro (Rangappa et al., 2000). We examined whether the microfilament bundles alone could enhance nerve cable formation and provide improved axonal growth across various gap lengths. The results demonstrate that bundled filaments enhance regeneration across 1.0-, 1.4-, and 1.8-cm gaps in the rat sciatic nerve model. Nerve cable formation and axonal growth were more consistent across these gaps with bundled filaments when compared to controls.

MATERIALS AND METHODS

PLLA Filament Preparation

Filament fabrication. Poly(L-lactic acid) (200 kD; Polysciences Inc., Warrington, PA) was dissolved in chloroform (Aldrich Chemical, St. Louis, MO) at a concentration of 0.1 g/ml of chloroform and the mixed solution loaded into a glass syringe (gas-tight syringes; Hamilton Co., Reno, NV) and placed in a syringe pump (model KDS200; KD Scientific, New Hope, PA). A Viton tube (Cole-Parmer, Vernon Hills, IL) connected the syringe to a 25-gauge needle dispenser by stainless steel Luer fittings. We used only blunt-tipped needles (Small Parts Inc., Miami Lakes, FL), as the bevel cut on sharp needles potentially could cause problems during extrusion.

The extrusion setup consisted of a glass tube, 25 mm in diameter by approximately 200 mm in length, immersed in a small plastic container full of the coagulating bath fluid. A rubber septum was placed on top of the glass tube, and the tube was filled completely with the coagulating bath by withdrawing air with a separate syringe. Once the glass tube was completely full (the bottom still immersed in the fluid in the container), the blunt-tipped needle was then pierced through the rubber septum and the syringe pump turned on to deliver 0.03 ml/min. The coagulation bath was a poor solvent for the polymer, yet highly miscible with the solvent used to dissolve the polymer. For the fibers used in this study, we used isopropyl alcohol as the coagulation bath fluid. When the fiber exits the coagulating bath it was wound on a 8.25-cm diameter bobbin attached to the jaws of a modified 5-inch Garden Lathe (Sears-Craftsman model 549-289000; Sears and Roebuck, Chicago, IL). A 24-V power supply (model 5005R; Power Designs Inc., NY) drove a 0.03-horsepower DC motor (Pittman Motors, Harleysville, Pennsylvania) that replaced the original motor on the lathe. We first wrapped the bobbin with paper so that at the completion of the extrusion run, we pulled the paper from the bobbin to remove the fiber intact. The angular velocity of the lathe was measured using an optical tachometer. The draw ratio was calculated as the ratio of the linear velocity of the fiber measured at the take-up bobbin to the calculated mean linear flow rate of the emulsion within the dispensing needle, because die-swell is not observed typically during these extrusions. At the end of the extrusion run, fibers were removed from the lathe, left on the paper-roll, and placed under vacuum at roughly 4°C for at least 4 hr at room temperature to help remove remaining solvents. The fiber was stored in a desiccator or in a $-20°C$ freezer until needed.

PLLA filament bundling and sterilization. PLLA filament bundles were created by inserting PLLA fibers through sterilized silicone tubes (A-M Systems, Carlsborg, WA; 1.4-, 1.8-, and 2.2-cm lengths with an outside diameter of 2.5 mm and inside diameter of 1.5 mm). They were washed three times in 1× phosphate buffered saline (PBS). Filaments were trimmed to 1.0, 1.4, and 1.8 cm and sterilized by 10× penicillin/streptomycin (Gibco, Carlsbad, CA) in PBS for 2 hr at 25°C with constant shaking. Filament bundles were rinsed and washed three times in PBS, and then stored in PBS at 4°C until use.

Experimental Groups

In the first set of experiments, PLLA filament bundles of various filament-packing densities were tested to bridge a 1.0-cm gap lesion. Four groups ($n = 50$) of animals were studied. The control group ($n = 17$) was implanted with silicone tubes without filaments. The experimental groups had implanted silicone tubes that were bundled as follows: 16–24 filaments (3.75% packing density, $n = 7$), 32–40 (7.5%, $n = 12$), 64–80 (15%, $n = 7$), and 128–160 (30%, $n = 7$). The filament-packing density was determined by comparing the cumulative cross-sectional area of the filaments to the available area of the silicone tube.

A second set of experiments was designed to test whether PLLA filament bundles could enhance regeneration across larger gap lesions. Experimental groups were implanted with bundled PLLA filaments (7.5% filament-packing density) loaded into silicone tubes to bridge 1.4-cm ($n = 8$) and 1.8-cm ($n = 8$) gaps. Similarly, control groups were implanted with silicone tubes without filaments to bridge 1.4-cm ($n = 8$) and 1.8-cm ($n = 8$) gaps.

Surgical Procedure

Adult female Sprague-Dawley rats (250–300 g) were anesthetized by intraperitoneal injection of ketamine (66 mg/ml)/xylazine (6.66 mg/ml) at 0.1 ml/100 g. A 3-cm incision was made parallel to the right femur. After blunt dissection to expose the sciatic nerve, a 2- to 5-mm section of the nerve was resected. PLLA filament bundles were then implanted to bridge either a 1.0-, 1.4-, or 1.8-cm gap between the proximal and distal nerve stumps. Superglue (Quicktite Loctite) was applied carefully to both ends of the tube to secure the implant to both nerve stumps. All tubes were filled with PBS upon deployment. Muscles were sutured with 4-0 coated VICRYL absorbable suture (Ethicon Inc., Piscataway, NJ), and stainless steel wound clips (Autoclip) were used to staple the skin together. Animals were monitored and maintained for 10 weeks with full access to food and water according to the animal care policy at the University of Kentucky Medical Center Division of Laboratory Animal Resources. Animals were monitored for any signs of infection and paw biting of the right limb. All animals recovered successfully from surgery. During the 10-week survival period, a few animals in each set of experiments developed mild autotomy. This included biting of the toenails or phalanges of the right hind paw. These animals were not excluded from the study because the injuries were not severe and they healed shortly after Elizabethan collars were applied and hind paws were treated.

Implant Retrieval and Histological Preparations

Animals were anesthetized using sodium pentobarbital (Nembutal, 0.1 ml/100 g), perfused transcardially with 0.9% sodium chloride and 0.5% sodium nitrite, and fixed with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.5. Implants were extracted and incubated in the same fixative for approximately 48 hr. Silicone tubes were then removed by longitudinal dissection to expose the regenerated nerves and filament bundles. Implants were washed with PB and prepared for microscopy studies.

Upon extraction of the implants after a 10-week implantation period, fibrous tissues ensheathed the outside of the silicone tubes. The sheaths were observed on all silicone tubes in every experiment. These tissues were removed along with the silicone tubes before histological preparations of the regenerating nerves that were encased in the tubes. Upon removal of the tissue sheath, fluid (clear to yellow in color) was seen inside empty and filament-filled silicone tubes. This fluid was drained as the silicone tubes were removed.

Specimen segments for light microscopy were incubated 3–4 hr in 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA). After PB wash and sequential alcohol dehydration, specimens were immersed 2 hr in propylene oxide (Electron Microscopy Sciences), then incubated overnight in a 1:1 mixture of propylene oxide and Spur resin (Polysciences).

Implants were embedded finally in 100% Spur resin and incubated overnight at 60°C.

Microscopy Studies

Semi-thin sections for light microscopy studies were cut with an ultramicrotome (Reichert-Jung) at a thickness of 0.6 μm and stained in 1% toluene blue in 1% sodium borate. For quantitation of axons, myelinated axons within the entire cross section were counted. This was done because axon density was not uniform throughout the section. Composite images for each section were taken at 100–400× magnification using the Nikon E800 light microscope Metamorph Imaging System 4.0 (Universal Imaging, Downington, PA) and transferred to Adobe Photoshop 5.5 software to generate a montage of the entire cross section. In Photoshop, a transparent layer with a grid pattern was superimposed on top of the image and the myelinated axons were noted by placing a dot on this layer. This process was repeated until all axons were confirmed on the image. Myelinated axons were seen as black ovoid rings. To differentiate myelinated axons from other organelles they were confirmed under 400× magnification. The layer with the "dotted axons" was transferred to Metamorph to count the number of dots (axons) automatically. Cable areas were measured for each sample using Metamorph Imaging System in pixels^2 and converted to mm^2 units. Cable areas were measured at the distal end for the 1.0-cm gap, at midsection and distal ends for the 1.4-cm gap, and at 2-mm intervals along the 1.8-cm gap. Note that animals having no nerve cables or having cables with no axons at the distal end were considered in the analysis as having zero number of axons.

Ultra-thin sections for transmission electron microscopy (TEM) analysis were cut at a thickness of 80–90 nm and stained in uranyl acetate and lead citrate. TEM studies were carried out using the Philips Tecnai transmission electron microscope at 80 kV. Images were captured at 500–30,000× magnification.

Statistical Analysis

Data were analyzed using the SPSS 10.1 statistical software (SPSS, Inc.). Fisher's exact tests were carried out to determine differences in success rates of nerve cable formation and axonal growth between control and test groups. One-way ANOVA was used to evaluate differences in nerve cable areas and myelinated axon counts for various filament-packing densities measured at the distal end. Dunnett's and Tukey HSD post-hoc analyses were used to determine differences within treatment groups. One-way ANOVA with repeated measures was used to determine differences in nerve cable areas and myelinated axon counts for various gap distances along the nerve cables of the 1.4- and 1.8-cm gap groups. The Bonferroni-Holmes method was used to correct for the possibilities of errors inherent in multiple test comparisons. $P < 0.05$ was the statistical significance criterion.

RESULTS

Filament-Packing Densities Across 1.0-cm Gap

The ability of microfilaments to organize cellular cable formation and increase axonal regeneration was examined initially across a 1.0-cm sciatic nerve gap. Our preliminary studies showed that PLLA filaments could
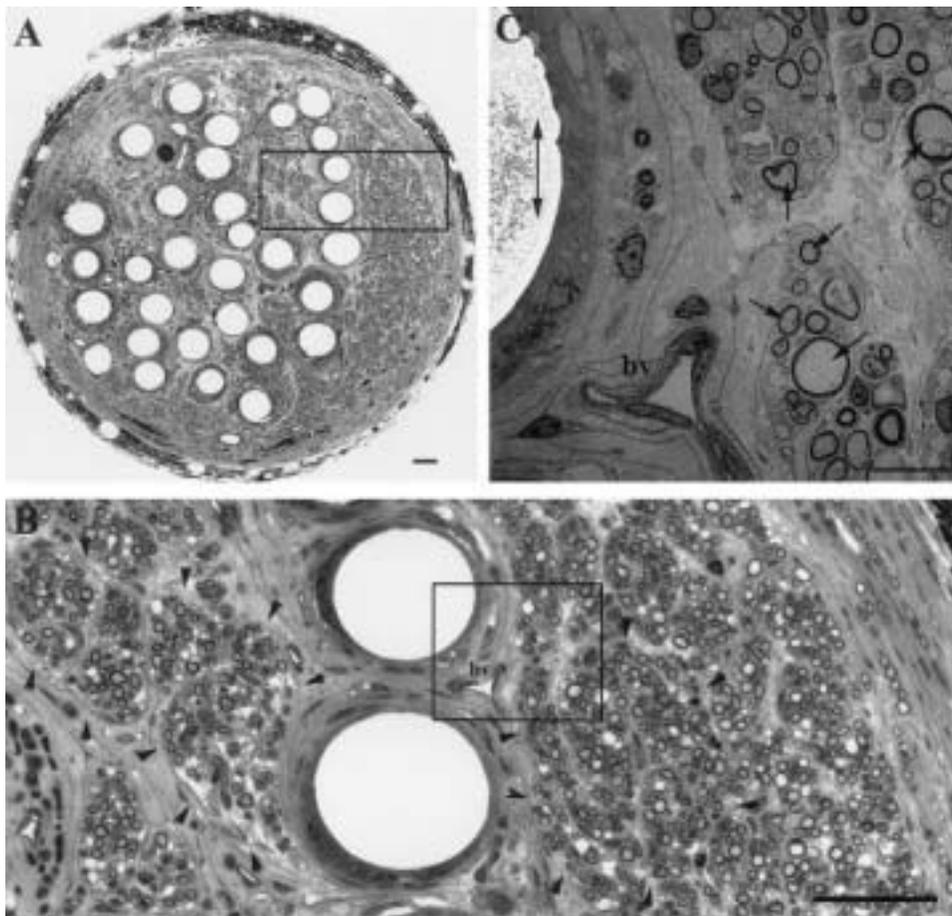
Fig. 1. Photomicrographs of regeneration across a 1.0-cm lesioned gap of the sciatic nerve in rats. Transverse cross-section of the distal end of a reconstructed nerve 10 weeks after implantation showed many well-defined fascicles (**A**). High magnification of one of the fascicles (box in A) showed bundles of myelinated axons surrounded by well-delineated perineuriums (**B**, arrowheads). Further examination of the nerve region (box in B) by TEM (**C**) showed a number of unmyelinated axons (★) present within the endoneurial sheaths of Schwann cells as well as blood vessels (bv). Myelinated axons (arrows) could also be seen a few microns away from the filament edge (double-headed arrow) which was surrounded by thin layers of cells (C). Scale bars = 50 μm, (A,B); 10 μm (C).

organize the growth of Schwann cells and axons in vitro and thus might display similar guidance ability in vivo. Examination of filament-loaded tubes 10 weeks after implantation showed a well-reconstructed nerve, displaying a well-defined epineurium, perineurium, numerous blood vessels, and large bundles of myelinated axons (Fig. 1A,B). At most locations examined, macrophages and fibroblasts were observed surrounding the microfilaments, similar to those observed in other systems using these polymers (Hooper et al., 1998). Only occasionally were Schwann cells or axons observed growing along the filament surface. Most myelin profiles were observed several cell layers away from the filament surface. Through TEM analysis, these nerves showed numerous unmyelinated axons within Schwann cell endoneurial sheaths surrounded by a collagen matrix (Fig. 1C). Ultrastructurally, nerve regeneration in the presence of microfilaments was very similar to that of normal nerve (data not shown). In regions where the filament density increased (in close proximity or contacting each other), few axon fascicles were observed. This observation suggested to us that the packing density of the filaments in the tube might affect axonal growth.

To examine the effect filament density had on nerve reconstruction and axonal growth across a 1.0-cm gap, silicone tubes were loaded with microfilaments equivalent to 3.75, 7.5, 15, and 30% of the internal cross-sectional diam-

eter. Light microscopy was used to evaluate the morphology of the regenerated nerve tissue at the distal stump (Fig. 2A–F). Nerve regeneration through empty tubes consisted of a sheath of perineurial-like cells surrounding the central group of axons (Fig. 2B). Nerve reconstruction through PLLA bundles with 3.75% filament-packing density (Fig. 2C), 7.5% (Fig. 2D), 15% (Fig. 2E), and 30% (Fig. 2F) showed less fibroblastic and collagen encapsulation. All of the reconstructed nerves were well vascularized, with many showing numerous myelinated axons. At the lowest packing density, filaments often seemed to concentrate toward one side of the tube. This was most likely due to gravitational settling of the filaments to the bottom of the tube. At mid-packing densities, filaments seemed more evenly spaced with larger distances between filaments, when compared to the highest filament-packing density.

Although 71% of the animals in the control group had a nerve cable bridging the 1.0-cm gap, all animals in the PLLA filament bundled group formed a cable across the lesion (Fig. 3A); however, this difference showed a trend toward significant ($P = 0.063$). This was expected, because a previous study showed regeneration of a severed nerve through silicone tubes could occur over gaps up to 1.0 cm (in a rat model) with little means of enhancement (Lundborg et al., 1982b). Evaluation of axonal regeneration showed that only 53% of the empty tubes, vs. 86–
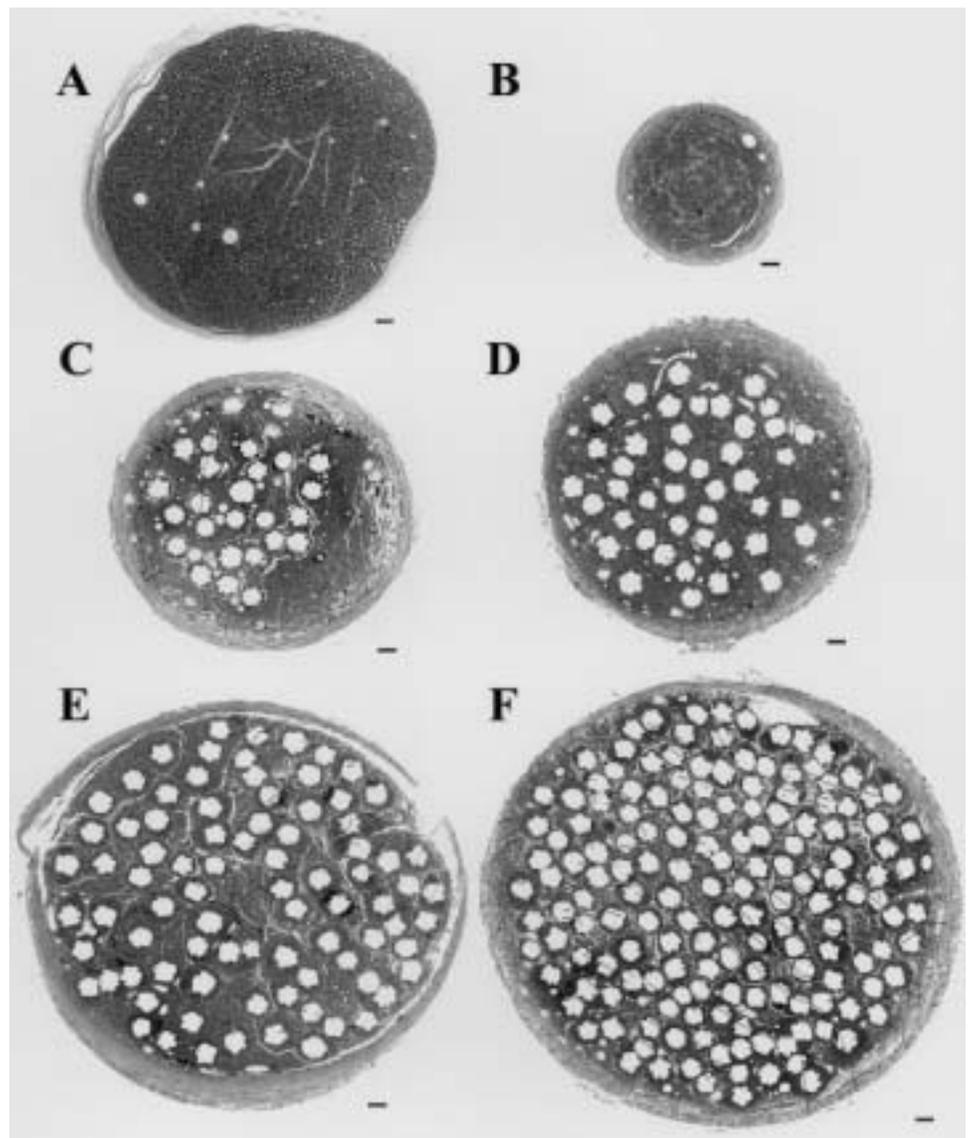
Fig. 2. Light photomicrographs of transverse cross-sections of normal sciatic nerve (**A**) through the distal end of sciatic nerve implants (**B–F**). Nerve regeneration through empty tubes resulted in a sheath consisting of perineurial-like cells surrounding the central group of axons (**B**). Regeneration through bundles with 3.75% filament-packing density (**C**), 7.5% (**D**), 15% (**E**), and 30% (**F**) showed less encapsulation of such cells. All nerve cables were well vascularized. Filaments seemed more dispersed at higher packing densities when compared to the lowest packing density where they tended to concentrate on one side of the tube. Scale bars = 50 μm for all sections.

100% of the PLLA filament bundled groups ($P = 0.020$), had myelinated axons at the distal end of the tubes (Fig. 3A). The cross-sectional area of each nerve tissue cable was examined to determine if filament density influenced the amount of tissue formed in the tube. Increasing the filament-packing density yielded a monotonic increase in the cross-sectional area of the regenerated nerve (Fig. 3B). Cross-sectional cable areas (not including filaments) of tubes with 15–30% filament densities showed a fourfold increase in tissue when compared to empty tube controls. Myelinated axon counts demonstrated an inverse proportional relationship between filament density and axonal regeneration, in which the number of myelinated axons at 30% filament density was similar to those for empty tubes. The PLLA-bundled group with 3.75% filament-packing density had the greatest number of myelinated axons when compared to all other groups (Fig. 3C). This group showed a significant difference in the number of myelin-

ated axons when compared to the control ($P = 0.008$) and to the 30% filament-packing density group ($P = 0.019$).

Nerve Regeneration Across Extending Gap Lesions

Regeneration across 1.4-cm gap. PLLA bundles with a 7.5% filament-packing density were chosen to determine whether axonal regeneration was improved over longer gap distances. Although the 3.75% filament-bundled group had a greater number of myelinated axons, filaments in this lower packing density group had the tendency to settle in the silicone tube (Fig. 2C). This was particularly apparent in initial studies with gap lengths of 1.4 and 1.8 cm. The higher filament-packing densities demonstrated less settling and more even distribution within the tube chamber (Fig. 2D).

Cellular cables formed across a 1.4-cm gap within 10 weeks post-implantation, for both empty tubes (Fig.

4A,C) and those containing PLLA filament bundles (Fig. 4B,D). The tissue cables seemed smaller for controls when compared to those formed using filament bundles. In addition, cross-sectional analysis at the midsection and distal end of the 1.4-cm cable showed that the average cable areas (not including filaments) for the PLLA-bundled group were approximately two and five times

A) Nerve Cable Formation & Axon Growth

B) Cable Area at Distal End

C) Myelinated Axons at Distal End

greater, respectively, than those for the control group (Fig. 4E). Analysis of the distal ends of these cables showed that the probability for myelinated axons increased from 25% for empty tube controls to 75% for the PLLA filament group (Table I). Examination of the midsection of these implants showed 100% of the filament group had myelinated axons, although there was an increased probability of axon regeneration for both groups. In addition, the average number of myelinated axons detected at the midsection and distal end for the PLLA-bundled group was nearly three times that of the control group (Table I). Even though the consistency of nerve cable formation, axonal growth, and the number of myelinated axons for the filament group was greater than that of the control group, the results were not statistically significant (Table I).

Regeneration across 1.8-cm gap.   To examine the possibility that filaments would enhance nerve repair over even greater gap distances, empty tubes and those containing 7.5% filament-packing density were implanted to create a 1.8-cm sciatic nerve gap. After 10 weeks, tissue cables formed between proximal and distal nerve stumps in 37.5% of the empty tubes (Fig. 5A,C,E) and 87.5% of the tubes containing PLLA filaments (Fig. 5B,D,F). Although the probability of nerve cable formation and axonal growth in the PLLA filament-bundled group was more consistent than in the control group (Fig. 6A), the differences showed a trend toward significance ($P = 0.063$ and $P = 0.080$, respectively). In three of eight empty tubes that showed cable formation, the reconstructed nerve tapered markedly and was a fraction of its proximal size at the distal end (Figs. 5E, 6B). In seven of eight reconstructed nerves within the filament-containing tubes, the nerve diameters were more uniform and contained much more tissue volume over the entire length of the cable (Figs. 5F, 6B). Nerve cable formation showed more of an "hourglass" formation in the PLLA filament group than in the control group. This pattern was most likely the result of increased Schwann cell migration from both the proximal and distal segments toward the middle of the implant. Nerve cable areas, as measured along the nerve segments
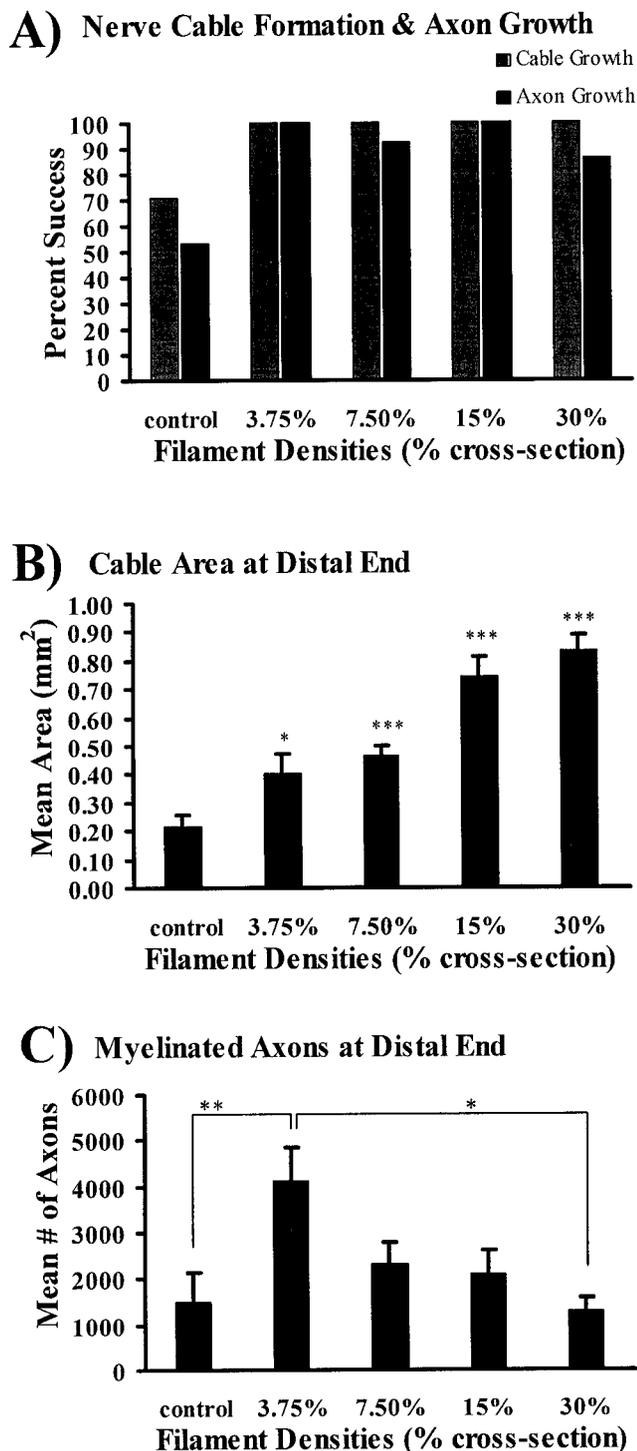
Fig. 3. Percent of implants showing successful nerve cable formation and axonal growth, average cross-sectional area of nerve cables, and average number of myelinated axon counts for various filament-packing densities. A nerve cable bridged the 1.0-cm gap in all animals in the PLLA filament-bundled group compared to only 71% of animals in the control group (Fisher's exact test, $P = 0.063$) (**A**). The success of axonal growth evaluated at the distal end of nerve cables was significantly greater for the PLLA filament-bundled (86–100%) groups than for the control group (53%) (Fisher's exact test, $P = 0.020$). There was a monotonic increase in the cross-sectional area as the packing density increased (**B**). Cross-sectional cable areas (not including filaments) between control and PLLA-bundled groups were significantly different (Dunnett's post-hoc test). A significant difference (Tukey HSD post-hoc test) in the number of myelinated axons was observed between the 3.75% filament-packing density group and the control group and with 3.75% and 30% filament-packing densities (**C**). Error bars = SEM values. ★$P < 0.05$, ★★$P < 0.01$, ★★★$P < 0.0001$.

TABLE I. Number of Implants Showing Successful Nerve Regeneration and Quantitation of Axonal Growth at the Midsection and Distal End of 1.4 cm Gaps

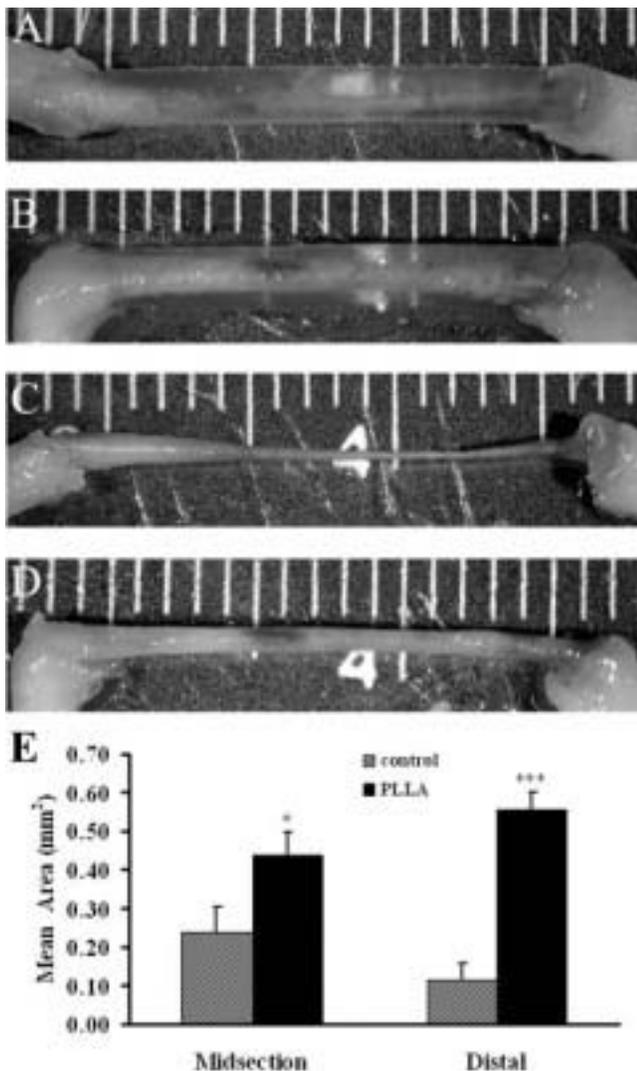|		Nerve Cable Formation	Axonal Growth	Mean # of Myelinated Axons
				(# $\pm$ SEM)
Midsection	Control	6 of 8 (75%)	5 of 8 (63%)	886 $\pm$ 431
	PLLA	8 of 8 (100%)	8 of 8 (100%)	2495 $\pm$ 719
Distal	Control	6 of 8 (75%)	2 of 8 (25%)	560 $\pm$ 424
	PLLA	8 of 8 (100%)	6 of 8 (75%)	1838 $\pm$ 696

Fig. 4. Implantation and growth through tubes containing PLLA filament bundles that bridge 1.4-cm gap lesions. Photomicrographs showing sciatic nerves 10 weeks after the implantation of silicone tubes with and without microfilament bundles (**A–D**). Tissue cables had formed between the proximal nerve stumps (left) and distal nerve stumps (right) after implantation with empty tubes (**A,C**) or those containing PLLA microfilaments (**B,D**). Nerve cables that bridged the 1.4-cm gap lesion seemed to be smaller for controls when compared to the filament bundles. Regenerated nerve segments are shown with (A, B) and without (C, D) silicone tubes. Ruler with 1-mm division scale is shown in the background. (**E**) Average cross-sectional area of nerve cables after implantation of PLLA filament bundles to bridge a 1.4-cm gap lesion. ANOVA indicated that the mean difference of cable areas between PLLA filament and control groups was significantly different ($P = 0.003$) as measured at the cable's midsection and distal end. Individual measurements between PLLA and control groups at the midsection ($\star P = 0.0405$) and distal end ($\star\star\star P = 0.00004$) also showed a significant difference. Error bars = SEM.

(Fig. 6B) between the PLLA filament and control groups, were significantly different ($P < 0.001$).

To determine if filament-containing tubes enhanced axonal regeneration, the number of myelinated axons was counted at 2-mm segments along the entire 1.8-cm gap. For both control and filament-containing tubes, axon numbers decreased along the length of the cable extending from the proximal to the distal end (Fig. 6A,C). The number of myelinated axons was 2.5-fold greater for each segment of the PLLA filament groups when compared to control groups. This difference was statistically significant ($P < 0.0001$) as measured along the nerve cables. For the PLLA filament group, myelinated axons were seen extending as far as 1.4 cm from the proximal end compared to the control group, where they extended up to 1.2 cm from the proximal stump. Although no myelinated axons were seen at the distal end of sections from either the control or the PLLA-bundled group, TEM analyses of sections from the PLLA group, but not control groups, showed numerous unmyelinated axons (Fig. 7). Interspersed among the filaments in these specimens were many Schwann cells, dense arrays of collagen, and occasional macrophages containing red blood cell debris.

DISCUSSION

Over the years, numerous methods have been developed to enhance axonal regeneration across large peripheral nerve gaps. To improve cable formation and increase the prospects of axonal regeneration, we have developed a method of wet-spinning bioresorbable polymeric microfilaments that can be loaded with up to 20% protein by weight. The overall goal of this technology was to develop microfilaments that enhance the physiochemical environment between the nerve stumps to approximate the bands of Bungner in the degenerating nerve. Another advantage is that filaments with different physiochemical characteristics, such as the release of specific chemoattractive or chemorepulsive factors, can be bundled together to aid the segregation of sensory and motor axons toward more appropriate nerve branches. We examined the ability of filaments with no drug load as structural organizers of nerve repair and the cellular responses they provoke.

Electron microscopic analysis showed a cell layer surrounding the filaments that might be a foreign body reaction. The cell types comprising this layer are presently being investigated. We have identified some of them by staining for ED-1, a marker for activated macrophages. Such macrophages and other inflammatory cells have been reported to be distributed throughout the fibrin matrix (Danielson et al., 1993; Zhao et al., 1993; Dahlin et al.,
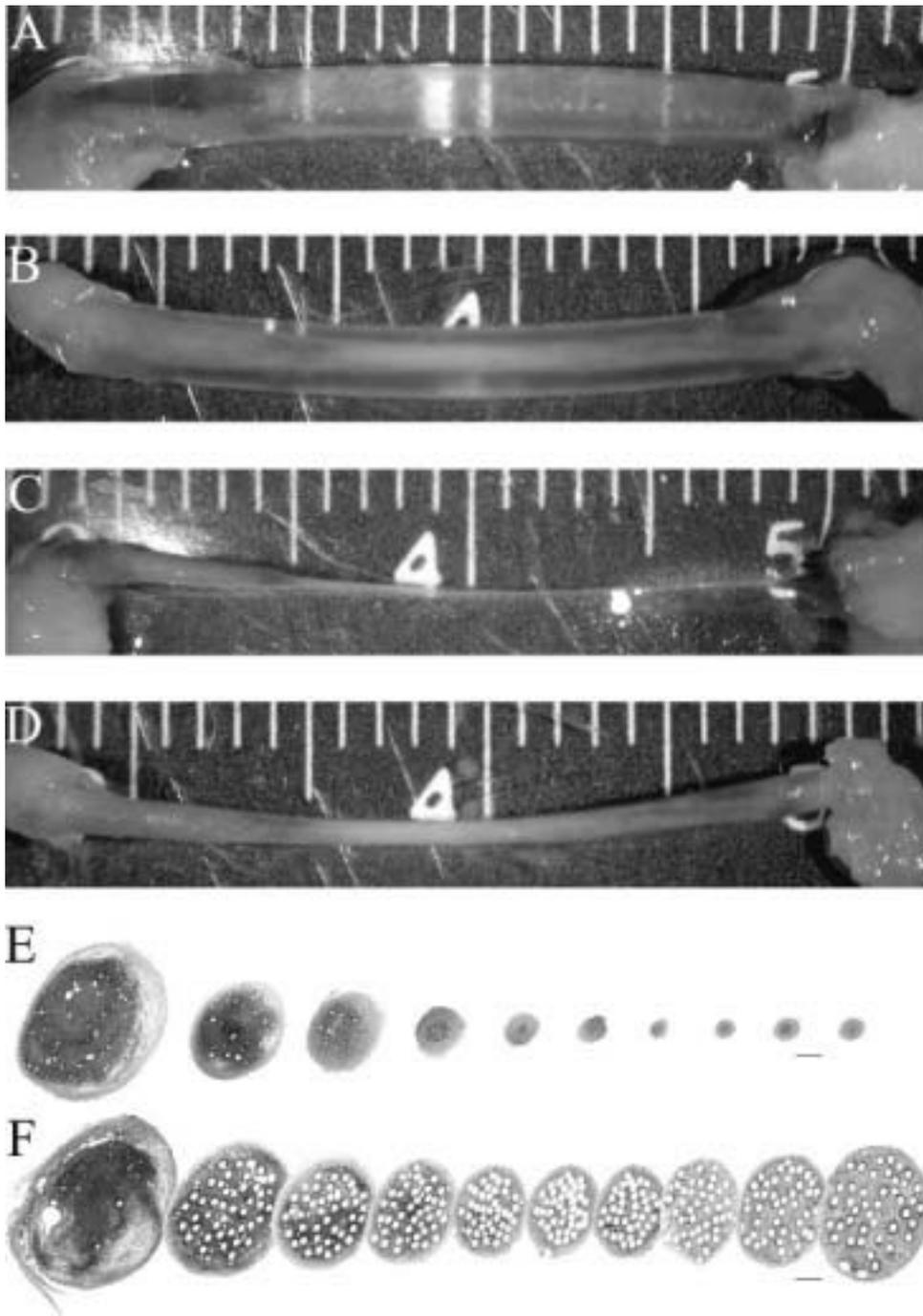
Fig. 5. Implantation and growth through tubes containing PLLA filament bundles that bridge 1.8 cm gap lesions. Photomicrographs and light micrographs showing sciatic nerves 10 weeks after implantation of silicone tubes with and without microfilament bundles. Tissue cables had formed between the proximal nerve stumps (left) and distal nerve stumps (right) after implantation with empty tubes (**A**,**C**,**E**) or those containing PLLA microfilaments (**B**,**D**,**F**). Although nerve cables formed across 1.8-cm gaps for both empty tubes (3/8) and filament bundled tubes (7/8), tubes containing filament bundles showed much better nerve growth than that observed within the 1.8-cm gap with empty tubes. Regenerated nerve segments are shown with (A,B) and without (C,D) silicone tubes. Ruler with 1-mm division scale is shown in the background of (A–D). Scale bars = 200 μm in light micrographs.

1995), and they may induce axonal outgrowth by releasing interleukin-1 (IL-1) to stimulate NGF production from nonneuronal cells such as Schwann cells (Lindholm et al., 1987; Dahlin et al., 1995; Miyauchi et al., 1997). In addition, filament degradation products may contribute slightly to inflammation. Although filament degradation was not apparent visually, PLLA pins implanted subcutaneously in rats displayed a small mass loss (<5%) within 100 days (Hooper et al., 1998). This suggests that PLLA filaments in the silicone chamber may undergo a degra-

dation process that alters the local environment and results in an inflammatory response (Piatelli et al., 1998). Because the degradation rate differs for various polymers, it is crucial that these materials degrade in a timely manner consistent with the regenerating nerve. For instance, if the material degrades too quickly, regeneration and maturation of the nerve within the chamber would not have consistent support (den Dunnen et al., 1995). In contrast, nerve compression may occur if the material does not degrade once nerve reconstruction is complete (den Dun-

A) Nerve Cable Formation & Axon Growth

B) Cable Area

C) Myelinated Axons

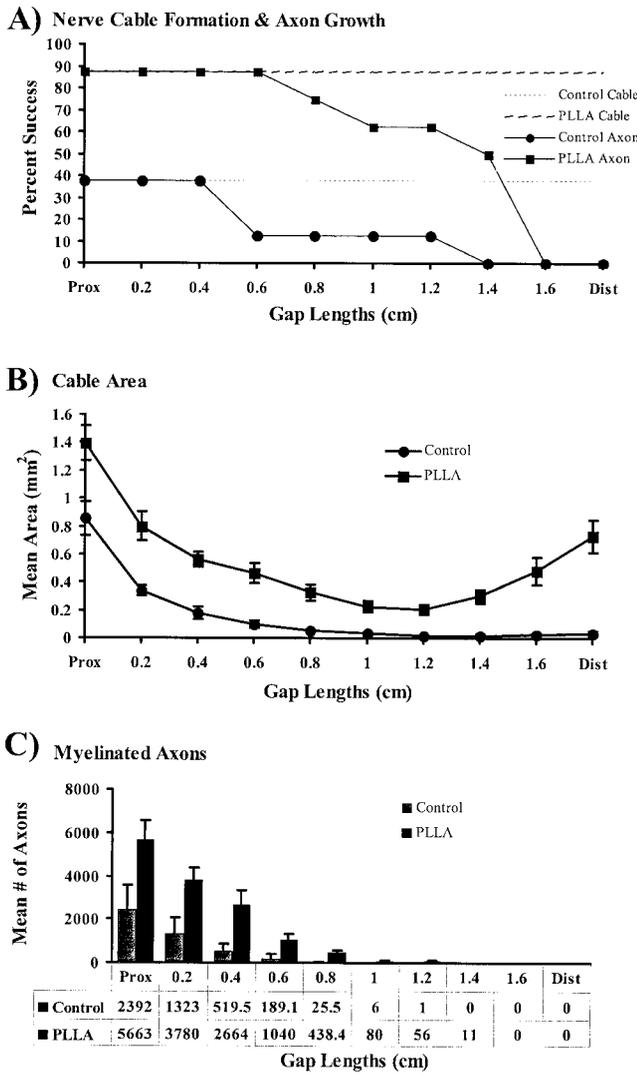|	Prox	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	Dist
■ Control	2392	1323	519.5	189.1	25.5	6	1	0	0	0
■ PLLA	5663	3780	2664	1040	438.4	80	56	11	0	0

Fig. 6. Percent of implants showing successful nerve cable formation and axonal growth, average cross-sectional area of nerve cables, and average number of myelinated axon counts for the 1.8-cm gap. The percent of successful nerve cable formation and axonal growth for the PLLA filament-bundled group was greater than that of the control group (**A**). Average nerve cable areas (**B**) between PLLA filament and control groups were significantly different (ANOVA, $P < 0.001$) as measured along the nerve segments. Regenerated nerve cables showed more of an "hourglass" formation for the PLLA filament group than for the control group. For both groups, the number of axons detected at 2-mm segments along the 1.8-cm gap decreased as regeneration progressed from proximal to distal end (A, **C**). The number of myelinated axons between the PLLA filament and control groups was significantly different (ANOVA, $P < 0.0001$) as measured along the nerve cables. In animals that showed axonal regeneration, however, the number of myelinated axons was similar between the two groups. Error bars represent SEM values.

nen et al., 1995). Presently, degradation rate studies for these and several other polymer filaments and their consequence on nerve repair are being conducted.

Nerve cable formation is important for axonal regeneration and dependent on the deposition of fibrin matrix to bridge the nerve gap in empty tubes (Williams et al., 1983; 1987; Williams and Varon, 1985). The matrix serves as a substrate to support the migration of nonneuronal cells, such as Schwann cells, endothelial cells, and fibroblasts, which precede the elongation of regenerating axons (Williams et al., 1983). Increasing the gap length from 1.0 to either 1.4 cm (Lundborg et al., 1982b; 1997; Zhao et al., 1993) or 1.8 cm significantly decreased the probability of nerve cable formation in the empty tube groups. One explanation is that the fibrin matrix either fails to form or deteriorates before migrating cells connect the nerve stumps (Lundborg and Kanje, 1996). Consequently, regeneration may fail because there is no substrate for the migration of nonneuronal cells and axons. Another possible reason is that there may be inadequate neurotrophic support, which is required for regeneration within the long gap chamber. A justification for using polymer filaments bundled or alternative bridging substrates, such as collagen, is that they may either stabilize the fibrin matrix or act to replace it, thus supporting migration of nonneuronal cells to reconnect the nerve stumps. The addition of approximately 32 microfilaments per tube markedly increased the probability of nerve cable formation and axonal regeneration at all gap distances studied. In all cases, filaments were surrounded by macrophages, fibroblasts, and numerous Schwann cells. Axons were rarely observed growing directly on filaments, but numerous axons were observed growing among Schwann cells near the surface of the filament. In previous tissue culture experiments, we observed the preference for these filaments to longitudinally direct Schwann cell migration followed by axonal growth (Rangappa et al., 2000). Thus, filament bundles serve to structurally organize the formation of the nerve cable by directing cell migration across the gap. Indeed, more consistent nerve cable formation and axonal growth was observed when PLLA filament bundles served as nerve guides when compared to empty tube controls. This was particularly apparent after implantation into 1.4- or 1.8-cm nerve gaps.

Cable formation and nerve repair observed using these PLLA microfilaments were similar to previous studies, in which layers of flattened cells were seen surrounding permanent polyamide (nylon) and bioabsorbable (catgut, polyglactin, polydioxanone) sutures that were bundled inside silicone tubes (Lundborg and Kanje, 1996; Lundborg et al., 1997; Terada et al., 1997; Dahlin and Lundborg, 1999; Arai et al., 2000). The microfilaments used in our study, however, have a number of advantages to those of other studies. First, they can be fabricated for drug or protein release, in which we have observed controllable release kinetics depending on the fabrication parameters and polymer blend. Second, they can be manufactured or coated with neurite growth-promoting molecules, such as laminin, to enhance cell migration and axonal regeneration (Rangappa et al., 2000). Third, degradation rates of the filaments can be controlled using specific blends of PLLA and poly (DL-lactide-co-glycolide) polymers (Nelson et al., in press). These advantages will
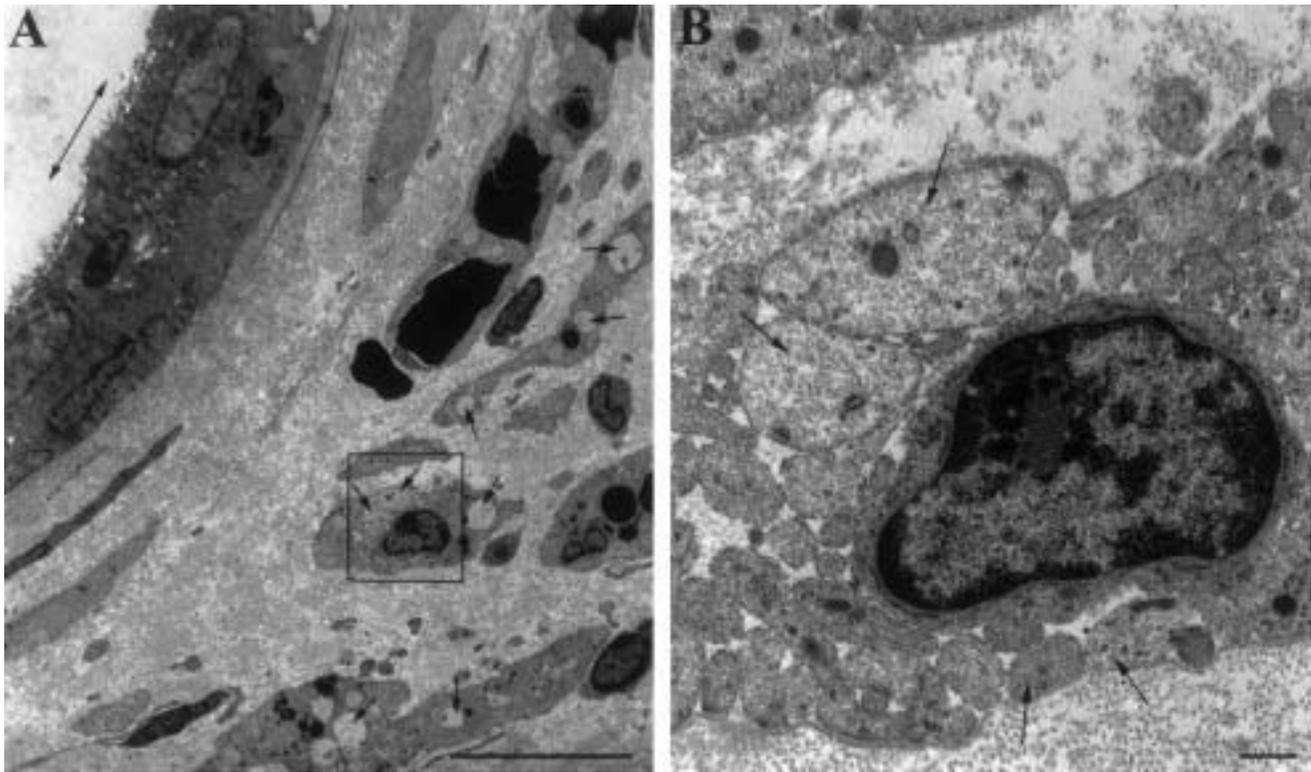
Fig. 7. TEM showed numerous unmyelinated axons (**A**, arrows) appearing at the distal end of 1.8-cm gap nerve cables from groups with PLLA filament bundles. Higher magnification (**B**) of A (boxed) shows numerous unmyelinated axon profiles. Double-headed arrow shows the edge of a filament. Scale bars = 10 μm in A; 500 nm in B.

permit better control of the regenerative process and potentially enhance overall functional recovery.

Before fabricating microfilaments for neurotrophin release, we needed to determine if packing density of the filaments effected regeneration. This is important because the number of filaments releasing the drug will have a significant influence on the overall size of the neurotrophin reserve and the amount released. Interestingly, the filament-packing density increased the thickness of the nerve cable but reduced the number of myelinated axons. At the highest packing density (30%), fewer axons were observed, despite the larger cable area when compared to empty controls or lower packing densities (3.75–15%). Too many filaments within a guidance channel might increase the number of macrophages or migration of fibroblasts while restricting the growth or migration of essential cellular components that support axonal regeneration. Too few filaments (below 3.75%), reduced the probability of successful cable formation at the 1.8-cm gap distance compared to that of control tubes. Even with suitable packing density (e.g., 3.75–7.5%), reduced axonal growth was observed in areas where the filaments juxtaposed each other. For example, in one of the animals in the 7.5% filament-packing density group, filaments coalesced in the center of the cable, and no axonal growth was observed at the distal end of a 1.0-cm gap cable (data not

shown). Therefore, clustering or compaction of filaments toward the center of the cable reduces the probability of axonal regeneration and becomes more apparent with longer gap distances. This illustrates the importance of ensuring that the filaments are dispersed in an organized fashion in the tube. Presently, we are examining methods to maintain even dispersion of filaments within the guidance chamber.

Numerous investigators have studied regeneration across extended gap lesions (longer than 1.4 cm) in the rat model (Williams et al., 1987; Yannas et al., 1987; Madison et al., 1988; Kakinoki et al., 1997, 1998; Hadlock et al., 1998; Arai et al., 2000). To our knowledge, none had reported regeneration across these gap distances with empty tube controls. Finding successful regeneration across a 1.8-cm gap in three of eight controls was unexpected, although the cables were very thin and fragile. Implantation of filaments reversed the thinning of the nerve cable, most likely by providing a suitable substrate for migration of Schwann cells, endothelial cells, and fibroblasts from both nerve stumps. This bidirectional migration might explain the altered "hourglass" formation observed for nerve cables with PLLA bundles, but absent in controls.

For all gap lengths, we observed a greater probability for successful regeneration and increased numbers of re-

generating axons with filament implants compared to controls. For gap lengths of 1.0 and 1.4 cm, the number of myelinated axons observed in the distal stump were 2.6- and 3.2-fold greater than those observed in empty tube controls. At a gap length of 1.8 cm, few myelinated axons, but multiple unmyelinated axons, were observed at the distal end. As sections of nerve cables were examined toward the proximal stump, myelinated axons were detected, with the number increasing toward the proximal stump. To determine the farthest length at which myelinated axons had regenerated, sections were examined at 2-mm increments from the distal to the proximal stump. The number of myelinated axons tapered off with increasing distance from the proximal stump, indicating that axonal regeneration and myelination were still taking place.

Although myelinated axons were not detected at the distal end for PLLA filament bundles with nerve cables spanning the entire 1.8-cm gap, unmyelinated axons were observed at this location. These structures were similar to those seen previously in similar studies (Morris et al., 1972; Williams et al., 1983), which referred to them as "regenerating units", representing groups of axons still in a regenerating phase. With time, these axons should mature and become myelinated, and axonal regeneration across 1.8-cm gap lengths would be successful. Future studies would include long-term implantation periods and functional studies with the PLLA filament bundles across 1.8-cm gaps to test the efficacy of these filament bundles as nerve guidance devices. For those studies, we have begun fabricating microporous bioresorbable tubes to replace the silicone tubes. Preliminary studies demonstrate a greater rate of axonal growth along filaments in these tubes compared to growth in silicone tubes. We also have fabricated PLLA/PGA filaments that dissolve more rapidly to release neurotrophin to enhance further regeneration across 1.8-cm long gaps.

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