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Regulation of Microglial Activities by Glial Cell Line Derived Neurotrophic Factor

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Abstract Much attention has been paid to the ability of glial cell line-derived neurotrophic factor (GDNF) to protect neurons from neurotoxic insults in the central nervous system (CNS). However, little is known about GDNF action on CNS glia that also can express GDNF receptor systems. In this study, we examined the effects of GDNF on primary rat microglia that function as resident macrophages in the CNS and as the source of proinflammatory mediators upon activation. We found that treatment of primary rat microglia with GDNF had no effect on the secretion of the proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), but it increased the nitric oxide (NO) production to some extent. In addition, GDNF increased the enzymatic activity of superoxide dismutase (SOD), the gene expression of surface antigen intercellular adhesion molecule-1 (ICAM-1), the production of the integrin α5 subunit, and the phagocytotic capability in primary rat microglia. Furthermore, inhibition of mitogen-activated protein kinase (Erk-MAPK) in the mouse microglial cell line BV2 by U0126 indicated that the MAP kinase signaling pathway may be involved in the regulation of NO and integrin α5 production by GDNF. In vivo evidence also showed that amoeboid cells with integrin α5 or with ED1 immunoreactivity appeared in GDNF-treated spinal cord tissues at the lesion site 1 week post spinal cord injury (SCI). Furthermore, inhibition of Erk-MAPK in the mouse microglial cell line BV2 by U0126 indicated that the MAP kinase signaling pathway may be involved in the regulation of NO and integrin α5 production by GDNF. Taken together, our results indicate that GDNF has a positive regulatory effect on microglial activities, such as phagocytosis and the upregulation of adhesion molecules. J. Cell. Biochem. 97: 501–511, 2006.

Key words: glial cell line-derived neurotrophic factor; microglia; inflammation

Microglia, a major immune cell population in the central nervous system (CNS), are inactive in healthy adult CNS, and become activated in acute and chronic neurodegenerative diseases [Kreutzberg, 1996; Streit et al., 2004; Kim and de Vellis, 2005]. Since activated microglia produce cytotoxic inflammatory mediators, such as nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), and IL-1β after CNS injury, these cells are believed to play a crucial role in triggering secondary tissue damage, which in turn leads to the progression of CNS diseases [Kreutzberg, 1996; Streit et al., 2004; Kim and de Vellis, 2005]. On the other hand, the activated microglia have a major effect on phagocytotic activities, which allow these cells to remove neural cell fragments and myelin debris efficiently [Elkabes et al., 1996; Kreutzberg, 1996; Liu and Hong, 2003]. These cells are also beneficial to neuronal survival in the damaged CNS via the production of neurotrophic factors [Giulian and Corpuz, 1993; Elkabes et al., 1996; Kreutzberg, 1996], and the regulation of the production of nonpermissive substrata [David et al., 1990].

Glial cell line-derived neurotrophic factor (GDNF), a transforming growth factor (TGF)-β superfamily, was originally isolated from a glial cell line, and serves as a trophic factor for dopaminergic neurons [Lin et al., 1993], as well as nondopaminergic neurons, including spinal cord motoneurons and peripheral ganglia. Thus, the molecule is thought to be a potential therapeutic agent for various CNS neurological diseases [Airaksinen and Saarma, 2002], such as cerebral ischemia/hypoxia [Ikeda et al.,

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2000], Parkinson's disease [Gash et al., 1996] and SCI [Grondin and Gash, 1998; Cheng et al., 2002; Tai et al., 2003].

Microglia express GDNF receptor complex-GDNF-family receptor α (GFRα) and transmembrane c-Ret tyrosine kinase that trigger the activation of extracellular signal-regulated kinase-mitogen-activated protein (Erk-MAPK) and phosphatidylinositol 3-kinase/Akt (PI3K/Akt) to regulate cellular functions [Batchelor et al., 1999; Honda et al., 1999]. Although the neurotrophic effect of GDNF on neurons is well established, its action on microglial activities remains to be clarified since this molecule has been considered as a potent agent for CNS treatment. In the present study, we used cultured primary rat microglia and various biochemical analyses to examine whether GDNF is able to mediate microglial activities, including the production of proinflammatory mediators (TNF-α and IL-1β), the activity of endogenous antioxidant enzyme superoxide dismutase (SOD), adhesion molecule expression and phagocytosis. We found that GDNF had no effect on the production of the microglial TNF-α and IL-1β, whereas the molecule increased NO metabolites, intercellular adhesion molecule-1 (ICAM-1) gene expression, integrin-α5 and the phagocytotic ability of primary rat microglia. We also used the mouse microglial cell line BV2, which was found to increase integrin α5 and Erk-MAPK activation in response to GDNF, to examine whether Erk-MAPK signaling is involved in GDNF action on microglial integrin α5 production. We also provide evidence showing that integrin α5 expression found in amoeboid cells of GDNF-treated spinal cord post SCI were ED1 immunoreactive. Our study provides evidence for GDNF action on microglial activities, and supports the idea of the diverse roles of GDNF in the improvement of CNS tissue repair.

MATERIALS AND METHODS

Cell Cultures

The mouse microglial cell line BV2, which was generated from primary mouse microglia transfected with a v-raf/v-myc oncogene [Blasi et al., 1990] kindly provided by Dr. J.S. Hong (NIEHS, NIH, USA), was maintained at 37°C in Dulbecco's modified Eagle's medium/F12 medium (DMEM/F-12) (Life Technologies, Grand Island, NY) with 5% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), 50 U/ml penicillin, and 50 mg/ml streptomycin (Sigma Chemical Co., St. Louis, MO) under a humidified atmosphere of 5% CO_2/95% air.

Primary rat microglial culture was prepared as previously described [Tzeng et al., 2002]. In brief, cerebral cortices from neonatal Sprague–Dawley rat brains (P1) were removed and carefully dissected. The tissue was dissociated in 0.0025% trypsin/EDTA (Life Technologies) and passed through a 70 μm pore nylon mesh (Falcon). After centrifugation, the cell pellet was resuspended in DMEM/F-12 containing 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. The cells (10^7 cells/flask) were then plated onto poly-D-lysine (PDL; Sigma) coated 75-cm tissue culture flasks. The medium was renewed every 2–3 days. Eight days later, primary rat microglia were collected using the shake-off method [McCarthy and de Vellis, 1980]. Primary rat microglia were replated either onto 96-well tissue culture plates or 35 mm tissue culture dishes at a density of 2×10^4 cells/well or 5×10^5 cells/dish, respectively. Eighteen to 24 h later, the cells were treated with rat recombinant GDNF (R&D, Minneapolis, MN) in DMEM/F-12 medium containing N1 serum supplement (Life Technologies). In general, there was 95% primary rat microglia with B4–isolectin (Sigma) positive staining in the microglial culture, whereas less than 5% of cells were GFAP-immunostained astrocytes and CNPase-immunostained oligodendrocytes in the culture.

Nitrite/Nitrate, TNF-α, and IL-1β Assay

Primary rat microglia at a density of 2×10^4 cells/well were replated onto 96-well plates. Twenty-four hours after treatment with GDNF, the culture media were collected and centrifuged at 1,500 rpm for 10 min to remove floating cells. The supernatant was then used for NO production assay and cytokine ELISA assay. The production of NO was assessed as the accumulation of nitrate and nitrite in the culture medium using a colorimetric reaction kit from R&D. TNF-α and IL-1β were measured using an enzyme immunoassay kit from R&D following the procedure provided by the vendor.

Reverse-Transcription PCR (RT-PCR)

Primary rat microglia at a density of 1×10^6 cells/dish were replated onto 60 mm culture petri dishes. Eighteen to 24 h after treatment with GDNF, total RNA was extracted from

microglia, and 1 μg of total RNA was used in the RT-PCR reaction mixed with SuperScript one-step RT-PCR system components (Gibco, Life Technologies, Grand Island, NY) with the following cycle parameters: 45°C, 30 min, 94°C, 2 min; 30 cycles, 94°C, 15 s; 55°C, 30 min, 72°C, 1 min; 72°C, 7 min, 4°C, ∞. Reaction products were then separated on a 1.5% agarose gel, stained with ethidium bromide, and photographed. Measurements were normalized to GAPDH mRNA levels. The RT-PCR primers were as follows: ICAM-1 (385 bp) sense 5′-ctggagagcacaaacagcagag-3′, antisense 5′-aaggccgcagagcaaaagaagc-3′; GAPDH (671 bp) sense 5′-ttcaccaccatggagaaggc-3′, antisense 5′-accaccctgttgctgtagcc.

Measurement of SOD Activity

Primary rat microglia at a density of 5×10^5 cells/dish were replated onto 35 mm tissue culture dishes. The cells were harvested at 18 h after treatment with GDNF at distinct concentrations, and homogenized in 5% monophosphoric acid. After centrifugation at 3,000 rpm for 2 min at 4°C, the supernatant was analyzed for total SOD activity using a BIOXYTECH SOD-525 kit from R&D. Total protein was measured using a Bio-Rad DC kit, and the specific activity was expressed as units per milligram of protein.

Western Blotting

Cells were washed twice with PBS after harvest, and lysed for 30 min on ice in the lysis buffer containing 1% Triton-X 100, 0.05% Tween-20, 10 μg/ml PMSF, 4 μg/ml pepstatin A, 2 mM 4-(2-aminothyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM EDTA, 130 μM bestatin, 1.4 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin, and 1 mM sodium orthovanadate (Sigma), and then centrifuged at 12,000 rpm for 10 min. Protein concentration was determined by a Bio-Rad DC kit (Bio-Rad, Hercules, CA). Proteins (10–20 μg) were separated by 7.5–12.5% SDS–PAGE, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham, Piscataway, NJ). The membrane was incubated with primary antibodies overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies and ECL solution (NEN LifeScience, Boston, MA). The antibodies used in this study included mouse anti-a-tubulin (Sigma), rabbit anti-integrin α5 (Chemicon, Temecula, CA), mouse anti-integrin β1 (BD PharMingen), anti-iNOS (Calbiochem, Cambridge, MA), anti-MnSOD (StreeGen Biotechnologies Corp., Victoria, Canada), rabbit anti-p-Erk42/44, rabbit anti-Erk, rabbit anti-p-Akt and rabbit anti-Akt (Cell Signaling, Beverly, MA).

Phagocytosis Assay

Primary rat microglia at a density of 5×10^4 cells/well were replated onto a 24-well plate followed by GDNF treatment for 24 h. A PKH26 red fluorescent phagocytic cell linker kit (Sigma) prepared following the procedure provided by the vendor was added to the culture, and incubated for 3 min followed by the addition of 1% bovine serum albumin for 1 min. Cells were fixed in 2% paraformaldehyde for 10 min, and then counterstained with 0.1% DAPI for 1 min. Four random fields of cells (approximately 80 cells) were counted using a 40× object lens under an epifluorescence microscope equipped with a cooling digital imaging system. The cells with bright, punctuated fluorescent appearance were referred to as phagocytic cells. The ratio of phagocytic cells to total counted cells was defined as the phagocytotic index.

Cell Count Assay

Primary rat microglia (5×10^4 cells/well) were plated onto 24-well plates. Eighteen to 24 h later, cells were treated for 24 h with GDNF at concentrations of 10, 20, or 50 ng/ml. The cultures were washed with PBS to remove cell debris and cells subsequently were harvested by adding 100 μl of 0.025% trypsin to each culture well for 2 min followed by adding 300 μl of PBS per well. Trypan blue staining method was used to identify live cells. Cell were immediately harvested and counted using a hemacytometer.

Animal Surgery

SCI was induced using the NYU weight-drop device [Constantini and Young, 1994; Basso et al., 1996]. The surgical procedures were followed as previously described [Cheng et al., 2002]. In brief, female adult (240–275 g) Sprague–Dawley rats that were supplied by the Animal Center of the National Science Council, Taiwan, were anesthetized with pentobarbital (50 g/kg, i.p.), and laminectomy was performed at T9-T10 levels. Within 1 h after anesthesia, the dorsal surface of the cord was

injured by dropping a 10 g rod from a height of 50 mm. A 5-μl Exmire micro-syringe with a 33-gauge needle was positioned at the midline of the spinal cord tissue 1–2 mm rostral and caudal to the epicenter and stereotaxically inserted 0.7–0.8 mm below the dura. At 5 min post injury, GDNF infusion (R&D) was started at a rate of 0.5 μl/min. After each injection, the 33-gauge needle was maintained in the spinal cord for an additional 5 min to reduce the possibility of leakage of the injected fluid from the site. The total volume of infused GDNF (0.5 μg/μl) to the injured spinal tissue was 8 μl (4 μl/injection; 4 μg/rat), and the infusion was completed in 20 min. The contused rats used as controls received the same amount of PBS solution. During surgery and infusion, each animal was placed on a temperature-controlled heating blanket to maintain body temperature. After injection, animals were housed in pairs, and manual bladder evacuation was performed at least twice a day. The injured animals received antibiotics (sodium ampicillin, 80 mg/kg/day) during the first week after injury. No autophagia was observed. Animal care was provided in accordance with the Laboratory Animal Welfare Act, Guide for the Care and Use of Laboratory Animals, National Cheng Kung University, Taiwan.

Animals were anesthetized at 1 week post-contusion injury, and then perfused with 0.9% cold NaCl followed with 4% paraformaldehyde in 0.1 M phosphate buffer. Spinal cord tissues approximately 2 cm in length containing the contusion center were removed, post-fixed with 4% paraformaldehyde overnight at 4°C, and then cryoprotected with 30% (w/v) sucrose in PBS overnight at 4°C.

Immunohistochemistry

Spinal cord tissues were embedded in optimum cutting temperature (OCT) matrix (Tissue-Tek, Torrance, CA), and longitudinally sectioned at 20 μm thickness. The tissue sections were incubated with 0.1% Triton X-100 in PBS for 30 min followed by mouse anti-ED1 (1:4,000; Serotec, Oxford, UK) or rabbit anti-integrin α5 (1:200; Chemicon) in PBS containing 5% horse serum overnight. The tissue sections were then incubated with biotinylated secondary antibodies (1:200) for 1 h, and fluorescein-avidin for 45 min. The results were observed under a fluorescence microscope (Nikon).

Statistical Analysis

Data are expressed as mean ± SD. Each experiment was repeated at least three times. Statistical significance of differences between the two groups of data (P-value < 0.05) was performed using unpaired t-test (Minitab, State College, PA).

RESULTS

Effect of GDNF on the Production of Proinflammatory Mediators in Primary Rat Microglia

To examine whether GDNF regulates primary microglial cytokine production, levels of IL-1β and TNF-α in cultured primary microglia were analyzed. As shown in Figure 1, no significant difference in secreted TNF-α and IL-1β levels was observed in the culture of primary

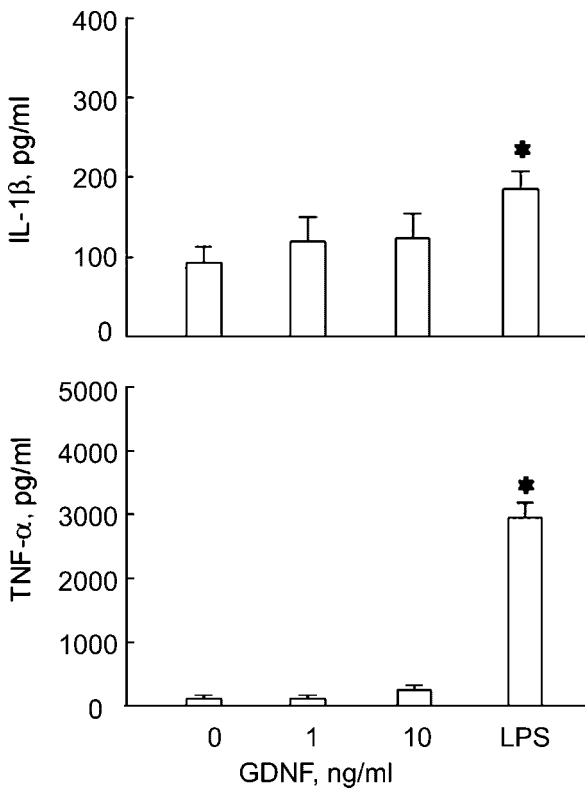

Fig. 1. Effect of GDNF on levels of secreted IL-1β and TNF-α in primary rat microglia. Microglia were treated with GDNF at the distinct concentrations as indicated above. The culture media were collected at 24 h, and subjected to IL-1β and TNF-α ELISA assays. The culture that was treated with 10 ng/ml of LPS was referred as a positive control. Values were determined using mean ± SD (n = 6 separate cultures per bar). The experiments were performed three times with similar results. *$P < 0.05$ (unpaired t-test) compared to control.

microglia treated with 1 and 10 ng/ml of GDNF for 24 h when compared to those of the control. We also found that there was no increase in microglial TNF-α and IL-1β levels at 48 h post-GDNF treatment (data not shown). Thus, our data demonstrated that GDNF has no effect on the induction of release of microglial proinflammatory cytokines.

Examination of microglial iNOS and NO metabolites (nitrate and nitrite) revealed that treatment with GDNF at the concentrations of 1 and 10 ng/ml caused no production of iNOS in microglia, but induced an increase in the level of NO metabolites (Fig. 2). Like other neurotrophic factors, GDNF can activate several signaling pathways, such as the Erk42/44-MAPK and PI3K/Akt pathways [Takahashi, 2001]. Since Erk-MAPK signaling is a common pathway for NO production via the induction of three NOS isoenzymes (iNOS, neuronal NOS, and endothelial NOS) [Schroeter et al., 2002], we examined whether MAPK is involved in nitrite/nitrate accumulation in GDNF-treated microglia using U0126, a selective inhibitor for p42/44 MAP kinase (MEK). Our results showed that the GDNF-induced increase in nitrite/nitrate accumulation was blocked by U0126 (Fig. 2), indicating that GDNF may activate the Erk-MAPK signaling pathway to increase NO production in microglia.

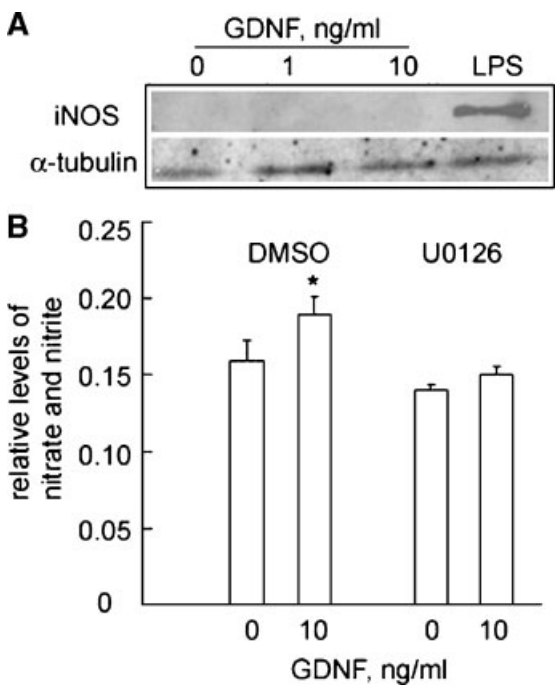

Fig. 2. Effect of GDNF on the accumulated amounts of nitrate and nitrite in primary rat microglial cultures. **A**: Microglia were treated with GDNF at 1 and 10 ng/ml for 24 h. Western blotting indicated that GDNF had no effect on the production of iNOS protein. The culture that was treated with 10 ng/ml of LPS was used as a positive control. Similar results were obtained in three independent experiments. **B**: Microglia were treated with 10 ng/ml of GDNF in the presence or absence of 10 μM U0126 for 24 h. Subsequently, the culture media were collected and submitted to the assays for nitrite and nitrate. Values were determined using mean ± SD (n = 6 separate cultures per bar). The experiments were performed twice with similar trends. $^*P < 0.05$ (unpaired *t*-test) compared to control.

Effect of GDNF on SOD Enzymatic Activity in Primary Rat Microglia

Microglia can generate ROS upon activation [Colton and Gilbert, 1987; Colton et al., 1994]. SOD is an endogenous enzyme to convert superoxide radicals to H_2O_2 and oxygen [McCord and Fridovich, 1969]. An increase in Cu/Zn-SOD and Mn-SOD immunoactivity in microglia after CNS injury has been reported [Liu et al., 1993]. Accordingly, we accessed the examination of SOD expression and activity in microglia post GDNF treatment. We found that treatment with GDNF caused a slight increase in MnSOD levels (Fig. 3A), but no change in Cu/ZnSOD levels (data not shown). Furthermore, examination of total SOD enzymatic activity indicated that 20 ng/ml of GDNF treatment induced an increase in SOD enzymatic activity (Fig. 3A). Since the primary rat microglia used in the two experiments were isolated from a different batch of rat pups, this may have caused a variation in the relative SOD activity induced by GDNF in the two experiments. It was also noted that GDNF at the lower concentrations failed to increase microglial SOD enzymatic activity (data not shown).

GDNF Action on the Expression of ICAM-1 in Primary Rat Microglia

Microglial activation is generally associated with the upregulation of microglial surface markers [Lee and Benveniste, 1999], such as ICAM-1, which is known to participate in the migration of microglia to an inflamed area [Streit et al., 2004]. To evaluate the regulation of microglial ICAM-1 expression in response to 20 ng/ml of GDNF, RT-PCR analysis was performed. The results indicated that treatment with GDNF increased mRNA expression of microglial ICAM-1 (Fig. 3B), implying that GDNF may have a regulatory effect on microglial migration.

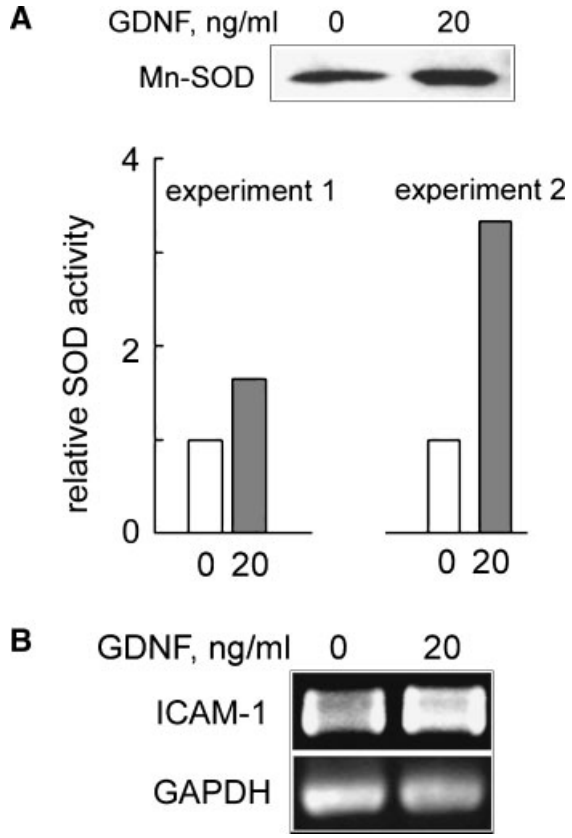

Fig. 3. Effect of GDNF on microglial SOD activity and ICAM-1 expression. **A**: Primary rat microglia were treated with 20 ng/ml of GDNF for 24 h. Western blotting indicated that 20 ng/ml of GDNF slightly increased the production of microglial MnSOD. The SOD activity assay indicated that treatment with GDNF at 20 ng/ml induced an increase in SOD activity. The relative SOD activity is indicated as the ratio of the SOD activity (unit/mg) in GDNF-treated microglia to control. **B**: Primary rat microglia were treated with GDNF at 20 ng/ml for 24 h followed by RNA extraction and RT-PCR analysis for ICAM-1 mRNA expression. Similar results were observed in three independent experiments.

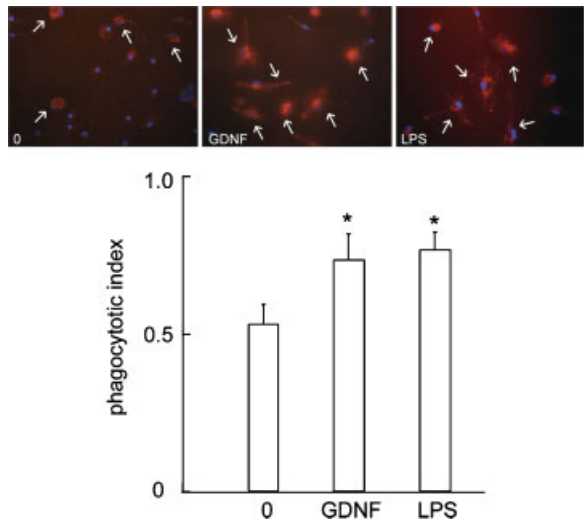

Fig. 4. Microglial phagocytotic activity improved by GDNF. Primary rat microglia were treated with 20 ng/ml of GDNF or 10 ng/ml of LPS for 24 h, and then incubated with a phagocytic cell linker kit for 3 min as described in Materials and Methods. Three independent experiments were done with similar results, and each experiment was performed in duplicate. Values were determined using mean ± SD of three experiments. $*P < 0.05$ (unpaired *t*-test) compared to control. Immunofluorescent images (400×) are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Microglial Phagocytosis Regulated by GDNF

GDNF has recently been reported to enhance the phagocytotic activity of macrophages [Hashimoto et al., 2005]. Since microglia exhibit typical macrophage behaviors, such as phagocytosis, the effect of GDNF on microglial phagocytotic ability was examined in this study. We found that the phagocytotic uptake of primary rat microglia was increased in response to GDNF (Fig. 4).

Production of Integrin α5 and β1 in Microglia Treated With GDNF

Recent studies have reported that integrin β1 regulates the phagocytotic uptake of bacteria by macrophages [Dersch and Isberg, 2000; Isberg et al., 2000]. Accordingly, we attempted to ascertain whether GDNF has a regulatory effect on integrin expression in primary rat microglia. We found that treatment with GDNF caused no change in the production of microglial β1, whereas 10 and 20 ng/ml of GDNF induced a significant increase in the levels of microglial integrin α5 (Fig. 5A). A mouse microglial cell line, BV2, has been shown to retain the morphological, phenotypical, and functional properties for primary rat microglia [Blasi et al., 1990]. Similar to the findings in primary rat microglia, our findings revealed that GDNF at concentrations of 10 and 20 ng/ml augmented the production of integrin α5 in BV2 cells (Fig. 5B).

Involvement of ERK Signaling in the Regulation of Integrin α5 Expression

Furthermore, we found that the phosphorylation of Erk-42/44 in primary rat microglia was increased by GDNF at 60 and at 120 min (Fig. 6A). Yet, treatment with GDNF had no effect on the phosphorylation of Akt when compared to that observed in the control during the entire experimental period (Fig. 6). Similar to primary rat microglia, GDNF induced a

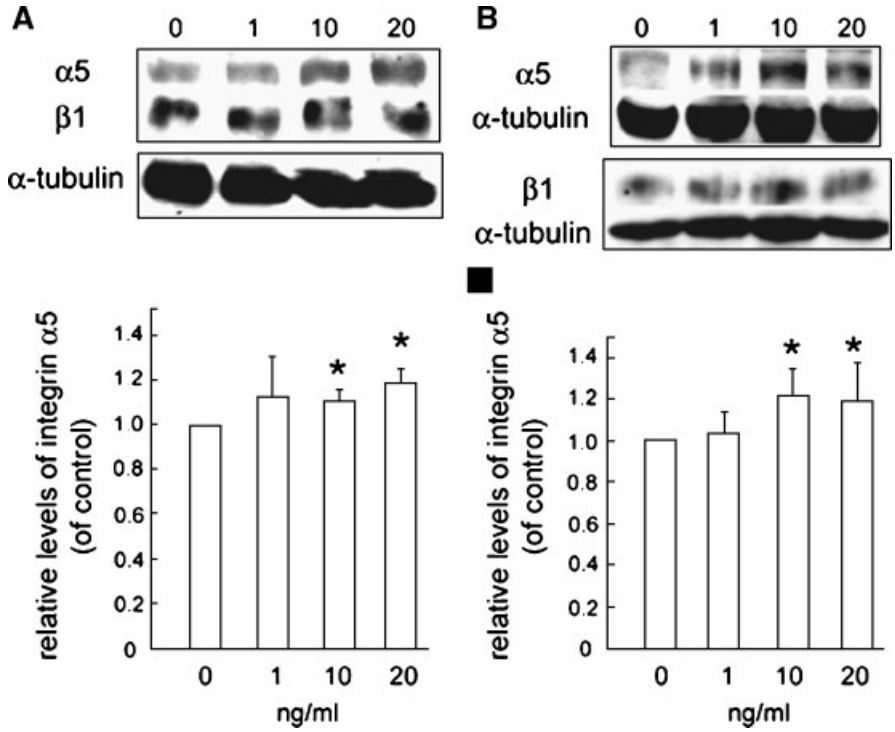

Fig. 5. The production of integrin α5 in microglia with GDNF treatment. Primary rat microglia (**A**) and BV2 cells (**B**) were treated for 24 h with GDNF at the different concentrations as indicated above. Cell extracts were analyzed by Western blotting as described in Materials and Methods. Data are representative of three similar experiments. Relative levels of integrin α5 were defined as the arbitrary unit in each treatment over the untreated control. The arbitrary unit was the level of integrin α5 in each treatment normalized to that of α-tubulin. Values were determined using mean ± SD of three experiments. $^*P < 0.05$ (unpaired *t*-test) compared to control.

delayed increase in the level of Erk-44 at 60 min in BV2 cells and this increase remained at 120 min (Fig. 6B), whereas treatment with GDNF caused no increase in the levels of pAkt. Accordingly, to determine the role of Erk42/44 signaling in the regulation of integrin α5 expression by GDNF, BV2 cells were preincubated with 10 μM of U0126 (a MEK inhibitor) for 5 min prior to treatment with 10 ng/ml of GDNF. As shown in Figure 6C, the level of integrin α5

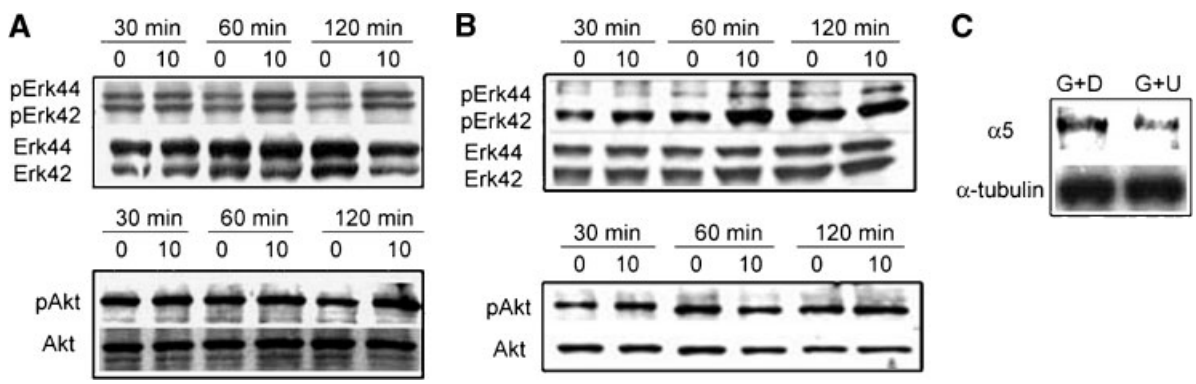

Fig. 6. Erk-MAPK signaling involved in integrin α5 production of GDNF-treated microglia. Primary rat microglia (**A**) and BV2 cells (**B**) were treated with 10 ng/ml of GDNF at the different time points as indicated above. In Western blotting, an increase in the level of pErk-42/44 or pErk-44 was observed at 60 and 120 min in GDNF-treated microglia (A) and BV2 cells (B), respectively. However, GDNF treatment did not cause any change in the phosphorylation of Akt. In each experiment, the same blot was stripped and reprobed with Erk42/44 or Akt antibodies. Data are representative of three similar experiments. **C**: Prior to treatment with 10 ng/ml of GDNF for 24 h, BV2 cells were incubated for 5 min with 0.1% of DMSO (G + D) or 10 μM of U0126 (G + D). The expression of integrin α5 was detected by Western blotting. The experiments were repeated twice with similar results.

was decreased by exposure of GDNF-treated BV2 to U0126.

Expression of Integrinα5 in GDNF-Treated Spinal Cord Following Spinal Cord Injury

We have previously shown that GDNF promoted neuronal survival and hindlimb functional recovery in rats with severe SCI [Cheng et al., 2002; Tai et al., 2003]. There is no appropriate antibody to distinguish between infiltrating round macrophages and activated amoeboid microglial populations. However, ED1 antibodies have been used to identify activated microglia or macrophages derived from resident microglial populations in the injured spinal cord [Popovich et al., 1997; Rosenberg et al., 2005]. Accordingly, we performed ED1$^+$ immunostaining and found that a large amount of ED1$^+$ amoeboid microglia/macrophages was observed in the GDNF-treated spinal cord at 1 week post SCI, while only a few ED1$^+$ cells appeared in the PBS-treated spinal cord (Fig. 7). Since macrophages have been reported to be classified as cells with round morphology with both OX42 and ED1 immunoreactivities [Popovich et al., 1997], to further define the type of ED1$^+$ amoeboid cells OX42 immunostaining was carried out. However, a weak OX42 immunostaining was observed in the injured spinal cord at 1 week post SCI (data not shown), indicating that ED1$^+$ amoeboid cells were activated microglia or macrophages-derived from activated microglia. In parallel, amoeboid cells with strong immunoreactivity for integrin α5 were found in GDNF-treated spinal cord tissues proximate to the lesion center. Yet, in the PBS-treated spinal cord tissue, cells with integrin α5 immunostaining were ramified or surrounding blood vessels. It has been reported that the number of apoptotic microglia was increased in the injured spinal cord rostral and caudal to the lesion center from 5 to 21 day after spinal cord contusion [Shuman et al., 1997]. ED1$^+$ amoeboid microglia/macrophages were found in GDNF-treated spinal cord tissues at 1 week post contusion (Fig. 7B,D). These findings suggest that administered GDNF may improve microglial cell survival or proliferation. Furthermore, examination of primary rat microglial cell growth by cell count assay indicated that the number of primary rat microglia was significantly increased at 24 h after the exposure to 20 and 50 ng/ml of GDNF when compared to that found in the control (Fig. 7G).

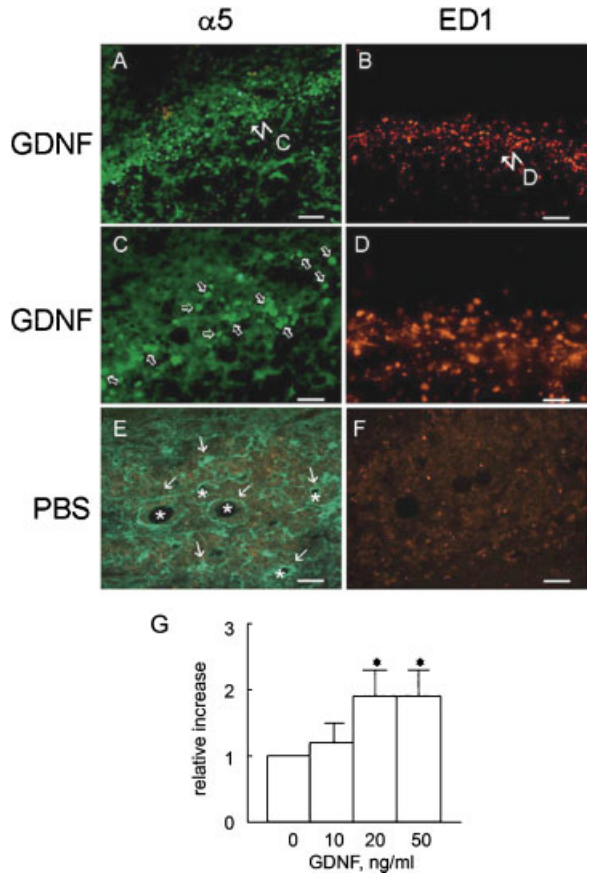

Fig. 7. Induction of integrin α5 expression by GDNF in the injured spinal cord 1 week after SCI. The adjacent horizontal section of the spinal cord was prepared for integrin α5 (**A**, **C**, **E**) or ED1 immunostaining (**B**, **D**, **F**). The amoeboid-like cells having a strong immunostaining for integrin α5 (arrows in A, C) were found in GDNF-treated spinal cord tissue 1 week after contusion injury. On the other hand, in PBS-treated control (E) integrin α5 immunostaining was localized in ramified cells or cells surrounding the blood vessels as indicated by *. ED1$^+$ amoeboid microglia/macrophages were visualized in the GDNF-treated spinal cord (B, D). However, rare ED1$^+$ immunostaining was detected in PBS-injected spinal cord tissue (F). The images were taken from the region of the injured spinal cord 2–3 mm rostral to the contusion center. A similar observation was noted in the spinal cord caudal to the contusion center. Scale bar in A, B, E, F = 100 μm; in C, D = 50 μm. Cell count assay was used to determine the effect of GDNF on the growth of primary rat microglia (**G**). Twenty-four hours after treatment with GDNF, cell count was performed using a hemacytometer. Relative increase in cell number was defined as the ratio of the total cell number in GDNF-treated cultures to the control. Data were expressed as mean ± SD (n = 4 separate culture per bar). *$P < 0.05$ (unpaired *t*-test) compared to control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Recent evidence has indicated that GDNF action is involved in the activation processes of macrophages [Hashimoto et al., 2005]. Additionally, several studies have demonstrated the

mitogenicity of GDNF on microglia in vivo [Shinoda et al., 1996] and in vitro [Salimi et al., 2003]. The principal aim of the present study was to understand whether GDNF has a regulatory role in microglial activities. Our results show that GDNF regulates ICAM-1 mRNA expression and increases SOD enzymatic activity in microglia. However, treatment with GDNF causes no change in the release of microglial TNF-α and IL-1β, which are believed to play a critical role in triggering secondary damage after CNS injury [Pocock and Liddle, 2001; Liu et al., 2002; Liu and Hong, 2003]. No production of iNOS was observed in GDNF-treated microglia, whereas a slight increase in the level of NO metabolites in the culture medium was detected. Interestingly, the phagocytotic ability of microglia can be enhanced by GDNF, which is also able to increase microglial integrin α5 production via the MAPK signaling pathway. In vivo evidence also showed that there were amoeboid cells with integrin α5 or ED1 immunostaining in the GDNF-treated spinal cord that had sustained contusive injury, implying that exogenous GDNF infusion may regulate integrin α5 expression in activated microglia/macrophages in the injured CNS. Moreover, our in vivo and in vitro observations also indicated that GDNF might have the regulatory effect on microglial survival/proliferation. Accordingly, GDNF may serve as a stimulatory molecule for the expression of microglial phagocytotic activity, growth and adhesion molecules.

It has been indicated that neuroprotective mechanisms of GDNF in the ischemic brain may participate in alleviating free radicals during injury [Ikeda et al., 2000]. In vivo study has also shown that GDNF is able to activate antioxidant enzyme systems in dopaminergic neurons [Chao and Lee, 1999]. According to our results, which show an increase in microglial SOD enzymatic activity by GDNF, the molecule may be able to activate the endogenous antioxidative system in microglia. Yet, our findings also show that a slight increase in the accumulation of NO metabolites was detected in GDNF-treated microglia, although microglia iNOS was not produced in GDNF-treated microglia. In comparison with iNOS, neuronal NOS and endothelial NOS produce much lower levels of NO in the cells [Bogdan, 2001]. Our observations have also indicated the inhibition of GDNF-induced increase in the accumulation of microglial NO metabolites by the inhibitor of MEK pathway, which is also known to involve in the regulation of neuronal NOS and endothelial NOS expression [Schroeter et al., 2002]. Therefore, microglial NO production increased by GDNF might be resulted from the effect of GDNF-induced or activated neuronal NOS and endothelial NOS.

Although microglial activation is associated with the neuropathogenesis in CNS neurodegeneration [Boje and Arora, 1992; Gonzalez-Scarano and Baltuch, 1999; Liu and Hong, 2003], activated microglia play an important role in CNS repair via phagocytosing neural/myelin debris which can hinder neuronal regeneration after CNS injury [Prewitt et al., 1997; Rabchevsky and Streit, 1997; Koenigsknecht and Landreth, 2004; Streit et al., 2004]. Integrin family members such as α5β1, α4β1, α6β1, αvβ5, αMβ2, and αXβ2 are known to be involved in microglial migration, adhesion, and phagocytosis [Berton and Lowell, 1999; Witting et al., 2000; Kloss et al., 2001; Milner and Campbell, 2003; Koenigsknecht and Landreth, 2004]. Nevertheless, whether the enhanced phagocytotic activity resulted from increased integrin α5 expression in GDNF-treated mcroglia needs to be further determined. On the other hand, our results indicating the effect of GDNF in promoting microglial phagocytotic ability provide evidence that administered GDNF in the injured CNS may not only induce neuronal survival, but also increase microglial phagocytosis. However, there was no significant upregulation of microglial integrin αv, α4, and α6 levels after GDNF treatment (data not shown). Nevertheless, whether GDNF modulates the expression of other integrin subunits in microglia and whether the increased integrin family affects microglial phagocytosis remain to be clarified.

GDNF is known to trigger multiple signaling pathways, including Ras/Erk-MAPK, PI3 kinase/AKT, p38MAPK, and JNK via interaction with GFRα and c-ret [Takahashi, 2001]. The inhibition of Erk-MAPK by U0126 causes the reduction of the accumulation of NO metabolites in the supernatant of GDNF-treated microglia, implying that treatment with GDNF may activate the Erk-MAPK signal pathway to enhance NO production in microglia. Furthermore, our evidence demonstrates the reduction of integrin α5 levels in GDNF-treated BV2 by U0126, suggesting that Erk-MAPK signaling

may be involved in GDNF action on the regulation of microglial activities.

In conclusion, we have demonstrated the stimulatory effect of GDNF on the production of microglial NO, the expression of adhesion molecules, and phagocytotic ability. Moreover, the Erk-MAPK signaling pathway may be involved in the GDNF-induced mechanism for the regulation of microglial activities. This study provides evidence that exogenous GDNF administration may not only have a protective effect on neurons, but may also have a modulatory role in microglial activities.

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