Glial Cell Line–Derived Neurotrophic Factor Gene Transfer Exerts Protective Effect on Axons in Sciatic Nerve Following Constriction-Induced Peripheral Nerve Injury

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Abstract
Damage to peripheral nerves following trauma or neurodegenerative diseases often results in various sensory and motor abnormalities and chronic neuropathic pain. The loss of neurotrophic factor support has been proposed to contribute to the development of peripheral neuropathy. The main objective of this study was to investigate the protective effect of glial cell line–derived neurotrophic factor (GDNF) using peripheral gene delivery in a rat model of constriction-induced peripheral nerve injury. In this study, it was shown that mechanical and thermal hypersensitivity increased on the injured limb at day 7 after chronic constrictive injury (CCI) was induced. The neurological changes were correlated with the structural changes and loss of GDNF/Akt signaling, particularly in the distal stump of the injured sciatic nerve. Subsequently, recombinant adenovirus was employed to evaluate the potential of intramuscular GDNF gene delivery to alleviate the CCI-induced nerve degeneration and neuropathic pain. After CCI for 3 days, intramuscular injection of adenovirus encoding GDNF (Ad-GDNF) restored the protein level and activity of GDNF/Akt signaling pathway in the sciatic nerve. This was associated with an improved myelination profile and behavioral outcomes in animals with CCI. In conclusion, the present study demonstrates the involvement of GDNF loss in the pathogenesis of CCI-induced neuropathic pain and the therapeutic potential of intramuscular GDNF gene delivery for the treatment of peripheral nerve degeneration.

Introduction
Injuries to peripheral nerves frequently result in considerable disabilities. In the extremities, such lesions may lead to the loss of sensory and motor functions. These effects can be severely debilitating, affecting individuals’ daily lives and resulting in many implications socially. Individuals often develop chronic neuropathic pain, which further exacerbates the negative impacts. Posttraumatic nerve therapy continues to be a major challenge in restorative medicine and reconstructive surgery. Despite many advances made in microsurgical techniques over the last 30 years, functional recovery after severe lesions of major nerve trunks often remain incomplete and unsatisfactory (Rochkind et al., 2007).

Many studies have demonstrated the clinical value of applying neurotrophic factors to peripheral nerves to encourage regeneration (Terenghi, 1999; Gordon, 2009). Several reports suggest that exogenous neurotrophic factors promote axonal growth in distal nerve stumps found in crush and transection injuries, and within nerve grafts and conduits used in the reconstruction of damaged nerves (Boyd and Gordon, 2003). Glial cell line–derived neurotrophic factor (GDNF), a member of the transforming growth factor β (TGF-β) superfamily, is perhaps the most potent neurotrophic factor influencing the development, survival, and maintenance of neurons in the central and peripheral nervous systems (Lin et al., 1993; Oppenheim et al., 1995; Ramer et al., 2000; Boyd and Gordon, 2003).
neurotrophic effects of GDNF are mediated by a multi-subunit receptor system consisting of the glycosyl-phosphatidylinositol–linked GDNF ligand-binding co-receptor, GFRα1; the transmembrane proto-oncogene, Ret; and neuronal adhesion molecule (Airaksinen and Saarma, 2002; Faratacha et al., 2003). It is known that nerve injury results in dysregulation of peptides, receptors, and channels, including a down-regulation of GDNF (Nagano et al., 2003). The continuous administration of GDNF by osmotic pump or gene delivery has been shown to promote the regeneration of sensory axons in the dorsal root entry zone and ameliorate neuropathic pain states in different animal models with nerve injury (Boucher et al., 2000; Ramer et al., 2000; Hao et al., 2003). More recently, GDNF has also been applied to the treatment of various types of neurodegenerative diseases including Parkinson’s disease (Gill et al., 2003; Eslamboli 2005) and amyotrophic lateral sclerosis (Manabe et al., 2002; Wang et al., 2002b) with promising results.

Our previous studies showed that GDNF gene transfer confers protection to neuronal cells and synaptic networks, thereby alleviating the paraplegia due to spinal ischemia and injury in rat models (Tai et al., 2003; Chou et al., 2005). Recently, we also demonstrated the therapeutic effect of peripheral GDNF gene delivery by intramuscular (IM) injection in animal models with diabetic neuropathy (Liu et al., 2009). In the present study, we began by defining chronologically the molecular events and behavioral changes that occur after inducing chronic constrictive injury (CCI) in a rodent model of peripheral nerve injury. This was followed by an attempt to evaluate the therapeutic effect of GDNF by administering it with adenoviral vectors via the IM route and measuring its outcome using immunohistological and molecular methods.

Materials and Methods

CCI animal model

All protocols were approved by the Animal Care and Use Committee at Kaohsiung Veterans General Hospital, Taiwan. Young adult male Sprague-Dawley rats (140 to 160 g at the time of surgery) were fed with standard lab rodent chow and water ad libitum and housed individually. The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally), and the left common sciatic nerve was cut into two segments from the region of ligation: the uninjured contralateral sciatic nerve in each animal was used as the control.

Measurement of size distribution of myelinated axons was performed by toluidine blue staining as recently described (Mazzer et al., 2008). Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and perfused through the left cardiac ventricle with PBS, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Both sciatic nerves were removed and a 5-mm section of each nerve (10 mm distal to the injury site) was processed for semi-thin (1 μm) cross-sections. All transverse sections of the sciatic nerve were stained with 1% toluidine blue and their images were projected by camera lucida directly onto a digitizing tablet on which the myelinated axons were quantified. Data were expressed as the average percentage of myelinated axons of different sizes.
FIG. 1. The chronological changes in behavioral assessments and morphology of nerve damage following chronic constrictive injury (CCI) in rats. (A) Prominent reductions in the mechanical withdrawal threshold and mean latency of paw withdrawal were noted in the sciatic nerve ligation. (B) Following the CCI, photomicrograph of permanganate-fixed sciatic nerve sections stained with toluidine blue in proximal and distal stump of sciatic nerve. (C, D) Fiber size distribution and numbers of myelinated axon fibers were counted and expressed as mean ± SEM from different high power fields. Scale bar, 100 μm. *p < 0.05, **p < 0.01.
Immunoblot analysis

The protein extract was isolated from tissues using buffer containing 150 mM NaCl, 50 mM HEPES pH 7, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, and complete protease inhibitors (Roche Applied Science, Indianapolis, IN). After separation on 12.5% SDS-PAGE, proteins were transferred onto a polyvinylidene fluoride membrane by using blotting apparatus. The membrane was blocked with 5% milk in TBS-T for 1 hr then incubated with GDNF (1:500 dilutions; R&D Systems Inc., Minneapolis, MN), P0 (1:500 dilutions; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and Neurofilament (1:500 dilutions; DAKO Inc., Carpinteria, CA), Akt (1:500 dilutions; Cell Signaling Technology Inc., Danvers, MA), and pAkt antibodies (1:500 dilutions; Cell Signaling Technology Inc.) for 1 hr at room temperature. After incubation with secondary antibody conjugated with horseradish peroxidase (1:5000 dilutions in 5% milk) for 1 hr, the blots were developed with chemiluminescence reagents and exposed to X-ray film for visualization.

FIG. 2. The effect of CCI on the density of Schwann cells and axon myelination in the axon fibers of sciatic nerve. After CCI at 7, 14, and 28 days, the density of Schwann cells around the myelinated axon fibers in proximal and distal stump of sciatic nerve were examined by S-100 (green) immunostaining. After CCI at 7, 14, and 28 days, the axonal demyelination and damage in the proximal and distal stump of sciatic nerve were analyzed by (A) myelin basic protein (P0) (red) and (B) neurofilament (NF; green) immunostaining and (C) Western blot analysis. (D) Quantitative analysis of Western blots is expressed as mean ± SEM. Scale bar, 20 μm. *p < 0.05, **p < 0.01. Scale bar, 20 μm. Color images available online at www.liebertonline.com/hum
30 min, the signals on membrane were detected using ECL-plus luminol solution (Pharmacia, Piscataway, NJ) and exposed to X-ray film for autoradiogram.

**Immunofluorescence analysis**

The transversal frozen sections (5 μm) of sciatic nerves were dried and incubated in blocking buffer containing 1.5% normal goat serum and 0.2% Triton X-100 in PBS. The slides were washed twice with PBS, incubated with the primary antibodies including GDNF (1:100 dilutions; Santa Cruz Biotechnology Inc.), GFRα1 (1:100 dilution; R&D Systems Inc.), S-100 (1:200 dilution; DAKO Inc.), P0 (1:100 dilution; Santa Cruz Biotechnology Inc.), and neurofilament antibody (1:200 dilution; DAKO Inc.) at 4°C overnight, followed by repeated washing with PBS, and replaced in secondary antibodies conjugated with Alexa 488 or Alexa 546 (1:1000 dilution; Invitrogen, Carlsbad, VA) for 1 hr at room temperature. The immunostained slides were observed and recorded under a confocal microscope (LSMPASCAL, Carl Zeiss Inc., Mannheim, Germany).

**Statistical analysis**

Comparisons within groups were made by using one-way analysis of variance (ANOVA). The comparisons across groups were accomplished with one-way ANOVA and, if significant, discrete comparisons were accomplished using post hoc tests. A p value of less than 0.05 was considered statistically significant. Data were expressed as mean ± SEM.
Results

Behavioral changes and axonal degeneration in the sciatic nerve following constriction injury

CCI of the sciatic nerve was used to induce chronic neuropathic pain in the hind paws of the rats. In the preliminary studies conducted, the paw withdrawal threshold in control (uninjured) animals in response to stimulation with von Frey filaments were 10.16 and 11.22 g on the left and right side of the paws, respectively. The latencies of withdrawal to heat stimulation in the control rats were 29.9 and 28.4 sec on the left and right side of the paws, respectively. These assessments were repeatedly conducted over a period of 28 days and there were no significant deviations from the initial values obtained.

In the rats induced with CCI in the unilateral limb, the threshold of paw withdrawal in response to mechanical or heat stimulus began to decrease on the injured side (left), but not the control side (sham-operated, right) after the operation. Threshold of both tests were observed to decrease significantly at day 7, 14, 21, and 28 after the CCI procedure (Fig. 1A, \(p < 0.001\)).

The proximal and distal stumps of the sciatic nerves in rats at various time intervals after CCI were examined by morphological analysis using toludine blue staining of the axon fibers in semi-thin sections. The morphologies of the axons in the control groups were consistent throughout different time points, with similar distribution of small- and large-diameter myelinated and unmyelinated nerve fibers and a regular proportion between myelin sheath thickness and fiber diameter. On the injured side, the nerve fibers showed signs of degeneration, as manifested by the predominance of smaller diameter nerve fibers at day 7, 14, and 28, in both the proximal and distal stump of the sciatic nerve, compared with the control side (Fig. 1B, C; \(n = 6\)). There was also a significant decrease in the number of axon fibers in both proximal and distal stump of the sciatic nerve in the injured side, compared with the control side (Fig. 1D; \(n = 6\)).

Loss of myelination and increased axonal fragmentation in the sciatic nerve following constriction injury

To investigate the profile of myelination and number of Schwann cells (SCs) following CCI, we evaluated the number of SCs in both proximal and distal stumps of the sciatic nerves in CCI rats with S-100 immunostaining. In both the proximal and distal stumps of the sciatic nerve, the S-100 immunostaining revealed that the loss of SCs around the nerve fibers occurred at day 7, 14, and 28 (Fig. 2A; \(n = 6\)). Furthermore, myelin basic protein (P0) and neurofilament (NF) immunostaining of transverse sections showed diminished thickness of the myelin sheath in the proximal stump of the sciatic nerve at day 7, 14, and 28 (Fig. 2B; \(n = 6\)). In the distal stump of sciatic nerve, there was significant reduction in myelin sheath thickness in the axon fibers. Additionally, axonal fragmentation was noted in the injured nerve accompanied by infiltration of Ox-42-positive microglia and GFAP-positive astrocytes (Supplementary Fig. S2). Western blot analysis similarly showed a decrease in P0 and NF expression in the distal part of injured nerves (Fig. 2C, D).
Deficiency of GDNF signaling complex in the sciatic nerve following constriction injury

To investigate whether GDNF signaling pathway is implicated in CCI, level of GDNF and its receptor, GFRα1, in the sciatic nerve were analyzed by Western blot analysis at day 7, 14, and 28 on the injured side, compared with the control side. At day 7, which is when nerve structural abnormalities that correlated with behavioral changes were initially detected, the expression of GDNF and its receptor (GFRα1) protein level in the sciatic nerve were significantly decreased in the injured side compared with the control side in the distal stump of the sciatic nerve (Fig. 3A; n = 6). Similar results were shown using ELISA and immunofluorescence analysis at day 28 (Fig. 3B, C).

The expression and phosphorylation of Akt, a downstream effector of GDNF signaling pathway, were investigated to analyze the influence of peripheral nerve injury on this pathway. It was shown that the extent of Akt phosphorylation, but not its protein level, was significantly decreased in the sciatic nerve at day 7, 14, and 28 in the distal stump of the sciatic nerve (Fig. 3D).

GDNF gene delivery restores GDNF/Akt signaling and improves mechanical allodynia and thermal sensitivity following constriction injury

Following a peripheral nerve injury, damaged neurons and DRG show a drastic dysregulation of their peptides, growth factors, and receptors. The studies above indicated that a deficiency in the GDNF signaling complex was involved in CCI-induced nerve degeneration. Our previous study showed IM GDNF gene delivery increased GDNF expression in both the muscle and in the circulation, which also led to the restoration of GDNF level in the sciatic nerve of diabetic rats by retrograde transport (Liu et al., 2009). Therefore, we investigated whether the muscle-produced GDNF could be transported to the sciatic nerve of CCI rats.

The animals were injected with adenoviral vectors via the muscle route at day 7 after the CCI procedure, when the pain behavior was established. GDNF expression in muscles was determined by immunohistochemistry and immunoblot analyses, which showed elevated GDNF expression only in Ad-GDNF-injected muscles (Supplementary Fig. S3). Besides, ED-1 immunostaining showed that there was no notable inflammation around the injection site. By using Western blot analysis, it was found that the Ad-GDNF–treated rats exhibited a significant increase in GDNF content when compared with the Ad-GFP–treated rats in both the proximal and distal stump of sciatic nerve at day 28 (Fig. 4A). In addition, the restored GDNF level in the proximal stump of the injured sciatic nerve of Ad-GDNF–treated CCI rats approximated the normal range found in uninjured sciatic nerve. GDNF gene delivery also significantly enhanced the Akt phosphorylation in the sciatic nerves as shown by Western blot analysis on distal stump of sciatic nerve in CCI rats (Fig. 4B). These results showed that IM GDNF gene delivery significantly restored the GDNF level and maintained GDNF/Akt signaling pathway in the peripheral nerves even at 28 days after CCI.

We subsequently evaluated the effect of GDNF gene delivery on the recovery of responses to both the mechanical and thermal stimuli. Rats were injected IM with adenovirus vectors at day 7 after the CCI procedure. Mechanical and thermal stimulation assessments were performed at various time points after treatment up to 28 days. Sham-operated rats showed no significant difference from baseline thresholds in both assessments after the surgery. However, in Ad-GDNF–treated rats, the animals exhibited significantly reduced mechanical sensitivity in the ipsilateral paws compared with the Ad-GFP–treated rats (7.63 ± 1.15 g in the Ad-GDNF–treated group versus 1.65 ± 0.84 g in the Ad-GFP–treated groups at day 28; Fig. 5A; p < 0.01). Significant improvement in thermal hyperalgesia was also observed in the Ad-GDNF–treated rats (20.89 ± 3.29 sec in the Ad-GDNF–treated group versus 11.65 ± 1.88 sec in the Ad-GFP–treated groups at day 28; Fig. 5B; p < 0.01). Therefore, we showed that the peripheral GDNF gene delivery improves mechanical allodynia and thermal hypersensitivity of CCI rats.

GDNF gene delivery attenuates the demyelination of nerve fibers and axonal fragmentation following constriction injury in rats

To elucidate whether GDNF gene delivery also confers protection against demyelination and axon loss after constriction-injury insults, we examined the extent of myelination...
of axon fibers in the sciatic nerve of rats with constriction injury after GDNF gene delivery. It was observed that Ad-GDNF–treated rats had significantly more large-diameter myelinated nerves in both proximal and distal stumps of the sciatic nerve compared with the sham-operated or Ad-GFP–treated rats at day 28 after CCI (Fig. 6A, B, n = 6; p < 0.01). In addition, it was found that there were significantly more myelinated axons in the sciatic nerves of Ad-GDNF–treated rats compared with those of the Ad-GFP–treated rats at day 28 after CCI (Fig. 6C; p < 0.01), indicating that degeneration of myelinated axons and the loss of axons were ameliorated by GDNF gene delivery.

Finally, we examined whether GDNF gene delivery alleviated demyelination by examining the profile of SCs and the level of basic myelin protein following constriction-injury insult. The extent of myelination in the sciatic nerves of different groups of CCI rats at 28 days was studied using S-100 and P0 immunostaining. It was found that IM GDNF gene delivery significantly attenuated the depletion of basic myelin protein of the nerve fibers and the loss of SCs in both the proximal and distal stumps of the sciatic nerves in CCI rats, compared with those of Ad-GFP–treated rats (Fig. 7A). In the distal stump of sciatic nerve, IM GDNF gene delivery also significantly alleviated axonal fragmentation (Fig. 7A), supported by Western blot analysis (Fig. 7B). These results indicate that GDNF transported from the transduced muscle cells exerts the neurotrophic effect to reduce demyelination and axonal loss of the peripheral nerves following constriction injury in rats.

**Discussion**

**Summary and novel findings of this study**

The present study chronologically studied the structural and functional changes in the peripheral nerves following constriction injury in rats, thereby confirming that changes in behavioral assessment (increased thermal and mechanical sensitivity) correlates with histological changes of nerve damage, which include demyelination and axonal degeneration, following the induction of nerve injury in a CCI animal model. It was observed through immunofluorescence staining that the distal stumps were severely damaged, with both SC demyelination and axonal degeneration occurring simultaneously. In contrast, in the proximal stumps, only SC demyelination was observed. Finally, we showed that IM GDNF gene delivery was effective in restoring GDNF contents, and hence, the downstream Akt signaling pathway, an important pro-survival signal for nerves. These, in turn, prevent the loss of SCs and axon fibers in peripheral nerves after constriction-injury insults. Most importantly, histological improvements were accompanied by a significant improvement in behavioral assessment.

**Role of GDNF for preventing SC demyelination and axonal degeneration in peripheral nerve injury**

In this study, we observed that GDNF gene delivery significantly increased the number of myelinated axons as well as the total number of axon fibers. The GDNF-mediated
effect on preventing the loss of SC myelination and reduction of axonal degeneration was further confirmed by P0, NF, and S-100 immunostaining in proximal and distal stumps of sciatic nerve. Previous studies showed that GDNF is rapidly up-regulated in denervated SCs after sciatic nerve injury (Hoke et al., 2002) and undergoes retrograde transport by motor neurons (Yan et al., 1995). Our results showed that the loss of SCs around the axon fibers was reversed by GDNF gene delivery in peripheral nerve injury. This may be due to the direct effect of GDNF on SCs, which also express GDNF receptors (Paratcha et al., 2003; Hase et al., 2005) since the application of GDNF causes SC proliferation and myelination of nerve fibers (Hoke et al., 2003). In the study by Hoke et al. (2003), it was shown that exogenous GDNF not only increased myelination, but it also increased the total myelinated axon numbers. Therefore, we cannot exclude the possibility that the increase in myelinated axons shown in our study was caused by the increase in total myelinated axon number by GDNF gene delivery.

Long-term continuous exogenous GDNF treatment significantly increased the number of motor neurons through regeneration of their axons by axonal sprouting in the distal nerve stump (Boyd and Gordon, 2003). Therefore, it is possible that, following CCI and IM GDNF gene delivery, GDNF may under retrograde transport along damaged axons to the responsive neurons to exert a direct neurotrophic effect. This, in turn, rescues injured neurons or increases the number of neurons that regenerate or the sprouting of their axons. In addition to recruiting more axons through regeneration and/or sprouting, GDNF gene delivery also increased the caliber of axon fibers. At morphometric analysis, GDNF gene delivery was shown to induce a shift from smaller axon diameter to larger ones, as compared with the sham-operated or Ad-GFP-treated group. Previous studies demonstrated that increased axon diameter is a determining factor for the initiation of myelination (Voyvodic 1989). Increased numbers of myelinated axons in GDNF-treated animals may be a result of either caliber increase of existing axon fibers or the recruitment of additional large-caliber axons. Our experiments did not allow differentiating between these two possibilities.

Role of GDNF for treatment of neuropathic pain due to peripheral nerve injury

In the present study, we demonstrated that IM injection of Ad-GDNF effectively alleviated the demyelination and
axonal degeneration in peripheral nerve injury, thereby reducing the duration of neuropathic pain in an animal model with pre-existing nerve injury. Apart from the neuroprotective mechanism, GDNF gene delivery may relieve the neuropathic pain by promoting the release of inhibitory peptide, somatostatin, in the dorsal horn (Charbel Issa et al., 2001), potentiating transmitter release and modulating synaptic function (Wang et al., 2001, 2002a), altering the expression of sodium channel subunits in sensory neurons (Boucher et al., 2000; Cummins et al., 2000), or regulating anti-nociceptive molecules and their receptors (Ogun-Muyiwa et al., 1999; Malcangio et al., 2000). Exogenous administration of GDNF or gene delivery by lentivirus or herpes simplex virus has previously been shown to be successful in treating neuropathic pain due to CCI (Hao et al., 2003; Pezet et al., 2006; Sakai et al., 2008). Together, these findings provide a rational basis for gene delivery of neurotrophic factor as a therapeutic alternative for treatment of neuropathic pain due to peripheral nerve injury.

Advantages and limitation of peripheral GDNF gene therapy

Gene transfer offers one approach to provide continuous production of short-lived trophic factors while restricting their distribution in the nervous system. The present study strongly supports the use of peripheral GDNF gene transfer to improve the neurological symptoms in CCI rats. Continuous GDNF production by gene transfer may constitute a promising alternative for the treatment of peripheral nerve injury (Gill et al., 2003). In our recent study, we found that peripheral gene delivery via IM injection improves the neurological function in rats with diabetic neuropathy (Liu et al., 2009). In our previous experience using both injection routes, IM gene delivery seemed to be a less invasive and safer route for delivering GDNF when compared with intrathecal injection into the nerve (Tai et al., unpublished data). However, there are still limitations for adeno-viral-mediated GDNF gene therapy such as inflammation responses and short-term expression. Previous clinical studies showed that individuals treated with adeno-viral vectors have experienced systemic reactions including fever, chill, and hypertension. The minimal effective dose and the duration of GDNF gene therapy remain to be characterized in the future.

Conclusion

In conclusion, our study showed for the first time that a single IM injection of Ad-GDNF improved the histological features of axonal damage in the sciatic nerve after CCI, which also correlate with the alleviation of neuropathic pain responses in an animal model. Therefore, GDNF signaling pathway facilitates promising therapeutic targets for treatment of peripheral nerve injury.

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