A Simple Technique for Augmentation of Axonal Ingrowth Into Chondroitinase-Treated Acellular Nerve Grafts Using Nerve Growth Factor

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Background and Purpose: Improvement in axonal regeneration may lead to the development of longer nerve grafts and improved outcomes for patients with peripheral nerve injury. Although the use of acellular nerve grafts has been well documented (Groves et al, *Exp Neurol.* 2005;195:278–292; Krekoski et al, *J Neurosci.* 2001;21:6206–6213; Massey et al, *Exp Neurol.* 2008;209:426–445; Neubauer et al, *Exp Neurol.* 2007;207:163–170; Zuo et al, *Exp Neurol.* 2002;176:221–228), less is known about the ability of neurotrophic factors to enhance axonal regeneration. This study evaluates axonal ingrowth augmentation using acellular, chondroitinase-treated nerve grafts doped with nerve growth factor (NGF).

Methods: Acellular chondroitinase-treated murine nerve grafts were placed in experimental (NGF-treated grafts) and control (carrier-only grafts) rats. Five days after implantation, axonal regeneration was assessed by immunocytochemistry along with digital image analysis.

Results: Higher axon count was observed throughout the length of the nerve in the NGF group ($P < 0.0001$), peaking at 3 mm from proximal repair ($P = 0.02$). Although the NGF group displayed a higher axon count per slice, the mean diameter of individual NGF axons was smaller ($P < 0.0001$), potentially consistent with induction of sensory axons (Rich et al, *J Neurocytol.* 1987;16:261–268; Sofroniew et al, *Annu Rev Neurosci.* 2001;24:1217–1128; Yip et al, *J Neurosci.* 1984;4:2986–2992).

Conclusion: The simple technique of doping acellular, chondroitinase-treated nerve grafts with NGF can augment axonal ingrowth and possibly preferentially induce sensory axons.

Key Words: acellular nerve grafts, chondroitinase, nerve growth factor, peripheral nerve regeneration

P eripheral nerve injury is common and frequently results in significant morbidity.[1,2] Although peripheral nerves are able to regenerate after injury, they require a complex interplay between cells, growth factors, and the extracellular matrix.[3,4] Surgical repair is often indicated in peripheral nerve injury and involves end-to-end, tension-free opposition of the proximal and distal ends of the transected nerve. In cases where a nerve gap must be bridged, autografts are the gold standard for reconstruction.[5] However, autograft use commonly involves donor site morbidity among other limitations.[6,7] An alternative is a processed allograft, which retains the biologic scaffold of nerve tissue but is nonimmunogenic due to lyophilization and detergent-based processing methods. Multiple animal studies[8,9] and 1 case series[10] have shown the efficacy of processed allografts for short nerve gap repair and their superiority over nerve conduits.[8]

In rendering the grafts acellular, one potential downside is that the processing disturbs the neural microenvironment, eliminating the neurotrophic factors and other cellular conditions that normally promote optimal nerve regeneration. Before proximal axons can begin growth into these processed acellular grafts, Schwann cells and other various inflammatory cells from the host nerve will migrate into and remodel the graft to promote regeneration.[11–13] This process is slow and, apart from restriction in longer grafts by the limits of Schwann cell proliferation and migration, may also be unpredictable.[11–13] Moreover, since axons grow slowly (1 mm/d), they may not reach their targets before the latter have atrophied because of chronic denervation. Because time and distance may ultimately defeat successful reinnervation, improved axonal ingrowth in peripheral nerve grafts represents an important advancement, especially since even small increases in reinnervation can mean significant improvements in function for patients.

Strategic modifications to these acellular allografts may enhance peripheral nerve regeneration. One well-known group of molecules with an important role in growth cone support are laminins, the most abundant noncollagenous structural glycoproteins in basement membranes.[4,14] The axon-promoting activity of the laminin subtype found in Schwann cells is inactivated in the intact nerve by chondroitin sulfate proteoglycans (CSPGs)[15,16]; however, during Wallerian degeneration, matrix metalloproteinases expressed by Schwann cells and macrophages degrade the CSPGs, freeing up the neurite-promoting activity of laminin to the ingrowing axons. Experimental application of chondroitinase ABC (ChABC), an enzyme that removes CSPGs, has been shown to markedly enhance axonal regeneration in acellular nerve grafts[17–21] as well as significantly increase the effective length of graft that may be used.[20] Degradation of the growth-inhibiting CSPGs with chondroitinase, thus, represents an important strategy in acellular allograft nerve graft therapy.

Moreover, it is known that neurotrophic factors may significantly increase the regeneration of injured nerves.[3,4,22–25] One of the earliest described neurotrophins, nerve growth factor (NGF) has been studied extensively and has been shown to effectively support axonal outgrowth in biocompatible matrices and acellular nerve grafts.[26,27–30] NGF induces regeneration along acellular nerve grafts by directing the action of budding growth cones.[22,28] Besides its direct activity on axons, NGF is also involved in the regulation of the proliferation and migration of Schwann cells.[31] Following injury

to mature peripheral nerves, denervated Schwann cells upregulate their NGF expression by 5-fold within 5 to 7 days. Increased NGF expression is maintained throughout the process of Schwann cell migration and organization until intimate axon–Schwann cell contact is established to mediate regenerating axon maturation. Consequently, NGF serves as a key guidance molecule for axonal regeneration through several mechanisms.

Despite its important translational implications, investigations of neurotrophic growth factors and their clinical application in peripheral nerve grafts are sparse. Clinical data examining the use of NTFs along with new advances in acellular nerve grafts are also limited. We hypothesize that the simple technique of doping commercially available acellular, chondroitinase-treated nerve grafts with well-known NTFs will further augment axonal regeneration, which in turn may allow for improvements in the clinical application of peripheral nerve grafts. Using a rat nerve injury model, this study sets out to characterize the ability of NGF to augment axonal ingrowth in acellular, chondroitinase-treated nerve grafts.

METHODS

Analysis of NGF Doped Nerve Grafts
Animals and Nerve Harvest
For our doping studies, sciatic nerves were harvested from culled rats from the Vanderbilt Division of Animal Care in accordance with the Vanderbilt Institutional Animal Care and Use Committee policies. Nerves were harvested from male and female rats of unknown age or origin. Obese rats were not used in this study. Segments of sciatic nerves were cleanly excised from the surrounding tissue and frozen in liquid nitrogen. Nerves were stored at −80°C until ready for doping procedure.

Preparation of Lyophilized Nerve Grafts
The specimens were freeze thawed 3 times from −80°C to 37°C, followed by a single wash with phosphate buffered saline (PBS). PBS wash was removed, and 200 μL of chondroitinase solution (2 U/mL in PBS, Sigma Aldrich, St. Louis, MO) was applied. Lyophilized nerves were incubated in chondroitinase solution overnight (~17 hours) at 37°C. The following day, the specimens were washed 3 times with PBS and then stored at −80°C until ready for NGF treatment. Fresh dilutions of mouse NGF beta (ProSpec Bio, East Brunswick, NJ) were made in distilled water (dH$_2$O) on the day of treatment. Following concentrations were used: 100 μg/mL, 10 μg/mL, 1.0 μg/mL, 0.1 μg/mL, 0.01 μg/mL, 0.001 μg/mL, and dH$_2$O (negative control). Each nerve segment (in triplicate) was incubated in 100 μL NGF solution or water for 2 hours at room temperature. Immediately following NGF incubation, they were washed 3 times with PBS. The final PBS wash was removed, and 100 to 200 μL of protein lysis buffer (9.5 M Urea/4% 3-[(3-cholamidopropyl dimethylammonio]-1 propanesulfonate (CHAPS)/Roche Protease Inhibitor Cocktail/2.5% tributylphosphine) was applied to each nerve segment. The specimens were then frozen at −80°C until ready for protein extraction.

Initially, 1 cm segments of nerve were used to identify the dynamic range of our NGF enzyme-linked immunosorbent assay (ELISA), and follow-up studies were carried out using two 2 cm nerve segments (4 cm of total nerve).

Protein Extraction and Quantification
Samples were freeze thawed 3 times from −80°C to 37°C. A sterile 5-mm stainless steel bead (Invitrogen, Carlsbad, CA) was then added to each sample. Samples were then placed in a TissueLyser II (Qiagen, Valencia, CA) and processed 4 × 2 minutes at 30 Hz (~1800 oscillations/min). Following tissue disruption, each sample

was centrifuged for 15 minutes at 10,000 × g at +4°C. Supernatants were removed to fresh tubes, beads were collected for resterilization, and cell debris was discarded. This centrifugation step was repeated once to ensure complete removal of cell debris.

Proteins were quantified by Bradford Assay using Coomassie Protein Assay Reagent (Thermo Fisher, Nashville, TN) and albumin standards (Pierce, Rockford, IL). Absorbances were measured at 595 nm on a NanoDrop (Thermo Scientific, Rockford, IL), a standard curve was generated, and replicates of each sample were plotted against the curve for quantitation.

Enzyme-Linked Immunosorbent Assay
To determine the percentage of NGF contributing to total protein in each sample, a precoated NGF Sandwich ELISA kit (Chemicon, Billerica, MA) was used. Standards were diluted as described in the product manual. Because of limited material, samples were diluted to specific concentrations for analysis.

Wash buffer (100 μL/well) was added to the wells for 5 minutes to prepare the surface for application of samples. Wash buffer was thoroughly removed by inverting the plate and blotting over paper towels. Standards and samples were then applied overnight (~17 hours) at +4°C with shaking. The following morning, they were removed, and each well was washed with 200 μL wash buffer. Washing was repeated 4 times for a total of 5 washes, with blotting between each wash. After the final wash and blotting, 100-μL mouse anti-NGF detection antibody (1:100) was applied for 2 hours at room temperature with shaking. The wash procedure was repeated, followed by application of 100-μL donkey anti-mouse, horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000) for 2 hours at room temperature with shaking. The washing procedure was repeated for the last time, and 100-μL room temperature 3,3′, 5,5″-tetramethylbenzidine (TMB) substrate was applied for 10 to 15 minutes. Stop solution (100 μL) was immediately applied, and absorbances were read at 450 nm. A standard curve was generated and sample absorbances were plotted against it. Percentages of NGF/total protein were calculated from the resulting data.

Acellular, Chondroitinase-Treated Nerve Graft Preparation
Approval for this part of the project was granted by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Rat experimentation and immunocytochemistry were performed at the University of Alabama at Birmingham Division of Plastic Surgery.

Adult female (180–200 g) Sprague Dawley rats (Harlan; Indianapolis, IN) were used as nerve donors and graft recipient hosts. Six donor rats were anesthetized with isoflurane and decapitated before harvest. Excised 10 mm segments of rat sciatic nerve were made acellular by lyophilization before treatment with chondroitinase as described in other studies.[18,20] Briefly, the lyophilization process involved freezing the grafts in liquid nitrogen for 2 minutes followed by thawing in a 37°C water bath for 2 minutes. This cycle was repeated 3 times to yield acellular nerve grafts, which were then washed in a PBS detergent solution containing 2 U/mL chondroitinase ABC (Sigma, St. Louis, MO) at 37.5°C for 16 hours, rinsed 3 times with PBS-only solution, then frozen in liquid nitrogen until needed.

NGF-Doping of Acellular, Chondroitinase-Treated Nerve Grafts
The acellular nerve grafts pretreated with chondroitinase were then soaked in a solution containing either a PBS-only solution or in a PBS solution with 10 μg/mL (5 μg/500 μL PBS) of NGF (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel). Specifically, the individual nerves were incubated in 96 well tissue culture plates

Percentage of NGF in total protein following doping with NGF
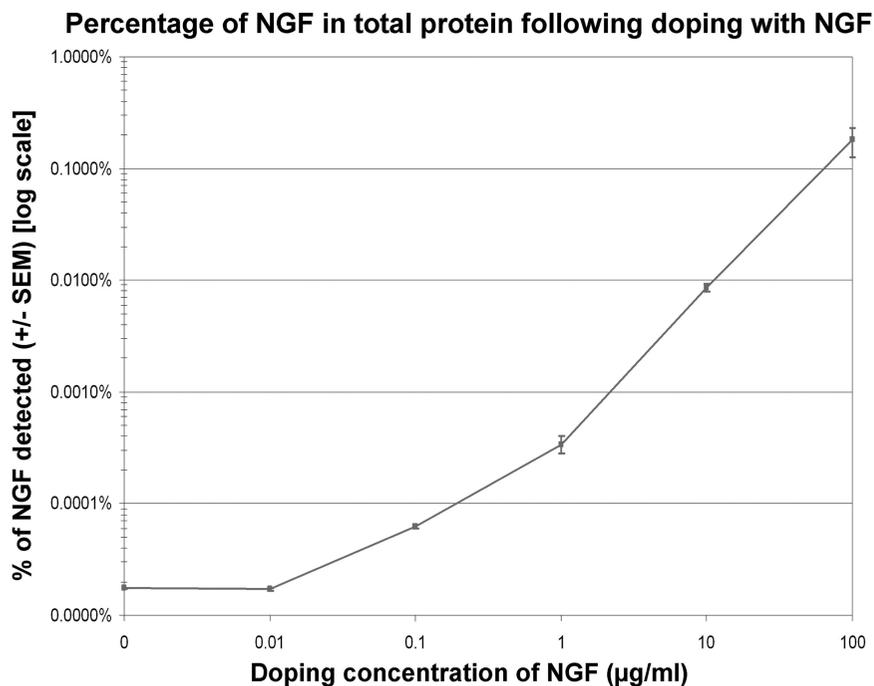
FIGURE 1. Graphical representation of a nonlinear increase in NGF composition of total protein after dosing with varying concentrations of murine NGF. Data labels shown are mean % NGF (±SEM).

(Sigma, St. Louis, MO) for 2 hours at room temperature with either PBS or 100 μL of 10 μg/mL NGF. They were then washed 3 times in PBS before implantation using standard microsurgical techniques.

In our current investigation, we chose 10 μg/mL as our study point based on our titration curve (Fig. 1) and based on published functional concentration data.[29]

Interpositional Nerve Grafting

Six recipient rats were deeply anesthetized for surgery with xylazine (8–10 mg/kg) and ketamine (80–100 mg/kg) and treated with Tylenol (300 mg/kg) and buprenorphine (0.01–0.02 mg/kg) perioperatively. Previously prepared grafts were thawed the day before engraftment, rinsed twice with Ringer's solution, and kept on ice before being subsequently used as interpositional grafts in a rat sciatic nerve injury model as described in other studies.[18,20] For the coaptation of the grafts to the cut ends of the recipient sciatic nerve, 10× surgical microscopic magnification and 9-0 Ethilon nylon sutures were used. The recipient rats were divided into 2 equal groups; in each group (n = 3), the rat received 1 graft, either treated with PBS-carrier only (control group) or with NGF (experimental group). After recovery from anesthetic, animals were returned to standard housing. Five days after implantation, all recipient rats were deeply anesthetized and decapitated. After euthanasia, the rat grafts were retrieved and frozen for cryosectioning.

Cryosection Protocol

The graft and the 2-mm proximal and distal host nerve were removed and immersed in PBS at 4°C. Using a dissecting microscope and the epineurial sutures as landmarks, each specimen was aligned and embedded. Specimens were stored at −80°C until ready for sectioning. The specimens were then sectioned into 12-μm transverse sections directly onto polarized glass slides for staining at −23°C. Acetone was applied, and the specimens were dried at 50°C for 5 minutes. Specimens were subsequently immediately stained for immunofluorescence.

Immunocytochemistry and Digital Imaging Analysis

Frozen nerve grafts were cryosectioned transversely at 0, 1, 2, 3, 4, and 5 mm from the site of proximal graft repair as noted earlier. At each incremental distance from proximal repair, 3 sequential 12-μm thick transverse slices were created. Axonal regeneration was assessed with growth-associated protein-43 (GAP-43) immunofluorescence and digital image analysis as described in other studies.[18] The specific GAP-43 antibody used is a commercially available rabbit polyclonal antibody specific to growth cone-expressed protein (Abcam ab16053).[32] Tissue sections mounted on slides were washed with PBS and then treated with 0.5% Triton ×-100 in PBS for 10 minutes. The sections were treated with blocking buffer (10% horse serum in PBS and 0.1% Triton ×-100) and then incubated 30 minutes at 4°C with primary antibodies (diluted in blocking buffer). After washing 3 times in PBS and 0.1% Triton ×-100, bound primary antibodies were labeled with fluorescein isothiocyanate anti-rabbit immunoglobulins (Dako, Carpinteria, CA). The anti-rabbit secondary antibody had been preabsorbed with rat serum. The sections were washed, rinsed, and coverslipped in fluorophore-stabilizing mounting media. Cells were then assessed using a Leitz microscope (Wetzlar, FRG) equipped with epifluorescence optics. Digital image analysis of stained slices was performed using Adobe Photoshop CS4 to determine the absolute axon counts as well as area calculations of the stained axon growth cones.

Axonal count analysis was performed on all slices at each sequential section from 0 to 5 mm. Axonal diameter analysis was performed only on the slices generated at 3 mm from proximal repair, where the greatest difference in axon counts was observed between the groups. Area calculations were verified by comparison with standardized area measurements.

Statistical Analysis

Descriptive statistics including counts, means, and standard error of the mean (±SEM) were used to summarize data as appropriate. Univariate analysis was performed on axon counts and areas using the Student t test for independent samples. Two-tailed P
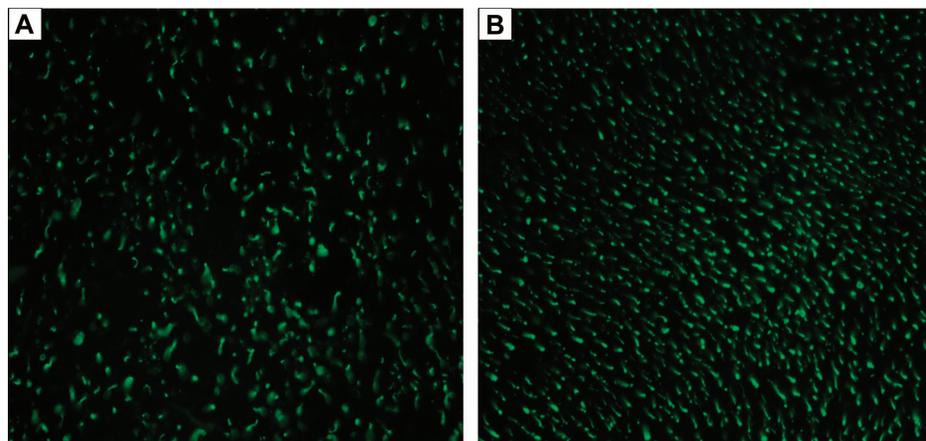
FIGURE 2. Fluorescein isothiocyanate (FITC) staining of GAP-43 immunolabeled nerve graft cross sections at 3 mm from proximal repair in control (A) and NGF-treated (B) graft groups at 40× magnification. Increased axonal regeneration is visualized in the NGF group compared with vehicle-treated control group.

values are reported wherever applicable with a significance level set at $P < 0.05$.

RESULTS

NGF Doping Study

ELISA studies of chondroitinase-treated, acellular nerve grafts doped with various concentrations of NGF demonstrated a nonlinear dose-dependent relationship between concentration of doping solution and percentage NGF of total protein (Fig. 1). The following pattern was observed: no difference between untreated and 0.01 μg/mL NGF dosed, only a 3.6-fold increase from 0.01 μg/mL to 0.1 μg/mL NGF dosed, only a 5.5-fold change up to 1.0 μg/mL dosed, and then a 25.3-fold change up to 10.0 μg/mL dosed, followed by a 20.9-fold increase up to 100.0 μg/mL.

Immunocytochemistry and Digital Imaging Analysis of Interpositional Grafts Harvested 5 Days After Neurorrhaphy

With immunofluorescent GAP-43 staining for axon growth cones, the experimental (NGF) rat group visibly showed a higher and more homogenous axon density in their nerve grafts 5 days after implantation (Fig. 2). Digital image analysis on histologic sections confirmed a higher axon count in the NGF group, consistent throughout the length of the nerve grafts with an average of 1035.9 ± 41.1 axons versus 552.3 ± 18.1 axons in the control grafts ($P < 0.0001$). At each section analyzed in sequential 1 mm increments up to 5 mm from proximal repair, the same trend of a higher NGF group axon count was observed (Fig. 3). Between the groups, the difference in axon counts peaked at 3 mm ($P = 0.02$), which was also the section with highest mean count (1214.2 axons) for the NGF group compared with 533.1 mean axons at 4 mm from proximal repair for the control group (Figs. 3, 4).

Looking more closely at the 3 mm from proximal repair slices, although the NGF group displayed a higher axon count per slice (Fig. 4), the mean diameter of the individual NGF axons was smaller at 2.40 ± 0.03 μm versus 3.46 ± 0.06 μm for the control grafts ($P < 0.0001$). The distributions of axon diameters in the 3 mm slices in both groups are shown in Figure 5.

DISCUSSION

Doping of Acellular Chondroitinase-Treated Nerve Grafts

The concentration of NGF detected in 1 cm grafts was not significantly different from that found in 2 cm long grafts, which

Axonal ingrowth in acellular chondroitinase-treated nerve grafts
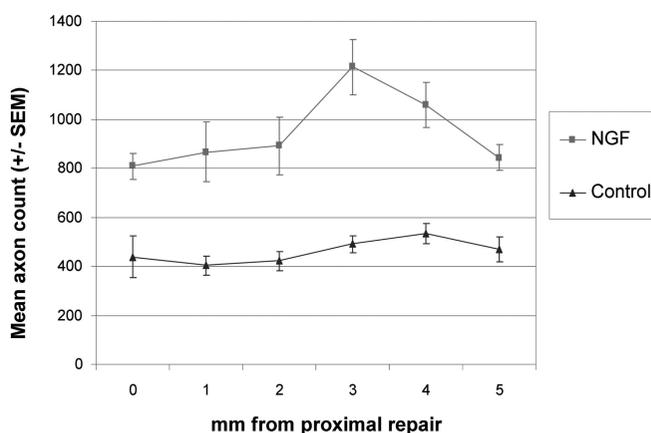
FIGURE 3. Axonal ingrowth throughout graft length in both NGF and control groups. Mean axon count ± SEM is plotted at each millimeter from proximal repair, demonstrating a higher mean count in the NGF group throughout the length of the grafts ($P < 0.0001$). The NGF grafts peak in count at 3 mm (1214.2 axons) versus at 4 mm (533.1 axons) from proximal repair for the control group.

suggests that absorption of NGF is uniform across the length of the graft. The authors suggest that the imbibition of NGF into the grafts involves a number of variables. This is evidenced by the fact that a linear relationship was not observed between concentration of doping solution and amount of NGF detected in the grafts, up to the point of saturation. Instead, a nonlinear relationship was observed (Fig. 1), whereby there was a >20-fold increase in detected NGF for a 10-fold increase in concentration of doping solution. The extracellular matrix microstructure is left intact in nerve allograft,[8] which results in a complex porous network. Imbibition of doping solution is therefore dependent on the complexity of the endoneurial microstructure and intermolecular forces within the doping solution.[33]

Additionally, the existing data do not identify whether the NGF is more concentrated at the sectioned ends of the graft or whether it is absorbed evenly along the graft. Further studies are required to further develop the dose concentration curve and determine whether the trend for increasing absorption of NGF is seen with increased concentration of doping solution. As other neu-
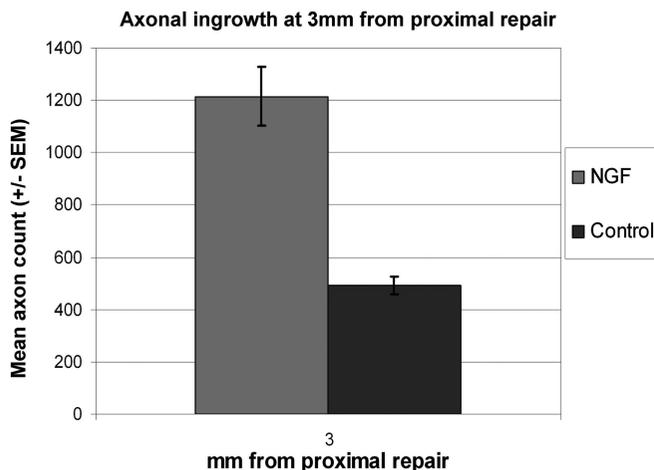
and highlight the availability of a simple technique to augment axonal ingrowth.

Driving Selective Sensory Nerve Ingrowth

In addition to promoting the ingrowth of axons, the ideal growth factor treatment would also allow for selectivity of the type of axons induced; specifically, sensory versus motor fibers. The mechanism by which different types of axonal growth cones select and grow into Schwann tubes has yet to be fully established. In a mixed nerve, the specificity of reinnervation of motor and sensory targets is thought to be the result of selective pruning of axon collaterals as they grow into neural tubes and may be influenced by gradient levels of target-derived neurotrophic factors and by Schwann cell signaling.[3,4,34,35] Needless to say, the ability to manipulate axonal outgrowth with specific concentrations or combinations of NTFs would represent a significant clinical advancement in peripheral nerve injury therapy and could potentially allow for grafts optimized for a specific target function.

Regarding NGF, several studies have suggested that it promotes growth of small sensory fibers[26,36,37] instead of larger motor axons. In our study, although the NGF group displayed a higher axon count per slice, the mean diameter of the individual NGF axons was 30.6% smaller compared with the control grafts ($P < 0.0001$) (Fig. 5). Although further studies would benefit from not only further histologic staining for specific types of axons[38] as well as functional assessment of the axons induced by NGF, the finding of smaller axons induced in the NGF group is consistent with the induction of a sensory axon, a fact that could help to therapeutically direct selective fiber ingrowth into these grafts.

Future Directions

Overall, our study shows the promising results of pretreating acellular, chondroitinase-treated nerve grafts with NGF. Further studies are needed not only to correlate the immunohistologic data with functional outcomes in rats but also to compare the axonal regeneration seen in these NGF-augmented acellular, chondroitinase-treated nerve grafts with that of autografting, the gold standard of nerve grafts. Moreover, the dosages applied in this pilot study are supratherapeutic and could have adverse effects if used clinically; therefore, future studies will further define the dose curve to optimize ingrowth and minimize dosing. Lastly, other studies have shown the enhancement of nerve regeneration as well as specific axon-type induction with other neurotrophic factors, including glial-derived neurotrophic factor, brain-derived growth factor, artemin, pleiotrophin, and ciliary neurotrophic factor.[22,24,39–42] Future research will include assessment of these other promising neurotrophic factors and assess their ability to both augment axon regeneration and to induce motor versus sensory fiber specificity in similar acellular, chondroitinase-treated nerve grafts.

CONCLUSIONS

NGF can augment axonal ingrowth as well as preferentially induce smaller caliber axons consistent with sensory fibers into acellular, chondroitinase-treated nerve grafts in an in vivo sciatic nerve injury model. Therefore, the simple technique of doping commercially available acellular, chondroitinase-treated nerve grafts with NGF has the potential for important implications in the clinical application of peripheral nerve grafts. Further research is needed to better characterize the different types of axons induced by NGF as well as assess other neurotrophic factors' potential in similarly augmenting axonal ingrowth and type specificity into these grafts.

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