EXPERIMENTAL PAPER

Exogenous administration of glial cell line-derived neurotrophic factor improves recovery after spinal cord injury

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KEYWORDS
Spinal cord injury; Neurotrophic factor; Apoptosis; Vascular endothelial growth factor

Summary
The aim of present study was to examine whether systemically delivered glial cell-derived neurotrophic factor (GDNF) was beneficial in reversing the spinal cord injury (SCI) in a spinal cord compression model. Rats were divided into three major groups: (1) sham operation (laminectomy only); (2) laminectomy + SCI + normal saline (1 ml/kg, i.v.); (3) laminectomy + SCI + GDNF (50 ng/kg, i.v.). Spinal cord injury was induced by compressing the spinal cord for 1 min with an aneurysm clip calibrated to a closing pressure of 55 g. GDNF or saline was administered immediately after SCI via the tail vein. Behavioral tests of motor function measured by maximal angle an animal could hold to the inclined plane were conducted at days 1–7 after SCI. The triphenyltetrazolium chloride staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling assay were also conducted after SCI to evaluate spinal cord infarction and apoptosis, respectively. Both GDNF and...
vascular endothelial growth factor (VEGF) in the injured spinal cord were assayed by immunofluorescence. It was found that systemically delivered GDNF, but not vehicle solution, significantly attenuated the SCI-induced hind limb dysfunction and spinal cord infarction and apoptosis. Both GDNF and VEGF could be detected in the injury spinal cord after GDNF, but not vehicle solution, therapy. The results indicate that GDNF treatment may be beneficial in reversing hind limb dysfunction by reducing spinal cord infarction and apoptosis in a spinal cord compression model.

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Introduction

Gliacl cell line-derived neurotrophic factor (GDNF), a distant member of the transforming growth factor-β family, is a protein that is essential for the survival of dopaminergic,1–3 motor,4–6 and peripheral neurons.7,8 It has been shown that topical application or intracerebral administration of GDNF decreases the size of ischemia-induced brain infarction.9,10

Acute spinal cord injury (SCI) is a two-step process that first involves the immediate mechanical damage and then the secondary tissue degeneration is induced by ischemia, hemorrhage, and edema.11–17 In a rodent model of spinal cord injury, human umbilical cord blood-derived CD34+ cells treatment significantly increased spinal cord levels of GDNF that correlated positively with reduced spinal cord infarcts and improved hind limb motor dysfunction.18 This raises the possibility that systemic administration of GDNF may be beneficial in reversing hind limb motor dysfunction in a rat spinal cord injury model.

In order to deal with the question, in the present study, we have used a standard rat compression SCI model to evaluate the neuroprotective potency of GDNF. Indeed, as expected, we provide new evidence to indicate that GDNF may cause restoration of hind limb motor function by reducing spinal cord infarction and apoptosis.

Materials and methods

Adult male Sprague–Dawley rats (weight, 270 ± 12 g), were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed in groups of four at an ambient temperature of 22 ± 1°C, with a 12 h light–dark cycle. Pellet rat chow and tap water were available ad libitum. It has been shown that topical application or intracerebral administration of GDNF decreases the size of ischemia-induced brain infarction.9,10

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A laminectomy, with removal of vertebral peduncle, was performed at T8 or T9 on rats anesthetized with sodium pentobarbital (25 mg/kg, intraperitoneally; Sigma Chemical Co., St. Louis, MO) and a mixture containing ketamine (44 mg/kg, i.m.; Nankang Pharmaceutical, Taipei, Taiwan), atropine (0.02633 mg/kg, i.m.; Sintong Chemical Industrial Co., Taoyuan, Taiwan), and xylazine (6.77 mg/kg, i.m.; Bayer, Leuerkusen, Germany). The jaws of a calibrated aneurysm clip with a closing pressure of 55 g were placed between the dorsal and ventral surfaces of the spinal cord and left in place for 1 min. The sham-operated controlled animals received the same laminectomy, but were not compressed by placing the clip. All animals were given 0.1 ml of Baytril (Bayer) antibiotic for 3 days after surgery. Animal with spinal cord injury were individually housed on special bedding to prevent skin breakdown, and had bowel and bladder manually expressed twice daily. Food and water were freely accessible at a lowered height in their cages.

Animals were assigned randomly to one of the following three groups. One group of rats were treated with laminectomy at T8 or T9 and followed immediately by i.v. infusion of normal saline solution (1 ml/kg). The second group of animals were treated with laminectomy at T8 or T9 and followed immediately by an i.v. infusion of GDNF (50 ng/kg; Research Diagnostics Inc., Flanders, NJ, USA). The third group of animals was used as sham-operated controls.

Experiment 1, SCI was performed randomly in rats treated with an i.v. dose of saline (n = 8) or GDNF (n = 8), and their effects on the maximal angle animals could cling to an inclined plane and spinal cord infarct zones were assessed 1–7 days after SCI. Another eight sham-operated rats were used as controls.

Experiment 2, SCI was performed randomly in rats treated with saline (n = 8) or GDNF (n = 8), and their effects on the amounts of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)-positive cells in the injured spinal cord were assessed 7 days after SCI. Another eight sham-operated rats were used as controls.

Experiment 3, SCI was performed randomly in rats treated with saline (n = 8) or GDNF (n = 8), and their effects on the amounts of vascular endothelial growth factor (VEGF)-positive cells in the injured spinal cord were assessed 7 days after SCI. Another eight sham-operated rats were used as controls.

Experiment 4, SCI was performed randomly in rats treated with saline (n = 8) or GDNF (n = 8), and their effects on the amounts of GDNF-positive cells in the injury spinal cord were assessed 7 days after SCI. Another eight sham-operated rats were used as controls.

The inclined plane was used to measure limb strength. Animals were placed, facing right and then left, perpendicular to the slope of a 20 cm × 20 cm cuff-ridged surface of an inclined plane starting at an angle of 55°. The angle was increased or decreased in 5° increments to determine the maximal angle an animal could hold to the plane. Data for each day were the mean of left and right side maximal angles. All behavioral tests were examined and independently scored by two observers who were unaware of prior treatment. These scores were averaged to arrive at one score for each animal for the behavioral session.

The triphenyltetrazolium chloride (TTC) staining procedures followed those described elsewhere.10 All animals were killed at day 7 after spinal cord injury. Under deep anesthesia (sodium pentobarbital, 100 mg/kg, intraperitoneally), animals were perfused intracardially with saline.
The spinal cord tissue was then removed, immersed in cold saline for 5 min, and sliced into 2.0-mm sections with a tissue slicer. The spinal cord slices were incubated in 2% TTC dissolved in PBS for 30 min at 37 °C, and then transferred to 5% formaldehyde solution for fixation. The lesion sites were evaluated by serial reconstructions, which was performed blindly. The volume of infarction, as revealed by pale TTC stain (whitish color) indicating dehydrogenase-deficient tissue, was measured in each slice and summed using computerized planimetry (PC-Based Image Tools Software). The volume of infarction was calculated as 2 mm (thickness of the slice) × (sum of the infarction area in all spinal cord slices [mm²]). The histological analyses were performed in a blinded manner.

The TUNEL assay was performed using the same spinal cord tissue used in histological verification. Color was developed using 3,3-diaminobenzidine tetrachloride (Sigma Chemical Co.). Sections were treated with xylene and ethanol to remove paraffin and for dehydration. They were then washed with PBS and incubated in 3% hydrogen peroxide solution for 20 min. The sections were treated with 5 μg/ml proteinase K for 2 min at room temperature, and rewarshed with PBS (0.1 M, pH7.4, PBS). The sections were then treated with a TUNEL reaction mixture (terminal deoxynucleotidyl transferase, nucleotide mixture, Roche, Mannheim, Germany) at 37 °C for 1 h, and then the sections were washed with distilled water. They were then reincubated in antifluorescein antibody-conjugated with horseradish peroxidase at room temperature for 30 min, rewarshed, and then visualized using the ABC technique and 0.05% 3,3′-diaminobenzidine tetrachloride as a chromogen. The numbers of TUNEL-positive cells were counted by a pathologist at 200× magnification, 30 fields per section. Blindness was performed for the pathologist’s grading of results.

Autofluorescence was first quenched using the method of Vendrame et al., after which the spinal cord sections were incubated with PBS containing anti-VEDF or anti-GDNF mouse antibody in 1:200 dilution and then detected with Alexa-Fluor 568 goat antimouse (IgG) antibody. Slides were examined under epifluorescence on an Olympus BX60 microscope.

Data are presented as the mean ± S.E.M. Repeated-measures analysis of variance (ANOVA) was conducted to test the treatment-by-time interactions and the effect of treatment over time on each score. The Duncan multiple range test was used for post-hoc multiple comparisons among means. *P < 0.05 was considered evidence of statistical significance.

**Results**

Behavioral tests of motor function were conducted at days 1, 4, and 7 after SCI to determine whether GDNF therapy adopted immediately after SCI would produce beneficial effects. As shown in Figure 1, although vehicle treatment was ineffective on all tests at these three time points, the SCI-induced motor deficits, measured by maximal angle an animal could hold to the inclined plane, were almost completely ameliorated at day 7 after SCI by a single i.v. dose of GDNF. At least 1 month after SCI, the improvement in motor performance exerted by GDNF therapy was still sustained. Immediately after the assessment of motor performance at days 1, 4, and 7 after SCI, animals were killed for TTC staining. Vehicle-treated SCI animals showed severe infarctions, characterized by pale TTC stains (whitish color) in the white matter of all injured spinal cord sections. A typical example was depicted in the top panels of Figure 2. Figure 2 also showed that intravenous administration of GDNF, but not vehicle solution, significantly limited the spinal cord infarct 4—7 days after SCI. In addition, day 7 after SCI, TUNEL stainings revealed that spinal cord apoptosis (evidenced by increasing numbers of TUNEL-positive cells in the injured spinal cord), which could be significantly reduced by GDNF therapy adopted immediately after SCI (Figure 3).

To elucidate whether VEGF can be secreted in spinal cord-injured area by GDNF, analysis of spinal cord homogenate by immunofluorescence for VEGF was done in another set of experiments. These revealed that the spinal cord-injured areas at 7 days after GDNF, but not vehicle solution, therapy displayed more VEGF-positive cells as compared with those of vehicle-treated groups (Figure 4).

As shown in Figure 5, immunofluorescence data revealed that GDNF-positive cells could be detected in the spinal cord-injured section 7 days after an i.v. dose of GDNF. However, 7 days after an i.v. dose of GDNF, GDNF-positive cells still could be detected in normal spinal cord sections (data not shown here).

**Discussion**

In a standard rat compression SCI model, the present study demonstrates that vehicle-treated SCI animals display severe infarctions, characterized by pale TTC stains (whitish color) in the white matters on all injured spinal cord sections examined. The SCI-induced spinal cord infarctions are accompanied by motor deficits. In contrast, SCI animals
Figure 2  TTC staining. (Upper) Schematic diagrams showing the examples of spinal cord infarct zones (striped areas) from a sham control (left), a vehicle-treated SCI rat (middle), and a GDNF-treated SCI rat (right). SCI rats were sacrificed at 7 days post-SCI. Sham-operated rats sacrificed at equivalent time were used as controls. (Lower) *Spinal cord infarct zone 4–7 days following SCI was significantly (P < 0.05; n = 8 for each day group) decreased for SCI animals treated with vehicle (ml/kg, i.v.) (□) compared to SCI sham control (■). #Spinal cord infarct zone 4–7 days following SCI was significantly (P < 0.05; n = 8 for each day group) increased for SCI animals treated with an i.v. dose of GDNF (■■) (50 ng/kg) compared to vehicle controls.

Figure 3  TUNEL-positive cells. *Numbers of the TUNEL-positive cells in spinal cord section 7 days following SCI was significantly (P < 0.05; n = 8) increased for SCI animals treated with vehicle (ml/kg, i.v.) (□) compared to SCI sham controls (■). #Numbers of TUNEL-positive cells in spinal cord sections 7 days following SCI was significantly (P < 0.05; n = 8 for each group) decreased for SCI animals treated with an i.v. dose of GDNF (■■) (50 ng/kg) compared to vehicle controls.

Figure 4  VEGF-positive cells. (Upper) The immunofluorescence stain reveals the VEGF-positive cells in the spinal cord injured section from a sham control (left), a vehicle-treated SCI rat (middle), and a GDNF-treated SCI rat (right). (Lower) *Numbers of VEGF-positive cells in spinal cord sections 7 days following SCI was significantly (P < 0.05; n = 8) increased for SCI animals treated with an i.v. dose of GDNF (■■) (50 ng/kg) compared to vehicle controls.

Figure 5  GDNF-positive cells. (Upper) The immunofluorescence stain indicates the GDNF-positive cells in the spinal cord-injured section from a sham control (left), a vehicle-treated SCI rat (middle), and a GDNF-treated SCI rat (right). (Lower) *Numbers of GDNF-positive cells in spinal cord sections 7 days following SCI was significantly (P < 0.05; n = 8) increased for SCI animals treated with an i.v. dose of GDNF (■■) (50 ng/kg) compared to vehicle controls.
treated with GDNF have a significant reduction in both the spinal cord infarction and motor deficits. Immunohistochemical examination also shows that systemically administered GDNF can be detected in the host spinal cord for at least 1 week after delivery. GDNF, when administered systemically, is never seen in corresponding areas of spinal cord of vehicle-treated animals. The results are consistent with the hypothesis that GDNF may migrate to and participate in the healing of neurological defects caused by traumatic assault. The hypothesis is consistent with the findings from many other investigators. For example, GDNF delivery to injured adult motor neurons has been shown to promote axonal growth and cellular protection.22 Topical application or intracerebral administration of GDNF reduces infarct size in a rat middle cerebral artery occlusion model.9,23,24 Neural stem cells protect against glutamate-induced excitotoxicity and promote survival of injured motor neurons through the secretion of GDNF.25 Human umbilical cord blood cells also significantly increases brain levels of GDNF and improve brain ischemia and neuronal damage in a rodent model of acute stroke26,27 or heatstroke.28 In addition, in a standard rat compression SCI model, CD34+ cells reverse spinal cord infarction and apoptosis and hind limb dysfunction by stimulating secretion of GDNF.18

Like cerebral ischemia,29–32 traumatic brain injury,33 and neurodegenerative diseases,32,34 neuronal and glial apoptosis or programmed cell death is involved in the spinal cord cell loss occurring after SCI. In addition to a burst of neuronal and glial apoptosis in the spinal cord lesion site within the first day, a delayed oligodendrocytes apoptosis in the spinal cord after traumatic insults occurred in distant white matter several days later.35 Since apoptosis is typically a rapid process (over in hours), the late apoptosis observed in the current study likely reflects a true wave of delayed oligodendrocytes death,35 which is associated with poor myelination of axons that persist long after experimental36 or human37 spinal cord injury. The present results further demonstrate that systemic administration of GDNF greatly ameliorates apoptosis in the injured spinal cord and subsequently improves hind limb motor function in spinal cord-injured rats. This indicates that measures aimed at blocking apoptosis may find therapeutic use in various central nervous system disease states. Indeed, repeated intraperitoneal injections of cycloheximide may improve neurological dysfunction during SCI in rat by reducing neuronal and glial cell loss.35

GDNF may reduce ischemia brain injury and apoptosis during middle cerebral artery occlusion in the rat by suppressing caspases-1 and -3.9,40 In addition, evidence has accumulated to suggest that interleukin-1beta (IL-1β) acts as an extracellular signal in the initiation of apoptosis in neurons and oligodendrocytes after SCI.31,41–43 Intrathecal injection of the IL-1 receptor antagonist significantly reduced the number of TUNEL-positive cells after SCI.44 Again, this raises the possibility that GDNF may reduce spinal cord apoptosis by ameliorating IL-1β overproduction in SCI. It has been shown that systemic administration of human umbilical cord blood-derived CD34+ cells to immunocompromised mice subjected to stroke 48 h earlier induces revascularization in the ischemic zone and provides a favorable environment for neuronal regeneration.40 VEGF is believed to act on the vasculature to have an important role in providing an environment conducive to neurogenesis,45,46 whereas GDNF is able to promote axonal growth and cellular protection in injured motor neurons.22 A more recent report also provides evidence showing that human umbilical cord blood-derived CD34+ cells may restore normal hind limb function in a SCI rat model by stimulating production of both VEGF and GDNF in the injured spinal cord.10 The present results further show that GDNF therapy after SCI reduces presence of both VEGF and GDNF in the spinal cord, attenuates spinal cord infarction and apoptosis, and restores normal hind limb motor function. These observations suggest an essential role for GDNF in promoting directly or indirectly an environment conducive to revascularization of ischemic spinal cord at neuronal regeneration can proceed.

Recent studies further demonstrate that GDNF may promote its neuroprotective effects by specifically binding to GDNF family receptor-α and its accessory receptor c-Ret and activating mitogen-activated protein (MAP) kinase and Bcl-2 signaling.47–49 Additionally, GDNF-activated MAP kinase and Bcl-2 signaling may contribute to neuronal survival after SCI. In summary, the current results demonstrate that GDNF, delivered systemically, have the ability to elevate both VEGF and GDNF levels in the spinal cord, to attenuate spinal cord infarction and apoptosis, and to restore the normal kind limb motor function in the rat.

Conflict of interest

None.

References