Growth factor enhancement of peripheral nerve regeneration through a novel synthetic hydrogel tube

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Object. The authors' long-term goal is repair of peripheral nerve injuries by using synthetic nerve guidance devices that improve both regeneration and functional outcome relative to an autograft. They report the in vitro processing and in vivo application of synthetic hydrogel tubes that are filled with collagen gel impregnated with growth factors.

Methods. Poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) (PHEMA-MMA) porous 12-mm-long tubes with an inner diameter of 1.3 mm and an outer diameter of 1.8 mm were used to repair surgically created 10-mm gaps in the rat sciatic nerve. The inner lumen of the tubes was filled with collagen matrix alone or matrix supplemented with either neurotropin-3 at 1 μg/ml, brain-derived neurotrophic factor at 1 μg/ml, or acidic fibroblast growth factor (FGF-1) at 1 or 10 μg/ml. Nerve regeneration through the growth factor–enhanced tubes was assessed at 8 weeks after repair by histomorphometric analysis at the midgraft level and in the nerve distal to the tube repair. The tubes were biostable and biocompatible, and supported nerve regeneration in more than 90% of cases. Nerve regeneration was improved in tubes in which growth factors were added, compared with empty tubes and those containing collagen gel alone (negative controls). Tubes filled with 10 μg/ml of FGF-1 dispersed in collagen demonstrated regeneration comparable to autografts (positive controls) and showed significantly better regeneration than the other groups.

Conclusions. The PHEMA-MMA tubes augmented with FGF-1 in their lumens appear to be a promising alternative to autografts for repair of nerve injuries. Studies are in progress to assess the long-term biocompatibility of these implants and to enhance regeneration further.

Key Words • **brain-derived neurotrophic factor** • **acidic fibroblast growth factor** • **neurotropin-3** • **peripheral nerve** • **sciatic nerve** • **poly(2-hydroxyethyl methacrylate-co-methyl methacrylate)** • **rat**

To date the best regenerative strategy to repair peripheral nerve injuries involves peripheral nerve autografts.[61] This strategy is inherently flawed, however, requiring that a second injury be created to harvest the tissue for repair of the primary injury. A better strategy would be to prepare a synthetic graft that mimics the properties of a peripheral nerve graft, thereby obviating the need for the secondary injury. Nonnerve biological tissue and synthetic biodegradable material have been used for more than a century (reviewed by Doolabh, et al.[17]) as bridges for neural repair. Modified biological tissues for nerve tube construction include laminin[17] and collagen,[2,3,41] whereas synthetic tubes have been constructed from biodegradable material such as polyglycolic acid[15,47,83] and polylactide-co-caprolactone[56] and nonbiodegradable materials such as silicone.[17,44,45,52]

Clinical use of nerve tubes has become increasingly popular.[55] Indeed, two tubes have recently received Food and Drug Administration approval for repair of nerve injuries: polyglycolic acid tubes (Neurotube; Neuroregen, LLC, Bel Air, MD), partially based on a favorable clinical randomized control trial for digital nerve injury repair,[83] and collagen nerve tubes (NeuraGen; Integra Neurosciences, Plainsboro, NJ), partially because of their effectiveness in nonhuman primates[3,41] and the results of Phase I–II clinical safety studies.

The PHEMA hydrogels form crosslinked macromolecular networks[33] and have been used for longer than a quarter of a century in soft contact lenses.[54] In addition to contact lens manufacture, PHEMA's favorable elastic properties, biostability, and relative inertness have led to its use in a variety of biomedical applications, including drug delivery and soft-tissue replacement.[18,22,67] We have invented a new process to make hollow nerve guide tubes from crosslinked PHEMA hydrogels.[13,46] This process, which is simple and reproducible, results in concentric, water-swollen, hydrogel tubes and permits precise control of their dimensions and

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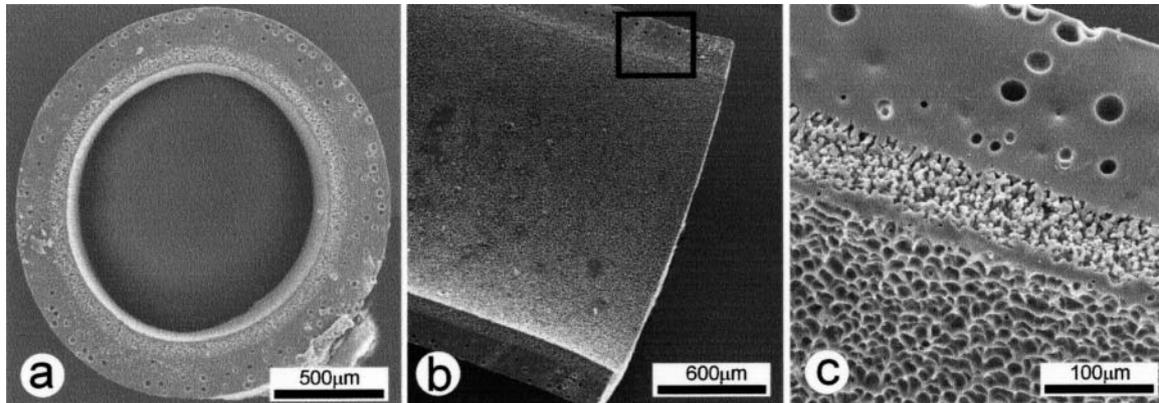
FIG. 1. Scanning electron microscopy images of nerve guide tubing made from PHEMA-MMA in cross section (a) and longitudinal section (b), with a magnified view (c) of the area in the box in 1b. The nerve tubes were concentric and round, with the wall having uniform thickness and structure (a). Note that the outer surface is smooth, whereas the inner lumen surface has a rough appearance (b and c).

their morphological and mechanical properties.[46] By adding MMA as a comonomer in the synthesis, the process can be adjusted to create tubes that meet the soft-tissue requirements of the peripheral nerve.

We report on the synthesis of PHEMA-MMA tubes and their use in a rat sciatic nerve injury and regeneration model. The tubes were investigated in terms of biocompatibility and effectiveness as guidance channels for nerve regeneration. Five animal groups (seven rats each) were compared with autograft controls: animals that received empty tubes, tubes filled with collagen matrix, and tubes filled with collagen matrix supplemented with either BDNF, NT-3, or FGF-1. The choice of these three growth factors from the neurotropin (BDNF and NT-3) and nonneurotropin (FGF-1) families was based on their promising effects on axonal regeneration, as is described in more detail in *Discussion* under *Growth Factors*.

Materials and Methods

Tube Manufacture

All chemicals, unless stated otherwise, were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used as received. Hydrogel nerve tubes were manufactured using a method previously described.[13,46] Briefly, a syringe-filtered, 0.45-μm poly(tetrafluoroethylene)–initiated monomer mixture was injected through a rubber septum into a silane-treated (Sigmacote; Sigma Chemical Co., St. Louis, MO) glass cylindrical mold with an ID of 1.8 mm, displacing all the air within the mold. The monomer mixture consisted of 2-hydroxyethyl methacrylate, ammonium persulfate, ethylene dimethacrylate, sodium metabisulfite, MMA, water, and ethylene glycol. The sealed mold was then rotated around its long axis in a horizontally mounted stirrer at 2500 rpm overnight at room temperature. Phase separation and gelation of the polymer at the periphery of the mold resulted in a crosslinked hydrogel tube. The ends of the mold were opened, excess water was tipped out, and the tube was removed from the mold. Hydrogel nerve tubes with an OD of 1.8 mm were then cut into 12-mm sections, placed into histological embedding cages, and Soxhlet-extracted overnight to remove all nonreacted molecules. After cooling, the nerve tubes were placed into filtered, deionized water, and autoclaved to achieve sterility.

Physical Characterization of Nerve Tubes

The morphological features of the wall of the gold-coated, freeze-dried tubes were examined with a scanning electron microscope (model S-570; Hitachi Corp., Tokyo, Japan) at 20 kV and a 15-mm working distance. Freeze-drying removed the water from the nerve tubes while maintaining their native structure. Representative images of the morphological investigations of the tube walls are reported (Fig. 1). The OD, ID, and wall thickness of the tubes were measured at two 90° cross sections per tube with a calibrated stereomicroscope (model MZ-6; Leica CO., Ltd., Nussloch, Germany). The Young modulus of four 12-mm-long tubes was determined using a micromechanical tester (Dynatek Dalta, Galena, MO) with the nerve tubes pulled in tension at a rate of 0.5%/second to a maximal displacement of less than 10%.

Matrix Preparation and Growth Factors

For use in vivo, the nerve tubes were left empty, filled with a buffered (pH 7.4) collagen matrix formed from 1.28 mg/ml collagen-1 (Vitrogen; Cohesion Technologies, Inc., Palo Alto, CA), or filled with a 1.28 mg/ml collagen matrix in which one of the following growth factors was distributed: 1 μg/ml FGF-1, NT-3, or BDNF (Promega, Madison, WI), or 10 μg/ml FGF-1. All collagen solutions were prepared at 0°C to prevent premature gelling. The nerve tubes were then filled with the collagen and growth factor solution and incubated at 37°C for at least 2 hours before surgery to allow the matrix to gel.

Surgical Methods and Study Groups

Forty-seven inbred adult male Lewis rats weighing 250 to 275 g each were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and housed in a standard animal facility with 12-hour on/off light conditions. The animals were acclimatized before surgery and allowed free access to standard rat chow and water. All surgical procedures were performed in an aseptic manner and standard microsurgical methods were used with an operating microscope (model M651; Wild Leitz, Willowdale, ON, Canada), as described previously.[59,60]

All experiments and animal interventions strictly adhered to Canadian Council on Animal Care guidelines. The anesthesia consisted of an intramuscular injection of 10 mg/kg xylazine (20 mg/ml; Bayer, Inc., Etobicoke, ON, Canada) and 100 mg/kg ketamine hydrochloride (0.1 ml/100 g Rogarestic; Rogra-STB, Montreal, QC, Canada) into the lumbar paraspinal musculature. After induction of anesthesia, surgical sites were shaved and prepared with Betadine and 70% surgical alcohol. After gluteal and posterior thigh incisions were made, the sciatic nerve was exposed deep to the biceps femoris muscle, a 10-mm segment of the nerve was excised, and then the appropriate 12-mm-long tube was sutured into the resulting gap.

To create a gap of 10 mm, the proximal and distal nerve stumps were inserted into the inner lumen of the tube at a distance of 1 mm
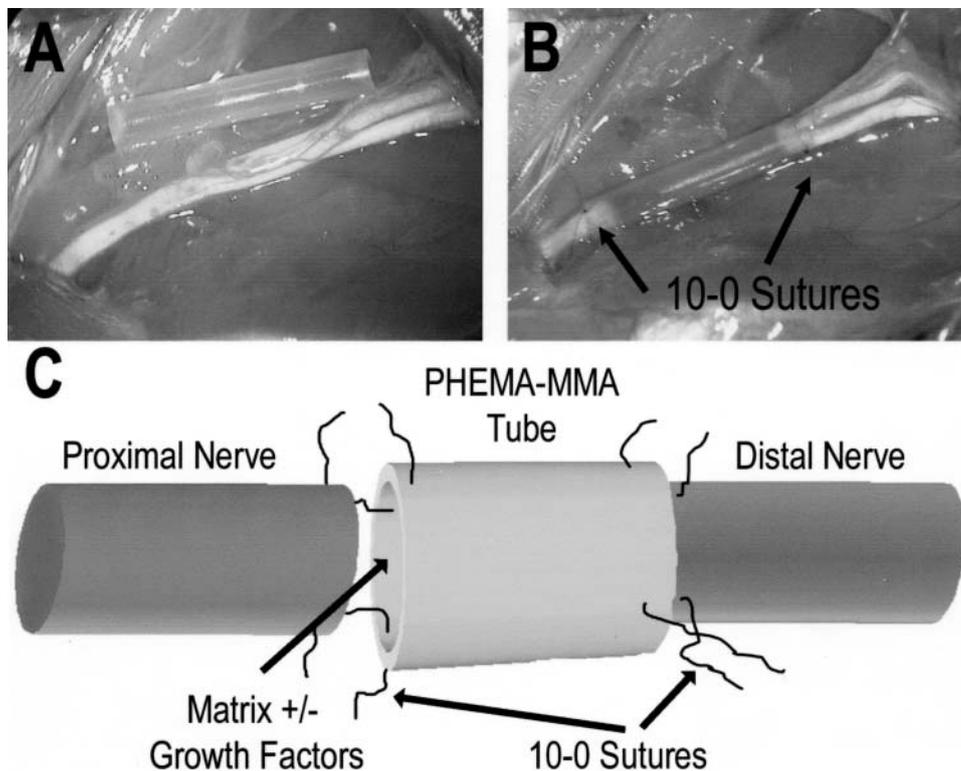
Fig. 2. Photographs showing the in situ appearance of tube implantation (A and B) and a schematic diagram of the surgical method (C). A: The 12-mm-long nerve guide tube was placed alongside the sciatic nerve. B: The tube was then placed across the surgically created 10-mm nerve gap. The proximal and distal nerve stumps were inserted 1 mm into the lumen of the tube and attached using 10-0 microsutures. At least 2 hours before surgery, the lumen of the tube was filled with a 1.28 mg/ml collagen matrix in which the various growth factors were incorporated.

from each end (Fig. 2) and sutured into place by using 10-0 nylon sutures (Dermalon; Davis & Geek, American Cyanamid Co., Danbury, CT). Nerve autograft segments (10 mm long) were harvested from isogeneic Lewis donor rats bilaterally from the sciatic nerves, after which the donor animals were killed. The harvested autograft segments were then placed into 10-mm-long surgically created nerve gaps in recipient rats and microsurgically repaired with 10-0 nylon epineurial sutures. Muscle and skin incisions were then approximated with interrupted 3-0 Polysorb sutures and continuous 3-0 silk sutures (both from Autosuture, Norwalk, CT). Characteristics of the tube and nerve autograft groups are summarized in Table 1.

Histological and Morphometric Studies

At the time of planned death (8 weeks after grafting), the animals were anesthetized and each midgraft or midtube segment and a portion of the distal host sciatic nerve (7–10 mm distal to the distal end of the graft) were harvested and fixed by immersion in Universal fixative. The tissue was postfixed with osmium tetroxide, embedded in Epon–Araldite, and sectioned on an ultramicrotome (model MT6000; Sorvall, Newtown, CT). Toluidine blue was used to stain 1-μm-thick cross sections for light microscopy.

Morphometric analysis was performed on the cross sections, as described previously,[59,60] by using a digital image analysis system linked to morphometry software (Vidas Image Processing System; Kontron Image Analysis Division, Eching, Germany). Five representative fields of known area (5041 μm²) at 1000× magnification were evaluated per nerve section for myelinated fiber counts from each regenerating nerve cable (Fig. 3A) by an observer (C.M.) working in a blinded fashion. The total number of nerve fibers present (an estimated number) was derived from the count, the sampled area, and the measured area of the regenerating nerve cable (corresponding to the gray zone in Fig. 3A). The nerve fibers were also evaluated for axon, myelin, and fiber area, and axon, myelin, and fiber di-

ameter (smallest sieve diameter) by the morphometry software. To assess the maturity of nerve fibers, axon/myelin ratios were also determined. Morphometric analysis for the regenerating axons in the distal nerve stump was more straightforward because the nerve fibers were distributed within the fascicles of the sciatic nerve; hence, the sampled fields and measured fascicular area corresponded to the largest tibial nerve fascicle. Data were analyzed using analysis of variance and post hoc t-tests (Statistica for Windows [1998]; StatSoft, Inc., Tulsa, OK).

Results

Tube Structure and Physical Properties

The PHEMA-MMA tubes were prepared by a new process that couples phase separation with centrifugal forces. The monomer mixture that is initially injected into the mold is a homogeneous solution. As the monomer is converted to a polymer, the polymer separates out of the solution. Because the polymerization occurs in a rotating cylindrical mold, the phase-separated polymer particles are pushed to the periphery of the mold by centrifugal forces, resulting in a tubular construct. As shown in Fig. 1, the PHEMA-MMA tube wall is biphasic, with an outer gel phase and an inner sponge phase. The gellike material that characterizes the majority of the tube is created by the coalescence of predominantly liquidlike phase-separated particles. Some closed-cell pores can be seen within this gel layer and are likely caused by water entrapment during polymerization (Fig. 1). As polymerization proceeds and phase-separated

TABLE 1
Characteristics of treatment groups of rats that underwent nerve gap repair

Treatment Group	Growth Factor Concentration	Collagen Matrix	No. of Rats	
			Initially	Finally*
growth factors				
FGF-1	1.0 μg/ml	1.28 mg/ml	7	7
NT-3	1.0 μg/ml	1.28 mg/ml	7	7
BDNF	1.0 μg/ml	1.28 mg/ml	7	7
high FGF-1	10.0 μg/ml	1.28 mg/ml	7	6
controls				
autograft	none	none	5	5
collagen only	none	1.28 mg/ml	7	6
empty tubes	none	none	7	6

* Number reflects the incidence of early death (two cases) and suture pullout (one case), which prevented end point analysis.

particles become more viscoelastic, they undergo less coalescence, thereby creating the porous inner sponge layer of the tube wall. The inner lumen surface likely results from the final stages of polymerization and is thin and rough, as seen in Fig. 1b and c.

The process provides standardized tubes that are concentric and have a constant wall thickness, as evidenced by the dimensional analysis: the OD of the tubes was 1.81 ± 0.02 mm (mean $\pm$ standard deviation for all measures of physical properties), whereas the ID was 1.28 ± 0.03 mm (eight samples). The equilibrium water content of the tubes was $40.88 \pm 0.53\%$ (six samples), reflecting the predominantly gellike morphological characteristics, and the elastic (Young) modulus was 2820 ± 72 kPa (four samples). From pilot studies, we determined that a minimum elastic modulus of 1200 kPa was required for the structural integrity of the tube to be maintained for in vivo peripheral nerve repair.

General Features and Gross Appearance of Implanted Tubes

The nerve tubes handled well at surgery; they were pliable but still firm enough for us to manipulate the nerve stumps into their lumen and they could hold a microsuture (Fig. 2). The inner lumen of the empty tubes appeared to fill with serum and tissue fluids during the microsurgical implantation procedures, which generally took 15 minutes each. The collagen gel, with or without growth factors, remained within the lumen of the tube during the surgical procedure.

The rats recovered well from surgery and all but two of the 47 survived to the study end point (8 weeks), when the tube implantation site was reexplored. In the overwhelming
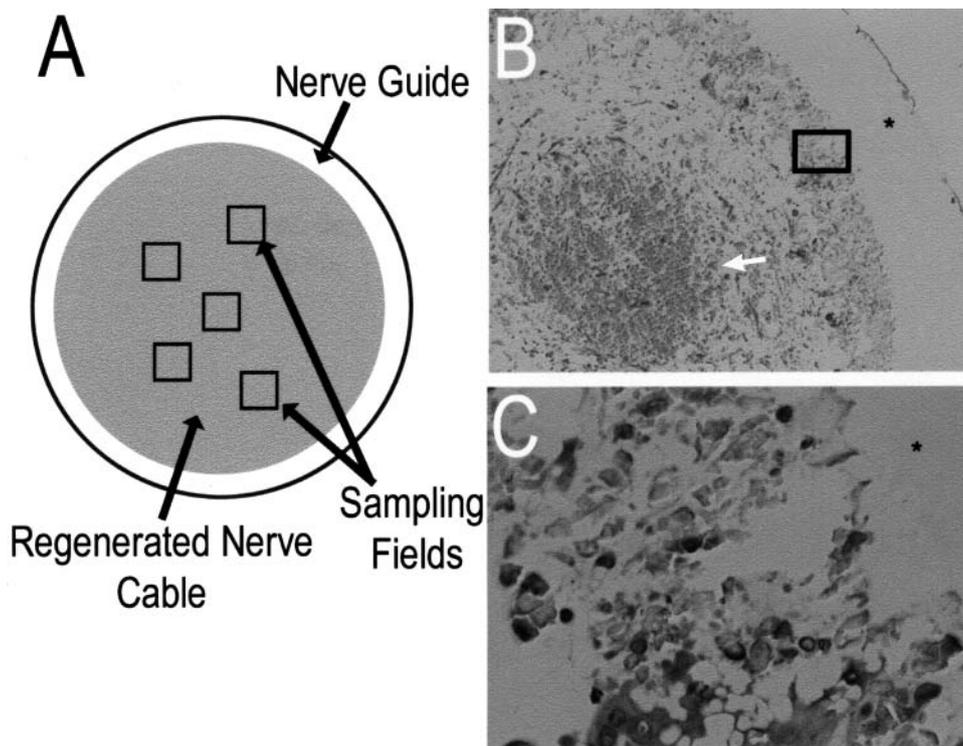
FIG. 3. Schematic diagram of a nerve tube cross section and the histomorphometric sampling method (A), and photomicrographs of toluidine blue–stained sections of the nerve tube (B and C) 8 weeks postimplantation.   A: Myelinated axons larger than 1 μm in diameter were assessed in five representative hpfs ($\times$ 1000; see Table 2) within the regenerating cable.   B: Nerve tubes maintained a round configuration with a cable of regenerating tissue that filled most of the cross-sectional lumen and contained regenerating axons concentrated (in this case) in the center of the tube *(arrow)*. The tube wall *(asterisks* in B and C) showed no cellular infiltration, even within its pores, and no or minimal evidence of an inflammatory reaction adjacent to it.   C: The tissue layer (corresponding to the peripheral area of the regenerating nerve cable) within the tube wall inner surface was a loose hypocellular fibrous matrix. Original magnification $\times$ 50 (B) and $\times$ 400 (C).

majority of cases the tubes appeared intact, lying between the nerve stumps (suture pullout occurred from the distal end in one case). These three cases with unfavorable outcomes (two early deaths and one suture pullout) were excluded from subsequent histomorphometric analysis (Table 1). In most cases, the tubes were round; in a few cases, the tubes were oval with a somewhat flattened appearance. The devices were minimally adherent to surrounding tissue lying within the intermuscular plane and were easy to dissect. Their consistency was firmer than at implantation, but not hard, and subsequent ultramicrotome sectioning proved to be straightforward. After sectioning the tube at surgery, a cable of regenerating nerve could be observed to extend into the middle of the tube with the aid of the operating microscope in the majority of cases. Moreover, the regenerating cable was round, with rare cases exhibiting tapering of the cable to a conelike structure with an hourglass shape.

General Histological Features

The tube was stable throughout the 8 weeks: it remained circular, with a wall of consistent thickness and a round lumen in the majority of cases (Fig. 3B). It also appeared to be biocompatible, with a thin layer of surrounding fibrous tissue and minimal inflammatory infiltrates along its outer wall. This layer of reactive tissue was thicker adjacent to the tube's inner lumen, whereas the wall and pores within the tube itself were devoid of infiltrating cells or elements of the regenerating cable (Figs. 3C and 4). A cable of regenerating nerve, composed of collagen and fibrous matrix, microvasculature, Schwann cells, and variable amounts of regenerating myelinated axons, sometimes in groups (regenerating units), was evident as far as the midportion of the tube in the majority of cases. The regenerating axons were scattered throughout but exhibited a tendency to be concentrated in the central portion of the regenerating cable of tissue and were not fasciculated. Regeneration was superior within the collagen matrix–containing implants compared with empty tubes and appeared to be further improved by the addition of growth factors.

Morphometric Analysis

Myelinated axons were counted in the midgraft segment and in the nerve distal to the suture line, and the morphometric parameters of the regenerating axonal population were determined (Tables 2 and 3 and Fig. 4 *lower row*). The morphometric analysis confirmed the qualitative assessment that the majority of tubes contained at least some regenerating axons (Tables 2 and 3). Nevertheless, some tubes failed to transmit axons into the distal stumps. This was most notable for the empty tubes, but was also observed in some tubes containing collagen supplemented with BDNF and the lower dose of FGF-1. Although there were significant differences in the number of myelinated regenerating axons between different conditions (as detailed later), the size distribution and myelination of axons was similar within the tubes (Tables 2 and 3 and Fig. 4 *lower row*). Counts of axons regenerating into the distal nerve stump were significantly improved by the higher dose of FGF-1 (see *Counts From Midtube*); moreover, this group's axonal maturity, as measured by the axon/myelin ratio, most closely approximated that of the normal autograft controls (Table 3).

Counts From Midtube

A significant main effect was seen ($F_{5,33} = 3.29$, $p < 0.05$) when comparing the myelinated nerve fiber counts in five hpfs from tubes containing one of the three growth factors with tubes containing a nongrowth factor–enhanced matrix and with empty tubes. Post hoc analysis revealed that when 10 μg/ml FGF-1 was incorporated into the collagen matrix, regeneration was superior to all other conditions, including the lower dose of FGF-1 ($p < 0.05$; Fig. 5).

Distal Nerve

When total myelinated fiber counts in the distal nerve were compared among 10 μg/ml FGF-1; 1 μg/ml FGF-1, NT-3, or BDNF; collagen; empty tubes; and autograft conditions, significant differences emerged ($F_{6,37} = 4.67$, $p < 0.001$). Post hoc analysis (least squares difference t-tests) revealed that the total counts in the autograft and in the 10-μg/ml FGF-1 group were similar to each other and significantly superior to all the other groups ($p < 0.05$; Fig. 6). The FGF-1, BDNF, or NT-3 at the 1 μg/ml level did not improve regeneration into the distal nerve stump compared with empty or collagen gel–filled tubes (Tables 2 and 3).

Discussion

Biomaterial Considerations

In adopting a synthetic material for biological use, several biomaterial considerations have to be taken into account, the foremost of which is biocompatibility. We used PHEMA, which has been shown to be noncytotoxic, noncarcinogenic, biocompatible,[35] and well tolerated when implanted subcutaneously in rats, with no necrosis, calcification, or infection observed for up to 6 months.[37] A further aspect of biocompatibility is that the polymer should be nonimmunogenic, causing no or minimal local tissue irritation and allergic response. The latter is especially important for a nerve guide tube, because even modest local tissue inflammation leading to adhesion and fibrosis in the area surrounding the tube or within its wall may collapse the tube and compress the nerve-regenerating cable.[17] In biocompatibility studies of subcutaneously and intraocularly implanted PHEMA sponges, there is no or minimal local tissue inflammation over weeks to months.[73] There is modest cell invasion by fibroblasts and capillaries into the implant for soft tissue replacement,[9] whereas sponge implants into the spinal cord and for optic nerve injuries are invaded by glial cells and ED1+ macrophage cells.[67] A minority of the long-term but not the short-term implants have demonstrated microcalcification.[81]

In our study, the PHEMA-MMA tubes provoked either no or minimal tissue reaction and thus were biocompatible. Before implantation, PHEMA-MMA tubes were first placed in boiling water for 24 hours to remove any residual or unreacted monomer and then autoclaved to achieve a sterile product. They appeared firmer and less pliable at harvesting than at implantation, but no gross calcification was observed on inspection or sectioning for general histological studies, although a von Kossa stain[38] would be needed to determine microcalcification. Furthermore, there was no significant cellular infiltrate observed within the tube wall on general histological analysis.
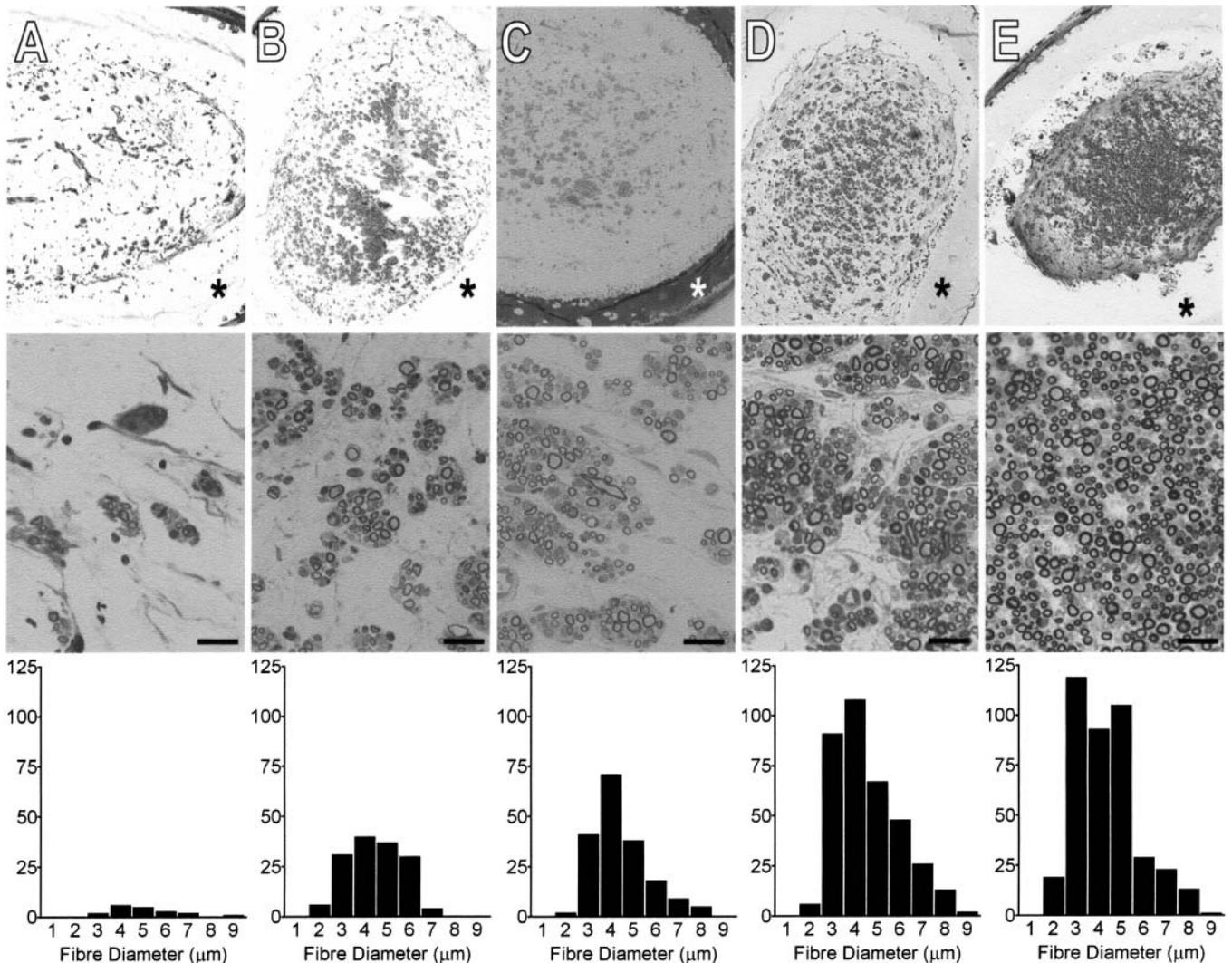
FIG. 4. *Upper* and *Center Rows:* Representative photomicrographs at low and high magnifications of sections cut through the center of the tube 8 weeks postsurgery: empty tube (A); collagen gel (B); 1 μg/ml BDNF in collagen (C); 1 μg/ml NT-3 in collagen (D); and 10 μg/ml FGF-1 in collagen (E). The tube wall *(asterisk)* lacked evidence of cell infiltration and showed a thin layer of fibrous tissue on its outer aspect. The cable of regenerating nerve tissue was oval to round and covered the entire cross-sectional area within the lumen (panel E shows artifactual postmortem separation of the cable from the inner lumen of the tube). The cable of regenerating nerve tissue contained myelinated axons scattered throughout, at times concentrated in the center, without fasciculation. In A, regeneration of axons was sparse; B and C show more abundant axons in small groups (regenerating units), whereas D and E show a more robust and uniform distribution of regenerating axons. All tubes supported at least some regeneration, which was improved with the incorporation of growth factors and was best in the 10-μg/ml FGF-1 group. *Lower Row:* Fiber diameter distribution plots calculated based on the samples illustrated in the *upper* and *center rows.* Although there were substantially higher counts in D and E, the distribution of fiber size did not differ. Toluidine blue, bars = 20 μm.

For nerve repair, biodegradability has been considered to be an important property, allowing the tube to degrade once it has fulfilled its role of permitting the nerve cable to regenerate through it.[47] We generally agree with this notion; however, currently available biodegradable materials release cytotoxic degradation products that provoke an inflammatory response and may cause injury to axons within the regenerating cable.[12,17] The PHEMA tubes used herein are nonbiodegradable, showing no appreciable breakdown with in vivo application over the 8-week study period. Biostable tubes, such as silicone, have been criticized because the cable of regenerating nerve may undergo a secondary compression injury in a delayed fashion, especially when the lumen of the tube is too small.[48] Nevertheless, silicone tubes of appropriate dimensions have been used successfully in the experimental and clinical repair of nerve injury.[12] Similarly, to minimize nerve compression, PHEMA tubes can be manufactured with optimal dimensions and stiffness, as reported here and elsewhere.[13,46] Whether PHEMA tubes maintain longer-term biostability and biocompatibility can only be determined by longer-duration in vivo studies, which are currently in progress in our laboratory.

Another important biomaterial property for nerve repair is the permeability of the tube. Generally, porous tubes that

TABLE 2
*Morphometric indices of regenerating axons measured midgraft (mean ± SEM) in rats that underwent nerve gap repair**

Group	Fiber Count in 5 hpfs	Fiber Diameter (μm)	Axon/Myelin Ratio	No. of Grafts†
FGF-1	130.70 ± 47.68	3.73 ± 0.081	0.709 ± 0.114	4 of 7
NT-3	284.22 ± 50.11	4.00 ± 0.180	0.647 ± 0.053	7 of 7
BDNF	137.31 ± 58.36	3.87 ± 0.120	0.686 ± 0.078	5 of 7
high FGF-1	419.50 ± 43.85	3.79 ± 0.082	0.583 ± 0.039	6 of 6
collagen only	187.83 ± 67.25	3.89 ± 0.070	0.639 ± 0.075	5 of 6
empty tubes	178.16 ± 89.34	4.04 ± 0.220	0.651 ± 0.034	5 of 6

* SEM = standard error of the mean.
† Reflects the number of grafts with any axons compared with the number tested. Note that the majority of tubes contained at least some regenerating axons. Failure to transmit axons into the distal stumps was most apparent for the empty tube group.

TABLE 3
*Morphometric indices of regenerating axons in the distal nerve (mean ± SEM) in rats that underwent nerve gap repair**

Group	Total Fiber Count	Fiber Diameter (μm)	Axon/Myelin Ratio	No. of Grafts*
FGF-1	740.70 ± 224.21	3.13 ± 0.11	0.530 ± 0.080	5 of 7
NT-3	507.61 ± 139.58	3.24 ± 0.13	0.431 ± 0.037	7 of 7
BDNF	867.77 ± 426.62	3.33 ± 0.14	0.494 ± 0.067	4 of 7
high FGF-1	2534.26 ± 933.76	3.26 ± 0.15	0.350 ± 0.028	5 of 6
autograft	2271.01 ± 137.87	3.58 ± 0.10	0.360 ± 0.014	5 of 5
collagen only	535.69 ± 209.37	3.19 ± 0.14	0.464 ± 0.055	5 of 6
empty tubes	219.66 ± 108.59	3.21 ± 0.17	0.340 ± 0.043	3 of 6

* Reflects the number of grafts with any axons compared with the number tested. Note that the majority of tubes contained at least some regenerating axons. Failure to transmit axons into the distal stumps was most apparent for the empty tube group.

are permeable to the surrounding tissue medium provide better nutritional support and improved regeneration.[12,15,17,47,48,69,79] The PHEMA-MMA tubes are permeable to tissue fluids and small molecules up to at least 10 kD in size, but not to macromolecules and cells.[46] Finally, the tube should be easy to handle and apply, should hold a microsuture, and be flexible enough to glide and bend with animal limb movement, yet remain stiff enough to prevent collapse. The PHEMA tubes have excellent handling characteristics, permitting ease of microsurgical application. When the appropriate amounts of MMA are incorporated (Young modulus ~ 2800 kPa), they appear to be firm enough to resist compressive and tensile forces and demonstrate minimal deformation, remaining circular after 8 weeks in vivo.

Regeneration Through the Nerve Guidance Tubes

When two nerve stumps are positioned within the proximal and distal parts of a hollow tube, the conduit fills within 1 day with serous fluid, which has neurotropic activity.[44] Matrix precursors accumulate and over several days a coaxial, acellular, fibronectin-positive, laminin-negative matrix forms, which acts as a scaffold for migrating cells, including Schwann cells, from the nerve stumps and leads to the formation of a tissue cable.[44] The axons sprouting from the proximal stump regenerate through this new tissue cable. The cable of regenerating nerve, as we also observed, is therefore composed of regenerating myelinated axons, often in groups, accompanied by Schwann cells, within a neovascularized collagenous matrix (Figs. 3B and C and 4).

There is a tendency of the nerve-regenerating cable to taper from both proximal and distal stumps toward the center in nerve tubes.[44] Unfortunately, the regeneration of axons is constrained by the preformed, tapered tissue cable.[72] This tapering decreases with smaller-diameter tubes and increases with longer tubes. As a result, regeneration over mainly short gaps (< 12 mm) in rats, but not longer gaps, has been comparable to nerve autografts (reviewed in Doolabh, et al.[17]). The 12-mm tubes in the present experiment were used to repair 10-mm-long gaps, and we observed minimal tapering of the regenerating nerve cable (Fig. 3B). In future studies we plan to investigate regeneration across longer gap lengths through PHEMA-MMA tubes that are further enhanced.

Conduit gap length limitations can be partially overcome by an inner scaffold providing an environment that is con-

ducive to axonal regeneration,[72] with some of the best results obtained by inserting an internal gel matrix.[10,50,72,84] The inner matrix confers structural stability to the tube, provides a growth-supportive environment that favors cell invasion, and augments the surface area available for regeneration.[75] Nevertheless, the matrix material itself may impede axonal outgrowth,[42,50,51,80] especially if it is too concentrated, as demonstrated by others[42,80] and us.[61] In the present study, a similarly low concentration (1.28 mg/ml) of collagen I matrix improved regeneration into the distal nerve stump compared with an empty tube (Tables 2 and 3). The axonal counts in collagen-containing tubes were significantly inferior to autografts, but an 8-week outcome (which is relatively early) may be insufficient to allow regeneration comparable to a nerve graft, unless the tube is enhanced further.[17] The collagen gel matrix allows relatively easy suspension of growth factors,[10,11] a strategy that was pursued in this experiment to enhance regeneration.

Growth Factors

In contrast to axonal outgrowth through nerve tubes, the axons regenerating through a nerve autograft encounter graft-derived Schwann cells and basal lamina endoneurial tubes.[34] These provide chemical (neurotropic) factors as
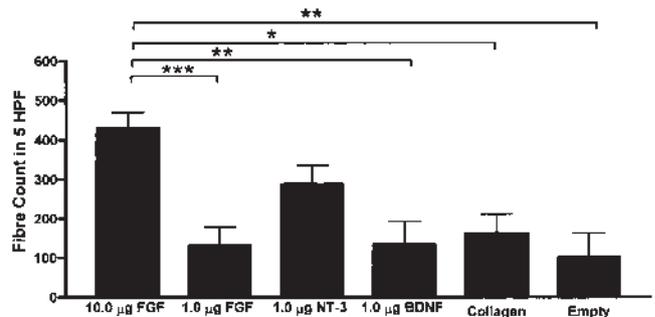
FIG. 5. Bar graph showing the number of myelinated fibers (mean ± standard error of the mean) in five hpfs counted within the tube center (see Fig. 3A for sampling method). Incorporation of 10 μg/ml FGF-1 into the collagen matrix significantly improved the number of fibers compared with 1 μg/ml FGF-1, 1 μg/ml NT-3, 1 μg/ml BDNF, or growth factor–unenhanced collagen matrix (*p < 0.05, **p < 0.01, ***p < 0.001).
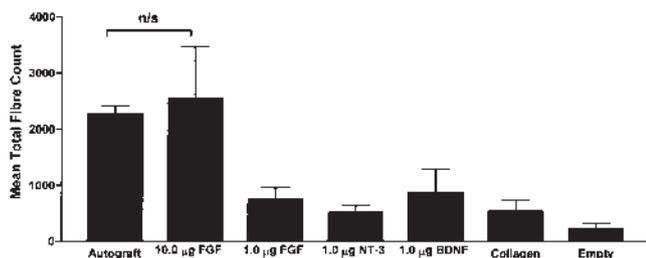
FIG. 6. Bar graph showing incorporation of 10 μg/ml FGF-1 into the collagen matrix, which resulted in nerve regeneration into the distal nerve stump comparable to that seen with nerve autografts. These two groups had a significantly higher mean number of myelinated fibers in the nerve distal to the graft repair compared with empty tubes, collagen matrix containing–tubes, or ones supplemented with growth factors at a concentration of 1 μg/ml (p < 0.05). n/s = not significant.

well as specific favorable cell and endoneurial tube surface adhesion molecules to the axons.[25] In particular, the critical role of viable Schwann cells within the nerve graft in supporting axonal regeneration has been stressed,[31] and their absence within nerve guide tubes is a potential cause for concern. Exogenous supplementation of tropic factors supplied by Schwann cells and other constituents of the nerve graft is one way to counter this deficiency, because many growth factors promote nerve regeneration (reviewed in Ebadi, et al.[19]).

The BDNF supports survival of embryonic sensory neurons[14] and is produced by peripheral glia,[1] target derived from skeletal muscle,[86] and retrogradely transported to motor neuron cell bodies, promoting their survival during development.[76] In adults, exogenous BDNF can replace the limited endogenous factor after peripheral axotomy,[24] thereby preventing the death of motor neurons[39,65] and promoting their regeneration[39,66,78] and remyelination.[29,40,86] The NT-3 is potently neurotrophic for sympathetic neurons[53] and for large sensory neurons that express high levels of tyrosine receptor kinase C,[63] particularly those that subserve muscle spindle and limb proprioceptive function.[4,20] Exogenous administration of NT-3 may be especially beneficial because there is less NT-3 available in the neuron after peripheral axotomy at the same time as the proximal nerve stump expresses increased levels of tyrosine receptor kinase C.[26] The NT-3 augments nerve regeneration, likely because of its tropic effects on large sensory[63] and motor neurons.[30,32]

The FGF family of polypeptides is composed of strong heparin-binding proteins, which are originally purified from bovine pituitary and brain.[21,27] The prototype family members, FGF-1 and FGF-2 (or basic FGF), are important regulators in the growth and development of mesodermal and neuroectodermal tissue, including angiogenesis and Schwann cell proliferation.[27] The FGF-1 is enriched in neurons,[27] produced within the cell body, and anterogradely transported along the axon, so that after axotomy there is a dramatic reduction in FGF-1 levels in the distal stump undergoing degeneration.[36] This polypeptide mediates in vitro survival and differentiation of several types of central and peripheral neurons,[8,11,70] whereas in vivo application of FGF-1 induces both peripheral[10,11,43] and spinal cord axonal[7,28] regeneration.

Based on the aforementioned considerations, we dis-

persed BDNF, NT-3, or FGF-1 into the collagen matrix within the tube lumen. All three growth factors enhanced regeneration into the nerve guide tube compared with collagen gel matrix alone, but statistically significant differences were observed only for the higher concentration of FGF-1. Indeed, the latter produced regeneration into the distal nerve stump similar to that observed with the positive controls (that is, nerve autograft repairs). One pitfall of using counts of myelinated axons alone for an outcome measure is that axonal counts reflect not only regenerating axons but also collateral sprouts growing from parent axons in the proximal nerve stump.[62] We speculate that the tropic effects of NT-3 may have caused increased collateral sprouting within the tube, as has been observed with the related neurotropin, nerve growth factor.[16] The increased axonal counts that were observed distal to the nerve guide tube in the group treated with 10 μg/ml FGF-1 are a much more robust finding, likely representing a significant regenerating pool of axons.[49] This improved regenerative response may have occurred from one or a combination of the known biological effects of FGF-1, including angiogenesis, Schwann cell mitogenesis,[27] and its neurotropic properties.[8,11,70] To exclude increased sprouting and branching within the nerve guide tube as the principal or the only positive effect of the growth factors, counts of retrogradely labeled parent sensory and motor neurons will be used[58] in future studies. This will allow improved evaluation of the effect of a given growth factor on regeneration from specific neuronal pools.[5]

The significant beneficial effect of FGF-1 on peripheral regeneration observed in our study is consistent with that reported in the literature.[10,82] In the first demonstration of the beneficial effect of FGF on in vivo nerve regeneration the author suggested the importance of appropriate dosing. In that study, Politis[68] cut rat peripheral nerves and then attached the proximal stumps to 6-mm-long, Y-shaped silastic implants in which the distal end of the implant was connected to different growth-promoting extracts as well as to placebo. In these experiments he observed improved axonal regeneration that was dose dependent with extracts containing FGF. This is consistent with the finding in our study, in which the higher (but not the lower) dose of FGF-1 improved axonal regeneration. Our findings may represent a dose–response relationship, but they also raise the possibility that therapeutic bioavailability was achieved only with the higher initial dose, as discussed later.

Growth Factor Delivery and Dosing

Tropic factors may be supplied systemically or locally. Local instillation often achieves adequate concentration and duration of activity of the growth factor in contrast to systemic administration, with bioequivalent systemic doses usually at least 100-fold greater than local doses.[43] The most common mode of local delivery has been with implantable osmotic pumps, which produce relatively constant rates of drug release and usually achieve reliable local concentrations.[40] Others have directly instilled the growth factor into the neural repair site by using a variety of carriers, including Gelfoam,[77] fibrin glue,[7,28,29] or genetically engineered cells that produce the factor.[29,64] Alternatively, the growth factor could be incorporated directly into the matrix substance that is instilled within the guidance conduit.[72] This

approach has several merits. First, it allows delivery of the factor directly into the local environment where axons are regenerating. In at least one study superior axonal regeneration was demonstrated with incorporation of factor into the matrix substrate compared with osmotic pump delivery.[78] Second, the method is simple to perform in that only one operation is required. Third, the method, if successful, may have significant clinical value, supplanting other methods of growth factor delivery.

A possible limitation of the delivery of factors within the matrix is that the drug levels may be inadequate. This may explain the reduced response with the lower concentrations (1 μg/ml) of growth factors used, although the lower concentration was consistent with the dosages used in previous studies by others and us.[10,28,57,60,71,74,85] Therefore, the reduced effect seen in our study with the 1-μg/ml doses may indicate a problem with growth factor bioavailability rather than inadequate initial dosing. In this context, it is possible that the growth factors may diffuse readily out of the semipermeable PHEMA tube. The release kinetics of neurotropins from the PHEMA tube lumen is currently being investigated in our laboratory. Recent reports indicate that prolonged release of growth factors can be achieved by incorporating the molecules into the nerve tube wall,[23] which we have demonstrated recently in preliminary in vitro studies in our laboratory. In future studies, we also plan to encapsulate the growth factors in biodegradable microspheres, which are in turn dispersed within the collagen gel matrix in the nerve tubes. This will improve the bioavailability and bioactivity[6] of the tropic factors over a prolonged period.

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