To reveal roles of neurotrophic factors in plastic changes of the primary afferent neurons following nerve injury, we investigated expression of glial cell line-derived neurotrophic factor (GDNF) as well as nerve growth factor (NGF) in a neuropathic pain model of the rat. The rats exhibited hyperalgesia and allodynia on the injured left side for at least 2 weeks after chronic constrictive injury to the sciatic nerve. Accompanied by the behavioral changes, expression of GDNF decreased in the dorsal root ganglia (DRGs) and the sciatic nerve on the injured side on the fourteenth day after the surgery. In contrast, the amount of NGF in DRGs was unchanged in spite of disturbance of NGF transport in the nerve. The present results suggest that decreased expression of GDNF takes some part in development and/or maintenance of neuropathic pain.

Key words: chronic constrictive injury; glial cell line-derived neurotrophic factor; nerve growth factor; neuropathic pain; two-site EIA

Original Article

Expression changes of glial cell line-derived neurotrophic factor in a rat model of neuropathic pain

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Introduction

Peripheral nerve injury often causes neuropathic pain characterized by intractable symptoms including spontaneous pain, allodynia and hyperalgesia. Considerable lines of evidence suggest that plastic changes occur in primary afferent neurons, sympathetic nerves and dorsal horn neurons. For example, injured primary afferents demonstrate spontaneous repetitive firing with low activation threshold, possibly due to expression changes in sodium channels and nociceptive neurotransmitters. In addition, central axon terminals of primary afferents sprout and make aberrant connections with spinal cord neurons. Sympathetic nerves also abnormally extend their axons and surround neurons in dorsal root ganglia (DRGs) after axotomy of primary afferents. However, how such plastic changes occur is still unclear.

Neurotrophic factors are responsible for maturation, survival and maintenance of distinct populations of neurons. In DRGs, about half of small-diameter unmyelinated primary afferents in adult rats are dependent in their survival on nerve growth factor (NGF) and the other half of afferents are on glial cell line-derived neurotrophic factor (GDNF). There has been accumulating evidence that NGF takes some part in development and/or maintenance of neuropathic pain. In animal models NGF decreases in injured DRGs but increases in the adjacent intact DRGs after spinal nerve ligation. Exogenously applied NGF increases release of substance P (SP), a major neurotransmitter mediating nociception, and treatment...
with anti-NGF antibody abolishes the sprouting of sympathetic nerves into DRGs following nerve injury\(^{23,24}\). In contrast to NGF, contribution of GDNF to neuropathic pain is largely unknown. GDNF-dependent neurons are also thought to mediate nociception. Vanilloid-1 receptor that has high affinity for capsaicin exists on cells expressing Ret\(^{25}\), a signal transducing component of GDNF receptor. There also exist on Ret-expressing cells receptors for ATP, another ligand for nociceptive sensation\(^{26}\). However, recent intriguing reports have revealed that GDNF ameliorated pain caused by spinal nerve ligation\(^{27}\) suggesting pain relieving effect of GDNF. In this regard, it is noteworthy that GDNF prevented neuronal sprouting after axotomy\(^{2}\), because neuronal sprouting in the spinal cord is one of neural basis for allodynia. It is, therefore, probable that GDNF expression in the primary afferents may change with development of the neuropathic pain in a manner distinct from NGF. However, there have been no reports analyzing quantitatively changes in expression of GDNF protein in the DRGs, the primary afferent fibers and the spinal cord. We, therefore, developed two-site enzyme immunoassay (EIA) for measurement of GDNF and NGF proteins and investigated chronic changes of GDNF expression, as well as NGF, after development of neuropathic pain.

We found that GDNF, but not NGF, decreased in the DRGs after the chronic constriction nerve injury, suggesting that GDNF may be responsible for development and/or maintenance of neuropathic pain.

### Materials and Methods

#### Experimental animals and production of chronic constriction injury (CCI)

Experimental procedures were approved by the institutional committee on laboratory animals and made under the guidelines of the International Association for the Study of Pain\(^{38}\). Rats were individually housed in plastic cages with soft bedding under a 12h light cycle. Young adult male Sprague-Dawley rats (140-160 g at the time of surgery) were used for all experiments. The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally), the left common sciatic nerve was then exposed in the left mid-thigh and loosely ligated with 4-0 silk thread in four regions at about 1-mm intervals\(^{39}\). The right sciatic nerve was left intact for control.

#### Behavioral test

Behavior of each rat was examined before operation and for fourteen days after operation. To test mechanical allodynia, the paw withdrawal in response to mechanical stimuli was measured using a set of von Frey filaments (Muromachi kikai, Tokyo, Japan) with bending forces ranging from 0.41 to 44.0 g. We placed each rat individually on the metallic mesh floor covered with a plastic box and applied von Frey monofilament from under the mesh floor to the plantar surface of both the right and the left hind paws. Each paw was stimulated with each filament 5 times at 2-second intervals in the individual trial. We referred as a threshold value to the weakest force (g) inducing withdrawal response of the paw at least 3 times in a trial. Plantar Test (Ugo Basile, VA, Italy) was used to examine heat hypersensitivity. We placed rats individually on glass plate with a radiant heat equipment underneath. After acclimating period the radiation heat was applied onto either the right or left hind paw pad independently. We measured the latency of paw withdrawal from noxious heat stimuli repetitively three times at 3-minute intervals, and adopted the average value. To minimize tissue damage by heat stimulus, the examination was done once a week.

#### Tissue preparation

The spinal cord, the forth lumbar (L4) and L5 DRGs and the sciatic nerves were dissected out at 14 days after the CCI operation, when we confirmed that neuropathic pain was established and maintained. The spinal cord of L4 to L5 segments was divided into the left (CCI side) and the right (control side) parts, and then further cut into the ventral and the dorsal parts. The left sciatic nerve was cut into three segments; the ligated, the adjacent proximal and distal segments. The ligated segments were about 5 mm long, and other segments were about 10 mm long. The sciatic nerve on the right side was also cut into three pieces at similar positions to those on the left side. Tissues were immediately frozen in liquid nitrogen and stored at - 80 °C. Extraction method of trophic factors from tissues was followed by Narisawa-Saito and Nawa\(^{30}\) except components of protease inhibitors. Each tissue sample was homogenized with Polytron homogenizer with 20-450 volumes of homogenizing buffer (50 mM Tris, 0.5 M NaCl, 0.3% Triton X-100, pH 7.5) containing a pre-made cocktail of protease inhibitors (Complete mini, Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged at 15000 rpm for 20 min at 4°C, and the supernatant was used for measurement
of neurotrophic factors.

Two-site EIA for NGF and GDNF

For measurements of NGF and GDNF we also principally followed procedures described by Narisawa-Saito and Nawa except we used antibodies commercially available. EIA titer plates (FluoroNunc plate, Nalgen Nunc International, NY, USA) were coated with primary polyclonal antibodies against NGF (20 ng/well, Promega, WI, USA) or GDNF (100 ng/well, Promega, WI, USA) for 18 h and then blocked with EIA buffer (50 mM Tris, 0.5 M NaCl, 0.3% Triton X-100, 1% bovine albumin and 1% gelatin, pH 7.5) at 4°C for more than 3 h. One hundred microliters of tissue extract or 1 to 1000 pg of standards (recombinant human NGF-β, Roche Diagnostics, Mannheim, Germany; recombinant human GDNF, Chemicon, CA, USA) was loaded into each well and incubated at room temperature for 8 h. After washing with Wash-buffer (EIA buffer without bovine serum albumin), 100 µl of biotinylated anti-GDNF monoclonal antibody (300 ng/ml, Genzyme/Techn, MN, USA) or β-galactosidase-conjugated anti-NGF monoclonal antibody (5 µg/ml, Roche Diagnostics, Mannheim, Germany) was added and further incubated for 18 h at room temperature. Biotinylated secondary antibody bound to GDNF was detected by incubation with streptavidin-β-galactosidase (1:5000 dilution, Roche Diagnostics, Mannheim, Germany) for 3 h. After extensive wash, activities of β-galactosidase were measured by incubation with 200 mM 4-methylumbelliferyl-β-galactoside (Sigma, MO, USA) in 50 mM sodium phosphate buffer (pH 7.3) containing 10 mM MgCl₂. The reactions were carried out in dark at room temperature for 3-5 h and the amount of fluorescent product was monitored by Spectrafluor Plus microplate reader (Tecan, Salzburg, Austria) with excitation at 360 nm and emission at 465 nm.

Statistical analysis

For each set of values, data were expressed as the mean ± s.e. of the mean. Two-way analysis of variance (ANOVA) was used to compare the values between the different groups. Values of P < 0.05 were considered to be significant.

Results

Behavioral studies

All rats that received CCI exhibited mechanical allodynia and thermal hyperalgesia representing symptoms of neuropathic pain. The time courses of the symptoms are presented in Fig. 1A and B. Before surgery the rats responded to the von Frey filament with 29.6 ± 4.6 g and 29.6 ± 4.6 g on the left and the right side of the paw, respectively. As time goes on, threshold of the paw withdrawal in response to mechanical stimuli began to decrease on the injuried left side from the next day after the surgery and the decrease persisted until the 14th day of sacrifice (3.72 ± 0.5 g, P < 0.001 compared with that before surgery, Fig. 1A). In contrast, control right paw showed slight decrease in the threshold, but then recovered to the pre-operative state on the day 14. In response to the infrared heat radiation, rats withdrew the left and the right hind paws with latencies of 10.8 ± 0.2 s and 11.7 ± 0.4 s, respectively (Fig. 1B), before the operation. After the injury the response latency significantly decreased to 8.0 ± 0.5 s at day 14 on the injured (P < 0.001) side, but not on the control side.
NGF protein expression

In the intact sciatic nerve on the right side, the nerve showed a gradient distribution of NGF, higher in the proximal section (636.8 ± 67 pg/mg of total protein) and lower in the distal section (436.9 ± 41 pg/mg of total protein), Fig. 2A). On the injured side, NGF decreased in the proximal (268.9 ± 50 pg/mg of total protein) and the ligated sections (245.4 ± 17 pg/mg of total protein), but not in the distal section (333.1 ± 55 pg/mg of total protein, Fig. 2A). However, apparent content of NGF in DRGs was unchanged on CCI side (283.1 ± 14 and 398.6 ± 48 pg/mg of total protein in left L4 and L5) compared with that on control side (279.3 ± 30 and 455.7 ± 66 pg/mg of total protein, Fig. 2B). The expression of NGF was very low in the spinal cord of both operated and non-operated sides (Fig. 2C). In the dorsal part of the spinal cord, NGF seemed a little higher on CCI side (19.1 ± 1.9 pg/mg of total protein) than on control side (14.1 ± 1.6 pg/mg of total protein), but the deference was not significant. Both sides of the ventral part (right, 22.4 ± 0.8; left, 24.3 ± 1.2 pg/mg of total protein) contained similar amounts of NGF.

GDNF protein expression

In the sciatic nerve of control side, the distal part contained more abundant GDNF (587.8 ± 154 pg/mg of total protein) than the proximal part (253.3 ± 32 pg/mg of total protein), contrasting with the NGF distribution (Fig. 3A). After the nerve injury, GDNF decreased in the proximal (133.3 ± 27 pg/mg of total protein) and the distal sections (157.3 ± 18 pg/mg of total protein), but not in the ligated section (255.3 ± 27 pg/mg of total protein). Contrary to NGF, GDNF decreased in L4 (310.9 ± 26 pg/mg of total protein) DRGs after CCI, although expression of GDNF in L5 DRG was unchanged (Fig. 3B). GDNF was highly expressed in the spinal cord (Fig. 3C), where the injured side did not show any change in GDNF expression.
Discussion

The present study showed that GDNF, a neurotrophic factor essential for the survival of a subgroup of the primary afferents, decreased in DRG at L4, but not L5, on the injured side 14 days after CCI. This may reflect partial nerve injury caused by the chronic constriction of the sciatic nerve in this model. In accordance with the reduction of GDNF in the DRG, adjacent segments to the ligated part of the sciatic nerve showed decrease in GDNF expression. These results suggest that GDNF supply and/or utilization decrease in the primary afferent neurons in the chronic pain states and that GDNF reduction may be responsible for maintenance of persistent pain. Reduced GDNF may reflect reduction in the number of GDNF-dependent primary afferents, although roles of these fibers in nociceptive transmission are still unclear. Of particular interest in this context is a report that administration of GDNF ameliorated pain-related behavior caused by the spinal ligation, another neuropathic pain model. Furthermore, intrathecal administration of GDNF was reported to abolish sprouting of Aβ fibers induced by axotomy of the sciatic nerve. Sprouting of the large-diameter fibers and their aberrant connections with dorsal horn neurons are thought to be morphological basis underlying mechanical allodynia. In the present study, reduction in GDNF occurred with decreased threshold of behavioral response to mechanical stimuli, a marker for allodynia. Taken together, partial nerve injury may cause decrease in the number of GDNF-dependent fibers, resulting in Aβ fiber sprouting in the spinal cord. Heat hypersensitivity seemed occur with slower time course, although we did not examined the early postoperative changes in the behavior. Therefore, additional mechanisms other than the fiber sprouting may also develop chronic pain. In the ligated section of sciatic nerve, GDNF was unchanged at day 14 after CCI. Schwann cells around the ligated nerve may produce GDNF and compensate its decrease, since it has been reported that axotomy of the sciatic nerve causes an increase in GDNF and its receptor mRNA in Schwann cells in the injured sciatic nerve. The axotomy also caused increase in GDNF mRNA in the DRG, which was inconsistent with our findings. Although the reason for this discrepancy is not known, it might be due to difference in procedure of nerve injury between models.

NGF is believed to be produced in the skin and transported retrogradely through the sciatic nerve to DRG cell bodies. Our results support the hypothesis, since the amount of NGF decreased in the ligated and its adjacent proximal sections on the injured side, suggesting disruption of NGF transport by the nerve ligation. The change in NGF expression varies among several neuropathic pain models. Herzberg et al. reported that NGF protein and mRNA increased in DRGs on the injured side at 14 days after CCI. Oh et al. reported that NGF protein levels of L3 to L6 DRGs were unchanged after the spinal nerve ligation at L5 and L6, while Fukuoka et al. reported increase in NGF protein at L4 DRG following spinal nerve ligation at L5. In the present study, apparent amount of NGF was unchanged in DRGs of either side in spite of disturbance of retrograde transport. These discrepancies may be explained by the difference in severity of nerve injury between neuropathic pain models. It is probable that NGF is produced in DRGs and such ectopic expression may play some part in induction and/or maintenance of neuropathic pain. Although identification of cells producing NGF is not yet determined, a candidate for the supply is satellite cells in DRGs. Increase in NGF mRNA was demonstrated in satellite cells surrounding DRG neurons after peripheral nerve injury. Since application of anti-NGF antibody abolishes sympathetic nerve sprouting after peripheral nerve injury, increased NGF in satellite cells may induce the nerve sprouting in the DRG.

The present study showed that GDNF and NGF changed distinctively in the rat exhibiting neuropathic pain after CCI. Most importantly, decreased expression of GDNF was observed in the DRG in the chronic pain state. These results could encourage us to search rational treatment with GDNF-related compounds to relieve intractable neuropathic pain.

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