GDNF From Human Periodontal Ligament Cells Treated With Pro-Inflammatory Cytokines Promotes Neurocytic Differentiation of PC12 Cells

Yoshida, Shinichiro
Department of Endodontology, Kyushu University Hospital

Yamamoto, Naohide
Section of Endodontology & Operative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University

Wada, Naohisa
Division of General Oral Care, Kyushu University Hospital

Tomokiyo, Atsushi
Department of Endodontology, Kyushu University Hospital

他

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Article

GDNF from human periodontal ligament cells treated with proinflammatory cytokines promotes neurocytic differentiation of PC12 cells†

Shinichiro Yoshida[1], Naohide Yamamoto[2], Naohisa Wada[3], Atsushi Tomokiyo[1], Daigaku Hasegawa[1], Sayuri Hamano[4], Hiromi Mitarai[2], Satoshi Monnouchi[2], Asuka Yuda[2] and Hidefumi Maeda[1,2*]

[1]Department of Endodontology, Kyushu University Hospital, Maidashi, Higashi-ku, Fukuoka, Japan
[2]Department of Endodontology and Operative Dentistry, Faculty of Dental Science, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan
[3]Division of General Oral Care, Kyushu University Hospital, Maidashi, Higashi-ku, Fukuoka, Japan
[4]OBT Research Center, Faculty of Dental Science, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan

[*]**Address corresponding to:** Hidefumi Maeda,
Department of Endodontology and Operative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Phone: +81-92-642-6432
Fax: +81-92-642-6366
E-mail: hide@dent.kyushu-u.ac.jp

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) is known to mediate multiple biological activities such as promotion of cell motility and proliferation, and morphogenesis. However, little is known about its effects on periodontal ligament (PDL) cells. Recently, we reported that GDNF expression is increased in wounded rat PDL tissue and human PDL cells (HPDLCs) treated with proinflammatory cytokines. Here, we investigated the associated expression of GDNF and the proinflammatory cytokine interleukin-1 beta (IL-1β) in wounded PDL tissue, and whether HPDLCs secrete GDNF which affects neurocytic differentiation. Rat PDL cells near the wounded area showed intense immunoreactions against an anti-GDNF antibody, where immunoreactivity was also increased against an anti-IL-1β antibody. Compared with untreated cells, HPDLCs treated with IL-1β or tumor necrosis factor-alpha showed an increase in the secretion of GDNF protein. Conditioned medium of IL-1β-treated HPDLCs (IL-1β-CM) increased neurite outgrowth of PC12 rat adrenal pheochromocytoma cells. The expression levels of two neural regeneration-associated genes, growth-associated protein-43 (Gap-43) and small proline-rich repeat protein 1A (Sprr1A), were also upregulated in IL-1β-CM-treated PC12 cells. These stimulatory effects of IL-1β-CM were significantly inhibited by a neutralizing antibody against GDNF. In addition, U0126, a MEK inhibitor, inhibited GDNF-induced neurite outgrowth of PC12 cells. These findings suggest that an increase of GDNF in wounded PDL tissue might play an important role in neural regeneration probably via the MEK/ERK signaling pathway.

Keywords: Glial cell line-derived neurotrophic factor; Nerve regeneration; Neural-like PC12 cell; Periodontal ligament cell

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor-beta family, which was originally isolated and purified from the supernatant of the B49 rat glial cell line [Lin et al., 1993]. GDNF binds to GDNF family receptor alpha1 (GFRα1) which is a specific glycosyl phosphatidylinositol-anchored receptor that signals in collaboration with its co-receptors, neural cell adhesion molecule (NCAM) or re-arranged during transformation (RET) [Airaksinen et al., 2002]. Numerous studies have shown that GDNF is involved in normal tissue development and recovery from pathophysiological events. GDNF promotes dopaminergic neuron survival and neural cell development [Lin et al., 1993]. Furthermore, GDNF plays a crucial role in ureteric bud branching during kidney development [Lu et al., 2009] and regulates differentiation of spermatogonia [Meng et al., 2000]. Recently, because of its neuroprotective and regenerative effects, GDNF has attracted attention as a therapeutic agent for treatment of Parkinson's disease [Anastasía et al., 2011].

Periodontal ligament (PDL) tissue originates from cranial neural crest-derived ectomesenchyme [Chai et al., 2000; Milletich et al., 2004] and consists of dense connective tissue anchoring the tooth root to the alveolar bone [Shi et al., 2005]. PDL tissue has a rich sensitive nerve supply [Maeda et al., 1999] and reacts to critical tissue damage as well as touch, pressure, and pain [Tadokoro et al., 1998]. Periodontal nerve endings are closely related to the epithelial cells rests of Malassez localized in PDL tissue [Lambrichts et al., 1993]. Previous studies have shown that inferior nerve

denervation leads to narrowing of the PDL width and dentoalveolar ankylosis [Fujiyama et al., 2004]. Furthermore, it has been reported that peripheral nerves might play a functional role in PDL tissue maintenance and regeneration [Fujiyama et al., 2004; Xiong et al., 2013]. Therefore, reinnervation of wounded PDL tissue is indispensable for PDL tissue regeneration to extend the tooth lifespan.

Robust regenerative reactions occur after nerve injury. One of the major events that play central roles in such regeneration is thought to be strong upregulation of regeneration-associated gene (RAG) expression [Chaudhary et al., 2012]. Two representative neural RAGs are growth-associated protein-43 (*Gap-43*) and small proline-rich repeat protein 1A (*Sprr1a*) [Chaudhary et al., 2012]. Gap-43 is a well-known specific marker of growth cones and axons [Morita et al., 2013], which is highly expressed in developing neurons and growing axons [Jacobson et al., 1986]. GDNF also enhances Gap-43 expression after sciatic nerve transection in adult rats [Chen et al., 2001]. Sprr1a was first identified as a RAG member in cDNA microarray analysis of genes induced during sciatic nerve regeneration [Bonilla et al., 2002]. Sprr1a, which is colocalized with Gap-43, is expressed in dorsal root ganglion neurons as well as primary sensory neurons and motoneurons of the spinal cord in adult mice after peripheral and central nerve injury [Starkey et al., 2009].

Recently, we reported that GDNF expression is increased in wounded PDL tissue and its gene expression was also promoted in human PDL cells (HPDLCs) stimulated with proinflammatory cytokines [Yamamoto et al., 2012]. However, the effects of GDNF on neurogenesis remain unclear.

The aim of this study was to investigate the association of GDNF and proinflammatory cytokines

in wounded PDL tissue and HPDLCs, and to furthermore examine the effects of GDNF derived from interleukin-1 beta (IL-1β)-treated HPDLCs on neurocytic differentiation of PC12 rat adrenal pheochromocytoma cells (a neural progenitor cell line) as well as the expression of RAGs in PC12 cells. In addition, the signaling pathway involved in these events was examined.

Materials and methods

Cell culture

HPDLCs were isolated from healthy molars of three patients, a 23-year-old female (HPDLC-3D cells), a 21-year-old female (HPDLC-3M cells) and a 26-year-old male (HPDLC-3Q cells), who visited the Dental Hospital of Kyushu University for tooth extraction according to our previous report (Fujii et al. 2006). Briefly, the middle region of the periodontal ligament tissue was stripped from the root surface of the extracted tooth. The tissues were washed with alpha-minimum essential medium (α-MEM; Gibco-BRL, Grand Island, NY, USA) and treated with 0.2% collagenase and 0.25% trypsin. The dispersed cells were maintained in α-MEM containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 50 μg/ml streptomycin, and 50 U/ml penicillin (Gibco-BRL) and cells from passages three through five were used as HPDLC in this study. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. PC12 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan) and maintained in Dulbecco's modified

Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% FBS, 10% horse serum (HS; Gibco-BRL), 50 μg/ml streptomycin, and 50 U/ml penicillin (10%FBS/10%HS/DMEM). Written informed consent was obtained from all participants. All procedures were approved by the Kyushu University Institutional Review Board for Human Genome/Gene Research (approval number: 20A-E)

Experimental animal model

Five-week-old male Sprague-Dawley rats weighing 140–150 g were purchased from Kyudo (Saga, Japan). The rats were allowed free access to food and water throughout the experimental period. On the day of surgery, the animals were anaesthetized by intraperitoneal injection of 2 mg/kg midazolam (Sandoz, Tokyo, Japan), 0.15 mg/kg medetomidine hydrochloride (Kyoritsu Seiyaku, Tokyo, Japan), and 2.5 mg/kg butorphanol tartrate (Meiji Seika Pharma, Tokyo, Japan). A periodontal defect of 2 mm in diameter and 2 mm in depth was formed using dental round burs #6 (MANI, Tochigi, Japan), which extended from the mesiopalatal submarginal portion to the palatal root of the left maxillary first molar. After irrigation of the cavity with sterile saline, hemostasis was confirmed. First molars on the right side were not wounded and used as controls.

At 1, 3, 5, and 7 days after surgery, the animals were sacrificed by transcardial perfusion with 4% paraformaldehyde (PFA; Merck Millipore, Darmstadt, Germany) in phosphate-buffered saline (PBS) under anesthesia as described above. Maxillae were removed and immersed in 4% PFA for 12 hours. The tissues were then washed with PBS and decalcified in 10%

ethylenediaminetetraacetic acid (Wako Pure Chemical Industries, Osaka, Japan) at 4 °C for 1 month before embedding in optimal cutting temperature compound (Sakura Fineteck, CA). All procedures were approved by the Animal Ethics Committee and conformed to the regulations of Kyushu University (approval number: A25-117-0).

Immunofluorescence analysis

Immunolocalization of GDNF and IL-1β in rat maxilla was examined using a goat polyclonal antibody against rat/human GDNF (1:100; Santa Cuz Biotechnology, Santa Cruz, CA, USA) and a rabbit polyclonal antibody against rat IL-1β (1:100; Santa Cruz Biotechnology), respectively. Samples were incubated with the primary antibodies at 4 °C overnight. The samples were washed with PBS and then incubated for 1 hour with an Alexa Fluor 568-conjugated donkey anti-goat antibody (1:200; Invitrogen) and Alexa Fluor 488-conjugated chicken anti-rabbit antibody (1:200; Invitrogen). Then, the samples were washed with PBS and mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Images of the samples were obtained under a Biozero digital microscope (Keyence, Osaka, Japan).

Enzyme-linked immunosorbent assay (ELISA)

To quantify the concentration of GDNF protein secreted from HPDLCs exposed to inflammatory stimuli, subconfluent HPDLCs were treated for 24 h with 10 ng/ml recombinant human IL-1β

(PeproTech EC, London, UK) or tumor necrosis factor-alpha (TNF-α; PeproTech EC). After treatment, the culture medium was collected and analyzed by a commercially available GDNF ELISA kit (Promega, Madison, WI, USA) to quantify the GDNF concentration. The absorbance was measured at 450 nm with an Immuno Mini NJ-2300 (Microtec, Chiba, Japan).

Western blotting analysis

PC12 cells were cultured in DMEM containing 10% FBS, 10% HS, and 50 ng/ml recombinant human GDNF (Peprotech EC) with or without U0126 which is a specific inhibitor of MEK1 and MEK2 (10^{-4} M; Merck Millipore), or U0124 which is a negative control for U0126 (10^{-4} M; Merck Millipore) for 10 min. The cells were lysed in Pierce RIPA Buffer (Thermo Fisher Scientific Inc., Waltham, MA) containing 1% Protease Inhibitor Cocktail (Thermo Fisher Scientific Inc.) and 1% Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc.). Aliquots containing 5 μg protein per lane were subjected to 10% sodium dodecylsulfate (SDS; Nacalaitesque, Kyoto Japan) polyacrylamide gel electrophoresis and subsequently transferred onto an Immuno-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA). After blocking with 5% skim milk (Yukijirushi, Tokyo, Japan) in Tris Buffered Saline with Tween 20 (TBST), the membranes were incubated overnight with indicated primary antibodies, mouse monoclonal anti-β-actin (1:1000 dilution; Santa Cruz Biotechnology), mouse monoclonal anti-total-ERK 1/2 (t-ERK; 1:1000 dilution; Santa Cruz Biotechnology), or goat polyclonal anti-p-ERK 1/2 (p-ERK; 1:1000 dilution; Santa Cruz Biotechnology). The reactions were followed by biotinylated anti-mouse IgG (Nichirei Biosciences,

Inc., Tokyo, Japan) or biotinylated anti-goat IgG (Nichirei Biosciences). Then the membranes were reacted with ExtrAvidin Peroxidase (Sigma-Aldrich) and visualized using ECL select Western blotting detection system (GE healthcare, Buckinghamshire, UK). Image capture was performed using ImageQuant LAS 500 (GE healthcare). Relative expression levels of bands were measured using ImageJ software (National Institute of Health).

Semi-quantitative RT-PCR

Total RNA was isolated with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was subjected to reverse transcription using a PrimeScript RT Reagent kit (Takara Bio, Shiga, Japan) according to manufacturer's protocol. PCR was performed using platinum Taq DNA polymerase (Invitrogen) in a PCR Thermal Cycler Dice TP600 (Takara Bio). PCR conditions were 94 °C for 2 min followed by an appropriate number of cycles at 94 °C for 30 sec, the appropriate annealing temperature for 30 sec, and then 72 °C for 30 sec with a final extension at 72 °C for 7 min. Rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as an internal control. PCR products were separated by electrophoresis on 2% agarose gels (Seakem ME; BioWhittacker Molecular Applications, Rockland, ME, USA) and photographed under ultraviolet light after staining with ethidium bromide. Specific primer sequences, annealing temperatures, cycle numbers, and product sizes for each gene are listed in Table 1. Primer sequences were designed using the GenBank database (National Center for Biotechnology Information), and a BLAST search of the primer sequences was performed to ensure specificity.

Quantitative RT-PCR

First-strand cDNA was synthesized with an ExScript RT Reagent kit (Takara Bio). Quantitative RT-PCR assays were performed with a SYBR Green II RT-PCR kit (Takara Bio) in a Thermal Cycler Dice Real Time System (Takara Bio) as described elsewhere (Maeda et al. 2010). Specific primer sequences, annealing temperatures, and product sizes for each gene are listed in Table 2. Rat G3PDH served as an internal control. Primer sequences were designed as described above. Expression levels of the target genes were calculated using ΔΔCt values.

IL-1β treatment

Subconfluent HPDLCs were treated with 10 ng/ml recombinant human IL-1β for 24 h. The cells were then washed with sterilie PBS at least three times and cultured in serum-free DMEM for a further 24 h. The conditioned medium (IL-1β-CM) was collected and passed through a 0.22-μm filter. Then, 10% FBS and 10% HS were added to the IL-1β-CM for use in subsequent experiments. Conditioned medium obtained from cells treated with PBS instead of IL-1β served as a control (CM).

Neuro-inductive effects of GDNF on PC12 cells

PC12 cells were cultured in IL-1β-CM or DMEM containing 10% FBS, 10% HS, and 50 ng/ml recombinant human GDNF (Peprotech EC) with or without a mouse neutralizing polyclonal

antibody against human GDNF (1 μg/ml; GeneTex, San Antonio, TX, USA), control mouse IgG (1 μg/ml; Santa Cruz Biotechnology), U0126 (10^{-4} M; Merck Millipore) or U0124 (10^{-4} M; Merck Millipore) for 2 weeks. Photographs were taken after 14 days of incubation under a Leica DM IRB inverted microscope (Leica Microsystems, Germany). According to our recent report (Tomokiyo et al. 2012), the cells were considered to exhibit neurite outgrowth when they were elongated by at least twice the original diameter of the round cells. Four fields were randomly chosen to count the differentiated cells in each condition. Neurite length of each cell was measured using ImageJ software (National Institute of Health). More than 30 cells were analyzed in each experiment. Then, the cells were lysed in TRIzol Reagent, and total RNA was extracted and subjected to gene expression analyses.

Statistical analysis

All values are expressed as the mean ± standard deviation. Statistical analysis was performed using the Student's unpaired *t*-test. A probability value of $p < 0.05$ was considered statistically significant.

Results

Expression of GDNF and IL-1β in normal and wounded rat PDL tissue

We compared immunohistochemical expression of GDNF and IL-1β in normal and wounded rat

PDL tissue on day 1, 3, 5 and 7 after surgery according to our previous study (Yamamoto et al., 2012). GDNF was expressed in normal tissue, but not intensely, and we observed no expression of IL-1β in normal rat PDL tissue (Fig. 1M, N, O) on 1 day after surgery. However, expression of GDNF was increased and IL-1β-immunoreactive cells were shown up near the wounded site (Fig. 1A, B). Then, 3 day after surgery, the number of GDNF- and IL-1β-immunoreactive cells was clearly increased near the wounded site (Fig. 1D, E). Furthermore, the expression levels of GDNF and IL-1β were gradually decreased at day 5 (Fig. 1G, H) and day 7 (Fig. 1J, K). On the other hand, the PDL tissues of second molars on the wounded side and first and second molars on the normal side did not show increased expression of GDNF or IL-1β (Fig. 1C, F, I, L, N, and O). Negative controls with omission of the primary antibody showed no staining (data not shown).

Proinflammatory cytokines promote GDNF protein expression in HPDLCs

In vivo analysis led us to examine the effect of proinflammatory cytokines on the expression levels of GDNF protein in HPDLCs. After treating HPDLC-3D, -3M and -3Q cells with IL-1β or TNF-α (10 ng/ml each) for 24 hours, GDNF concentrations in the culture supernatants were determined by an ELISA. In the culture supernatants of IL-1β-treated HPDLC-3D, -3M and -3Q cells, we found 57.56 ± 10.21, 69.85 ± 3.68 and 70.53 ± 8.08 pg/ml GDNF, respectively. In culture supernatants of untreated HPDLC-3D, -3M and -3Q cells, we found 15.48 ± 2.57, 7.15 ± 1.39 and 28.47 ± 3.16 pg/ml GDNF, respectively (Fig. 2B). The GDNF concentrations in culture supernatants of TNF-α-treated HPDLC-3D, -3M and -3Q cells were 29.19 ± 1.67, 38.77 ± 3.47 and 60.53 ± 5.77

pg/ml, respectively, whereas those in culture supernatants of untreated HPDLC-3D, -3M and -3Q cells were 19.40 ± 1.53, 16.69 ± 4.17 and 28.33 ± 5.77 pg/ml, respectively (Fig. 2C). In contrast, HPDLCs treated with recombinant human GDNF (50 ng/ml) showed no upregulation of the mRNA expression of IL-1β and TNF-α (data not shown). These results indicated that proinflammatory cytokines increased the expression levels of GDNF protein in HPDLCs. In addition, because of the noticeable effect of IL-1β-treatment, we performed subsequent experiments using IL-1β. Before starting ELISA analysis, standard curve of GDNF was drawn (Fig. 2A).

Effects of GDNF from HPDLCs on neurite outgrowth of PC12 cells

First, PC12 cells were confirmed to express *GFRα1*, *RET*, and *NCAM* genes by semi-quantitative RT-PCR (Fig. 3A). PC12 cells treated with recombinant human GDNF protein showed the neurocytic phenotype of neurite outgrowth (Fig. 3B, C, D). These findings prompted us to examine the effects of GDNF derived from IL-1β-treated HPDLCs on neurite outgrowth of PC12 cells. PC12 cells were cultured for 2 weeks in IL-1β-CM derived from HPDLC-3D, -3M and -3Q cells. PC12 cells cultured in IL-1β-CM showed a change in cellular morphology and neurite outgrowth (Fig. 3B, C, D). The percentage of differentiated PC12 cells (Fig. 3E, F, G) and their neurite length (Fig. 3H, I, J) were significantly higher than those of CM-cultured control cells. To confirm the contribution of GDNF in IL-1β-CM to neurite outgrowth of PC12 cells, we used a specific neutralizing antibody against GDNF. As a result, the neurocyte-inducing effect of IL-1β-CM was clearly inhibited whereas IL-1β-CM containing control IgG had the same inductive effect as IL-1β-CM (Fig. 3B-J).

Expression of Gap-43 and Sprr1A genes in PC12 cells stimulated with GDNF

To further evaluate the effects of GDNF on PC12 cells, we investigated the expression of two neural regeneration-associated genes *Gap-43* and *Sprr1A* by quantitative RT-PCR. The results showed that the mRNA expression of *Gap-43* (Fig. 4A-C) and *Sprr1A* (Fig. 4D-F) were significantly upregulated in GDNF-treated and IL-1β-CM groups. Such upregulation was completely inhibited by the neutralizing antibody against GDNF (Fig. 4).

GDNF induces neurite outgrowth of PC12 cells through the MEK/ERK signaling.

To investigate the signaling mechanism of neurocyte-inducing effects of GDNF on PC12 cells, we performed western blotting analysis to clarify whether GDNF activates the MEK/ERK signaling pathway. GDNF-induced phosphorylation of ERK 1/2 was observed at 5 and 10 min. after stimulation and decreased thereafter (Fig. 5A). Next we tried to investigate the effects of the MEK/ERK signaling pathway on neurocytic differentiation of PC12 cells. U0126, a MEK inhibitor, treatment changed their cellular morphology (Fig. 5B), significantly decreased the percentage of differentiated PC12 cells (Fig. 5C), and reduced their neurite growth (Fig. 5D). U0126 treatment also inhibited the GDNF- induced phosphorylation of ERK 1/2 (Fig. 5E). U0124, a negative control for U0126, treatment did not affect the inducible activity of GDNF on neurocytic differentiation and phosphorylation of ERK 1/2 in PC12 cells (Fig. 5B-E).

Discussion

In this study, we first found the immune-reaction for both GDNF and IL-1β induced in wounded PDL tissues and upregulated secretion of GDNF in IL-1β- or TNF-α-treated HPDLCs. In addition, we elucidated the inducible activity of GDNF secreted from IL-1β-treated HPDLCs on neurite outgrowth and RAG expression of PC12 cells, and the involvement of the MEK/ERK signaling pathway in these events.

A previous report has shown up-regulation of IL-1β and TNF-α expression in the early stage of periodontitis, which play a crucial role in regulating inflammatory responses during periodontal diseases [Bartold et al., 2006]. In addition, it has been reported that wound healing processes consist of three phases, inflammation, proliferation, and tissue remodeling [Campos et al., 2008; Guo et al., 2010]. Thus, a rapid inflammatory response is likely to occur in PDL tissue after wounding. In this study, we first found upregulated GDNF expression with increased production of IL-1β in surgically wounded rat PDL tissue after injury. Furthermore, our results revealed that HPDLCs treated by proinflammatory cytokines increased GDNF secretion. Some studies also have shown that IL-1β and TNF-α increase GDNF expression in astrocytes [Appel et al., 1997; Kuno et al., 2006] and enteric glia cells [Von Voyen et al., 2006]. GDNF reduced neuropathic pain after sciatic nerve injury [Boucher et al., 2000], and continuous administration of GDNF exerted anti-inflammatory effects on ulcerative colitis [Zhang et al., 2010]. Taken together, our data suggest that GDNF expression increases in PDL tissue under pathological conditions, and GDNF might play a role in wound healing of PDL tissue.

Neurite outgrowth was induced by several extracellular matrix and neurotrophic factors [Lamoureux et al., 1992]. Moreover, sensory nerves innervated in PDL tissue are considered to play an important role in maintenance of PDL homeostasis [Fujiyama et al., 2004]. Several studies have reported that GDNF can be used as a therapeutic agent for nerve regeneration. Continuous injection of GDNF through an osmotic minipump promoted regrowth of damaged sensory axons in the dorsal root and spinal cord [Ramer et al., 2000]. Furthermore, long-term treatment with exogenous GDNF increased the number of regenerated axons after tibial nerve transection [Boyd et al., 2003]. Therefore, we examined whether GDNF derived from proinflammatory cytokine-treated HPDLCs affected neurocytic differentiation of PC12 cells. Previous studies have reported that PC12 cells show neurite outgrowth and neurocytic differentiation in the presence of GDNF [Garbayo et al., 2007; Wissel et al., 2008]. In this study, we validated that PC12 cells cultured in IL-1β-CM showed neurite outgrowth. This neurocyte-inducing effect of GDNF in IL-1β-CM was inhibited by a specific neutralizing antibody against GDNF. These findings suggested that GDNF expression in HPDLCs was increased by proinflammatory cytokines and affected neurocytic differentiation.

GDNF derived IL-1β-treated HPDLCs also upregulated the mRNA expression of *Gap-43* and *Sprr1A* in PC12 cells.Gap-43 is intensely expressed in growing axonal terminals and plays a role in synaptogenesis [Morita et al., 2013]. Expression of Gap-43 was increased in injured peripheral nerves and considered to participate in neurite outgrowth and nerve regeneration [Huebner et al., 2009]. Sppr1A is also involved in axonal outgrowth [Bonilla et al., 2002] and regeneration of injured nerves [Starkey et al., 2009]. Considering the above findings, GDNF from HPDLCs treated

with IL-1β may promote neurite outgrowth and neurocytic differentiation of PC12 cells through *Gap-43* and *Sprr1A* expression.

It has been reported that a MEK inhibitor suppressed the GDNF-induced Gap-43 expression in human neuroblastoma cell line [Murakami et al., 2011]. In the present study, U0126 significantly reduced neurite outgrowth of PC12 cells and inhibited GDNF-induced phosphorylation of ERK 1/2. Although further study on signaling pathways will be needed, our results suggested that GDNF induces the differentiation of PC12 cells into neurocytes via the MEK/ERK signaling pathway.

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Conflict of Interest

The authors indicate no conflicts of interest.

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Figure legends

Figure 1. Immunofluorescence analysis of GDNF and IL-1β in wounded rat PDL tissue. (A-O) Horizontal sections through the first and second molars in rat maxilla were prepared. Immunopositive cells were visualized by anti-GDNF (red) and anti-IL-1β (green) antibodies. Merged images are shown. **(A-C)** Immunostaining of GDNF and IL-1β were recognizable near the wounded area **(A, B)** 1 day after surgery. **(D-F)** The expression levels of GDNF and IL-1β near the wounded site **(D, E)** was clearly increased at day 3. **(G-L)** The number of GDNF- and IL-1β-immunoreactive cells near the wonded site were gradually decreased at day 5 **(G, H)** and day 7 **(J, K)**. **(C, F, I, L)** The PDL tissues of second molars on the wounded side showed no increased expression of GDNF and IL-1β. **(M-O)** Untreated PDL tissue was served as a control side. The tissues were counterstained with DAPI (blue). **(A-O)** All sections were subjected to hematoxylin and eosin staining after immunostaining. Scale bars = 100 μm. M1, first molar; M2, second molar; Bu, buccal; Dis, distal; Mes, mesial; Pa, palatal; AB, alveolar bone; Def, defect site; PDL, periodontal ligament; R, tooth root.

Figure 2. Expression of GDNF in HPDLC-3D, -3M and -3Q cells treated with proinflammatory cytokines. (A) The ELISA standard curve was drawn for each GDNF standard concentration. **(B, C)** HPDLCs were treated with PBS, IL-1β (10 ng/ml, **B**), or TNF-α (10 ng/ml, **C**) for 24 hours. Secretion of GDNF was examined by an ELISA (n = 3; error bars, s.d.). $^{**}p < 0.01$ vs. control

(PBS).

Figure 3. Effects of GDNF secreted from HPDLC-3D, -3M and -3Q on neurocytic differentiation of PC12 cells. (A) Semi-quantitative RT-PCR analysis of the expression of genes encoding GDNF receptor GFRα1 and its co-receptors, NCAM and RET, in PC12 cells. **(B-G)** Neurocytic differentiation of PC12 cells was examined after culturing in 10%FBS/10%HS/DMEM (control), 10%FBS/10%HS/DMEM containing GDNF (50 ng/ml) with or without normal mouse control IgG (cIgG) or an anti-GDNF neutralizing antibody (1 μg/ml), CM, or IL-1β-CM with or without normal mouse cIgG or anti-GDNF neutralizing antibody for 14 days. **(B, C, D)** Phase-contrast microscopic images of each culture condition. **(E, F, G)** The percentage of differentiated PC12 cells was determined in each culture condition (n = 3; error bars, s.d.). $^{**}p < 0.01$; $^{*}p < 0.05$. **(H-J)** The quantification of total neurite length (n = 3; error bars, s.d.). $^{**}p < 0.01$. Arrowheads show differentiated PC12 cells with neurite processes. Results are representative of three separate experiments. Scale bars = 100 μm.

Figure 4. Expression of genes encoding *Gap-43* and *Sprr1A* in PC12 cells. (A-F) HPDLC-3D (**A**, **D**), HPDLC-3M (**B**, **E**) and HPDLC-3Q (**C**, **F**) cells were cultured with 10%FBS/10%HS/DMEM (control), 10%FBS/10%HS/DMEM containing GDNF (50 ng/ml) with or without normal mouse control IgG (cIgG) or an anti-GDNF neutralizing antibody (1 μg/ml), CM, or IL-1β-CM with or without normal mouse control IgG (cIgG) or the anti-GDNF neutralizing antibody for 14 days.

Gene expression of *Gap-43* (**A**-**C**) and *Sprr1A* (**D**-**F**) was examined by quantitative RT-PCR (n=3; error bars, s.d.). $^{**}p < 0.01$; $^{*}p < 0.05$.

Figure 5. Involvement of the MEK/ERK signaling pathway in GDNF-induced neurocytic differentiation of PC12 cells. (A) The expression of p-ERK 1/2, t-ERK 1/2, and β-actin in PC12 cells treated with GDNF (50 ng/ml) for 0, 5, 10, 15, 20, 30, 45, and 60 min. was examined by western blotting analysis. **(B-D)** PC12 cells were cultured in 10%FBS/10%HS/DMEM containing GDNF (50 ng/ml) with or without U0124 (10^{-4} M) or U0126 (10^{-4} M) for 14 days. **(B)** Phase-contrast microscopic images of each culture condition. Scale bars = 100 μm. **(C)** The percentage of differentiated PC12 cells was determined in each culture condition (n = 3; error bars, s.d.). $^{**}p < 0.01$; $^{*}p < 0.05$. **(D)** The quantification of total neurite length under the same culture condition (n = 3; error bars, s.d.). $^{**}p < 0.01$. **(E)** PC12 cells were cultured in 10%FBS/10%HS/DMEM containing GDNF (50 ng/ml) with or without U0124 (10^{-4} M) or U0126 (10^{-4} M) for 10 min. The expression of p-ERK 1/2, t-ERK 1/2, and β-actin were examined by western blotting analysis. The graph shows the quantification of the expression of p-ERK 1/2 (n = 3; error bars, s.d.). $^{**}p < 0.01$.

TABLE 1 Specific primer sequences, annealing temperatures, cycle numbers, product sizes, and sequence IDs for semi-quantitative RT-PCR

Target gene (abbreviation)	Forward (top) and reverse (bottom) primer sequences	Annealing temperature (°C)	Cycle Numbers	Size of products (bp)	Sequence ID
G3PDH	5'- TCGGCACCACCAACTGCTT -3'	60	40	96	NM_017008.4
	5'- TGGCAGTGATGGCATGGAC -3'				
GFRα1	5'- TGGCTCAGATGTGACCATGT -3'	64	33	195	NM_012959.1
	5'- TGTGTGCTACCCGACACATT -3'				
NCAM	5'- AAAGGATGGGGAACCCATAG -3'	64	33	195	NM_031521.1
	5'- TAGGTGATTTTGGGCTTTGC -3'				
RET	5'- TGGCACACCTCTGCTCTATG -3'	64	33	186	NM_012643.2
	5'- TGCTCCCAGGAACTATGGTC -3'				

TABLE 2 Specific primer sequences, annealing temperatures, cycle numbers, product sizes, and sequence IDs for quantitative RT-PCR

Target gene (abbreviation)	Forward (top) and reverse (bottom) primer sequences	Annealing temperature (°C)	Cycle Numbers	Size of products (bp)	Sequence ID
G3PDH	5'- TCGGCACCACCAACTGCTT -3' 5'- TGGCAGTGATGGCATGGAC -3'	60	40	96	NM_017008.4
Gap-43	5'- GGCTCTGCTACTACCGATGC -3' 5'- GGCTTGTTTAGGCTCCTCCT -3'	60	40	225	NM_017195.3
Sprr1A	5'- AGCCCTGTCACCCTATTCCT -3' 5'- CTCAGGTGCCTTGGGATG -3'	60	40	215	NM_021864.1

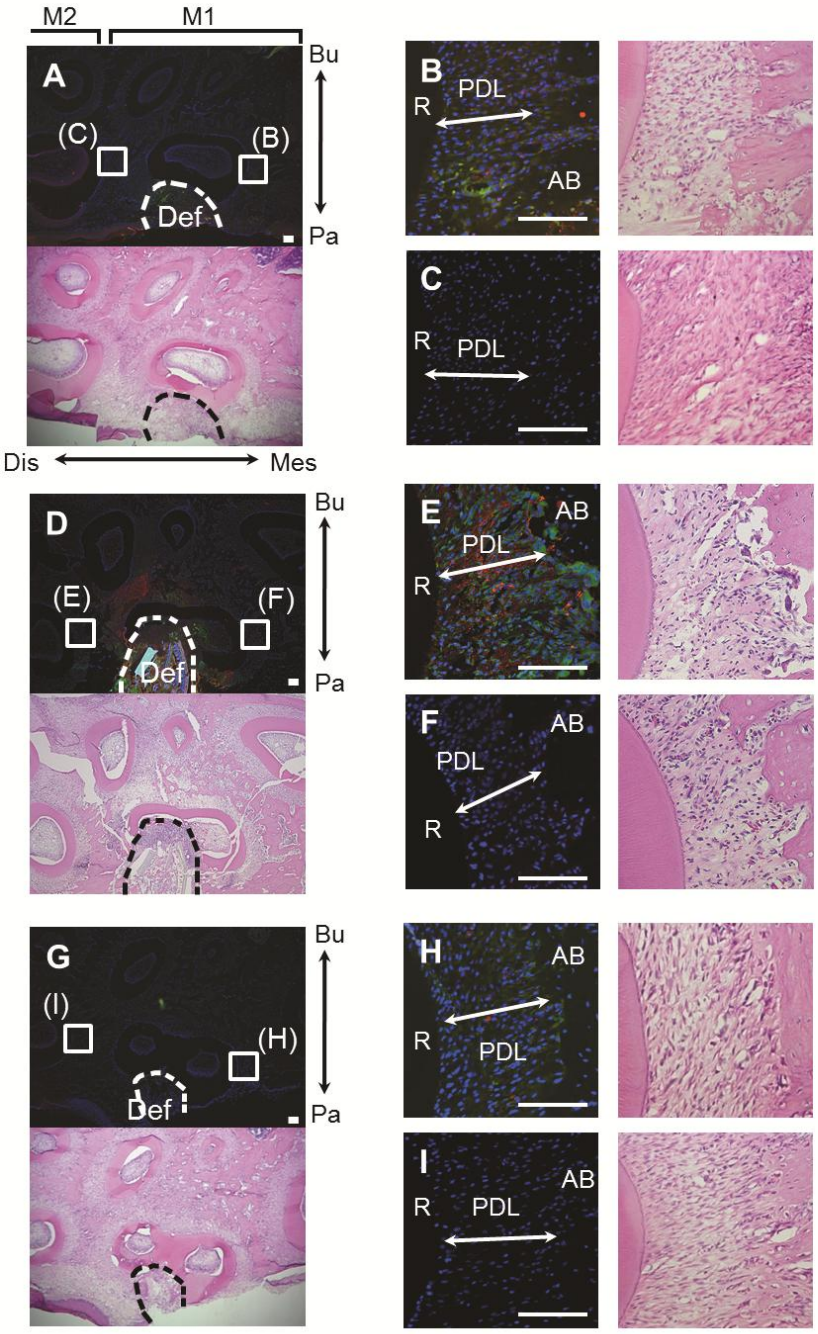

Figure 1

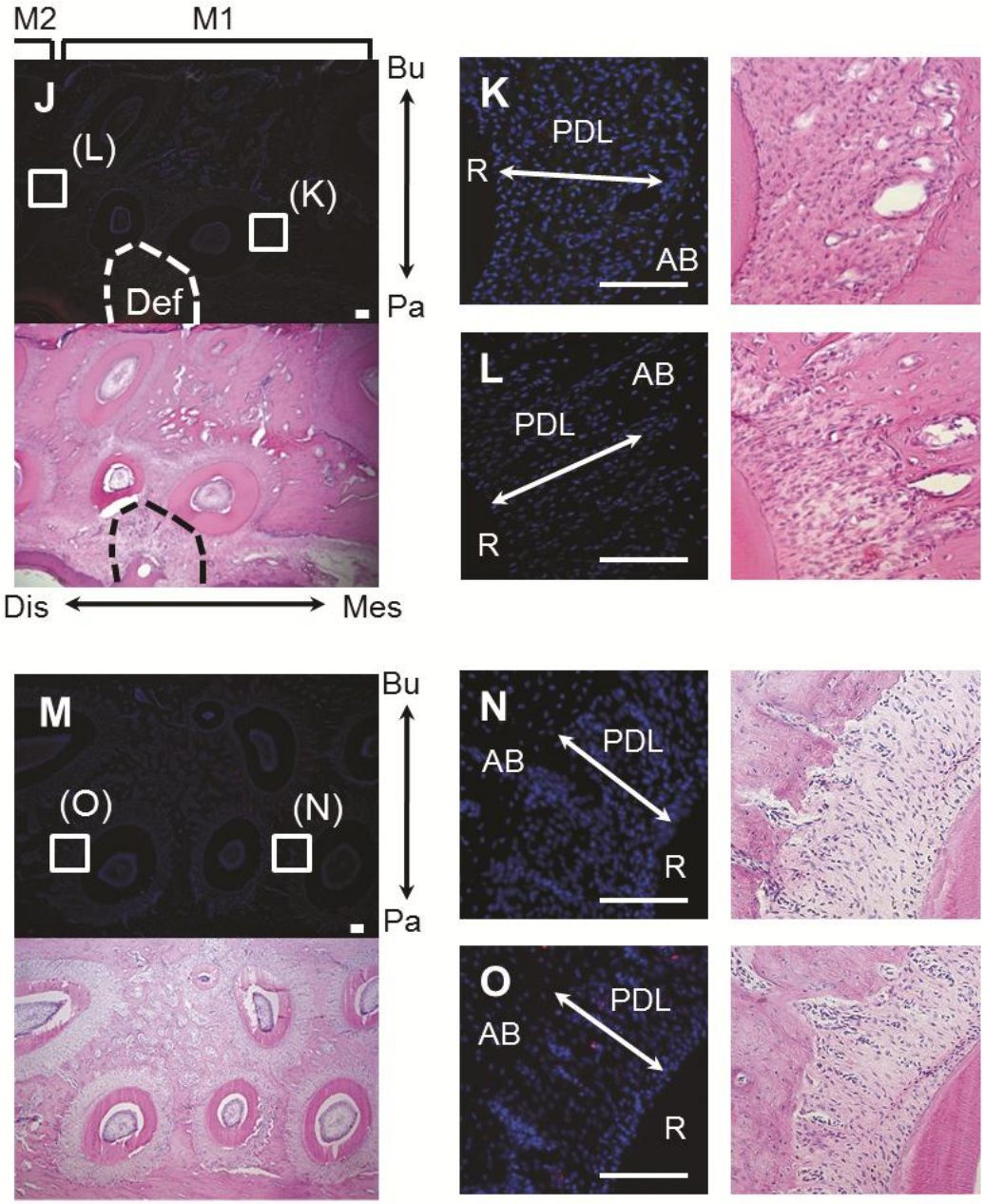

Figure 1

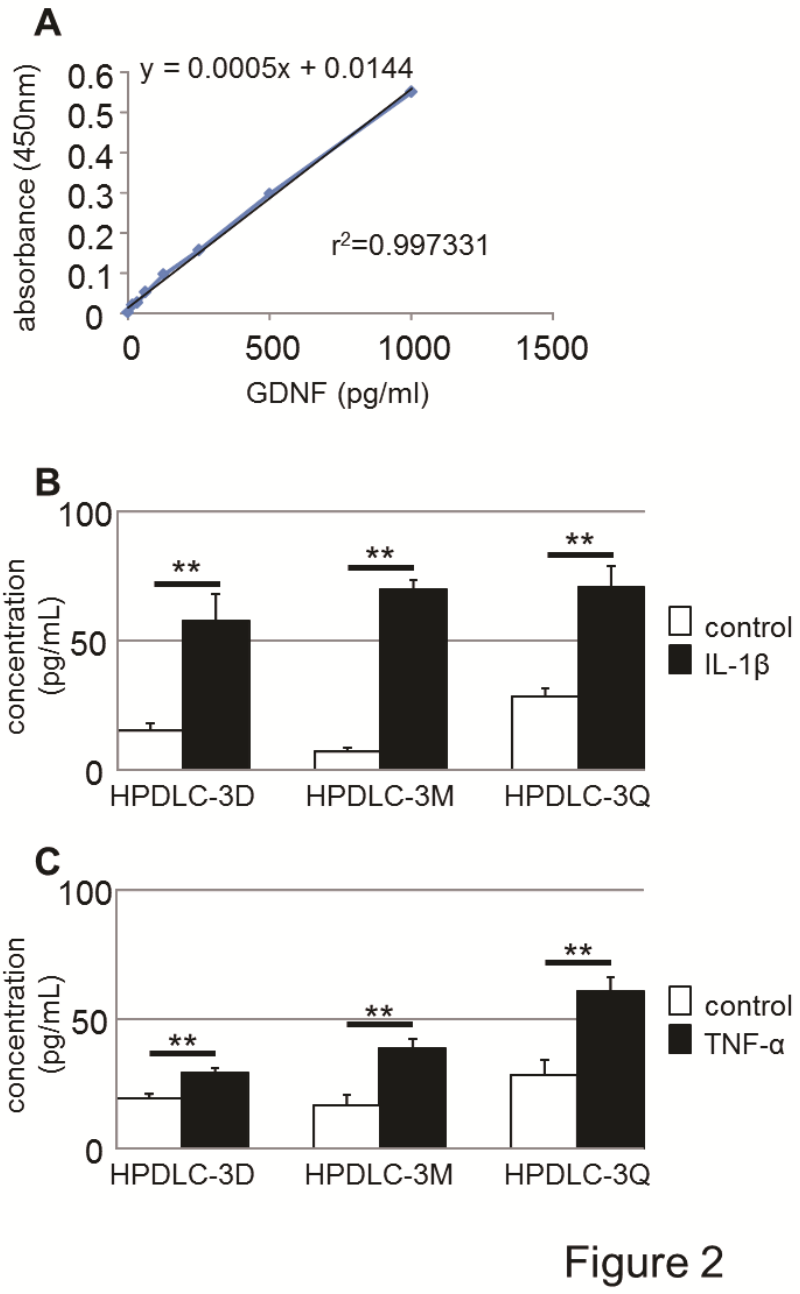

Figure 2

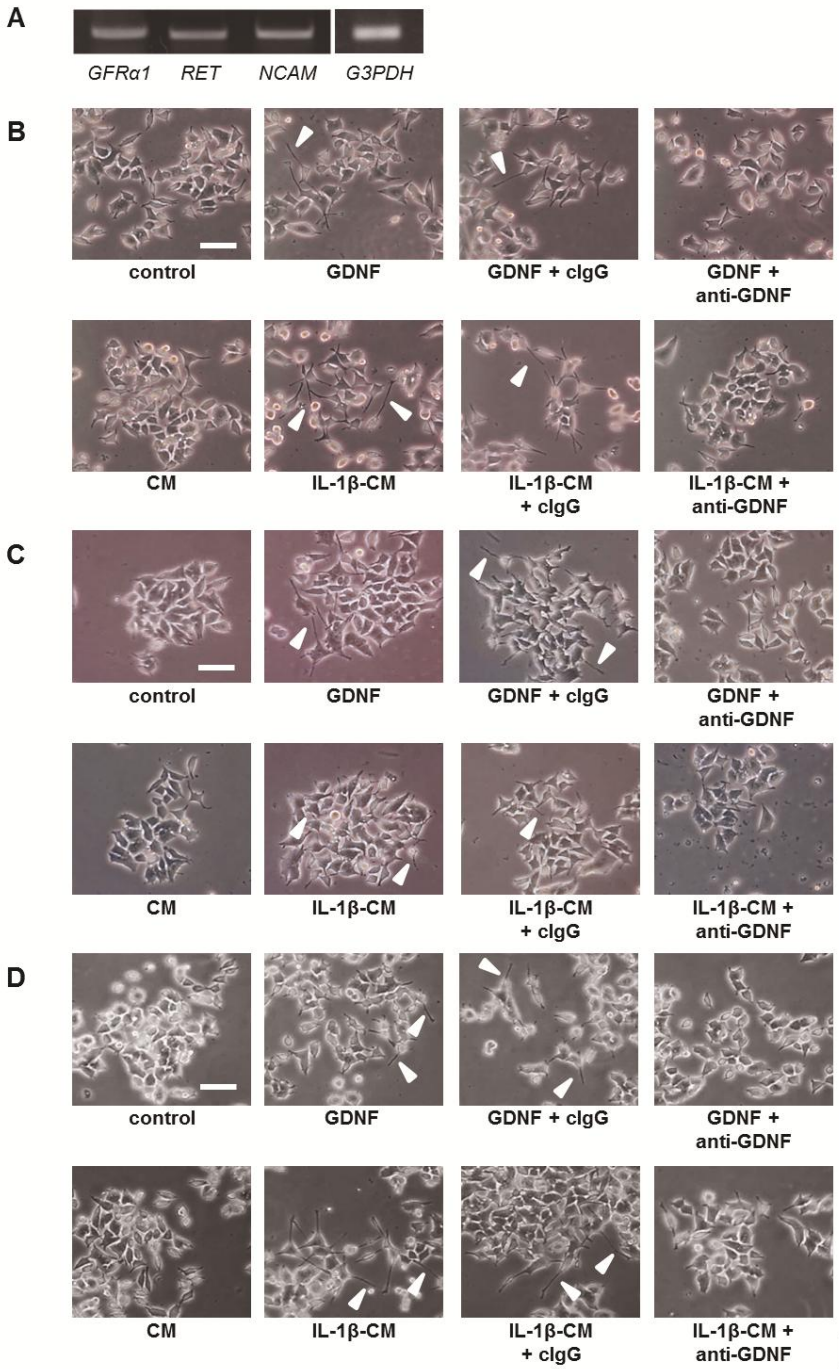

Figure 3

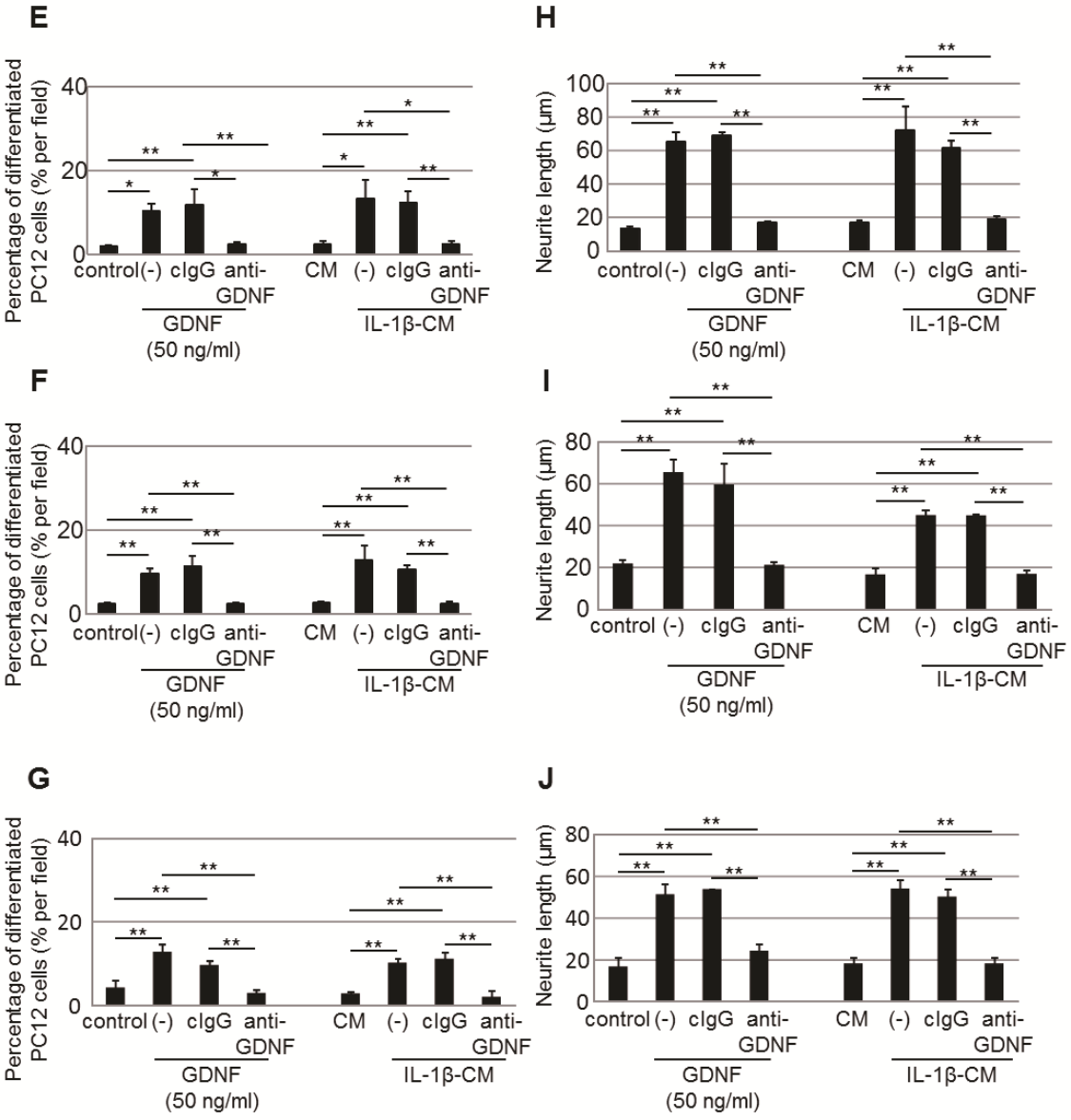

Figure 3

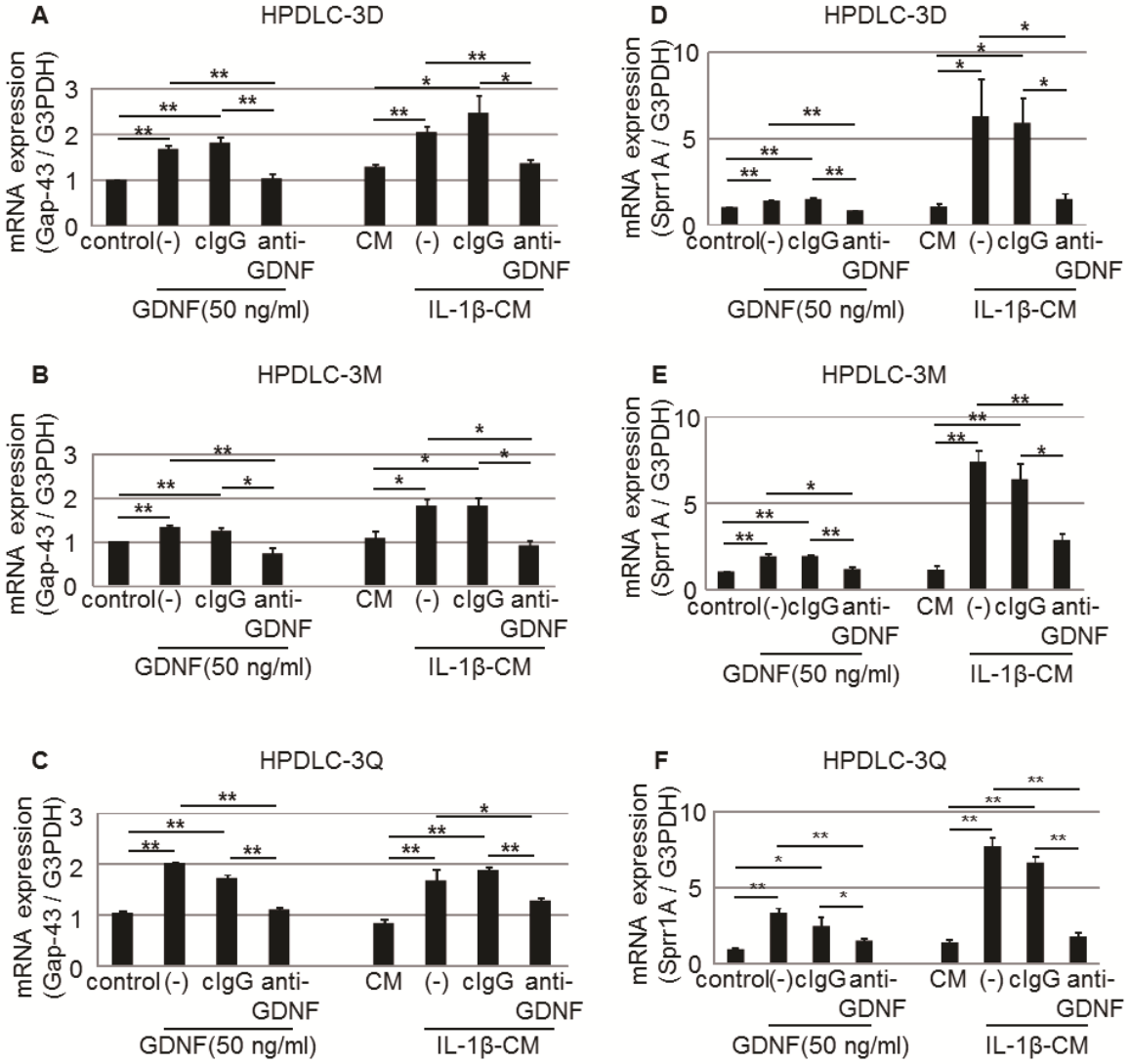

Figure 4

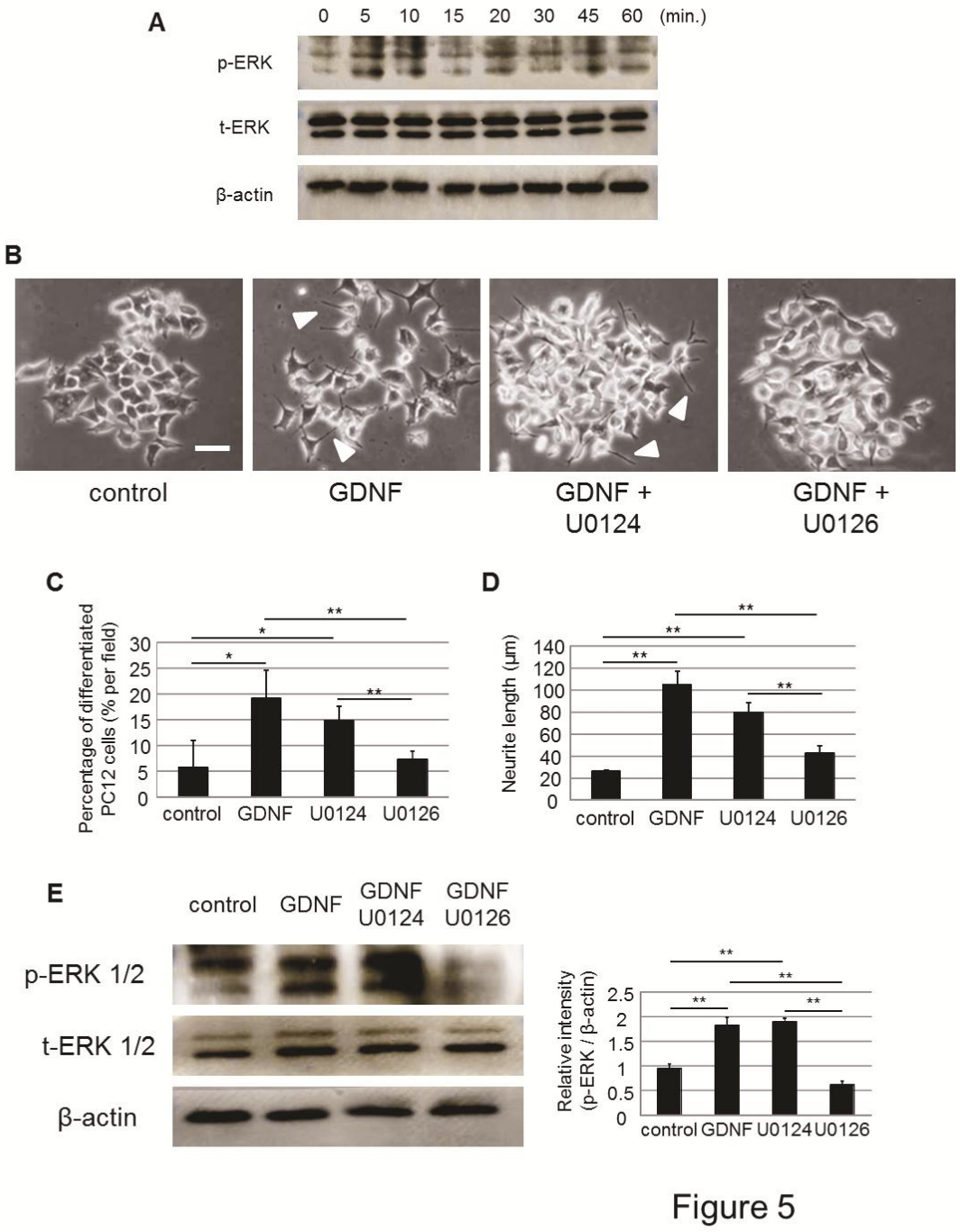

Figure 5