Original Article

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

Bunpei Kakinoki,[1] Sumito Sekimoto,[1] Satoshi Yuki,[2] Tetsuya Ohgami,[1] Mikiko Sejima,[1] Keiji Yamagami,[1] and Ken-ichi Saito[1]

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 strepotozotocin-induced diabetic animals. More importantly, MCC-257 ameliorated small fiber dysfunctions, including thermal hypoalgesia, substance P content, and histopthological 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.

From the [1]Research Laboratory I, Pharmaceutical Research Unit, Research and Development Division, Mitsubishi Pharma, Yokohama, Japan; and [2]Research Laboratory II, Pharmaceutical Research Unit, Research and Development Division, Mitsubishi Pharma, Yokohama, Japan.

Address correspondence and reprint requests to Bunpei Kakinoki, Research Laboratory I, Pharmaceutical Research Unit, Research and Development Division, Mitsubishi Pharma Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama 227-0033, Japan. E-mail address: kakinoki.bunpei@mf.m-pharma.co.jp.

Received for publication 22 August 2005 and accepted in revised form 2 December 2005.

MCV, motor nerve conduction velocity; NGF, nerve growth factor; PGP9.5, protein gene product 9.5; PKC, protein kinase C; SCV, sensory nerve conduction velocity; STZ, streptozotocin; Trk, tyrosine receptor kinase.

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 cholesterols, 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 cholesterols 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 Wister 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-+*Lepr*^{db}/+*Lepr*^{db}/Jcl (*db/db*) and BKS.Cg-*m* +/+ *Lepr*^{db}/Jcl (*db*/+m) were used (CLEA Japan, Tokyo, Japan). At 9 weeks of age, body weight, value in a hot plate test (predosing), and plasma glucose level were measured for all animals before the beginning of drug administration. The *db/db* mice with plasma glucose levels >250 mg/100 ml were used as diabetic animals. Diabetic *db/db* mice were divided into three experimental groups (diabetic-vehicle and 3 or 10 mg/kg MCC-257 daily) of 20 animals, and then a 7-week treatment with MCC-257 was started.

MCC-257 [*N*-(5α-cholestan-3α-yl)-5-acetamido-3,5-dideoxy-2-*O*-methyl-α-D-*glycero*-D-*galacto*-2-nonulopyranosonamide, solid dispersion form 1:3 = MCC-257, polymer] was synthesized and manufactured by Mitsubishi Pharma. The compound was suspended in purified water and administered orally. Vehicle-treated diabetic animals were administered purified water. Normoglycemic animals (sham or *db*/+m) were administered purified water.

Dorsal root ganglion culture. Dorsal root ganglion neurons prepared from 14-day-old Wistar-ST rats were grown as dissociated cultures as previously described (29) with minor modification. These cells were plated onto poly-L-lysine–coated culture dishes in serum-free Ham's F12 medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 20 nmol/l progesterone, 0.1 mmol/l putrescine, 30 nmol/l sodium selenite, 50 units/ml penicillin, and 50 units/ml streptomycin. Cytosine arabinoside (100 μmol/l) was added to the culture medium from day 1 in culture for 24 h. Treatment with MCC-257 in the presence or absence of NGF (1 ng/ml; Toyobo, Osaka, Japan) was started immediately after plating. As a positive control, the other wells were treated with 100 ng/ml NGF.

PC12 cell culture. Pheochromocytoma 12 (PC12) cells (American *Type Culture* Collection) were cultured in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, 50 unit/ml penicillin G, and 50 μg/ml streptomycin sulfate in culture dishes. After the medium was exchanged for a serum-free RPMI medium, treatment of cultured cells with different doses of MCC-257 without or with NGF (50 ng/ml) was started immediately.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide assay. Dorsal root ganglia or PC12 cells were tested at 2 days in culture. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide solution (5 mg/ml in PBS) was added to each well, and the plates were incubated at 37°C for 4 h in a humidified 5% CO_2 atmosphere. After the incubation, a stop/solubilization solution consisting of 20% SDS in 50% dimethylformamide/50% distilled water was added. At 1 h after the addition of the stop/solubilization solution, the contents of the wells were mixed, and the absorbance at the 570-nm wavelength was measured.

TrkA tyrosine autophosphorylation. When PC12 cells were near confluent, the cells were preincubated with serum-free Dulbecco's modified Eagle's medium overnight and stimulated for 5 min at 37°C with MCC-257 in the presence or the absence of NGF (50 ng/ml). The cells were lysed with lysis buffer (20 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 10 mmol/l NaF, 5 mmol/l EDTA, 2 mmol/l Na_3VO_4, 80 units/ml aprotinin, and 1 mmol/l phenylmethylsulfonyl fluoride). The protein levels in the lysates were measured and equalized accordingly. Immunoprecipitation was performed with a Trk antibody (C-14; Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were washed with lysis buffer and then resuspended in Laemmli-SDS sample buffer (30) and boiled for 5 min. The samples and several doses of the positive control (epidermal growth factor–stimulated A431 cell lysate; Invitrogen, Charlottesville, VA) were subjected to SDS-PAGE and transferred to polyvinylidine difluoride membrane (Millipore, Billerica, MA). The membranes were probed with anti-phosphotyrosine antibody (4G10; Upstate Biotechnology). TrkA tyrosine phosphorylation on the membrane was detected by an enhanced chemiluminescent detection system (Amersham Biosciences, Piscataway, NJ). The densities of blots for the samples were measured with a densitometer (Personal Densitometer; Molecular Dynamics, Sunnyvale, CA). To compare the measured value of the samples among the trials, the amount of tyrosine phosphorylation of TrkA for an individual sample (namely, the density of each blot) was converted into the sample buffer volume of the positive control (μl) that was loaded on the same electrophoretic gel.

Measurement of nerve conduction velocity. At the end of the treatment period, the rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.; Abbott Laboratories). Rectal temperature was monitored during the procedure, and the rats were placed on a heating mat to diminish the hypothermic effects of anesthesia. The left sciatic nerve was stimulated at the sciatic notch and then at the Achilles tendon. Stimulations were delivered via concentric bipolar needle electrodes with a Synax 1100 (NEC San-ei Instruments, Tokyo, Japan). The M waves and H-reflexes to each stimulus were recorded via a needle electrode from the second interosseous muscle using Synax 1100. The temporal separation of the peaks of the M waves and H-reflexes was calculated with a Synax 1100. Nerve length from the sciatic notch to the Achilles tendon was measured and used to calculate motor nerve conduction velocity (MCV) and sensory nerve conduction velocity (SCV).

Determination of NGF content in the sciatic nerves. At the end of the treatment period, all of 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 containing 0.4 mol/l NaCl, 0.02% NaN_3, 0.1% BSA, and 1 mmol/l $MgCl_2$). 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-methyumbelliferyl-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-350A; 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 treatment period, all of the mice were anesthetized with diethyl ether and decapitated, and the plantar skins were dissected bilaterally. Left planter 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 dithiothreitol. 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 antisera (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 rehydration, each section was blocked with 1% BSA. The sections were incubated with the polyclonal primary antibody PGP9.5 (1:1,000; Ultraclone, 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) 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 computer. The digital images were saved as TIFF 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 (dermis within a 50-μm radius from the epidermal/dermal junction) was traced, and its area was

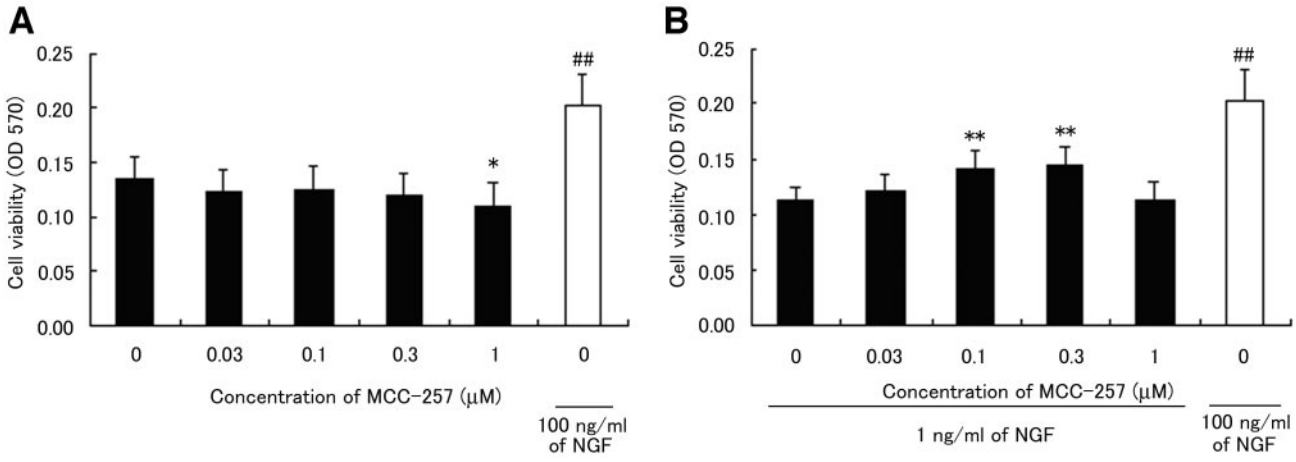

FIG. 1. Effect of MCC-257 on cell survival in cultured dorsal root ganglion neurons. *A*: MCC-257 had no effect on cell viability in cultured dorsal root ganglion neurons without NGF ($n = 6$). *$P < 0.05$ (ANOVA with Dunnett's test); ##$P < 0.01$ vs. 0 μmol/l (paired *t* test). *B*: MCC-257 protected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

measured using Image-Pro plus. The number of PGP9.5-immunoreactive nerve fibers with a cross-sectional area of ≤20 μm^2 was counted, and it was normalized with skin length (epidermal/dermal junction), using the following: nerve fibers (≤20 μm^2) per unit skin length (counts/mm) = PGP9.5-immunoreactive nerve fibers (≤20 μm^2) (counts)/skin length (μm) × 1,000.

Statistical analysis. Unless otherwise noted, all data are the means ± 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. 1*A*). MCC-257 (0.1, 0.3 μmol/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. 1*B*), 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 μmol/l. MCC-257 significantly deceased the viability of dorsal root ganglion neurons at a concentration of 1 μmol/l because of its insolubility ($P < 0.05$) (Fig. 1*A*).

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. 2*A*). The exposure of PC12 cells to MCC-257 in the presence of NGF (50 ng/ml) significantly enhanced TrkA autophosphorylation (0.1 μmol/l, $P < 0.01$) (Fig. 2*A*), whereas in the absence of NGF, treatment with MCC-257 had no effect on the tyrosine phosphorylation of TrkA (Fig. 2*A*). MCC-257 (0.1 μmol/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 μmol/l (Fig. 2*B*).

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

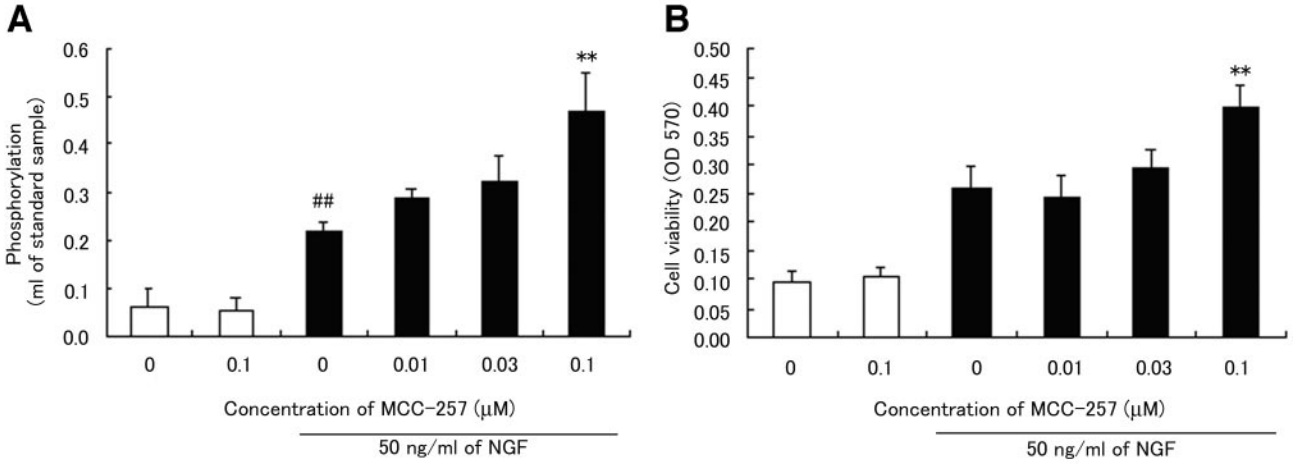

FIG. 2. Effect of MCC-257 on the tyrosine phosphorylation of TrkA. *A*: Effect of MCC-257 on the tyrosine phosphorylation of TrkA in PC12 cells with or without NGF ($n = 5$). ##$P < 0.01$ vs. 0 μmol/l without NGF (paired *t* test); **$P < 0.01$ vs. 0 μmol/l with NGF (Dunnett's test). *B*: Effect of MCC-257 on cell viability in serum-deprived PC12 cells with 50 ng/ml of NGF in the medium ($n = 5$). **$P < 0.01$ vs. 0 μmol/l (ANOVA with Dunnett's test). Data are the means ± SE. OD, optical density.

TABLE 1
MCV and SCV and NGF content in normal and diabetic rats treated with or without MCC-257

	Normal sham	Diabetic vehicle	MCC-257			
			0.1 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg
MCV (m/s)	61.6 ± 0.4	54.4 ± 1.0*	56.0 ± 1.0	57.3 ± 0.7	57.0 ± 0.4	57.7 ± 0.9†
SCV (m/s)	60.8 ± 0.6	49.9 ± 1.1*	51.5 ± 0.9	55.1 ± 0.7†	56.2 ± 0.8†	57.2 ± 1.6†
NGF content (pg/mg of sciatic nerve)	1.142 ± 0.106	0.450 ± 0.0551‡	0.516 ± 0.0609	0.623 ± 0.0856	0.740 ± 0.0980†	0.783 ± 0.0634†

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 hypoalgesia. 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 μm^2 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. 5*D*). 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. 5*D*). 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-

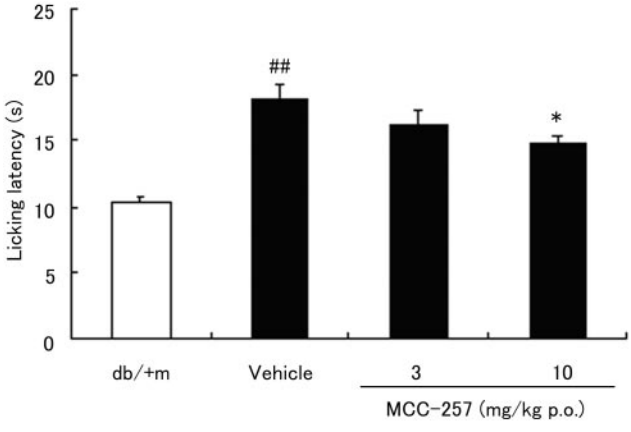

FIG. 3. Effect of MCC-257 on thermal hypoalgesia in genetically diabetic mice. A 7-week treatment with MCC-257 improved licking latencies in *db/db* mice ($n = 20$). ##$P < 0.01$ vs. *db*/+m (normoglycemic, unpaired t test); *$P < 0.05$ vs. *db/db* vehicle (Dunnett's test). Data are the means ± SE.

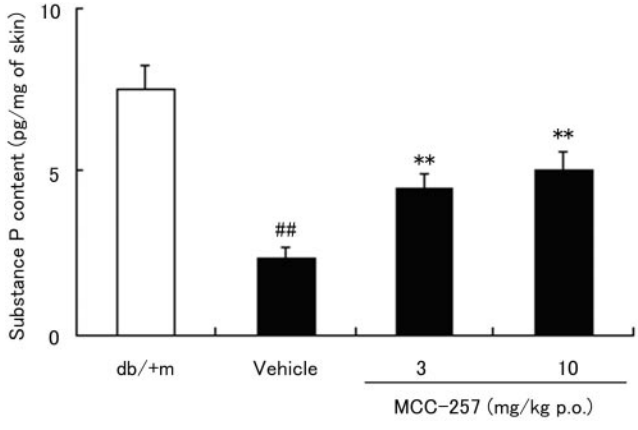

FIG. 4. Effect of MCC-257 on the substance P level in genetic diabetic mice. MCC-257 improved substance P content in the plantar skin ($n = 20$). ##$P < 0.01$ vs. *db*/+m (normoglycemic, unpaired t test); **$P < 0.01$ vs. *db/db* vehicle (Dunnett's test). Data are the means ± SE.

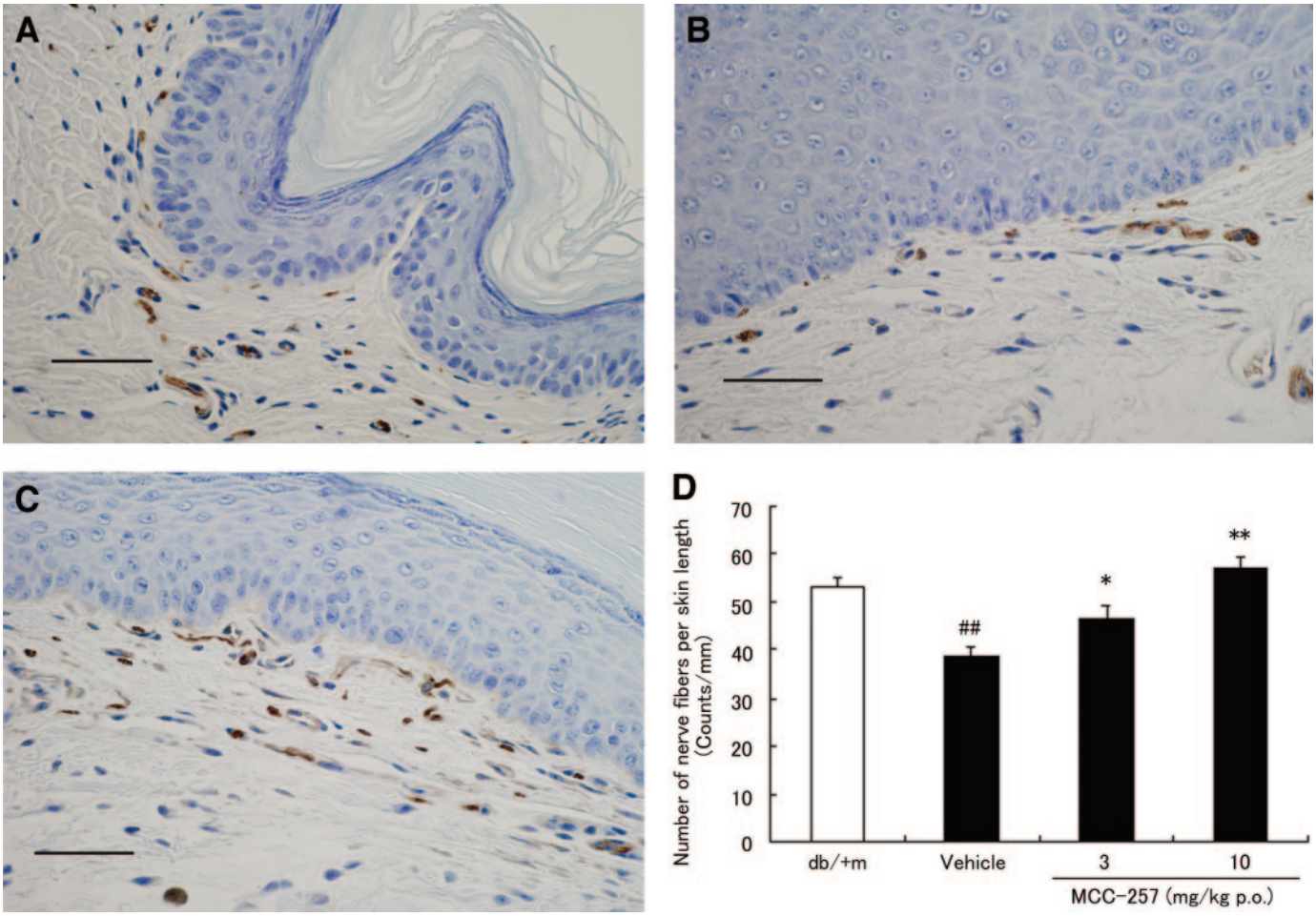

FIG. 5. *A–C*: Effect of MCC-257 on the cutaneous innervation in genetic diabetic mice. Immunohistochemistry for PGP9.5 in the mouse plantar skin. PGP9.5-positive fiber (dark brown) that has a cross-sectional area of ≤20 μm² was counted in the target field (dermis within a 50-μm radius from the epidermal/dermal 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.

inally 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

area is <20 μm^2 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 trails 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 E_2 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 sialic 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

1. Simmons Z, Feldman EL: Update on diabetic neuropathy. *Curr Opin Neurol* 15:595–603, 2002
2. Vincent AM, Brownlee M, Russell JW: Oxidative stress and programmed cell death in diabetic neuropathy. *Ann N Y Acad Sci* 959:368–383, 2002
3. Sheetz MJ, King GL: Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* 288:2579–2588, 2002
4. Greene DA, Arezzo JC, Brown MB: Effect of aldose reductase inhibition on nerve conduction and morphometry in diabetic neuropathy. *Neurology* 53:580–591, 1999
5. Greene DA, Stevens MJ, Obrosova I, Feldman EL: Glucose-induced oxidative stress and programmed cell death in diabetic neuropathy. *Eur J Pharmacol* 375:217–223, 1999
6. Koya D, King GL: Protein kinase C and the development of diabetic complications (Review). *Diabetes* 47:859–866, 1998
7. Anand P, Terenghi G, Warner G, Kopelman P, Williams-Chestnut RE, Sinicropi DV: The role of endogenous nerve growth factor in human diabetic neuropathy. *Nat Med* 2:703–707, 1996
8. Fernyhough P, Diemel LT, Hardy J, Brewster WJ, Mohiuddin L, Tomlinson DR: Human recombinant nerve growth factor replaces deficient neurotrophic support in the diabetic rat. *Eur J Neurosci* 7:1107–1110, 1995
9. Apfel SC, Arezzo JC, Brownlee M, Federoff H, Kessler JA: Nerve growth factor administration protects against experimental diabetic sensory neuropathy. *Brain Res* 634:7–12, 1994
10. Unger JW, Klitzsch T, Pera S, Reiter R: Nerve growth factor (NGF) and diabetic neuropathy in the rat: morphological investigations of the sural nerve, dorsal root ganglion, and spinal cord. *Exp Neurol* 153:23–34, 1998
11. Bennett GS, Garrett NE, Diemel LT, Brain SD, Tomlinson DR: Neurogenic cutaneous vasodilatation and plasma extravasation in diabetic rats: effect of insulin and nerve growth factor. *Br J Pharmacol* 124:1573–1579, 1998
12. Apfel SC, Kessler JA, Adornato BT, Litchy WJ, Sanders C, Rask CA: Recombinant human nerve growth factor in the treatment of diabetic polyneuropathy: NGF Study Group. *Neurology* 51:695–702, 1998
13. Ceccarelli B, Aporti L, Finesso M: Effects of brain gangliosides on functional recovery in experimental regeneration and reinnervation. *Adv Exp Med Biol* 71:275–293, 1976
14. Purpura KP, Baker HJ: Neurite induction in mature cortical neurones in feline GM1-ganglioside storage disease. *Nature* 266:553–554, 1977
15. Roisen FJ, Bartfeld H, Nagele R, Yorke G: Ganglioside stimulation of axonal sprouting in vitro. *Science* 214:577–578, 1981
16. Sabel BA, Dunbar GL, Stein DG: Gangliosides minimize behavioral deficits and enhance structural repair after brain injury. *J Neurosci Res* 12:429–443, 1984
17. Sabel BA, DelMastro R, Dunbar GL, Stein DG: Reduction of anterograde degeneration in brain damaged rats by GM1-gangliosides. *Neurosci Lett* 77:360–366, 1987
18. Sabel BA, Slavin MD, Stein DG: GM1 ganglioside treatment facilitates behavioral recovery from bilateral brain damage. *Science* 225:340–342, 1984
19. Toffano G, Savoini GE, Moroni F, Lombardi G, Calza L, Agnati LF: Chronic GM1 ganglioside treatment reduces dopamine cell body degeneration in the substantia nigra after unilateral hemitransection in rat. *Brain Res* 296:233–239, 1984
20. Chan KF: Ganglioside-modulated protein phosphorylation: partial purification and characterization of a ganglioside-inhibited protein kinase in brain. *J Biol Chem* 263:568–574, 1988
21. Mutoh T, Tokuda A, Miyadai T, Hamaguchi M, Fujiki N: Ganglioside GM1 binds to the Trk A protein and regulates receptor function. *Proc Natl Acad Sci U S A* 92:5087–5091, 1995
22. Crepaldi G, Fedele D, Tiengo A, Battistin L, Negrin P, Pozza G, Canal N, Comi GC, Lenti G, Pagano G, Bergamini L, Troni W, Frigato F, Ravenna C, Mezzina C, Gallato R, Massari D, Massarotti M, Matano R, Grigoletto F, Davis H, Klein M: Ganglioside treatment in diabetic peripheral neuropathy: a multicenter trial. *Acta Diabetol Lat* 20:265–276, 1983
23. Abraham RR, Abraham RM, Wynn V: A double blind placebo controlled trial of mixed gangliosides in diabetic peripheral and autonomic neuropathy. *Adv Exp Med Biol* 174:607–624, 1984
24. Tsuji S, Yamashita T, Tanaka M, Nagai Y: Synthetic sialyl compounds as well as natural gangliosides induce neuritogenesis in a mouse neuroblastoma cell line (Neuro2a). *J Neurochem* 50:414–423, 1988
25. Yamashita T, Tsuji S, Nagai Y: Sialyl cholesterol is translocated into cell nuclei and it promotes neurite outgrowth in a mouse neuroblastoma cell line. *Glycobiology* 1:149–154, 1991
26. Yamashita T, Tsuji S: Nuclear sialyl cholesterol causes changes in the structure of chromatin and its transcription level followed by the promotion of neuritogenesis. *J Neurochem* 58:1360–1364, 1992
27. Hashitani T, Furuyama F, Kumazaki M, Zhou XR, Geller HM, Toyomaki Y, Okamoto K, Oomura Y, Nishino H: Sialyl cholesterol enhances the development of grafted neurons and motor recovery. *Brain Res Bull* 29:795–806, 1992
28. Apfel SC, Schwartz S, Adornato BT, Freeman R, Biton V, Rendell M, Vinik A, Giuliani M, Stevens JC, Barbano R, Dyck PJ: Efficacy and safety of recombinant human nerve growth factor in patients with diabetic polyneuropathy: a randomized controlled trial: rhNGF Clinical Investigator Group. *JAMA* 284:2215–2221, 2000
29. Sekimoto S, Tashiro T, Komiya Y: Two stages in neurite formation distinguished by differences in tubulin metabolism. *J Neurochem* 64:354–363, 1995
30. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
31. Hory-Lee F, Russell M, Lindsay RM, Frank E: Neurotrophin-3 supports the survival of developing muscle sensory neurons in culture. *Proc Natl Acad Sci U S A* 90:2613–2617, 1993
32. Ernfors P, Lee KF, Kucera J, Jaenisch R: Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* 77:503–512, 1994
33. Leeman SE. Gamse R: Substance P in sensory neurons. *Trend Phramacol Sci* 2:119–121, 1981
34. Apfel SC: Neurotrophic factors in the therapy of diabetic neuropathy (Review). *Am J Med* 107:34S–42S, 1999
35. Lewin GR, Rueff A, Mendell LM: Peripheral and central mechanisms of NGF-induced hyperalgesia. *Eur J Neurosci* 6:1903–1912, 1994
36. Rueff A, Dawson AJ, Mendell LM: Characteristics of nerve growth factor induced hyperalgesia in adult rats: dependence on enhanced bradykinin-1 receptor activity but not neurokinin-1 receptor activation. *Pain* 66:359–372, 1996