ABSTRACT: Nerve root compression produces persistent behavioral sensitivity in models of painful neck injury. This study utilized degradable poly(ethylene glycol) hydrogels to deliver glial cell line-derived neurotrophic factor (GDNF) to an injured nerve root. Hydrogels delivered >98% of encapsulated GDNF over 7 days in an in vitro release assay without the presence of neurons and produced enhanced outgrowth of processes in cortical neural cell primary cultures. The efficacy of a GDNF hydrogel placed on the root immediately after injury was assessed in a rat pain model of C7 dorsal root compression. Control groups included painful injury followed by: (1) vehicle hydrogel treatment (no GDNF), (2) a bolus injection of GDNF, or (3) no treatment. After injury, mechanical allodynia (n=6/group) was significantly decreased with GDNF delivered by the hydrogel compared to the three injury control groups (p<0.03). The bolus GDNF treatment did not reduce allodynia at any time point. The GDNF receptor (GFRα-1) decreased in small, nociceptive neurons of the affected dorsal root ganglion, suggesting a decrease in receptor expression following injury. GDNF receptor immunoreactivity was significantly greater in these neurons following GDNF hydrogel treatment relative to GDNF bolus treated and untreated rats (p<0.05). These data suggest efficacy for degradable hydrogel delivery of GDNF and support this treatment approach for nerve root-mediated pain. © 2008 Orthopaedic Research Society.

Keywords: radiculopathy; neurotrophic factor; GDNF; hydrogel; allodynia

Chronic neck pain affects as many as 71% of adults at some point during their lives.1,2 Painful cervical spine injuries can result from nonphysiologic loading of the neck as occurs in recreational accidents and contact sports,3,4 when nerve roots can be compressed.5 Nerve root compression induces persistent behavioral hypersensitivity in rat models of radiculopathy, in which painful responses are elicited in the affected dermatome by stimulation that does not normally provoke pain (mechanical allodynia).3–5 Further, hypersensitivity to a stimulus has been used as a sensitive clinical indicator of pain.10 Compression of primary afferent neurons also produces increased neuronal excitability, ectopic axonal firing, Wallerian degeneration, endoneurial edema, inflammatory responses, and decreased spinal substance P.7,8,11–16

Current treatments for neuropathic pain include opioids, nonsteroidal anti-inflammatory agents, antagonists to ion channels, neuropeptides, cytokines, and trophic factors to promote cell survival and regeneration.17–23 Neurotrophic factors can prevent secondary neuronal degeneration and reduce spontaneous firing. In particular, glial cell line-derived neurotrophic factor (GDNF) has analgesic effects and modulates nociceptive signaling by altering sodium channel subtype expression and reducing aberrant A-fiber sprouting into the cord.18,19,24–26 However, in neuropathic pain models, GDNF is decreased after injury which may initiate nociceptive mechanisms.19,27 GDNF also upregulates somatostatin, directly opposing the nociceptive action of substance P.24,26,28 GDNF is a member of the TGF-β superfamily and binds the GDNF family receptor (GFRα-1), initiating an intracellular MAP kinase cascade that enhances neuronal survival via inhibition of apoptosis proteins.20 Continuous GDNF delivery prevents behavioral and electrophysiological abnormalities in neuropathic pain and partially reverses increased GFRα-1 in large dorsal root ganglion (DRG) neurons if administered by an osmotic minipump.18,29 However, implantation of osmotic minipumps,18,22 repeated injections,30 or gene therapy31 all have inherent clinical limitations.

The delivery of neurotrophic factors from degradable polymers, such as hydrogels, obviates clinical issues, and may provide significant analgesia compared to an equivalent dosing in a single injection treatment. A variety of studies have utilized hydrogel matrices for tissue engineering and drug delivery,32–34 but few have applied trophic factor release from hydrogels in an in vivo model of neuronal injury.35 Degradable hydrogels can be designed for a range of release profiles, based on crosslinking density, susceptibility to degradation, and hydrophilicity.36,37 Degradable poly(ethylene glycol) (PEG) has been used to deliver neurotrophins and improve neurite outgrowth from retinal explants.38 Trophic factor delivery in vivo to injured neural tissue significantly increased fiber sprouting and motor recovery for many hydrogels and trophic factor systems, including PEG.35,39–41 However, no study has compared behavioral hypersensitivity following neural injury for controlled release of GDNF from a hydrogel system versus a single injection of an equivalent quantity of GDNF.

In our model of dorsal root compression, transient loading of the root produces behavioral hypersensitivity that persists for 7 days.15,42 In other pain studies, neural compression reduces GDNF-immunoreactivity in the DRG,19,27 induces axonal degeneration and macrophage infiltration in the dorsal root, and significantly decreases spinal neuropeptides.43 To our knowledge, no study has
investigated controlled release of GDNF from degradable PEG hydrogels for reducing behavioral hypersensitivity and restoring GDNF-immunoreactivity in the DRG following painful dorsal root injury.

MATERIALS AND METHODS

Hydrogel Formulation and GDNF Bioassay

In vitro assays established the temporal release and bioactivity of degradable PEG-encapsulated GDNF prior to in vivo implantation. The hydrogel was formed from a macromer of acrylated polyactic acid and PEG (PLA-b-PEG-b-PLA, Polysciences, Warrington, PA). The macromer was fabricated from 4Da PEG (Sigma, St. Louis, MO) capped with 1-4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, I2959, Ciba Specialty Chemicals, Basal, Switzerland) was prepared. For polymerization, GDNF was suspended in the polymer solution at the desired concentration (250 µg/ml for determination of release profile, 5 µg/ml). The solution (20 µl) was pipetted into a cylindrical mold and exposed to ultraviolet light for 5 min using a long-wave ultraviolet lamp (FT85BB, Topbulb, East Chicago, IN).

To characterize the release profile, hydrogels (n = 3) were everted into Eppendorf tubes containing 1 ml PBS and placed in a 37°C circulator with gentle agitation. On days 1, 2, 4, 7, and 15, PBS containing released GDNF was sampled and stored at −20°C; tubes containing the hydrogels were refilled with fresh PBS, until complete gel degradation. GDNF content of each PBS sample was determined by ELISA (R&D Systems, Minneapolis, MN).

To establish the biological activity of the released GDNF, primary cultures of dissociated embryonic rat (E18) cortical cells were incubated with hydrogels containing GDNF. Cortical cells were plated at a density of 10,000 cells/well in neurobasal media containing Penicillin-Streptomycin (Invitrogen, Carlsbad, CA). At 48 h, media was exchanged, and hydrogels containing 100 ng of GDNF were suspended in triplicate wells. Control wells containing no hydrogels, hydrogels containing no GDNF, or 50 ng of free GDNF in the media were also analyzed in triplicate. At 72 h after adding GDNF, 200 × images were taken of each well at five randomly spaced regions (334 × 422 µm) using an inverted light microscope. Images (n = 15/group) were analyzed using ImageJ to determine total cell counts and percentage of cells with processes. At 7 days, new images were analyzed to assess cell count, number of cells containing at least one process, average number of processes per cell, average process length, and longest process length.

In Vivo Hydrogel Behavioral Studies

In vivo studies were performed using male Holtzman rats (250–350 g) (Harlan Sprague-Dawley, Indianapolis, IN). Rats were housed with a 12–12 h light–dark cycle and had free access to food and water. All surgical procedures were performed under isofluorane inhalation anesthesia and were approved by the Institutional Animal Care and Use Committee. A C6/C7 hemilaminectomy and facetectomy exposed the C7 nerve roots on the right side, and a 100-nM microvascular clip was applied to the dorsal root midway between the DRG and the dorsal root entry zone. After compression for 15 min, one of the following treatments was applied directly to the injured root: (1) 20 µl hydrogel containing 5 µg GDNF (250 µg/ml GDNF gel; n = 6); (2) 20 µl vehicle hydrogel containing PBS (vehicle gel; n = 6); (3) 20 µl bolus of PBS containing 5 µg GDNF (250 µg/ml GDNF bolus; n = 6); or (4) no treatment (sham; n = 6). The incision was closed with sutures and surgical staples.

Rats were evaluated for bilateral forepaw mechanical allodynia on postoperative days 1, 3, 5, and 7. Preoperatively, baseline measurements of paw withdrawal responses were recorded on consecutive days. A single blinded tester performed all allodynia testing. For each session, after 20 min of acclimation to the environment, rats were stimulated on the plantar surface of each forepaw using von Frey filaments (1.4, 2, 4 g) (Stoelting, Wood Dale, IL). Each session consisted of three rounds of 10 stimulations, 10 min apart. The number of withdrawal responses was recorded for each filament after each round. Allodynia, determined by an increase in withdrawals over baseline, was averaged by group on each day.

Assessment of GDNF and GFRα-1 in the DRG

Rats were euthanized on day 7 by an overdose of sodium pentobarbital (40 mg/kg) and transcardially perfused with 200 ml of PBS followed by 200 ml of 4% paraformaldehyde in PBS. The C7 ipsilateral and contralateral DRGs were harvested and placed in 4% paraformaldehyde for 1 h followed by 50% ethanol overnight. Tissue was dehydrated in graded ethanols and embedded in paraffin for longitudinal sectioning at 10 µm. DRG sections were labeled for GDNF and the GFRα-1 receptor by immunohistochemistry using polyclonal antibodies against GDNF (1:100; Santa Cruz, CA) and GFRα-1 (1:100; Neuromics, Bloomington, MN). Horse anti-goat or goat anti-rabbit secondary antibodies (Vector, Burlingame, CA) were used at a dilution of 1:200. All antibody dilutions were previously optimized. Sections were exposed to 3,3-diaminobenzidine for color development (Vector) and cover-slipped using a nonaqueous mounting medium. Immunostained sections were imaged at 200 ×. Neuron populations were identified by cross sectional area; small neurons (<600 µm²) in the DRG were identified as a measure of nociceptive neurons, and large neurons (>600 µm²) were taken as mechanceptive/proprioceptive. The number of small and large neurons positive for each of GDNF or GFRα-1 was reported as a percentage of all small or large neurons in each DRG.

Data and Statistical Analyses

Cell counts and process length measurements from the primary neural cell cultures were averaged across the wells for each group and analyzed by one-way ANOVA with post-hoc Bonferroni correction. Paw withdrawal frequencies were compared using a two-way ANOVA with repeated measures to determine significant effects of treatment over time, followed by a one-way ANOVA with post-hoc Bonferroni correction for pairwise comparisons between experimental and control groups on each day. Differences in small and large GDNF- or GFRα-1-positive neurons among groups were compared by one-way ANOVA with post-hoc Bonferroni correction. Statistical analyses were performed using SYSTAT v10.2 (SYSTAT, Richmond, CA) with data presented as mean ± SD and significance at p < 0.05.

RESULTS

GDNF Release Profile and Bioassay

Over 15 days, degradation of the lactic acid units in the PLA-b-PEG-b-PLA macromer allowed the encapsulated GDNF to be released from the hydrogel, at which
time the hydrogels were fully degraded (Fig. 1). The hydrolytic nature of degradation allowed for an assessment of GDNF release in a cell- and degradative enzyme-free system, thereby eliminating cell binding or enzymatic degradation as possible sources of measurement error. After 24 h, 57.8% ± 3.0% of the GDNF was released. Hydrogels were nearly completely degraded within 7 days, releasing 98.2% ± 1.7% of the GDNF (Fig. 1).

Addition of hydrogel-encapsulated GDNF to primary cortical cell cultures increased the cell count and outgrowth of processes at day 7 (Figs. 2 and 3A–C). Also, after 72 h, free GDNF and GDNF released from the hydrogel similarly increased the cell numbers and percentage of cells with processes over wells incubated without GDNF, indicating no significant loss of bioactivity from hydrogel polymerization (Fig. 3D). The number of viable neural cells, average process lengths, and lengths of the longest process were all significantly increased ($p < 0.043$) in cultures incubated with a GDNF hydrogel compared to those with no hydrogel (Fig. 3A–C). No significant increases occurred for wells containing gels with no GDNF (Fig. 3). Cultures incubated with the GDNF hydrogel averaged 15.1 ± 9.2 cortical cells per image (Fig. 3A), with 40.8% containing at least one process. Average process length was 74.8 ± 19.6 μm (Fig. 3B), the longest being 106.5 ± 74.4 μm (Fig. 3C). Cultures incubated without a hydrogel had 7.5 ± 5.0 cells, with only 25.8% containing at least one process. Processes averaged 40.8 ± 7.6 μm, the longest being 43.4 ± 37.2 μm. No significant differences were observed in the number of cells containing processes or the average number of processes per cell among any of the groups.

**Behavioral Studies**

Hydrogel-encapsulated GDNF treatment to the compressed root significantly reduced mechanical allodynia (Fig. 4). Qualitative trends in allodynia were similar and most robust for the 4-g filament; as such, responses to the 4-g filament are reported. For all groups receiving an injury except the GDNF gel group, allodynia in the ipsilateral forepaw was significantly greater than sham for 7 days ($p < 0.05$; Fig. 4A). Allodynia in the GDNF gel group was evident on day 1, but did not remain elevated above sham and significantly decreased relative to both injury and vehicle gel by day 5 ($p < 0.03$). Allodynia for the GDNF gel group was also significantly reduced relative to the GDNF bolus group by days 5 and 7 ($p < 0.03$; Fig. 4A).

Contralateral mechanical allodynia was produced by day 7 for all groups except the GDNF gel group (Fig. 4B). Contralateral allodynia in this latter group was significantly reduced relative to both the injury and vehicle gel groups ($p < 0.045$). GDNF treatment with the sham procedures did not alter ipsilateral or contralateral allodynia from baseline at any time point.

**GDNF and GFR-1 in the DRG**

Root compression did not significantly affect the number or size distribution of DRG neurons. GDNF-immunoreactivity in ipsilateral DRG neurons was not significantly affected for any treatment group at day 7. *Sham* rats exhibited GDNF-immunoreactivity in 9.2% ± 3.3% of the small and 25.9% ± 4.1% of the large neurons, which were not significantly different than GDNF-immunoreactivity in normal rats (Table 1). Four tissue samples from the *injury* group were unusable for analysis; accordingly, additional matched samples were generated by the same injury procedure ($n = 4$ rats) to provide a complete data set. Those rats exhibited similar mechanical allodynia (data not shown) to those in the *injury* group. *Vehicle gel* and *injury* rats displayed
GDNF-immunoreactivity in 5.1% ± 1.0% and 5.8% ± 1.8% of the small neurons and 20.6% ± 5.5% and 13.8% ± 6.7% of large neurons, respectively, a moderate decrease relative to sham, GDNF gel, and GDNF bolus (Table 1). No differences in GDNF were detected in the contralateral DRG among any groups.

GFRα-1-immunoreactivity decreased significantly in small DRG neurons in the absence of a GDNF hydrogel (Fig. 5A). In small neurons of the ipsilateral DRG, GFRα-1-immunoreactivity was not significantly decreased for the GDNF gel group relative to sham. Yet, for vehicle gel rats, GFRα-1-immunoreactivity in small neurons (8.14% ± 3.2%) was significantly decreased relative to the GDNF gel group (16.0% ± 2.8%; p < 0.006). Additionally, for injury rats, GFRα-1-immunoreactivity in small neurons (7.4% ± 3.5%) was significantly decreased relative to the GDNF gel group (p < 0.001). The GDNF bolus group (10.1% ± 3.8%) had significantly fewer GFRα-1-positive small neurons than the GDNF gel group (p < 0.05; Fig. 5A). Compression decreased GFRα-1-immunoreactivity in the contralateral DRG when untreated, but this trend was not significant (Fig. 5B).

GDNF REDUCES NERVE ROOT-MEDIATED PAIN

GFRα-1 was moderately increased in large neurons of the ipsilateral DRG for the injury group (21.7% ± 5.1%) compared to the GDNF gel (16.4% ± 4.4%) group, and was significantly increased compared to sham (13.5% ± 2.5%) (Fig. 5C). No trends in GFRα-1 were observed among groups in the contralateral DRG (Fig. 5D).

DISCUSSION

Controlled release of GDNF from degradable PEG hydrogels following compression of the cervical dorsal root reduced behavioral hypersensitivity and prevented decreases in GFRα-1 in small neurons of the ipsilateral DRG (Figs. 4 and 5). GDNF release is controlled by degradation and diffusion mechanisms that can be altered through hydrogel design (e.g., number of lactic acid units, macromer concentration). Osmotic minipump delivery rates as high as 12 μg/day did not produce toxic side effects18,22, so the release profile was optimized to deliver about half of the encapsulated GDNF (<3 μg) within the 1st day, corresponding to the largest decrease in GDNF in the DRG (unpublished data) and the most robust behavioral hypersensitivity.15 Over
98% of encapsulated GDNF was released by day 7 in the in vitro assay, a time point relevant for chronic behavioral symptoms and degenerative pathology in this model.8,9,42 When treated with the 10 wt% degradable PEG hydrogel containing 5 μg GDNF, the allodynia normally observed following root compression was significantly attenuated and did not differ from sham after day 1. GDNF-immunoreactivity in the DRG was not significantly decreased following dorsal root compression (Table 1). Exogenous GDNF internalization by large and small DRG neurons may have increased slightly following GDNF application by bolus injection or hydrogel, yet this trend was not significant at day 7. GDNF-immunoreactivity was expected to increase in the DRG with application of exogenous GDNF;47 however, the measured GDNF-immunoreactivity could not be specifically determined to be from cellular internalization or changes in endogenous expression. Also, after binding the GFRz-1 receptor, GDNF might have been metabolized and cleared from the DRG rather than becoming internalized by DRG neurons. In contrast, GFRz-1-immunoreactivity was significantly decreased in small DRG neurons and increased in large DRG neurons following injury, with no significant loss of small or large neurons. Continuous GDNF administration via the hydrogel maintained GFRz-1 expression at normal levels in the ipsilateral DRG (Fig. 5A, C). These results suggest that a decrease in GFRz-1 expression in small neurons and an increase in large neurons may facilitate aberrant neuronal behavior causing radicular pain, and that preventing these changes may decrease behavioral hypersensitivity.18 A decrease in GFRz-1 expression in small, nociceptive neurons reduces the binding of GDNF-GFRz-1 to Ret, which subsequently decreases the action of the inhibitor of apoptosis proteins.20 Therefore, aberrant neuronal firing in nociceptive neurons and increased pain sensitivity18,24 may result from decreased cell viability due to decreased GFRz-1 expression. We also identified an increase in GFRz-1 expression in large DRG neurons that was prevented by continuous GDNF delivery, a phenomenon also observed after sciatic nerve transection.29 Together, these studies suggest that continuous application of exogenous GDNF may reduce allodynia by promoting enhanced receptor expression and GDNF binding in small neurons that likely mediate nociception and by preventing increased expression in large, normally nonnociceptive neurons. Additional

### Table 1. GDNF Immunoreactivity in the Ipsilateral DRG

<table>
<thead>
<tr>
<th></th>
<th>Small Neurons (%) (GDNF-Positive)</th>
<th>Large Neurons (%) (GDNF-Positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8.0 ± 3.4</td>
<td>33.2 ± 9.7</td>
</tr>
<tr>
<td>Sham</td>
<td>9.2 ± 3.3</td>
<td>25.9 ± 4.1</td>
</tr>
<tr>
<td>GDNF gel</td>
<td>12.4 ± 7.5</td>
<td>29.3 ± 15.2</td>
</tr>
<tr>
<td>GDNF bolus</td>
<td>10.3 ± 3.9</td>
<td>27.6 ± 8.7</td>
</tr>
<tr>
<td>Vehicle gel</td>
<td>5.1 ± 1.0</td>
<td>20.6 ± 5.5</td>
</tr>
<tr>
<td>Injury</td>
<td>5.8 ± 1.8</td>
<td>13.8 ± 6.7</td>
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*Mean ± SD.

Figure 5. GFRz-1 immunohistochemistry in the (A,C) ipsilateral and (B,D) contralateral DRG, reported as a percent of total (A,B) small (<600 mm²) or (C,D) large (>600 mm²) neurons (+SD). The percent of immunopositive neurons in normal rat DRGs is indicated by the horizontal dashed line. (A) In the ipsilateral DRG, the percent of GFRz-1-positive small neurons was significantly reduced (+) for the GDNF bolus, vehicle gel, and injury groups relative to the GDNF gel group. GFRz-1-immunoreactivity small neurons of the vehicle gel and injury groups was also significantly decreased (*) relative to sham. (C) GFRz-1-immunoreactivity in large neurons significantly increased (*) for the injury group relative to sham.
Mechanical allodynia and GFRα-1 immunoreactivity for the GDNF bolus group was significantly different from the GDNF gel group in small neurons of the ipsilateral DRG, suggesting that continuous GDNF delivery is necessary to maintain its analgesic effect. Given the short half-life of GDNF in vivo and rapid diffusion into adjacent tissues, hydrogel delivery allows GDNF to remain at the injury site during hydrogel degradation, whereas even high doses administered by bolus injection are quickly cleared or metabolized. For example, while the burst release of ~3 μg of GDNF from a hydrogel over the 1st day provides continuous dosing, a 5 μg bolus injection provides twice the dosage within minutes. Furthermore, bolus injection poses the risk of toxic side effects such as focal cell loss, pia thickening, Schwann cell hyperplasia, and ingrowth of sympathetic fibers resulting from a high dose of local delivery. Repeated administration of lower doses could replicate mechanical allodynia produced by the sustained delivery of a hydrogel or minipump. While repeated injections would more clearly define temporal profiles in vivo, the hydrogel delivery provides simplified treatment with alterable release kinetics eliminating multiple treatments or implantation of minipumps. Hydrogels can be delivered with light-initiated photopolymerization or through two-component redox initiating systems, where gelation occurs in vivo after the two initiators are mixed, avoiding the need for surgical implantation. This is important when light cannot reach the injury site. Degradable PEG hydrogels have been used to deliver neurotrophic factors to stimulate neurite growth and restore motor function in spinal cord injury models without toxic effects but, to our knowledge, this study is the first to use the controlled release of GDNF to alleviate nerve root-mediated pain.

Although allodynia was significantly reduced by GDNF, early allodynia was unaffected. Continuous GDNF delivery via osmotic minipumps reduces behavioral hypersensitivity as early as day 1. This disparity may be explained by differences in delivery and dosing paradigms. In our study, 5 μg of GDNF was released over 7 days, with less than 3 μg delivered in the 1st day. Boucher et al. delivered 12 μg per day continuously at a rate of 0.5 μg/h. This fourfold increase over the 1st day may have been sufficient to reduce early hypersensitivity, while the cumulative effect in our study did not reduce allodynia until the 3rd day. Cell numbers and process formation in cells incubated in free GDNF or hydrogel-encapsulated GDNF were comparable (Fig. 3D), indicating biological activity of encapsulated GDNF over 1 week. However, in vitro bioactivity evaluation must be interpreted as an estimated comparison because of the continuous release of GDNF from hydrogels over time. The release profile and biological activity of GDNF determined in vitro may not match in vivo release kinetics and activity. Nonetheless, hydrogel delivery substantially reduced bilateral behavioral sensitivity (Fig. 4). The improved analgesia following hydrogel delivery compared to a bolus injection further suggests efficacy of this method. Future studies involving repeated GDNF injections at different doses may indirectly provide insight into the in vivo release kinetics of GDNF.

In summary, controlled GDNF release from degradable hydrogels following transient compression of the cervical dorsal root alleviated bilateral behavioral hypersensitivity and prevented GFRα-1 receptor depletion in the DRG. Despite no evidence of altered GDNF-immunoreactivity in the DRG 7 days following injury or treatment, increased GFRα-1 expression in large neurons and decreased GFRα-1 expression in nociceptive primary afferents may have been responsible for the pain symptoms. This shift in receptor expression may have decreased the analgesic potential of GDNF, which was reversed by administration of excess exogenous GDNF from a degradable hydrogel. Controlled GDNF release from degradable PEG hydrogels in this radiculopathy model proved effective as a simple technique to deliver trophic factors continuously and directly to the injury site. These data suggest that hydrogel delivery provides significant trophic support to damaged primary afferents and is a promising treatment modality for nerve root compression-mediated pain.

ACKNOWLEDGMENTS

We thank Dr. Meaney and his lab for neuron cultures, and recognize funding from NIH (#AR047564), NSF, and the Sharpe and Ashton Foundations.

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126

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