SHORT REPORT

GDNF RELEASED FROM MICROSPHERES ENHANCES NERVE REGENERATION AFTER DELAYED REPAIR

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ABSTRACT: *Introduction*: Delays in surgical repair following nerve transection produce progressively inferior motor nerve regeneration. Regeneration can be improved with delivery of exogenous growth factor. We developed a delivery system that could be applied at the nerve repair site to deliver growth factors locally to regenerating nerve. *Methods*: Poly(lactic-co-glycolic acid) microspheres containing glial-derived neurotrophic factor (GDNF) suspended within fibrin were developed into a delivery system for local application surrounding nerve at a repair site in an experimental rat model. *Results*: The system containing GDNF remained at the injury site for up to 2 weeks and improved motor nerve regeneration following chronic axotomy and denervation. *Conclusions*: Based on the positive outcome of the delivery system, we plan to study the delivery system over longer time courses of release and nerve regeneration.

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Immediately following nerve injury, the expression of neurotrophic factors, such as glial cell-line derived neurotrophic factor (GDNF), and their receptors are transiently upregulated in Schwann cells and neurons before returning to basal levels.[1] Delays in surgical repair of peripheral nerve injuries lead to states of chronic axotomy, in which neurons are no longer connected to their end-organ targets, and chronic denervation, in which Schwann cells in the distal stump are no longer in contact with axonal processes. Both states progressively reduce the capability of axons to regenerate.[2,3] Reduced expression of neurotrophic factors and their receptors is in part responsible for poor nerve regeneration in delayed nerve repair.[4]

Local extended delivery of GDNF using osmotic mini-pumps has been shown to promote axonal regeneration of motoneurons following chronic axotomy.[5] Alternatively, microspheres that have demonstrated the ability to release drug from days to months[6] may serve as a possible alternative to osmotic pumps. We report the development of a delivery system applied at the nerve repair site that can deliver growth factors locally to regenerating nerve. This delivery system was constructed by loading microspheres within fibrin glue, which allowed the delivery system to be applied locally at the nerve repair site. Furthermore, this delivery system was stable at the repair site for at least 2 weeks and was capable of delivering GDNF to enhance motor nerve regeneration following delayed nerve repair.

Abbreviations: CF, common fibular; DS, delivery system; GDNF, glial-derived neurotrophic factor; PLGA, Poly(lactic-co-glycolic acid)

Key words: chronic axotomy, chronic denervation, common fibular nerve, drug delivery, fibrin, nerve injury

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MATERIALS AND METHODS

Microsphere Characterization and Delivery System (DS) Construction. Poly(lactic-co-glycolic acid) (PLGA) (50/50;inherent viscosity 0.15-0.25 dl/g; Lactel Absorbable Polymers, Cupertino, CA) microspheres were prepared as previously described[7] with and without GDNF (Peprotech, Rocky Hill, NJ). Microsphere size was characterized using static light scattering. The delivery system was constructed by mixing 5 mg of microspheres (containing 1.8 ± 0.2 μg GDNF) with 80 μl of fibrin, formed by the manufacturer's instructions from equal parts fibrinogen (75-115 mg/ml) and thrombin (500 IU/ml) (Tisseel®, Baxter Healthcare, Deerfield, IL). The time course of release was measured by incubating the delivery system in 1 ml of phosphate buffered saline (PBS) at 37°C. Each day the PBS was collected and replaced. Enzyme-linked immunosorbent assays were performed to measure the biological activity of GDNF collected from the time course release studies according to the manufacturer's instructions (R&D systems, Minneapolis, MN). GDNF release was measured after 1 and 7 days.

***In Vivo* Delayed Nerve Repair Model.** Thirty-six Sprague-Dawley rats (n=6 per group) underwent common fibular (CF) nerve transection and repair with or without chronic axotomy and denervation (delayed repair) following institutional animal care

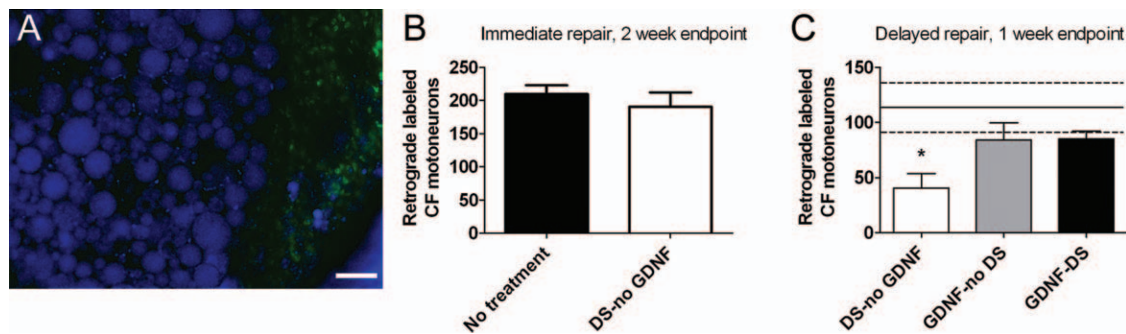

FIGURE 1. Visualization and nerve regeneration following application of the delivery system (DS). (**A**) Photomicrograph of microspheres containing glial-derived neurotrophic factor (GDNF) (blue) that were mixed with fibrin to construct the delivery system (DS) that was localized at the nerve (green) for at least 2 weeks. Scale bar = 100 μm. (**B**) The DS without GDNF (DS-no GDNF) was implanted on the nerve repair site immediately following nerve injury with epineural suture repair and did not impair motor nerve regeneration compared with repair without the DS (No treatment) 2 weeks following nerve repair. (**C**) The DS was placed on the nerve repair site following delayed epineural suture of the nerve ends after a 2-month period of chronic axotomy and denervation. GDNF treatment, with (GDNF-DS; black bar) or without microspheres (GDNF-no DS; gray bar), resulted in motor nerve regeneration similar to immediately repaired nerve (solid line in figure; $P < 0.05$) 1 week following nerve repair, while lack of GDNF (white bar) resulted in inferior motor nerve regeneration. Mean ± SEM shown where *indicates $P < 0.05$ compared with immediately repaired nerve (mean shown with a solid line and SEM shown with dotted lines in C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

guidelines. The CF nerve was exposed and transected near the sciatic trifurcation. In the delayed repair rats the CF stumps were sutured back to surrounding muscle for 2 months. CF nerves following delayed nerve repair were first "freshened" by resecting ~1 mm from both nerve ends before repair using 2 10-0 nylon sutures followed by applicable treatment. The freshening of the nerve ends resulted in small but minimal tension, and immediate nerve repairs were not resected back before repair. The nerve was surrounded by 1 of the following treatments at the suture site: fibrin loaded with microspheres containing GDNF (GDNF-DS; group I), fibrin loaded with microspheres without GDNF (no GDNF-DS; groups II & V), or fibrin loaded with GDNF but no microspheres (GDNF-no DS; containing 1.8 μg free GDNF; group III). Additionally, a control group received no fibrin, GDNF, or microspheres and was repaired without delay (no treatment; groups IV & VI).

A 4% solution of FluoroGold (Sigma-Aldrich, St. Louis, MO) was applied to the transected CF nerve 10 mm distal to the injury site 2 weeks following immediate nerve repair in experiments (groups V-VI; Fig. 1B) and 1 week after delayed nerve repair (groups I-IV; Fig. 1C) to count motoneurons that regenerated their axons. One and 2 weeks were chosen to (1) ensure that the delivery system vehicle of GDNF was not toxic (2 weeks after immediate repair) and (2) to evaluate the effect of the GDNF after 1 week of delivery when GDNF release was measured. Seven days after FluoroGold application, the rats were perfused with saline and 4% paraformaldehyde. Tissues were harvested for cryosectioning as 50 μm sagittal sections of spinal cord and 10 μm nerve cross-sections. Nerve specimens were stained with neurofilament (Sigma-Aldrich; 1:250 dilution) and GDNF (Peprotech; 1:500) primary antibodies followed by Alexa Fluor secondary antibodies (Invitrogen, Burlington, ON; 1:500) using standard immunohistochemistry techniques.

Means with standard error of the mean are reported. Statistical analysis was evaluated using analysis of variance (ANOVA) with *post hoc* tests including Bonferroni correction for determining differences between groups where $P < 0.05$ was considered statistically significant.

RESULTS

The PLGA microspheres constructed for use in the delivery system demonstrated a moderately high GDNF encapsulation efficiency of 72 ± 8% (i.e., the percentage of GDNF successfully encapsulated during construction), with a microsphere diameter of 47 ± 10 μm (large enough to avoid internalization by macrophages).[8] The fully constructed delivery system (GDNF microspheres loaded in fibrin) released 0.22 ± 0.09 μg and 0.74 ± 0.04 μg cumulative GDNF to PBS by 1 and 7 days, respectively.

In the *in vivo* study, implanted GDNF microspheres remained at the nerve repair site for at least 2 weeks (Fig. 1A). Following immediate nerve repair (no chronic axotomy and denervation), placement of the delivery system (DS) without GDNF (DS-no GDNF) at the repair site was not detrimental to motor nerve regeneration as evidenced by the similar retrograde labeled counts of motoneurons between groups with and without the delivery system (no treatment) 2 weeks following immediate nerve repair (Fig. 1B).

Following chronic axotomy and denervation, motor axon regeneration was significantly increased by GDNF treatment with (GDNF-DS) or without microspheres (GDNF-no DS) to levels equal to that of immediate nerve repair as illustrated by the data obtained 1 week following repair (Fig. 1C). Omitting GDNF (DS-no GDNF) or omitting the delivery system (no treatment; data not shown) decreased the number of motoneurons regenerating their axons by ∼100% compared with the immediate nerve repair group ($P < 0.05$).

DISCUSSION

In this study, we demonstrated that microspheres and fibrin can be constructed into an implantable delivery system for localized drug delivery at a nerve repair site. We also chose to assess early nerve regeneration well before any functional improvement would be expected to first establish the feasibility of the delivery system. Fibrin glue has previously been used to successfully deliver growth factors such as nerve growth factor (NGF) and GDNF to regenerating axons following transection injury.[9] We determined that the delivery of GDNF from fibrin alone (GDNF-no DS) or from microspheres (GDNF-DS) were equally effective in promoting early motor nerve regeneration (1 week) following delayed nerve repair. However, free GDNF suspended in fibrin is released by 7 days *in vivo*.[10,11] Therefore, for extended release of growth factors, a material, such as the polymer microspheres used here, will be needed to extend the time course of release. In the future, we plan to design microspheres capable of releasing growth factors within fibrin glue for periods of weeks to design a delivery system that can provide growth factors longer than fibrin glue with free growth factors alone. These microspheres will allow us to determine the contribution of extended GDNF delivery to recovery by measuring nerve regeneration after several weeks to months. At later time points we will also be able to measure isometric tetanic contractile muscle force and other metrics of functional recovery.

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