ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Fibrin Matrices With Affinity-Based Delivery Systems and Neurotrophic Factors Promote Functional Nerve Regeneration

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ABSTRACT: Glial-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) have both been shown to enhance peripheral nerve regeneration following injury and target different neuronal populations. The delivery of either growth factor at the site of injury may, therefore, result in quantitative differences in motor nerve regeneration and functional recovery. In this study we evaluated the effect of affinity-based delivery of GDNF or NGF from fibrin-filled nerve guidance conduits (NGCs) on motor nerve regeneration and functional recovery in a 13 mm rat sciatic nerve defect. Seven experimental groups were evaluated consisting of GDNF or NGF and the affinity-based delivery system (DS) within NGCs, control groups excluding the DS and/or growth factor, and nerve isografts. Groups with growth factor in the conduit demonstrated equivalent or superior performance in behavioral tests and relative muscle mass measurements compared to isografts at 12 weeks. Additionally, groups with GDNF demonstrated greater specific twitch and tetanic force production in extensor digitorum longus (EDL) muscle than the isograft control, while groups with NGF produced demonstrated similar force production compared to the isograft control. Assessment of motor axon regeneration by retrograde labeling further revealed that the number of ventral horn neurons regenerating across NGCs containing GDNF and NGF DS was similar to the isograft group and these counts were greater than the groups without growth factor. Overall, the GDNF DS group demonstrated superior functional recovery and equivalent motor nerve regeneration compared to the isograft control, suggesting it has potential as a treatment for motor nerve injury.

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KEYWORDS: drug delivery; growth factor; nerve guidance conduit; peripheral nerve injury; rat sciatic nerve

Introduction

Despite advances in the treatment of peripheral nerve injury, the low incidence of complete functional recovery suggests that an alternative to the current standard of care (nerve autografts) is greatly needed (Beazley et al., 1984; Dellon and Mackinnon, 1988). An alternative repair strategy involves the use of nerve guidance conduits (NGCs), which can be filled with a biomaterial matrix and/or growth factors, to bridge nerve defects and enhance axonal regeneration (Schmidt and Leach, 2003). NGCs have demonstrated results similar to autografts in histological assessments of regenerated nerve, but data supporting the ability of NGCs to promote motor nerve regeneration and functional recovery are limited.

Achieving functional recovery following peripheral nerve injury is greatly hindered by the lack of knowledge concerning the mechanism of motor nerve regeneration and muscle reinnervation. Studies have shown that target end-organs, such as muscle (Madison et al., 2007; Robinson and Madison, 2004), and components within the nerve branches, such as Schwann cells (SCs) (Hoke et al., 2006), provide different levels of trophic support, and that specific growth factors may stimulate superior motor versus sensory nerve regeneration. Additionally, motor and sensory neurons express varying levels of growth factor receptors,

for example, motor neurons and their axons express receptors for glial-derived neurotrophic factor (GDNF (GDNFRα1 and RET)) but not NGF (TrkA) (Boyd and Gordon, 2003). Therefore, the inclusion of growth factors in NGCs that target motor neurons may stimulate motor-specific nerve regeneration and, in turn, facilitate superior functional recovery.

Differences in motor nerve regeneration have been observed between NGCs delivering different growth factors that target different neuronal populations. Studies utilizing NGCs capable of delivering motor-specific growth factors from the conduit walls have demonstrated improved motor nerve regeneration in vivo (Barras et al., 2002; Fine et al., 2002). Based on these studies we hypothesized that an alternative method of growth factor delivery, *affinity-based* delivery from luminal matrices within NGCs, could further stimulate motor nerve regeneration through the release of motor-specific growth factor.

In contrast to diffusion-based release, an *affinity-based* DS allows the release of growth factors to be controlled by cell-based degradation of the DS and surrounding fibrin matrix (Sakiyama-Elbert and Hubbell, 2000a). Our lab has developed an affinity-based DS that sequesters growth factors inside a fibrin matrix (Sakiyama-Elbert and Hubbell, 2000a,b) through the use of bi-domain heparin-binding peptide. One domain within this heparin-binding peptide consists of a transglutaminase substrate (Ichinose et al., 1983; Kimura et al., 1985), to facilitate peptide cross-linking into fibrin matrices, while the other domain consists of a heparin-binding domain (Sakiyama et al., 1999; Tyler-Cross et al., 1994, 1996) that allows non-covalent immobilization of heparin to the peptide. As a result, electrostatic interactions between various growth factors with peptide-bound heparin effectively immobilizes these neurotrophic factors within the fibrin matrix in a manner similar to growth factor sequestration to heparan sulfate in the extracellular matrix (Yamada, 1983). Delivery of NGF and GDNF from fibrin matrices containing our affinity-based DS has previously been found to promote peripheral nerve regeneration in short-term in vivo studies (Lee et al., 2003; Wood et al., 2009b, 2010). Yet, previous studies did not examine the ability of NGCs loaded with affinity-based DS containing various growth factors to selectively promote motor neuron regeneration and functional recovery. The present study was therefore designed to evaluate the effects of the controlled release of GDNF and NGF from fibrin matrices containing our *affinity-based* DS on motor nerve regeneration and functional recovery in a rat sciatic nerve model 12 weeks postoperatively.

Materials and Methods

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless specified otherwise.

Experimental Animals

Adult male Lewis rats (Harlan, Indianapolis, IN), each weighing 250–300 g were used in this study. All surgical procedures and perioperative care measures were performed in strict accordance with the National Institutes of Health Guidelines and were approved by the Washington University Animal Studies Committee.

Experimental Design

One hundred twelve animals were randomized into seven groups ($n = 16$) as shown in Table I. Fifty-six of those animals (all seven groups; $n = 8$) were used for the evoked motor response portion of the study, while the remaining animals (all seven groups; $n = 8$) were used for the retrograde labeling portion of the study. An additional eight animals served as sciatic nerve isograft donors. Empty conduits served as the negative control group (Empty). Additional control groups received NGCs containing fibrin with the DS and without growth factor (DS (no GF)) or fibrin with growth factor and without DS (NGF/GDNF (no DS)) (Fig. 1). Reversed nerve isografts from syngeneic donor animals served as a positive control (Isograft).

Preparation of Fibrin Matrices

Fibrin matrices were prepared as previously described (Sakiyama-Elbert and Hubbell, 2000a). The DS was constructed by incorporating heparin and a bi-domain peptide

Table I. Experimental design.

		Motor response study		Retrograde study	
Group name	Group description	Number of rats	Number with regeneration	Number of rats	Number with regeneration
Isograft	Isograft	8	8	8	8
GDNF DS	Fibrin + DS + GDNF (100 ng/mL)	8	5	8	5
GDNF (no DS)	Fibrin + GDNF (100 ng/mL)	8	2	8	0
NGF DS	Fibrin + DS + NGF (50 ng/mL)	8	5	8	6
NGF (no DS)	Fibrin + NGF (50 ng/mL)	8	4	8	1
DS (no GF)	Fibrin + DS (no GF)	8	3	8	3
Empty	Empty	8	3	8	2

DS, delivery system; GF, growth factor; GDNF, glial-derived neurotrophic factor; NGF, nerve growth factor.

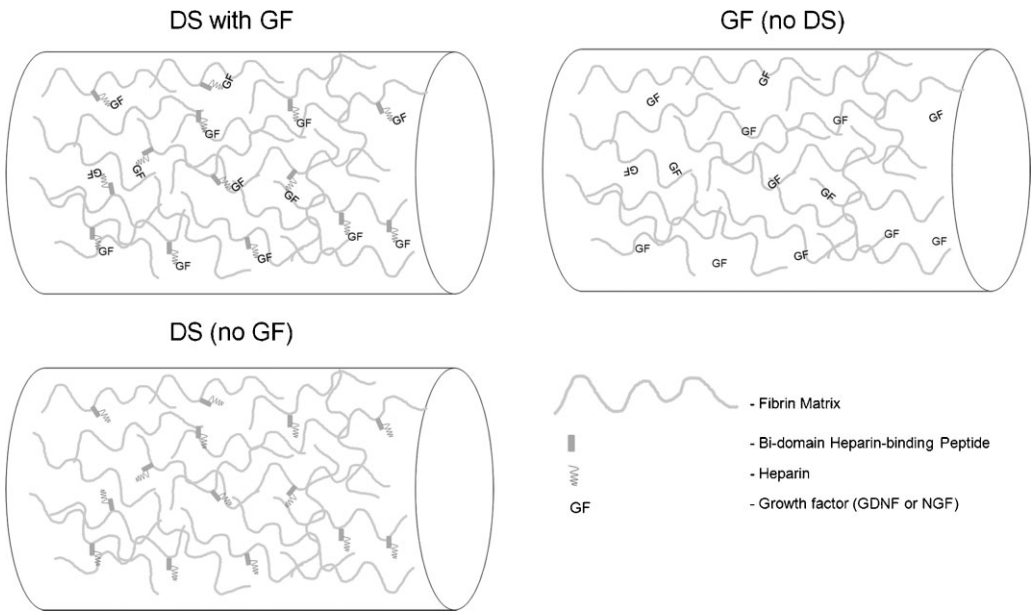

Figure 1. Schematic representation of nerve guidance conduit containing the affinity-based delivery system and its controls. The delivery system consisted of a bi-domain peptide cross-linked into the fibrin matrix at one domain while the other binds heparin by electrostatic interactions. The growth factor can then bind to the bound heparin, creating a matrix-bound, non-diffusible complex. Applicable controls (DS (no GF) and GF (no DS)) were constructed by omitting either the GF or the heparin and peptide, respectively, while still including the fibrin matrix.

(ATIII), based on a modified version of the antithrombin III-heparin binding domain ((*AcG*)*NQEQVSPK*(βA) FAKLAARLYRKA, where AcG denotes *N*-acetyl-glycine and the transglutaminase substrate is given in italics) (Sakiyama et al., 1999; Tyler-Cross et al., 1994), into fibrin matrices during polymerization. The bi-domain peptide was synthesized as described previously (Wood et al., 2009a). Components were mixed to obtain the following final solution concentrations (for DS + GF groups): 4 mg/mL fibrinogen, 2.5 mM Ca^{++}, 2 NIH units/mL of thrombin, 0.25 mM peptide (which results in 8 mol of cross-linked peptide per mole fibrinogen, Sakiyama et al., 1999; Schense and Hubbell, 1999), 62.5 μM heparin (sodium salt), and recombinant human GDNF or β-NGF (100 and 50 ng/mL, respectively; Peprotech, Inc., Rocky Hill, NJ; doses based upon previous in vivo studies; Wood et al., 2009b).

Silicone tubing (SF Medical, Hudson, MA) (1.5 mm inside diameter and 2.1 mm outer diameter) was cut into 15 mm segments and autoclaved. The fibrinogen solution was drawn into the silicone tube using a pipette and allowed to polymerize for 10 min prior to implantation.

Operative Procedure

All surgical procedures were performed using aseptic technique. Four percent isoflurane gas (Vedco, Inc., St Josephs, MO) anesthesia was used for animal induction followed by 2% isoflurane gas for maintenance. The hind leg of the rat was prepared and the sciatic nerve was exposed through a dorsolateral-gluteal muscle splitting incision. A 5 mm nerve segment was excised proximal to the trifurcation of the sciatic nerve and a 15 mm silicone NGC, containing fibrin with or without DS and with or without growth factor, was then sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end (resulting in a 13 mm gap). Four 9-0 nylon interrupted microepineurial sutures were used to secure the NGC. In animals receiving the isograft control, a 13 mm segment of sciatic nerve was harvested from a syngeneic donor animal and sutured into the recipient animal in reverse orientation. Wounds were irrigated with saline, dried, and closed in two layers, utilizing 5-0 vicryl suture to close the muscle layers, and 4-0 nylon suture to close the skin. Experimental animals were recovered in a warm environment prior to returning to the housing facility.

Behavioral Analysis

Two behavioral tests were performed to assess functional recovery at baseline and at 4, 8, and 12 weeks post-operatively. Walking track analysis, as developed by de Medinaceli et al. (1982), was utilized to assess the recovery of hindlimb function through the calculation of sciatic functional index (SFI) (Bain et al., 1989). A grid-grip test modified from Metz et al. was also utilized to measure functional recovery in the hindlimb (Johnson et al., 2010; Metz et al., 2000). Investigators blinded to the treatment groups observed each animal walking on a fixed grid of bars

spaced 1.5 in. apart for a period of 3 min. Successful grips of the grid, defined as two or more toes of the injured foot gripping the bar and facilitating movement to another bar without slipping, and the total number of steps with the injured foot were counted during the allotted time period. Animals were required to walk at least 20 steps during the allotted observation time in order to account for variability in both the activity level and motility of various animals.

Measurement of Evoked Motor Responses

Twelve weeks postoperatively, sciatic nerve function was assessed by examining the evoked motor response in reinnervated EDL muscle upon electrical stimulation of the sciatic nerve. Prior to testing, animals were re-anesthetized and the distal segment of the EDL muscle was surgically isolated. Animals were immobilized in a custom-designed force measurement jig and the distal tendons of the EDL muscle were connected to a 5 N load cell (S100; Strain Measurement Devices, Inc., Meriden, CT). Cathodic, monophasic electrical impulses (duration = 200 μs, frequency = single–200 Hz, burst width = 300 ms, amplitude = 0–3 V) were then applied to the sciatic nerve proximal to the regenerated nerve segment via silver wire electrodes while resulting force production in the EDL was recorded using custom Matlab software.

Twitch contractions measured using the custom force recording system were utilized to determine the optimal stimulus amplitude (V_o) and optimal muscle length (L_o) for isometric force production in the EDL muscle. All subsequent isometric force measurements were made at V_o and L_o. Single twitch contractions were recorded, and peak twitch force (F_t) was calculated. Tetanic contractions were recorded at increasing frequencies (5–200 Hz) of stimulation; allowing 2 min intervals between stimuli to preserve muscle recovery. Maximum isometric tetanic force (F_o) was subsequently calculated from the resulting sets of recorded force traces.

Physiological cross-sectional area (PCSA) of the EDL muscle was calculated using the following equation (Urbanchek et al., 1999):

$$\mathrm{PCSA} = \frac{M \times \cos\theta}{(\rho)(L_o)(0.44)} \quad (1)$$

where PCSA is the physiological muscle cross-sectional area (cm^2), M the EDL muscle mass (g), $\cos\theta$ the angle of connected EDL muscle relative to load cell (0°), ρ the density of mammalian skeletal muscle (1.06 g/cm^3), L_o the optimal muscle length (cm), 0.44 = ratio of fiber length to muscle length (L_f/L_m) in rat EDL muscle. Maximum specific isometric force was calculated as the maximum isometric force normalized to muscle PCSA. Values were reported relative to specific isometric forces measured in healthy, Unoperated (naive). Following assessment both denervated/reinnervated and unoperative EDL muscles were harvested and weighed. Muscle mass recorded for denervated/reinnervated EDL muscles was normalized to that of unoperative EDL muscles to quantify the relative degree of muscle atrophy.

Retrograde Labeling of Spinal Motor Neurons

Twelve weeks postoperatively, the surgical site was reopened under general anesthesia. The sciatic nerve was then transected 5 mm distal to the implanted conduit or isograft and the proximal nerve stump was immediately placed in a silicone well containing 4% Fluorogold in sterile saline for 1 h. The silicone wells and Fluorogol solutions were removed, incisions were closed, and animals were recovered as described previously. Contralateral sciatic nerves were labeled in a similar manner. Ten days following the procedure animals were euthanized and perfused in 4% paraformaldehyde prior to harvesting the lumbar region (L3–L6) of the spinal cord for frozen sectioning. Twenty micron thick axial sections of the lumbar spinal cord were obtained on a cryostat. The number of labeled cell bodies within the ventral horn of the control and experimental sides of each spinal cord section was counted using an optical dissector technique facilitated by MicroBrite Field stereology software (MBF Bioscience StereoInvestigator version 7.0, Williston, VT) using a 10× objective (NA = 0.30, 100× overall magnification). Count estimates were accepted if the Gundersen coefficient of error was less than 0.08. Values were reported as the percentage of cell bodies labeled on the experimental side relative to the contralateral, uninjured side.

Statistical Analysis

All results are reported as mean ± standard error of the mean. All "n" values represent the number of animals analyzed within each group. Only animals demonstrating positive nerve regeneration (presence of a regenerating nerve cable within the NGC or isograft upon harvest) were included for analysis. Statistical analyses were performed using Statistica version 6 (Statsoft, Inc., Tulsa, OK). All data were evaluated for differences between groups using the analysis of variance (ANOVA) with post hoc LSD tests with Bonferroni correction with significance set at $\alpha = 0.05$ ($P < 0.05$).

Results

Nerve Guidance Conduit Harvest

The effectiveness of NGF or GDNF delivery in promoting nerve regeneration across a critical nerve gap was evaluated in vivo after sciatic nerve transection and NGC implantation (effectiveness is the number of animals with a neural structure spanning the 13 mm gap, also called positive nerve

regeneration; see Table I). After 12 weeks, groups with the DS and either growth factor resulted in a more effective nerve regeneration (>50%) while groups without both the DS and growth factor were less effective (<50%). All animals (100%) in the isograft group demonstrated effectiveness (positive nerve regeneration). The regenerated nerves in the NGF DS and GDNF DS groups exhibited a larger, more robust nerve cable compared to other conduit groups by gross observation. Nerve cables were centered compactly within the NGC away from the walls, in all conduit groups. Furthermore, all nerve cables observed in conduit groups demonstrated intact connections to the proximal and distal segments of the sciatic nerve.

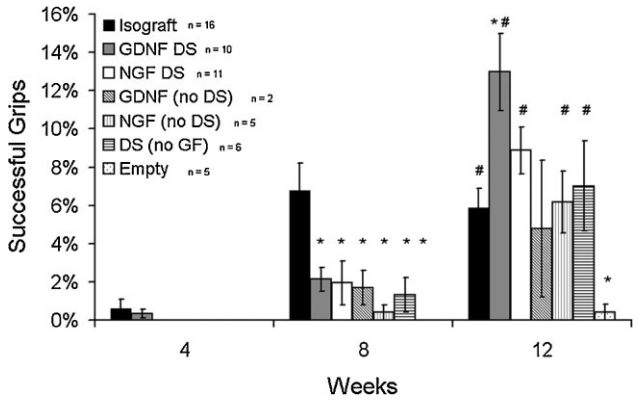

Figure 2. Successful grid-grip percentages for groups with nerve regeneration 12 weeks after injury. Animals were placed on a wire mesh to assess the number of times the animal successful placed its toes to grip the wire mesh without slipping and the total number of steps taken with the injured foot. The isograft had improved recovery compared to all groups at 8 weeks; however, GDNF with the delivery system (GDNF DS) had more successful grid-grips compared to the isograft at 12 weeks. All other experimental groups performed equal to the isograft except the empty conduit group at 12 weeks. Data represent mean ± SEM, * indicates statistical significance ($P < 0.05$) compared to the isograft, and # indicates statistical significance ($P < 0.05$) compared to empty.

Behavioral Testing

Functional recovery was serially assessed in all animals using non-invasive behavioral testing over a period of 12 weeks. Only animals demonstrating positive nerve regeneration at the time of harvest were included in the behavioral analysis, which included animals pooled from both portions of the study. The average preoperative SFI score was -7.3 ± 1.1, demonstrating normal nerve function prior to injury. Subsequent SFI scores did not reveal any differences between experimental groups at 4, 8, or 12 weeks after injury, as SFI scores did not differ between weeks (data not shown). Toe and ankle contractures (reactive distortions in the toes and ankles) were observed in some animals at 8 and 12 weeks after injury, precluding accurate assessment of locomotor function through walking track analysis. However, animals without contractures showed no difference in SFI scores at 4, 8, or 12 weeks after injury or between experimental groups.

Animals were assessed using a grid-grip test every 4 weeks postoperatively to measure gradual changes in motor behavior. As with SFI measurements, only animals demonstrating positive nerve regeneration were included in the post hoc analysis, although all animals underwent behavioral testing. Preoperatively all groups demonstrated $79 \pm 13\%$ successful grid-grips. Animals tested but later found to lack positive nerve regeneration (a neural structure in the conduit) did not exhibit any improvement in the grid-grip task (<1% regardless of group). Four weeks after injury, all experimental groups demonstrated poor grid-gripping ability (Fig. 2). At 8 weeks, animals in the isograft group demonstrated improved functional recovery compared to all other groups, suggesting earlier reinnervation of distal musculature. No other groups were different at 8 weeks. However, at 12 weeks animals in the GDNF DS group demonstrated superior functional recovery compared to the isograft group and all other experimental groups except the NGF DS group. GDNF (no DS), NGF (no DS), and NGF DS were all similar to one another. All experimental groups, except the empty conduit group, were equivalent to the isograft group at 12 weeks in regard to successful grid-grip percentages. These observations suggest that isografts promote more rapid functional recovery, while NGCs containing the GDNF DS treatment facilitate superior functional recovery in long-term settings.

Evoked Motor Response

Exposed sciatic nerve was stimulated proximal to implanted isografts and NGCs in order to assess functional nerve regeneration and reinnervation of distal musculature. The number of animals with positive nerve regeneration tested in this portion of the study is summarized in Table I. Measurements of force production in the EDL muscle were normalized to the cross-sectional area of the muscle to calculate specific force production, a measure of the functional capacity of reinnervated muscle independent of muscle mass. Electrical stimulation of uninjured nerve was found to elicit specific twitch and tetanic forces of 5.0 ± 0.48 and $16 \pm 1.6\ N/cm^2$, respectively. No experimental group was found to be equivalent to healthy EDL muscle in either measure.

All groups except those employing NGCs containing GDNF produced similar specific twitch and tetanic forces compared to isograft controls, including groups utilizing NGCs without growth factor (Empty and DS) (Fig. 3). However, this effect may be due to the high degree of variance observed in groups without growth factor and low number of animals with positive nerve regeneration, which diminished statistical power for comparison (only animals with positive regeneration were included). Both groups employing NGCs containing GDNF demonstrated increased specific twitch and tetanic force measurements compared to isograft, empty NGC (Empty), NGC without growth factor

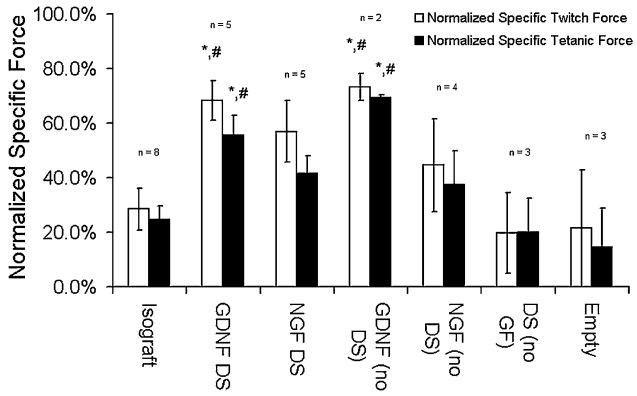

Figure 3. Normalized specific EDL force measurements for groups with nerve regeneration 12 weeks after injury. EDL muscles were stimulated proximal to the graft or conduit to the produce maximal twitch and tetanic muscle force, which were divided into the muscle cross-sectional area for specific force. These values were normalized to uninjured, normal EDL muscle-specific forces. All groups, except GDNF groups, produced similar specific forces compared to the isograft. Specific twitch and tetanic forces increased for GDNF groups, compared to empty and DS (no GF) groups and the isograft. Data represent mean ± SEM, * indicates statistical significance ($P < 0.05$) compared to the isograft, and # indicates statistical significance ($P < 0.05$) compared to empty or DS (no GF) groups.

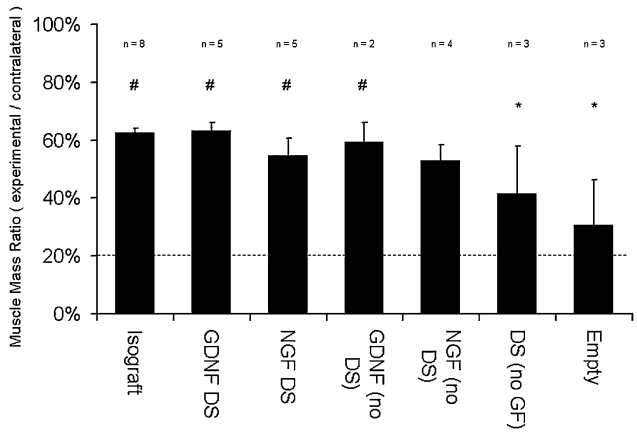

Figure 4. Relative muscle mass of EDL for groups with nerve regeneration 12 weeks after injury. EDL muscles (experimental and contralateral sides) were harvested and weighed, and the experimental muscle mass normalized to the contralateral mass. Groups with growth factor were similar to the isograft in relative muscle mass. Data represent mean ± SEM, dotted line indicates relative muscle mass with no neural regeneration, * indicates statistical significance ($P < 0.05$) compared to the isograft, and # indicates statistical significance ($P < 0.05$) compared to empty.

(DS) groups. There was no difference between the GDNF DS and GDNF (no DS) groups. The NGF DS and NGF (no DS) groups were equivalent to the isograft as well as both GDNF groups and did not differ from one another. It is of note that the GDNF groups were different from the isograft group, while the NGF groups were not. Overall, these results suggest that the presence of GDNF within NGCs promotes superior muscle reinnervation and increased functional recovery following nerve injury.

EDL muscles were harvested and weighed following assessment of evoked motor responses to assess the level of muscle atrophy during denervation. Animals with no neural regeneration had increased levels of muscle atrophy. Groups with NGCs containing growth factor demonstrated decreased levels of muscle atrophy similar to the isograft groups. In contrast, groups employing NGCs without growth factor demonstrated increased muscle atrophy compared to isograft controls (Fig. 4). There were no differences between DS and no DS growth factor groups for GDNF and NGF. These observations suggest that growth factor delivery from NGCs generally reduces the degree of muscle atrophy experienced in distal musculature.

Retrograde Labeling

Experimental and uninjured sciatic nerves were labeled using a Fluorogold solution as a means of quantifying the number of surviving motor neurons in the ventral horn (see Table I for animal numbers). Retrograde labeling was selected as the primary histological measure of nerve regeneration due to the fact that histomorphometric nerve fiber counts do not allow discrimination between regenerating motor and sensory nerve fibers and are commonly biased by large-scale collateral sprouting. Within this portion of the study neither NGF (no DS) nor GDNF (no DS) groups had more than one animal with positive nerve regeneration. As a result, these groups were omitted from further analysis as statistics measures could not be performed.

Examination of stained motor neurons in acquired frozen sections revealed no differences in cell size, diameter, or anatomical location within the spinal cord between experimental groups (Fig. 5). Staining of healthy, uninjured sciatic nerve demonstrated 3,200 ± 180 labeled ventral horn neurons. GDNF DS and NGF DS groups were comparable to isograft controls in the percentage of ventral horn neurons labeled; all of which demonstrated greater neuron counts than groups employing empty NGCs or DS alone (Fig. 6). Overall, these results suggest that local *affinity-based* growth factor delivery improves motor nerve survival and regeneration.

Discussion

This study investigated the effect of growth factor delivery using an affinity-based delivery on motor-specific nerve regeneration and functional recovery. Release of growth factors by diffusion-based delivery systems has previously been shown to promote motor-specific nerve regeneration in vivo (Fine et al., 2002). We evaluated an affinity-based DS, using non-covalent interactions to sequester and slow the release of growth factor prior to cell-mediated release, to assess whether affinity-based release can be used to promote

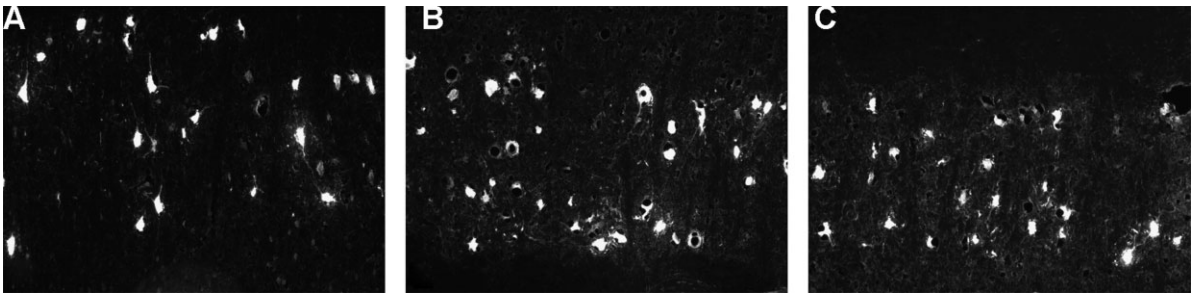

Figure 5. Representative sections of retrograde labeled ventral horn SC neurons 12 weeks after injury. The experimental and contralateral sciatic nerves were labeled with 4% Fluorogold to stain motor (ventral horn SC) neurons. No qualitative differences in cell sizes within the SC (**A**–**C**) between experimental groups were observed between isograft (A), GDNF DS (B), or NGF DS (C) groups. Scale bar represents 200 μm.

motor-specific nerve regeneration. Growth factor release from fibrin matrices can be regulated by cell-mediated processes such as neurite outgrowth-induced protease activation (e.g., plasminogen to plasmin) (Alvarez-Buylla and Valinsky, 1985; Herbert et al., 1996; Kalderon, 1984; Krystosek and Seeds, 1984; Pittman and Buettner, 1989; Pittman et al., 1989). Yet, long-term in vivo studies of the efficacy of such affinity-based delivery systems have yet to be accomplished. Therefore, the present study was designed to examine whether local delivery of different neurotrophic factors via an affinity-based DS could stimulate motor nerve regeneration following peripheral nerve injury.

Regardless of the growth factor used, NGCs containing the DS loaded with growth factor promoted nerve regeneration across the 13 mm nerve gap in more than 50% of animals (see Table I). These results mirror previous observations that controlled release increases the effectiveness of nerve regeneration (Barras et al., 2002; Dodla and Bellamkonda, 2008; Fine et al., 2002). Previous studies conducted in our laboratory further corroborate the positive effect of the DS on nerve regeneration in vivo, as the presence of the DS and growth factor within the fibrin matrix increased neurite extension in primary neuronal cultures compared to free growth factor with no DS (Sakiyama-Elbert and Hubbell, 2000a; Wood and Sakiyama-Elbert, 2008; Wood et al., 2009a). One possible explanation of this effect may be that, in the presence of the DS, the initial burst of growth factor from the conduit is greatly reduced. In vitro studies conducted in our laboratory confirm this hypothesis, in that an initial burst of growth factors observed from fibrin matrices without DS was dramatically reduced upon the addition of the DS (Wood et al., 2009a; Wood and Sakiyama-Elbert, 2008). In vivo, this reduction in the initial burst of growth factor from the conduit would minimize the detrimental effect of supra-physiologic levels of neurotrophic factors on nerve regeneration (Barras et al., 2002). Reducing the initial burst release from implanted conduits would also increase the amount of growth factor present in the system during infiltration of cells and axons, which may require sustained neurotrophic support.

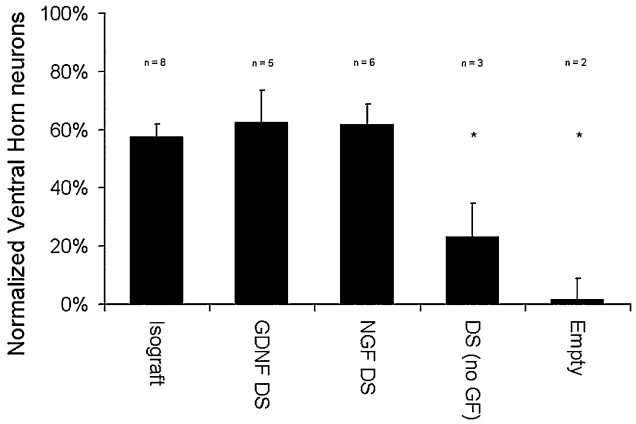

Figure 6. Normalized ventral horn SC neurons retrograde labeled 12 weeks after injury. The experimental and contralateral sciatic nerves were labeled distal to the conduit or graft with 4% Fluorogold to stain regenerating motor (ventral horn SC) neurons. Spinal cords (ventral horn region) were harvested 10 days after labeling, and 20 μm sections were evaluated for total cell numbers using stereological techniques. Cell numbers were normalized to the contralateral cell counts for each animal. GDNF DS and NGF DS were comparable to the isograft in percentages of normalized ventral horn neurons. Additionally, these three groups contained more labeled ventral horn neurons compared to empty conduit groups. Data represent mean ± SEM where all cell counts were normalized to the contralateral side, and * indicates statistical significance ($P < 0.05$) compared to the isograft.

Behavioral analyses were performed to determine whether differences in motor recovery between groups translated to improvements in function. No differences in behavioral recovery were observed between groups using SFI scores due to the prevalence of ankle contractures, observed previously in multiple studies (Hare et al., 1992, 1993). Conversely, grid-grip analysis, unaffected by contracture formation, demonstrated that groups employing fibrin-filled NGCs were functionally equivalent to isograft groups in terms of behavioral recovery. The improvement in grid-grip observed in groups with fibrin-filled NGCs could result from the presence of a permissive substrate for cell adhesion, which is missing in the empty conduit. Fibrin has previously been demonstrated to support nerve regeneration within

NGCs (Galla et al., 2004; Lee et al., 2003; Marcol et al., 2005), and can promote cell adhesion and ingrowth as a result of its numerous binding sites for integrins (Thiagarajan et al., 1996) and SCs.

Earlier behavioral recovery was noted in the isograft group compared to all NGC groups, as the isograft had a higher grid-grip percentage 8 weeks postoperatively. However, the GDNF DS group demonstrated improved behavioral recovery compared to the isograft group after 12 weeks. The faster rate of recovery observed in the isograft group may be due to the presence of a greater number of regenerating nerve fibers compared to NGCs at earlier time points (Wood et al., 2009b). Early increases in nerve fibers have been demonstrated to positively correlate with improved function (Aydin et al., 2004; Lien et al., 2008). However, NGCs containing GDNF and DS supported a greater number of larger (5–7 μm), mature nerve fibers than isografts (Wood et al., 2009b), which may correlate with the superior functional recovery observed in the GDNF DS group at the terminal time point (Fraher and Dockery, 1998; Williams and Wendell-Smith, 1971).

Measurements of specific force production in EDL muscle generally supported the results of the grid-grip analysis, as NGCs containing growth factor were found to promote levels of motor recovery comparable to nerve isografts. Simultaneously, groups employing NGCs containing growth factor (except NGF without DS) demonstrated decreased muscle atrophy compared to groups employing NGCs without growth factor. Taken together, these results indicate that controlled release of neurotrophic factors from NGCs generally increases muscle reinnervation and prevents atrophy. Empty NGCs and DS NGCs also demonstrated levels of specific force production equivalent to nerve isograft. This result is likely due to a high degree of spontaneous nerve regeneration and variation in regeneration between animals (Mackinnon et al., 1985). Together, the high biological variance and low effectiveness exhibited by negative controls in this injury model tended to result in poor statistical comparisons.

Significantly, groups employing NGCs containing GDNF, with or without DS, elicited increased recovery of specific twitch and tetanic force compared to isograft groups or NGC groups without growth factor. This observation further suggests that controlled GDNF release at the site of nerve injury facilitates superior muscle reinnervation and function. GDNF is upregulated in SCs in the distal nerve stump and skeletal muscle after injury (Nagano and Suzuki, 2003; Zhao et al., 2004) and regulates presynaptic differentiation and neuromuscular junction connections (Nagano and Suzuki, 2003; Yang and Nelson, 2004). Exogenous delivery of GDNF from NGCs at the site of injury may therefore amplify the existing endogenous mechanism of motor nerve regeneration, resulting in the improved functional reinnervation of distal musculature. This hypothesis may explain why NGCs loaded with GDNF, with and without DS, enabled increased specific twitch and tetanic force compared to isograft controls. Equivalent motor neuron counts for GDNF DS and isograft groups further suggest that the increase in functional recovery may be a result of more effective reinnervation of motor targets by motor nerve fibers regenerating through NGCs in the presence of GDNF.

In contrast, NGF DS groups were not anticipated to demonstrate similar levels of functional recovery and motor neuron regeneration compared to isograft groups due to the absence of the high-affinity NGF receptor, TrkA, on motor neurons. Previous studies using NGCs to deliver neurotrophic factors specific to motor neurons, such as GDNF, have demonstrated increased motor axon counts (Barras et al., 2002; Fine et al., 2002) compared to other growth factors, such as NGF and NT-3. In the present study no differences were observed in retrograde labeled motor neuron counts between GDNF or NGF delivered with the DS despite differences in functional recovery. This discrepancy may be the result of the inclusion of a fibrin matrix within the lumen of NGCs. Delivery of growth factors from this luminal material may yield different outcomes compared to diffusion-based delivery from the wall of NGCs, utilized in previous studies. Furthermore, it is hypothesized that the inclusion of fibrin matrices with DS and either growth factor may act to enhance SC migration into NGCs, thus indirectly stimulating axonal regeneration. Previous studies support the positive role of local SCs in nerve regeneration, which are largely responsible for the increased regenerative potential of nerve isografts (Whitlock et al., 2009). Additionally, SCs have been noted to express receptors for both NGF (Anton et al., 1994; Taniuchi et al., 1988; Tomita et al., 2007) and GDNF (Iwase et al., 2005), providing a viable basis for this mechanism of cell migration, proliferation (Anton et al., 1994), and remyelination (Iwase et al., 2005; Tomita et al., 2007). Overall, this theory would suggest that while both GDNF and NGF stimulate SC migration and support, resulting in significant nerve regeneration and recovery, only GDNF encourages both SC activation and specific regeneration of motor axons, resulting in superior functional outcomes compared to nerve isograft postoperatively. Silicone conduits serve as a useful model to study nerve regeneration, but clinically have associated morbidities (Battiston et al., 2005; Dellon, 1994; Merle et al., 1989). The combination of our DS with a biodegradable conduit would be more desirable for clinical use. Based on the positive functional outcomes observed in our study, work directed toward the controlled delivery of GDNF for motor nerve injuries may be beneficial in translating better functional outcomes into clinical practice.

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