Orally Active Neurotrophin-Enhancing Agent Protects Against Dysfunctions of the Peripheral Nerves in Hyperglycemic Animals

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Biological substances with neurotrophic activities, such as nerve growth factor (NGF) and monosialoganglioside GM1, have been considered as agents for diabetic peripheral neuropathy. Because recent studies have suggested that decreased availability of these substances might contribute to the pathogenesis of diabetic peripheral neuropathy, some clinical trials of NGF for diabetic peripheral neuropathy have been conducted and have led to mixed conclusions. The major reasons were its limited delivery to the nervous system and adverse effects induced by subcutaneous injection, which was necessary because NGF is a polypeptide. The current study investigates whether an orally active sialic acid derivative, MCC-257, has neuroprotective properties in diabetic peripheral nerves. MCC-257 augmented NGF activity in cultured dorsal root ganglia and PC12 (pheochromocytoma 12) cells. Treatment with MCC-257 elevated NGF levels in the sciatic nerve, accompanied by improvement in nerve conduction velocity in streptozotocin-induced diabetic animals. More importantly, MCC-257 ameliorated small fiber dysfunctions, including thermal hypoalgesia, substance P content, and histopathological innervation in the plantar skin of diabetic animals. Thus, the orally active neurotrophin enhancer provides a new option for the clinical treatment of diabetic peripheral neuropathy. Diabetes 55:616–621, 2006

Peripheral neuropathy is one of the most common complications in diabetic patients. Although recent studies suggest that a disorder of the polyol pathway (1), oxidative stress (2), and/or the abnormal activation of protein kinase C (PKC) (3–6) underlie the pathogenesis of diabetic peripheral neuropathy, this has not been fully clarified. Currently, a significant number of aldose reductase inhibitors are under development. In diabetic peripheral neuropathy, it has also been reported that nerve growth factor (NGF) and substance P levels in the skin decrease and that their decrease is correlated with an impairment of sensory functions (7). NGF affects the survival of small-diameter sensory nerve fibers and sympathetic neurons as well as the maintenance of their phenotypic properties, such as producing substance P and calcitonin gene–related peptide. The degeneration of small fibers has a great impact on the quality of life of diabetic patients because most of the symptoms in diabetic peripheral neuropathy are caused by small fiber dysfunctions. Regardless of the pathogenesis, supplying the neurotrophin to neurons has been considered a potential treatment for neurodegenerative disease, including diabetic peripheral neuropathy. In experimental diabetic rats, treatment with NGF ameliorates decreased substance P and calcitonin gene–related peptide levels in the peripheral nerves (8), dorsal root ganglia (9,10), and spinal cords (10). In addition, NGF restores the nociceptive threshold to thermal noxious stimuli (9), myelinated nerve fiber morphology (10), and neurogenic vasodilatation (11) in streptozotocin (STZ)-induced diabetic rats. Exogenous recombinant human NGF actually showed significant beneficial effects on diabetic peripheral neuropathy in a phase II clinical trial (12).

It is noteworthy that monosialoganglioside GM1 enhances the activity of NGF. For instance, it facilitates neurite outgrowth (13–15), reduces neural degeneration (16–19), and modulates protein kinase activity in injured brain tissue (20). GM1 also enhances NGF-induced tyrosine receptor kinase (Trk)A autophosphorylation (21). Treatment with mixed gangliosides, including GM1, was found to improve both paresthesias and some electrophysiological parameters in previous clinical trials (22,23). Not only GM1 but also synthetic sialyl cholesterol, whose structure is similar to that of gangliosides, have been reported to promote neurite outgrowth (24–26) and enhance the development of grafted neurons (27), which suggests that sialyl cholesterol might be agents that have potential neuroprotective and neurorestorative properties.

NGF itself has been considered an option for the treatment of diabetic peripheral neuropathy. Unfortunately, the efficacy was not demonstrated in a recently completed phase III trial (28). The reasons for the failure were its limited delivery to the nervous system and adverse effects after subcutaneous injection (28), which was necessary because NGF is a polypeptide. To avoid this problem, orally active small molecules that potentiate the expression and activity of NGF may be required. In the current study, we demonstrate that...
the orally active sialyl cholesterol mimetic MCC-257 potentiates NGF actions and ameliorates dysfunction of the peripheral nerves in hyperglycemic animals, which suggests MCC-257 has a beneficial effect in the clinical treatment of diabetic peripheral neuropathy.

RESEARCH DESIGN AND METHODS

All animal experiments in these studies were performed in accordance with the guidelines of Mitsubishi Pharma and were approved by the animal investigation committee of our institute. In the experiment with rats, diabetes was induced by intravenous injection of STZ (50 mg/kg, dissolved in 0.01 mol/l citrate buffer at pH 5.5) in 12- to 14-week-old male Wistar rats (Japan Laboratory Animals, Tokyo, Japan). An additional group of rats was injected with citrate buffer to act as a sham control. Hyperglycemia was verified 4 weeks after STZ injection, and the animals were divided into six groups (sham-vehicle, diabetic-vehicle, and 0.1, 0.3, 1, or 3 mg/kg MCC-257 daily), and then a 4-week treatment with MCC-257 was started. The number of animals in each group was 10, except for 9 in the 0.1-mg/kg group in the nerve conduction study and 8 in the 0.1-mg/kg group in the study of NGF content. In the experiment with mice, male BKS.Cg-Leprdb/Leprdb (db/db) and BKS.Cg-m/+ (Lepr+/+), each group was 10, except for 9 in the 0.1-mg/kg group in the nerve conduction study and 8 in the 0.1-mg/kg group in the study of NGF content. In all the treatment period, the rats were killed, and the sciatic nerves (both sides of each animal) were dissected. NGF was extracted by repetitive freezing-thawing cycles and soaked in washing buffer (0.1 mol/l Tris-HCl buffer pH 7.5, 0.1% BSA, 0.1% NaN3). NGF was measured by the enzyme immunoassay system as follows. First, 96-well immunoplates were coated with anti-mouse NGF monoclonal antibody or anti-mouse digoxigenin antibody (Roche Diagnostics, penzberg, Germany) and then washed and blocked. After a 5-h incubation of the extracted samples or NGF standards, the plates were washed, and β-galactosidase–conjugated anti-NGF antibody (0.1 unit/ml in washing buffer; Roche Diagnostics) was added. After overnight incubation and washing, a substrate for the enzyme (4-methylumbelliferyl-β-D-galactoside, 20 μg/ml in washing buffer; Sigma) was then added to the plates, which was incubated for 5 h at room temperature in the dark. The reaction was stopped with 0.1 mol/l glycine-NaOH buffer (pH 10.3). Fluorescence intensity was measured at two wavelengths of 355 and 460 nm.

Hot plate test. A hot plate test was performed before and after drug treatment. Each animal was habituated to the test apparatus (MK-350; Muromachi Kikai, Tokyo) before the nociception assay. The mice were placed on the hot plate maintained at 53°C, and the latency to response (licking of front or hind paw) was recorded. If the response did not occur within 45 s, the animal was taken out, and the result was recorded as 45 s.

Tissue preparation. On the day after the hot plate test, which followed the determination of NGF, the rats were killed by anesthetizing with Diethyl ether and decapitated, and the plantar skins were dissected bilaterally. Left plantar skins were stored at −80°C as the samples for biochemical evaluation. Right plantar skins were fixed in 10% neutral buffered formalin. The sectioned skin was embedded in paraffin and cut into ~5-μm-thick sections for histopathological evaluation.

Measurement of substance P. The tissues were boiled for 15 min in 0.5 ml of boiling 2 mol/l acetic acid containing 10 mmol/l hydrochloride, 1 mmol/l EDTA, and 1 mmol/l dihydrothreitol. After boiling, the tissues were homogenized, the remaining solution was centrifuged (9,000 g, for 5 min), and the supernatant was freeze-dried overnight. Substance P was determined by enzyme-linked immunosorbent assay, using commercially available antiserum (Peninsula Laboratories, San Carlos, CA).

Immunohistochemistry. Paraffin blocks of plantar skins were stained for protein gene product 9.5 (PGP9.5) by immunohistochemistry. After deparaffinization and dehydration, each section was blocked with 1% BSA. The sections were incubated with the polyclonal primary antibody PGP9.5 (1:1,000; Ultracron, Isle of Wight, U.K.) for 60 min at 37°C. The sections were then incubated with the biotinylated link secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min and peroxidase-labeled streptavidin (Vector Laboratories) for 60 min according to the manufacturer’s instructions. 3′,5′-diaminobenzidine was used as the substrate-chromogen solution. Counterstaining was performed with hematoxylin.

Histopathological evaluation. The tissue sections stained with PGP9.5 were observed by optical microscopy (AX-80; Olympus, Tokyo, Japan). Digital images were captured with a charged-coupled device camera (DP70, Olympus). Image acquisition was controlled through DP control (Olympus) running on a Windows computer. The digital images were saved as TIF files. The length of the epidermal/dermal junction was measured as skin length, using image analysis software (Image-Pro Plus, version 4.5; Media Cybernetics, Silver Spring, MD). Every PGP9.5-positive nerve fiber in a target field (diameter within a 50-μm radius from the epidermal/dermal junction) was traced, and its area was
measured using Image-Pro plus. The number of PGP9.5-immunoreactive nerve fibers with a cross-sectional area of $\leq 20 \mu m^2$ was counted, and it was normalized with skin length (epidermal/dermal junction), using the following:

$$\text{nerve fibers (count/mm)} / \text{skim length (mm)}.$$

Statistical analysis. Unless otherwise noted, all data are the means $\pm SE$. Differences among experimental group were detected by ANOVA, and the significance of differences between groups was assessed by paired or unpaired $t$ test or Dunnett’s multiple comparison. Significance was defined as $P < 0.05$.

RESULTS

Potentiation of the neuroprotective effect of NGF on dorsal root ganglion neurons. A high concentration of NGF (100 ng/ml) had a protective effect on the survival of dorsal root ganglion neurons compared with nontreated cells ($P < 0.01$) (Fig. 1A). MCC-257 (0.1, 0.3 $\mu$mol/l; $P < 0.01$, respectively) significantly increased the cell viability of cultured dorsal root ganglion neurons in the presence of a low concentration of NGF (1 ng/ml) (Fig. 1B), whereas in the absence of NGF in the media, MCC-257 had no effect on the viability of dorsal root ganglion neurons up to 0.3 $\mu$mol/l. MCC-257 significantly deceased the viability of dorsal root ganglion neurons at a concentration of 1 $\mu$mol/l because of its insolubility ($P < 0.05$) (Fig. 1A).

Facilitation of TrkA autophosphorylation and survival of PC12 cells. Exposure to NGF (50 ng/ml) for 5 min significantly increased the tyrosine phosphorylation of TrkA in PC12 cells compared with nontreated cells ($P < 0.01$) (Fig. 2A). The exposure of PC12 cells to MCC-257 in the presence of NGF (50 ng/ml) significantly enhanced TrkA autophosphorylation (0.1 $\mu$mol/l, $P < 0.01$) (Fig. 2A), whereas in the absence of NGF, treatment with MCC-257 had no effect on the tyrosine phosphorylation of TrkA (Fig. 2A). MCC-257 (0.1 $\mu$mol/l, $P < 0.01$) significantly increased the cell viability of serum-deprived PC12 cells in the presence of NGF (50 ng/ml). In the absence of NGF in the media, MCC-257 had no effect on PC12 cell viabilities up to 0.1 $\mu$mol/l (Fig. 2B).

MCV and NGF content in STZ-induced diabetic rats. The MCV and SCV of 8-week hyperglycemic rats were reduced compared with those of animals in the sham group by 7.2 and 10.9 m/s, respectively (MCV $P < 0.05$, SCV $P < 0.05$) (Table 1). These impairments were significantly

![FIG. 1. Effect of MCC-257 on cell survival in cultured dorsal root ganglion neurons. A: MCC-257 had no effect on cell viability in cultured dorsal root ganglion neurons without NGF ($n = 6$). *$P < 0.05$ (ANOVA with Dunnett’s test); **$P < 0.01$ vs. 0 $\mu$mol/l (paired $t$ test). B: MCC-257 protected cultured dorsal root ganglion neurons with 1 ng/ml of NGF in the medium ($n = 6$). ***$P < 0.01$ vs. 0 $\mu$mol/l (ANOVA with Dunnett’s test). #$P < 0.01$ vs. 0 $\mu$mol/l (paired $t$ test). Data are the means $\pm SE$. OD, optical density.](image1)

![FIG. 2. Effect of MCC-257 on the tyrosine phosphorylation of TrkA. A: Effect of MCC-257 on the tyrosine phosphorylation of TrkA in PC12 cells with or without NGF ($n = 5$). **$P < 0.01$ vs. 0 $\mu$mol/l without NGF (paired $t$ test); ***$P < 0.01$ vs. 0 $\mu$mol/l with NGF (Dunnett’s test). B: Effect of MCC-257 on cell viability in serum-deprived PC12 cells with 50 ng/ml of NGF in the medium ($n = 5$). **$P < 0.01$ vs. 0 $\mu$mol/l (ANOVA with Dunnett’s test). Data are the means $\pm SE$. OD, optical density.](image2)
TABLE 1
MCV and SCV and NGF content in normal and diabetic rats treated with or without MCC-257

<table>
<thead>
<tr>
<th></th>
<th>Normal sham</th>
<th>Diabetic vehicle</th>
<th>0.1 mg/kg</th>
<th>0.3 mg/kg</th>
<th>1 mg/kg</th>
<th>3 mg/kg</th>
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<tr>
<td>MCV (m/s)</td>
<td>61.6 ± 0.4</td>
<td>54.4 ± 1.0*</td>
<td>56.0 ± 1.0</td>
<td>57.3 ± 0.7</td>
<td>57.0 ± 0.4</td>
<td>57.7 ± 0.9†</td>
</tr>
<tr>
<td>SCV (m/s)</td>
<td>60.8 ± 0.6</td>
<td>49.9 ± 1.1*</td>
<td>51.5 ± 0.9</td>
<td>55.1 ± 0.7†</td>
<td>56.2 ± 0.8†</td>
<td>57.2 ± 1.6†</td>
</tr>
<tr>
<td>NGF content (pg/mg of sciatic nerve)</td>
<td>1.142 ± 0.106</td>
<td>0.450 ± 0.0551‡</td>
<td>0.516 ± 0.0609</td>
<td>0.623 ± 0.0856</td>
<td>0.740 ± 0.0890†</td>
<td>0.783 ± 0.0634†</td>
</tr>
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Data are means ± SE. The number of animals is 10 except for 9 in the 0.1-mg/kg group in the nerve conduction study and 8 in the 0.1-mg/kg group in the study of NGF content. *P < 0.05 vs. sham (unpaired t test); †P < 0.05 vs. vehicle (ANOVA with Dunnett’s test); ‡P < 0.01 vs. sham (unpaired t test).

improved by 4 weeks of treatment with MCC-257 (MCV: 3 mg/kg orally, P < 0.05; SCV: 0.3, 1, and 3 mg/kg orally, P < 0.05, respectively) (Table 1).

The effect of MCC-257 on NGF content in the sciatic nerves was also examined in diabetic rats. Compared with sham-treated rats, the NGF content in the sciatic nerves of hyperglycemic rats (vehicle group) was significantly decreased (P < 0.01) (Table 1). MCC-257 (1 and 3 mg/kg orally, P < 0.05) significantly increased NGF content in the sciatic nerves in STZ-induced hyperglycemic rats compared with untreated hyperglycemic rats (Table 1).

Behavioral evaluation of thermal nociception in diabetic mice. Compared with nondiabetic db/+m mice, the licking latency of db/db mice was significantly longer after 7 weeks of drug administration (10.26 s in db/+m vs. 18.13 s in db/db vehicle, P < 0.01) (Fig. 3), suggesting that the db/db mice showed thermal hyperalgesia. Compared with the db/db vehicle-treated group, the licking latency of the db/db MCC-257-treated group (10 mg/kg orally) was significantly shorter after 7 weeks of administration (18.13 s in db/db vehicle vs. 14.77 s in db/db MCC-257 10 mg/kg orally, P < 0.05) (Fig. 3), which suggests that MCC-257 has a protective effect on thermal nociceptive function.

Biochemical evaluation of subcutaneous innervation in diabetic mice. The substance P level was measured in plantar skins obtained from the animals used in the behavioral test. The substance P level was significantly reduced in the skin from db/db mice compared with that of db/+m mice (P < 0.01) (Fig. 4). Treatment with MCC-257 for 7 weeks significantly elevated substance P level at doses of 3 and 10 mg/kg orally (P < 0.01, respectively) (Fig. 4). This result suggests that MCC-257 protects small fiber innervations in diabetic plantar skins.

Histopathological evaluation of subcutaneous innervation in diabetic mice. The nerve fiber density (number of PGP9.5-positive nerve fibers that have an area <20 μm² per unit length of skin) for db/+m mice was 52.8 ± 2.1 counts per skin length. Reduced cutaneous innervation was observed in the plantar skin of db/db mice compared with that of db/+m mice (38.7 ± 1.9 counts per skin length in db/db vehicle, P < 0.01) (Fig. 5D). This reduction was attenuated by a 7-week treatment with MCC-257 at doses of 3 and 10 mg/kg orally (P < 0.05 and P < 0.01, respectively) (Fig. 5D). This result is consistent with results obtained from behavioral and biochemical evaluations.

DISCUSSION

We have developed a novel compound, MCC-257, that potentiates the action of neurotrophins on central and peripheral neurons, which leads to the protection of neuronal cells from some fatal conditions. Furthermore, the compound, a sialic acid derivative, exerts protective effects on both the sciatic nerve and the primary afferent nerve fibers in diabetic animals. The compound prevented neuronal cell death only in the presence of a small amount of NGF, whereas this effect was not elicited when NGF was not provided to the neuronal cells. These findings suggest that the compound does not in itself have a neurotrophic effect but enhances NGF signal transduction. Mutoh et al. (21) reported that GM1 preferentially activates the TrkA receptor. In that report GM1 was tightly associated with TrkA and increased the NGF-induced autophosphorylation of TrkA. Because MCC-257 was orig-
Finally synthesized as a GM1 mimetic, direct or indirect association of MCC-257 with Trk proteins may result in the stimulation of NGF-induced TrkA phosphorylation and enhancement of neurotrophin activity. Moreover, because the compound activated the autophosphorylation of TrkB in a primary culture of embryonic rat cerebral cortex neurons (data not shown), it may have broad activity on Trk proteins. It is hypothesized that the cells that can produce neurotrophins and the cells expressing TrkA or TrkB may be the main targets of this compound. The compound also increased NGF content in the sciatic nerve of diabetic rats. This result suggests that the compound facilitated NGF production in the peripheral tissues and/or Schwann cells and improved the availability of NGF.

In addition, the compound protected against impairment of nerve conduction velocity in STZ-induced diabetic rats. Nerve conduction velocity is an indicator of large fiber function. Because neurotrophin-3 is trophic for large-caliber sensory neurons and motor neurons (31,32), the effect of this compound on nerve conduction velocity may be partially attributable to the enhancement of neurotrophin-3 production or autophosphorylation of TrkC. The effect of MCC-257 on TrkC is also under investigation.

From a clinical point of view, it is especially noteworthy that the compound ameliorated small-caliber sensory nerve function and its cutaneous innervation. This is the first report that a nonpeptide small molecular compound was able to improve sensory dysfunction and cutaneous innervation in diabetic animals. These results of behavioral, biochemical, and histopathological studies were consistent with each other. Because thermal nociceptive stimuli are mainly transmitted through unmyelinated C-fiber afferents and NGF has trophic effects on small fibers, MCC-257 acted on small fibers in experimental thermal hypoalgesia. Substance P content in peripheral skins could be a good marker of the presence of active small fibers or cutaneous innervation because a population of small fibers expresses substance P (33). The elevation of substance P level in diabetic skins seems to be responsible for the amelioration of thermal sensory functions. In the biochemical study, however, we could not deny the possibility that MCC-257–induced NGF increased substance P expression independently of the cutaneous innervation. Hence, we conducted a histopathological quantification of cutaneous nerves with PGP9.5 antibody, which is generally used for the evaluation of clinical and experimental cutaneous innervation and is well characterized as a pan-neuronal marker. Taking this into account, for quantitative analysis, we focused on nerve fibers with a cross-sectional area <20 μm². Although nerve fibers whose cross-sectional

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**FIG. 5.** A–C: Effect of MCC-257 on the cutaneous innervation in genetic diabetic mice. Immunohistochemistry for PGP9.5 in the mouse plantar skin. PGP9.5-positive fiber (dark brown) that has a cross-sectional area of ≤20 μm² was counted in the target field (dermis within a 50-μm radius from the epidermal/dermal junction). Scale bar = 50 μm. Magnification 560×. In hyperglycemic mice (B), PGP9.5 immunoreactivity was lower than that of normoglycemic mice (A). Treatment of hyperglycemic mice with MCC-257 was associated with an increase in PGP9.5 immunoreactivity (10 mg/kg) (C). D: MCC-257 restored the cutaneous innervation in the plantar skin (n = 20). ##P < 0.01 vs. db/+m (normoglycemic, unpaired t test); *P < 0.05, **P < 0.01 vs. db/db vehicle (Dunnett’s test). Data are the means ± SE.
area is <20 μm² are not always small fibers, this compound increased smaller-caliber nerve fibers, including small fibers. Recent studies have suggested that reduction in availability of neurotrophic factors also contributes to diabetic peripheral neuropathy (34). However, the efficacy of NGF or GM1 was mixed in clinical trials for the treatment of diabetic peripheral neuropathy. One of the reasons that NGF or GM1 did not show efficacy in the latest clinical trials is their pharmacokinetic properties. Because NGF is a polypeptide, the route of administration was restricted to subcutaneous injection. Therefore, the side effects associated with direct action on mast cells (35) in the skin or potentiation of bradykinin and prostaglandin E₂ activities (36) were inevitable. Mixed gangliosides, including GM1, were also given parenterally for clinical trials because of their poor absorption. Because MCC-257 is a nonpeptide small molecule potentiating the action of neurotrophins, we can adopt oral administration, which may prevent local adverse effects related to the injection. Once this pharmacokinetic problem has been overcome, supplemental therapy with neurotrophins may be a realistic option in treating diabetic peripheral neuropathy. The pathogenesis of diabetic peripheral neuropathy seems to be composed of multiple factors, including a disorder of the polyol pathway, oxidative stress, and the abnormal activation of PKC, and therefore aldose reductase inhibitors alone or PKC inhibitors alone may be less effective against diabetic peripheral neuropathy. For this reason, supplemental therapy is a meaningful option for the treatment of diabetic peripheral neuropathy.

Our results showed that a new salicylic acid derivative, MCC-257, enhanced signal transduction of NGF. Moreover, this compound improved both nerve conduction velocity and hot plate response accompanied by cutaneous innervation in diabetic animals. These results indicate that MCC-257 may have a beneficial effect on the clinical treatment of diabetic peripheral neuropathy. Further investigation of this compound as an agent for diabetic peripheral neuropathy is underway.

REFERENCES