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Glial cell line-derived neurotrophic factor induces cell migration and matrix metalloproteinase-13 expression in glioma cells

Dah-Yuu Lu [a,**], Yuk-Man Leung [a], Chi-Wai Cheung [b], Yun-Ru Chen [a], Kar-Lok Wong [b,c,*]

[a] Graduate Institute of Neural and Cognitive Sciences, China Medical University, Taichung, Taiwan
[b] Department of Anesthesiology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong, China
[c] Department of Anesthesiology, China Medical University and Hospital, Taichung, Taiwan

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ABSTRACT

Malignant gliomas are the most common primary brain tumors in adults and the second most common tumor in children. Gliomas are associated high morbidity and mortality because these tumors are highly invasive into surrounding brain tissue, making complete surgical resection impossible. Glial cell line-derived neurotrophic factor (GDNF) has been identified as a potent neurotrophic factor in a variety of neuronal cell populations. However, the molecular mechanisms and pathologic roles underlying GDNF-induced glioma migration remain unclear. In this study, we found that application of recombinant human GDNF enhances the migration of U87 and U251 cells but not C6 cells. In addition, we found that the expression of matrix metalloproteinase-13 (MMP-13) mRNA, protein and secretion increase in response to GDNF stimulation. The GDNF-induced increase in cell migration was antagonized by MMP-13 neutralizing antibody or silencing MMP-13. We then examined the involvement of mitogen-activated protein kinases (MAPKs) in glioma cell migration induced by GDNF. GDNF-induced MMP-13 expression and glioma migration were attenuated by MEK/extracellular signal-regulating kinase (ERK) and c-Jun N-terminal protein kinase (JNK) inhibitors, as well as ERK and JNK dominant-negative mutants. Treatment with GDNF-induced MEK/ERK and JNK/c-Jun activation and increased AP-1 DNA binding activity in a time-dependent manner. Treatment with AP-1 inhibitors (tanshinone IIA and curcumin) also reduced GDNF-induced glioma cell migration. In migration-prone sublines, cells with greater migration ability had higher GDNF expression. These results indicate that GDNF enhances migration of glioma cells through the increase of MMP-13 production and is mainly regulated by the MEK/ERK and JNK, c-Jun and AP-1 pathways.

1. Introduction

Most primary brain tumors are derived from glial cells and are collectively referred to as gliomas. Malignant gliomas, the most common type of brain tumors in adults, are particularly invasive into surrounding brain tissue [1]. Migrative capacity is an essential prerequisite for invasion and precedes malignant tumor formation [2]. Glioblastomas are one of the most common primary central nervous system tumors and their biology makes successful treatment very difficult. The biggest problem is the aggressive invasion of malignant cells from the original tumor; metastasis or invasion into the surrounding brain tissue renders complete surgical resection impossible. Radiation and chemotherapy, alone or in combination, have produced only modest increases in median survival due to problems both with the effective targeting of the invading cells and resistance to conventional radiotherapy and chemotherapy [3,4]. Commonly used treatment procedures, such as chemotherapy, radiation and surgery [5], have failed to improve survival in patients with gliomas [6]. Effective treatment will ultimately require a deeper understanding of the signaling pathways that drive glioma invasion as well as the identification and specific targeting of the critical signaling effectors.

GDNF is a neurotrophic factor that promotes survival and development of various neural cells in the central and peripheral nervous systems. Importantly, GDNF promotes the survival, proliferation and activation of glioma cells [7,8]. Previous reports have shown that GDNF promotes pancreatic cancer cell migration and invasion [9–11]. Recently, we showed that GDNF increases chondrosarcoma cell migration [12]. However, little is known about the effect of GDNF on glioma cell migration. We hypothesized that GDNF release from neural tissue within and around the glioma is important for the growth and invasive potential of glioma cells.

* Corresponding author at: Department of Anesthesiology, China Medical University and Hospital, No. 2, Yude Rd., North Dist., Taichung, Taiwan. Tel.: +886 4 22052121x3550.
** Corresponding author at: Graduate Institute of Neural and Cognitive Sciences, China Medical University, No. 91 Hsueh-Shih Road, Taichung, Taiwan. Tel.: +886 4 22053366x8206; fax: +886 4 22071507.
E-mail addresses: dahyuu@mail.cmu.edu.tw (D.-Y. Lu), klwong@mail.cmuh.org.tw (K.-L. Wong).

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Glioblastoma multiforme is the most common malignant tumor of the CNS. At early stages, while the tumor mass is limited in macroscopic appearance, tumor cells have already invaded the surrounding brain [13]. This pathologic characteristic of their insidious infiltration of the brain leads to the poor prognosis after surgery and/or radiation therapy. Invasion of glioma cells into adjacent brain structures occurs through the activation of multigenic programs, including matrix metalloproteinases (MMPs), which play a important role in tumor invasion because of their ability to degrade many extracellular matrix components and other substrates [14], enabling tumor cells to invade the surrounding stroma. The MMPs can be divided into subgroups of collagenases, gelatinases, and other MMPs according to their substrate specificity and function [15]. We have previously reported that leptin enhanced migration and invasion of C6 glioma cells by increasing MMP-13 production [16]. This study therefore sought to determine whether GDNF affects the expression of MMP-13 in human glioma cells. We also investigated the intracellular signaling pathways involved in GDNF-induced up-regulation of MMP-13 expression. Our findings, revealing molecular mechanisms promoting GDNF in glioma cell migration, may lead to a better understanding of the malignant progression of human gliomas.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and OPTI-MEM were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA). Goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgG, primary antibodies against c-Jun, phospho-c-Jun (Ser^{63}), MEK1, phospho-MEK, β-actin, MMP-13, JNK, ERK2, and phospho-ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNA against human MMP-13, GPRα1 and GPRα2, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant GDNF was purchased from PeproTech (Rocky Hill, NJ). Monoclonal antibody specific for GDNF and polyclonal antibody specific for human phospho-Ret were purchased from R&D Systems (Minneapolis, MN). MEK and JNK kinase assay kits were purchased from Cell Signaling and Neuroscience (Danvers, MA). Curcumin and PD98059 were obtained from Sigma–Aldrich (St. Louis, MO). SP600125 was purchased from Tocris Bioscience (Ellisville, MO). Tanshinone IIA was purchased from Biomol (Plymouth Meeting, PA). The dominant-negative mutants of ERK (DN-ERK) and JNK (DN-JNK) were provided by Dr. C.-H. Tang (Department of Pharmacology, China Medical University).

2.2. Cell cultures

C6 cells originated from a rat brain glioma. U87 and U251 cells originated from a human brain glioma. All cell lines were purchased from the American Type Culture Collection (Manassas, VA). C6 cells were maintained with F12 medium (Invitrogen Life Technologies, Carlsbad, CA), while U87 and U251 cells were maintained in 75 cm^2 flasks with DMEM. All cells were cultured in medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C, incubated in a humidified atmosphere consisting of 5% CO_2 and 95% air.

2.3. Transfection

U87 cells were transiently transfected with 0.8 μg DN-ERK2, DN-JNK or pcDNA3 empty vectors, or 100 nM GPRα1, GPRα2, MMP-13, or control siRNA by Lipofectamine 2000 (LF2000; Invitrogen) for 24 h. Plasmid DNA and LF2000 were premixed in OPTI-medium (Invitrogen Life Technologies, Carlsbad, CA) for 20 min and then applied to the cells. An equal volume of medium containing 20% FBS was added 6 h later. After transfection for 24 h, LF2000-containing medium was replaced with fresh serum-free medium and treated with GDNF for another 24 h.

2.4. Reverse transcriptase-PCR and quantitative real time-PCR

Total RNA was extracted from cells using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using the oligo(dT) primer, then amplified using oligonucleotide primers:

MMP-13 5′-TGCTCGCATTCTCCTTCAGGA-3′; 5′-ATGCATCCAGGGGTCCTGGC-3′
GAPDH 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

Each PCR cycle was carried out for 30 s at 95 °C, for 30 s at 55 °C, and for 1 min at 68 °C. PCR products were then separated electrophoretically in a 2% agarose gel and stained with ethidium bromide. The band intensity was quantified with a densitometric scanner and presented as the relative level of GAPDH.

2.5. Western blot analysis

Cells were treated with GDNF for various time periods and then washed with cold PBS that had been lysed for 30 min on ice with radioimmunoprecipitation assay buffer (50 mM HEPES (PH 7.4), 150 mM NaCl, 4 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% Triton X-100, 0.25% sodium deoxycholate, 50 mM 4-(2-aminoethyl) benzene sulfonylfluoride, 50 μg/ml leupeptin and 20 μg/ml aprotinin).

The nuclear extracts were prepared as described previously [17]. Cells were rinsed with PBS and suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and vortexed for 10 s. The lysates were separated into cytosolic and nuclear fractions by centrifugation at 12,000 × *g* for 10 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclear fraction was resuspended in buffer C (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 13,000 × *g* for 20 min and stored at −80 °C.

Protein samples were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS for 1 h at room temperature and then probed overnight with primary antibody at 4°C. After undergoing three PBS washes, the membranes were incubated with secondary antibodies. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). The blots were subsequently stripped through incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 0.1 M β-mercaptoethanol) and reprobed for β-actin as a loading control. Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.6. Zymographic analysis

MMP-13 secreted into the culture medium of glioma cells was evaluated after GDNF application. The culture medium was mixed

with non-reducing sample buffer and electrophoresed by 10% SDS-polyacrylamide gel containing 1% bovine type B gelatin (Sigma–Aldrich). The gel was then washed with 2.5% Triton X-100 to remove SDS, rinsed with 50 mM Tris-HCl, pH 7.5, and then incubated overnight at room temperature in the developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% NaN_3, 1% Triton X-100). The MMP-13 enzyme activities were revealed by staining with 1% Coomassie Blue. The sample was also loaded into SDS-polyacrylamide gel and stained with 1% Coomassie Blue, to serve as the loading control [18,19].

2.7. Protein kinase assays

MEK and JNK protein kinase assays were performed according to the manufacturer's protocols. Equal amounts of protein were incubated with recombinant Elk or c-Jun fusion protein agarose for 16–18 h at 4 °C with gentle rotation. The beads were washed three times with wash buffer and kept on ice. The kinase reactions were performed by incubating immunoprecipitated beads with 50 μl of kinase buffer contained within 200 μM ATP at 30 °C for 30 min. To assess MEK and JNK activities, the reaction mixtures were analyzed by SDS-PAGE using specific antibodies against Elk phosphorylation and c-Jun phosphorylation.

2.8. Migration assay

In vitro migration assays were performed using Costar Transwell inserts (Costar, NY, USA; pore size, 8-μm) in 24-well plates. Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, or transfected with various dominant-negative mutants for 24 h. According to a cell viability assay, the various concentrations of inhibitors used did not affect glioma cell death (data not shown). Approximately 1×10^4 cells in 100 μl of serum-free medium were placed in the upper chamber, and 400 μl of the same medium containing GDNF was placed in the lower chamber. The plates were incubated for 24 h at 37 °C in 5% CO_2, then the cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper sides of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the undersides of the filters were examined and counted under a microscope. Each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of GDNF treatment (corrected migration cell number = counted migration cell number/percentage of viable cells) [20–22].

2.9. Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay was performed using gel shift kit (Panomics, Redwood City, CA) according to the manufacturer's protocol. Nuclear extract (2 μg) from U87 glioma cells was incubated with poly d(I-C) at room temperature for 5 min. The nuclear extract was then incubated with biotin labeled probes, followed by incubation at room temperature for 30 min. After electrophoresis on an 8% polyacrylamide gel, the samples on gel were transferred onto a presoaked Immobilon-Nyt membrane (Millipore, Billerica, MA). The membrane was cross-linked in an oven for 3 min and then developed by adding the blocking buffer and streptavidin–horseradish peroxidase conjugate, and then subjected to Western blot analysis.

2.10. Establishment of migration-prone sublines

Subpopulations from U87 glioma cells were selected according to their differential migration ability [16], using cell culture insert system as described earlier. After 24 h of migration, cells that penetrated through pores and migrated to the underside of the filters were trypsinized and harvested for a second-round selection. The original cells that did not pass through membrane pores were designated as P0. After 4 rounds of selection, the migration-prone subline was designated as P4.

2.11. Measurement of cell viability

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells cultured in 24-well plates were treated with various concentrations of GDNF for 24 h. After incubation, MTT (0.5 mg/ml) was added for 60 min, the culture medium was removed and cells were dissolved in dimethyl sulfoxide and shaken for 10 min. The absorbency (OD) values at 550 nm were immediately measured in a microplate reader.

2.12. Statistical analysis

Statistical analysis was performed using software Graphpad Prism 4.01 (Graph Pad Software Inc., San Diego, CA). Values are means ± S.E.M. Statistical analysis of the difference between two samples was performed using Student's *t*-test. Statistical comparisons involving more than two groups were performed using one-way ANOVA with Dunnett's post hoc test. In all cases, a value of p less than 0.05 was considered to be significant.

3. Results

3.1. GPRα1 and GPRα2 involved in GDNF-directed migration of glioma cells

GDNF has been identified as a potent neurotrophic factor for a variety of neuronal cell populations. Furthermore, GDNF affects the survival, proliferation and activation of glioma cells [7,8]. Previous reports have shown that GDNF increases migration of human colorectal cancer cells [23], chondrosarcoma cells [12] and pancreatic cancer cells [10,24,25]. However, whether GDNF affects the migration of glioma cells remains unclear. In this study, GDNF-regulated glioma cell migration was examined using the Transwell assay with correction of GDNF-induced proliferation effects [12,26]. GDNF directed the migration of both human glioma U87 and U251 cells but not rat glioma C6 cells (Fig. 1A). Furthermore, GDNF increased the migration of U87 and U251 glioma cells in a concentration-dependent manner (Fig. 1B). GDNF-directed enhancement of the glioma cell migration was abolished by treatment with GDNF-neutralizing antibody (Fig. 1C). The receptor for GDNF is thought to be a complex of GFRα-1, which acts as a ligand-binding domain, and RET, which acts as the signal-transducing domain. In addition, GDNF may also be able to act via GFRα-2, particularly in the presence of RET [27]. In order to investigate the role of the GDNF receptor involved in GDNF-mediated glioma cell migration, we transfected GPRα1 and GPRα2 siRNA into U87 glioma cells. GDNF-induced U87 cell migration was antagonized by pretransfection with GFPα1 and GFPα2 siRNA (Fig. 1D). GDNF has been identified as a ligand for Ret, a receptor tyrosine kinase required during embryogenesis for the survival of neuroblasts. We therefore examined whether any interaction between GDNF and its receptor is involved in the signal transduction pathways leading to GDNF-induced glioma cell migration. The phosphorylation level of the Ret receptor after GDNF stimulation of U87 cells was assessed by Western blot analysis. As shown in Fig. 1E, GDNF-induced Ret phosphorylation in a time-dependent manner. These data suggest that GDNF-induced glioma cell migration may occur via activated Ret, GPRα1 and GPRα2 receptors.

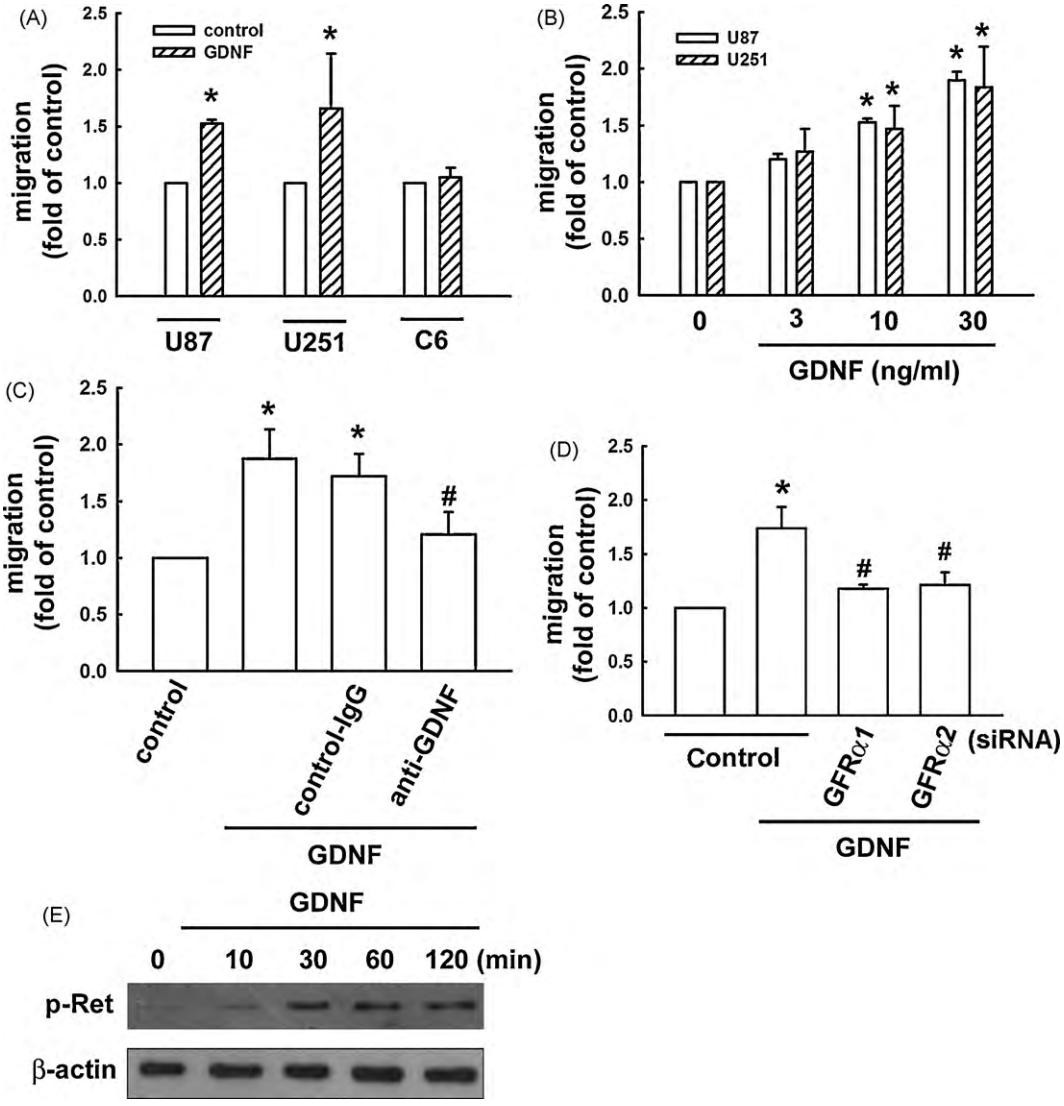

Fig. 1. GDNF induces the migratory activity of glioma cells. (A) U87, U251 and C6 cells were incubated with GDNF (30 ng/ml). *In vitro* migration activities, as measured with the Transwell assay after 24 h, showed that GDNF-induced migratory activity in U87 and U251 cells. (B) U87 and U251 cells were treated with various concentrations of GDNF. (C) U87 cells were treated with control IgG or GDNF antibody (4 μg) followed by stimulation with GDNF. (D) U87 cells were pretransfected with GPRα1, GPRα2 siRNA, or Control siRNA for 24 h. *In vitro* migration activity measured with the Transwell assay after GDNF treatment for 24 h. Results are expressed as the means ± S.E.M. of at least four independent experiments. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the GDNF treatment group. (E) U87 cells were treated with GDNF for indicated time periods (10, 30, 60 and 120 min), protein extract of Ret phosphorylation was determined by Western blot analysis. Results are the representative of three independent experiments.

3.2. GDNF-directed glioma cell migration involves MMP-13 expression

It has been reported that MMP-13 expression is involved in cancer cell migration and metastasis [28–30]. Our recently published data have also shown that leptin directs glioma cell carcinoma migration through MMP-13 up-regulation [16]. We therefore hypothesized that MMP-13 may be involved in GDNF-directed migration of glioma cells. Following GDNF stimulation, MMP-13 secretion levels, protein and mRNA expression, were assessed by gelatin zymography, Western blot and RT-PCR analysis. As shown in Fig. 2A–C, GDNF increased MMP-13 secretion, protein expression and RNA expression. Pretreatment of cells with the MMP-13 neutralizing antibody markedly inhibited GDNF-induced cell migration compared with control IgG (Fig. 2D). Furthermore, pretransfection of cells with MMP-13 siRNA effectively inhibited GDNF-induced glioma cell migration (Fig. 2E). These data suggest that GDNF-induced glioma cell migration may occur via MMP-13 up-regulation.

3.3. MEK/ERK and JNK/c-Jun signaling pathways are involved in GDNF-mediated MMP-13 up-regulation and glioma cell migration

It has been reported that GDNF increases the phosphorylation of MEK and ERK [12,31]. It has also been reported that activation of JNK and ERK were observed in the GDNF-treated cells. To examine the role of MEK/ERK in cancer migration and MMP-13 up-regulation, U87 cells were pretreated with PD98059 (30 μM) or SP6100125 (10 μM) for 30 min, followed by incubation with GDNF. As shown in Fig. 3A and B, GDNF-induced MMP-13 protein and RNA expression were inhibited by treatment with the specific MEK inhibitor PD98059 and the JNK inhibitor SP600125. In addition, GDNF-induced migration activity of glioma cells was greatly reduced by PD98059 and SP600125 (Fig. 3C). Transfection with DN-ERK or DN-JNK for 24 h also inhibited GDNF-directed glioma cell migration (Fig. 3D). Stimulation of cells with GDNF increased the phosphorylation of MEK and ERK (Fig. 4A and B). In parallel, by using an Elk-agarose fusion protein as the MEK substrate, an increase in MEK activity was also observed in

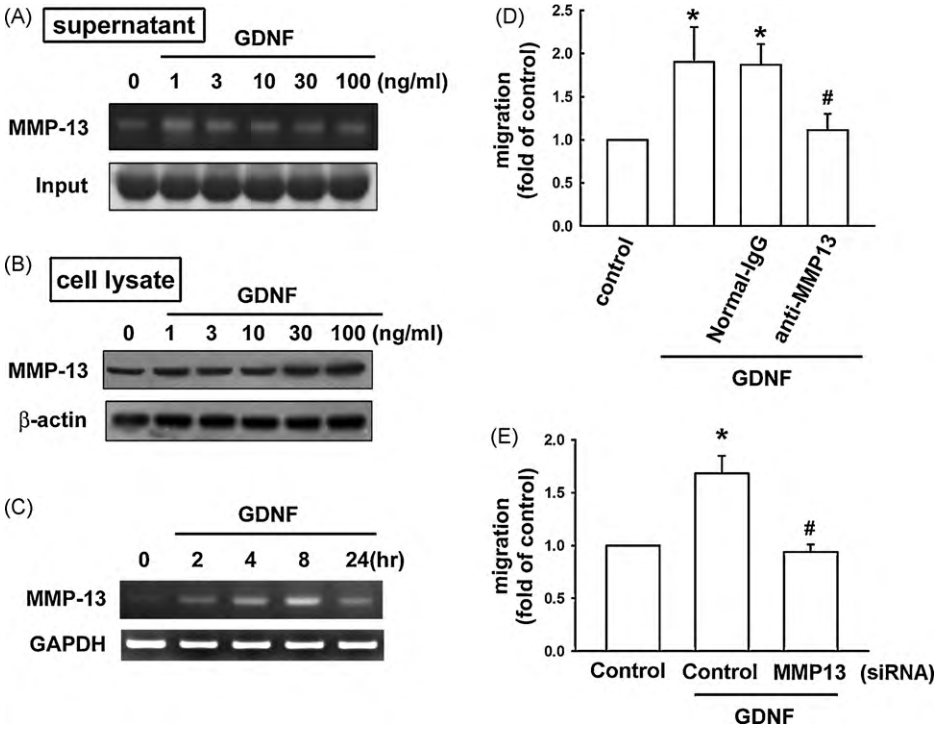

Fig. 2. GDNF-directed migration activity involves up-regulation of MMP-13 in U87 glioma cells. Cells were incubated with various concentrations of GDNF (1, 3, 10, 30 or 100 ng/ml) for 24 h, after which the supernatant (A) and cell lysate extracts (B) were collected, and MMP-13 protein levels determined using gelatin zymography and Western blot analysis. (C) Cells were treated with GDNF (30 ng/ml) for indicated time periods (2, 4, 8 and 24 h), and MMP-13 mRNA expression was analyzed by RT-PCR. Results are the representative of three independent experiments. Cells were incubated with control IgG or MMP-13 antibody (4 μg, D), or pretransfected with Control siRNA or MMP-13 siRNA (E) for 24 h, followed by stimulation with 30 ng/ml GDNF. *In vitro* assessments after 24 h showed that MMP-13 antibody and MMP-13 siRNA are capable of inhibiting migration activity. Results are expressed as the means ± S.E.M. of four independent experiments. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the GDNF treatment group.

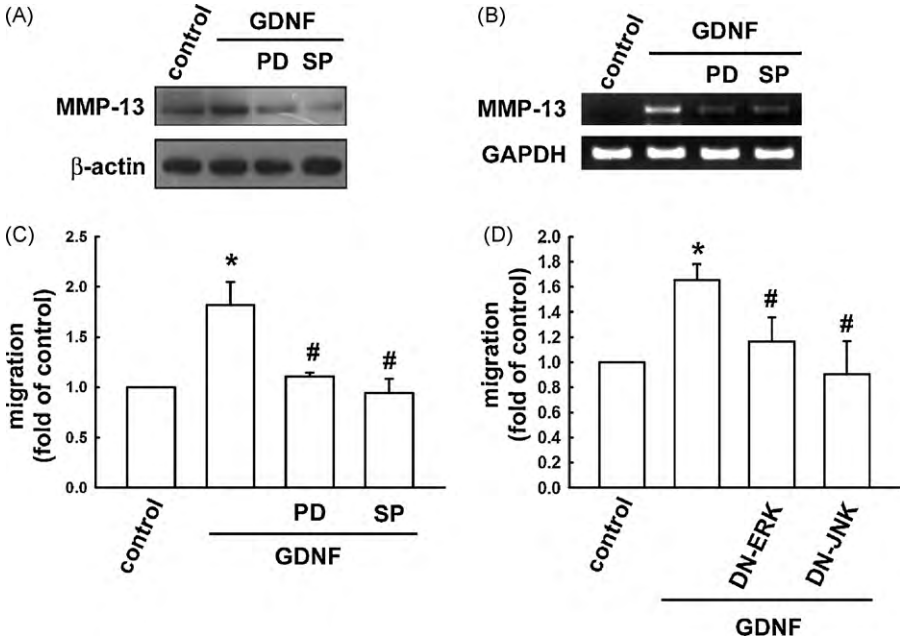

Fig. 3. Involvement of ERK and JNK in GDNF-induced MMP-13 expression and gliomal cell migration in U87 glioma cells. Cells were pretreated with PD98059 (30 μM) or SP600125 (10 μM) for 30 min, followed by incubation with GDNF. The cell lysated extract of MMP-13 was determined by Western blot (A) and RT-PCR (B) analysis. Results are the representative of three independent experiments. Cells were pretreated with PD98059 or SP600125 for 30 min (C), or pretransfected with DN-ERK or DN-JNK for 24 h (D) and stimulated with GDNF. *In vitro* cell migration was determined by Transwell assay after 24 h of GDNF treatment. Results are expressed as the means ± S.E.M. of three independent experiments. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the GDNF treatment group.

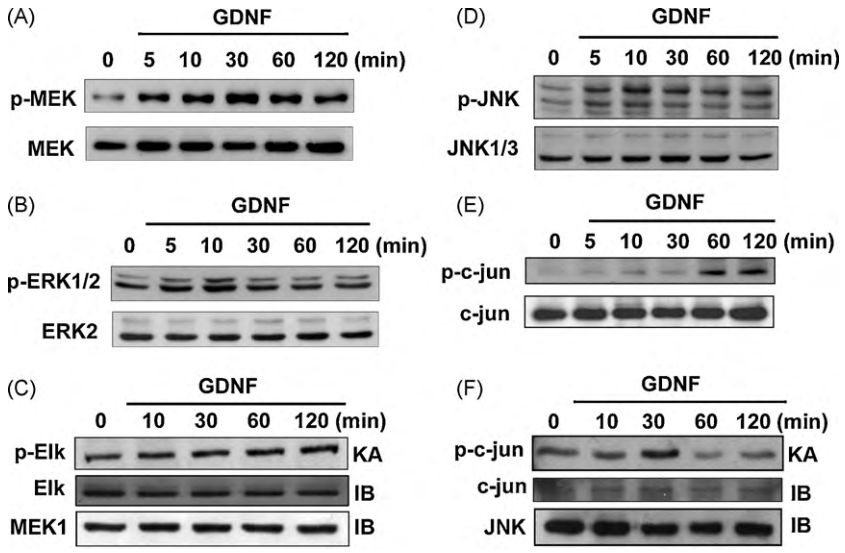

Fig. 4. GDNF induces MEK/ERK and JNK/c-Jun signaling activation in U87 glioma cells. Cells were incubated with GDNF for indicated time periods. The phosphorylation of MEK (A) and ERK (B) were determined by Western blot analysis. (C) Cells were incubated with GDNF for indicated time periods, and cell lysates were then immunoprecipitated with Elk fusion protein agarose beads. One set of immunoprecipitates was subjected to SDS-PAGE and analyzed by immunoblotting with the anti-phospho-Elk.antibody. Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for MEK1. Cells were incubated with GDNF for indicated time periods. The phosphorylation of JNK (D) and c-Jun (E) were determined by Western blot analysis. (F) Cells were incubated with GDNF for indicated time periods, and cell lysates were then immunoprecipitated with c-Jun fusion protein agarose beads. One set of immunoprecipitates was subjected to SDS-PAGE and analyzed by immunoblotting with the anti-phospho-c-Jun.antibody. Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for JNK. Results are expressed as four independent experiments.

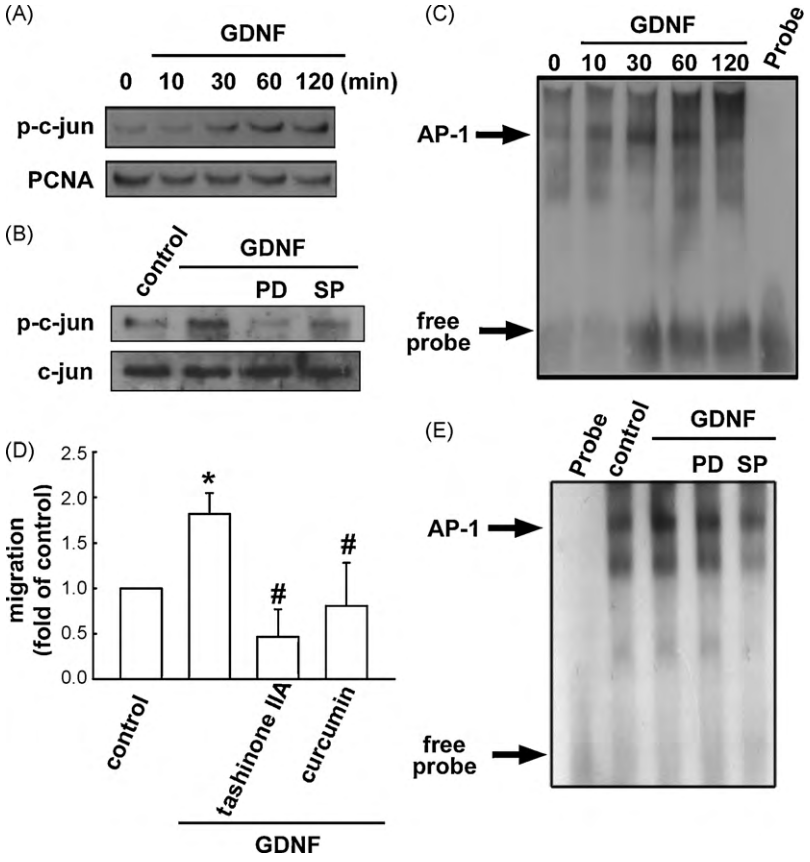

Fig. 5. GDNF induces cell migration through activation of AP-1 in U87 glioma cells. (A) Cells were treated with GDNF for the indicated time periods, and the level of nuclear c-Jun phosphorylation was determined by immunoblotting with phospho-c-Jun-specific antibody. (B) Pretreatment of PD98059 and SP600125 for 30 min was followed by stimulation with GDNF for 60 min, c-Jun phosphorylation was determined by immunoblotting with phospho-c-Jun-specific antibody. (C) Nuclear extracts from cells treated with GDNF for various time periods were incubated with an AP-1 probe and analyzed by EMSA. The AP-1-specific complex is indicated by an arrow. Results are the representative of three independent experiments. (D) Cells were pretreated with the AP-1 inhibitor tanshinone IIA or curcumin for 30 min, followed by stimulation with GDNF. *In vitro* migration was measured with the Transwell assay after GDNF treatment for 24 h. Results are expressed as the mean ± S.E.M. of three independent experiments. *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the GDNF treatment group. (E) Pretreatment of PD98059 and SP600125 was followed by stimulation with GDNF for 120 min, incubation with an AP-1 probe and then analysis by EMSA. Results are the representative of three independent experiments.

GDNF-treated U87 glioma cells (Fig. 4C). GDNF also increased JNK and c-Jun phosporylation in a time-dependent manner (Fig. 4D and E). Using a c-Jun-agarose fusion protein as the JNK substrate, an increase in JNK activity was also observed in GDNF-treated cells (Fig. 4F). These results indicate that the MEK/ERK and JNK/c-Jun pathways are involved in GDNF-induced migration activity and MMP-13 up-regulation in glioma cells.

3.4. Involvement of AP-1 in GDNF-induced cell migration in human U87 glioma cells

As previously mentioned, c-Jun activation is a necessary component in the signaling of GDNF-induced glioma cell migration and MMP-13 expression. We sought to determine whether c-Jun activation is involved in GDNF-induced transcription activity. As shown in Fig. 5A, GDNF increased expression of phosphorylated c-Jun at Ser^{63} accumulation in the nucleus. As shown in Fig. 5B, treatment of cells with the specific MEK inhibitor PD98059 and the JNK inhibitor SP600125 reduced the GDNF-increased c-Jun phosphorylated at Ser^{63}. We then assessed whether GDNF affects the transcription factor binding to DNA. GDNF stimulation significantly increased the DNA binding activity of AP-1 in a time-dependent manner, as determined by EMSA (Fig. 5C). Moreover, the AP-1 inhibitors tanshinone IIA and curcumin significantly reduced GDNF-enhanced glioma cell migration (Fig. 5D). GDNF-indued increases in AP-1 binding activity were also attenuated by PD98059 or SP600125 (Fig. 5E). These data suggest that activation of MEK/ERK, JNK, c-Jun and AP-1 is important for GDNF-induced glioma cell migration.

3.5. Increased GDNF expression in migration-prone cells

We selected U87 sublines with higher cell mobility, according to the procedures as described in Section 2. The migration-prone subline P4 had higher cell mobility and migrated more easily through the membrane of cell culture inserts basement membrane matrix as compared with original U87 cells designated as P0 (the difference was approximately 2.2-fold; Fig. 6A). Our previous results suggest that migration-prone sublines expressing more MMP-13 have higher migration and invasion activity [16]. Here, we found that P4 markedly increased the expression of GDNF protein levels, by approximately 3-fold.

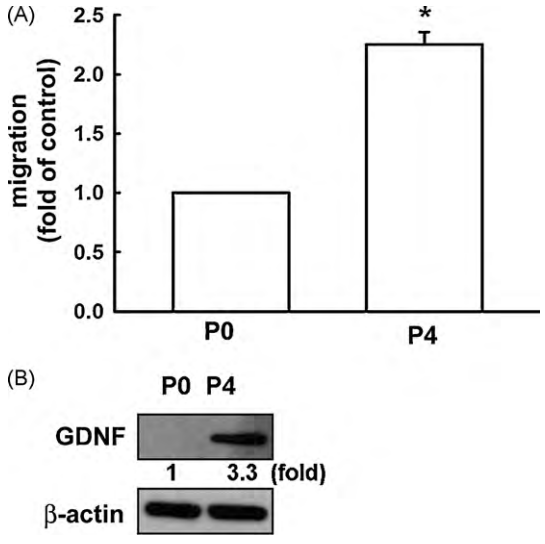

Fig. 6. Up-regulation of GDNF expression in migration-prone cells. (A) After 4 rounds of selection of U87 cells by cell culture insert system, the migration-prone subline (P4) exhibited more migration than the original U87 cells. (B) The supernatant of P4 and the original U87 glioma cells (P0) were collected after 24 h of culturing, and GDNF protein levels determined using Western blot analysis. Note that P4 expressed higher GDNF protein levels.

4. Discussion

The elucidation of the molecular biology of cancer cells in recent years has identified alterations in various molecular pathways in different cancers. This information is currently being exploited to develop potential therapeutic targets. It has been reported that GDNF induces cell migration of lung [32] and pancreatic cancer cells [10,11,25]. However, the molecular mechanisms and pathologic roles underlying GDNF-induced glioma migration remain unclear. In this study, we found that exogenous application of GDNF enhanced the migratory ability of human glioma cell lines U87 and U251.

Different MMP families have been found to be involved in cancer cell migration and invasion. MMP-9 expression is elevated by GDNF in human pancreatic cancer [25]. Our previous report demonstrates that leptin induced glioma cell migration. In addition, it has been reported that bone morphogenetic protein-2 [28], Cyr61 [30] and CTGF [29] enhance the motility of human cancer cells via activation of MMP-13. Our present study found that production and activity of MMP-13 markedly increased in response to GDNF application in human glioma cells. The GDNF-induced cell mobility was correlated to the elevated MMP-13 production by using MMP-13 neutralizing antibody and MMP-13 silencing. In contrast, migration-prone sublines were selected and showed higher migration ability. Our previous results suggest that migration-prone sublines expressing higher levels of MMP-13 have higher migratory and invasion activity [16]. The more prominent expression of GDNF in migration-prone cells further indicates that GDNF may be involved in autocrine or paracrine functions that enhance migration and invasion. GDNF enhances the migratory ability of U87 and U251 cells to a greater extent than that of C6 cells. In addition, we found that U87 and U251 cells secrete more GDNF than do C6 cells (data not shown). Our results are correlated with previous study, showing that high-grade glioma cells (C6 cells) have greater migratory ability and secrete more GDNF than low-grade glioma cells (Hs683 cells) [33]. Notably, GDNF treatment of the low-grade glioma cells (Hs683 cells) significantly increased migration to levels comparable to those of high-grade glioma cells (C6 cells) [33]. These results reveal an autocrine and/or paracrine effect of GDNF in promoting human glioma cell migration.

GDNF was initially discovered to be a potent survival factor for midbrain dopaminergic neurons [34]. It has also been reported that GDNF affects the survival, proliferation and activation of glioma cells [7,8]. The receptor for GDNF has recently been identified to be a complex of GFRα-1 [35,36], which acts as a ligand-binding domain, and of a heterodimeric complex of a proto-oncogene, RET [36,37]. In addition, RET acts as the signal-transducing domain [37,38]. Clinical studies have shown an association between expression of RET and GFRα1 and poorer survival of patients with pancreatic and bile duct carcinomas [32,39]. Higher expression of RET receptors has been found in patients with prostate and breast carcinoma [31,40]. Furthermore, GDNF may also be able to act via GFRα-2, particularly in the presence of RET [27]. GDNF has been identified as a ligand for Ret, a receptor tyrosine kinase required during embryogenesis for the survival of neuroblasts. In addition, previous report showed that GDNF induces Ret-mediated formation of lamellipodia in SK-N-MC cells [31]. It has also been reported that the expression of GDNF and its receptor are significantly higher in human gliomas than in normal human brain [8]. In 1998, Bennett and colleagues showed that GDNF and its receptor components RET, GFRα-1, and GFRα-2 signaling protect against nerve injury [41]. This present study showed that GDNF-induced

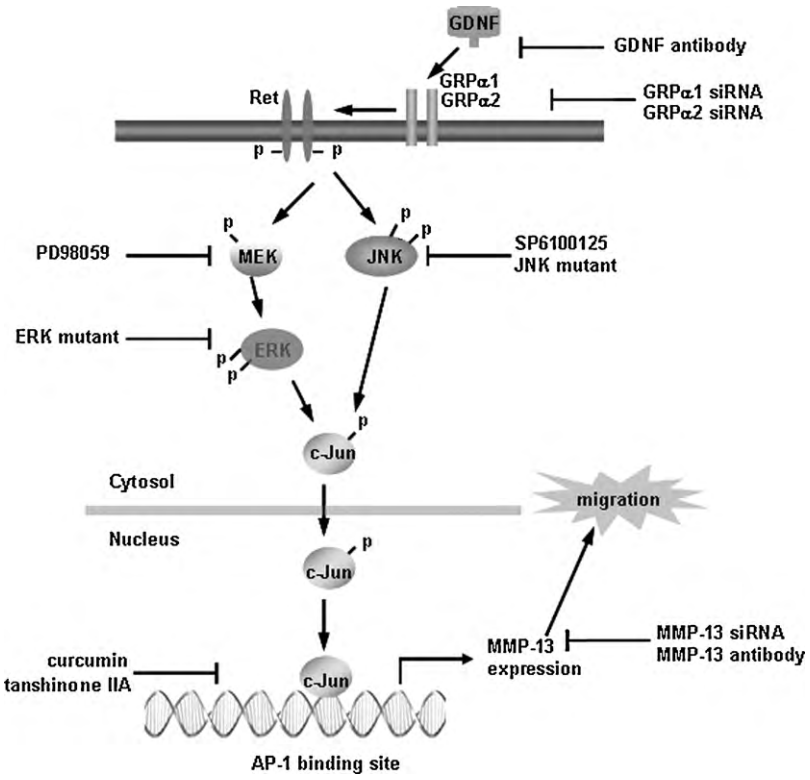

Fig. 7. Schematic diagram of the signaling pathways involved in GDNF-induced MMP-13 expression and cell migration in glioma cells. GDNF binds to the GDNF receptor (GRPα1 and GRPα2) and activation of Ret, MEK/ERK and JNK/c-Jun pathways enhances binding of c-Jun to the AP-1 site, resulting in the transactivation of MMP-13 expression, leading to glioma cell migration.

glioma cell migration was antagonized by pretransfection with GFRα1 and GFRα2 siRNA (Fig. 1D). We also showed that GDNF induces Ret phosphorylation in a time-dependent manner (Fig. 1E). Inhibition of either GFRα receptor produced similar effects on migration. These data suggest that GDNF-induced glioma cell migration may occur via the activated Ret receptor, and GFRα1 and GFRα2 receptors. Indeed, GFRα1 and GFRα2 might act through the same key receptor, Ret, as the most important in GDNF-induced motility.

Activation of Ret by GDNF results in activation of the MAPK pathways.[31] Multiple lines of evidence indicate the involvement of GDNF in brain tumor motility. Signaling molecules including ERK and JNK have been demonstrated to play important roles in glioma cell invasion and migration [42,43]. Song and Moon [33] have shown that GDNF promotes low-grade Hs683 glioma cell migration through JNK and ERK-1/2 signaling pathways. It has been reported that RET activates ERK and JNK signaling pathways [44,45]. PD98059 and SP600125 effectively antagonized GDNF-induced MMP-13 protein expression (Fig. 3A) and reduced GDNF-induced MMP-13 RNA expression (Fig. 3B). The inhibition of ERK and JNK also reduced GDNF-induced glioma cell migration (Fig. 3C and D). Our results showed that GDNF-induced MEK/ERK and JNK phosphorylation. In addition, GDNF also increased MEK and JNK kinase activity in human glioma cells (Fig. 4).

It has been reported that astrocyte-derived GDNF promotes complete and long-term survival of adult facial motoneurons following avulsion and differentially regulates the expression of AP-1 transcription factors [46]. Our recent report showed that bradykinin enhances migration of glioma cells through AP-1 activation [22]. We have also shown that MMP-13 transcription is activated in astrocytes responding to hypoxia via AP-1 activation [19]. It has been reported that MMP-13 transcription may be activated via AP-1 in cells responding to CXCL12 [47] and ultrasound [48]. GDNF time-dependently increases c-Jun Ser^{63} phosphorylation accumulation in the nucleus (Fig. 6A). GDNF-induced phosphorylation of c-Jun (Ser^{63}) was reduced by the MEK/ERK inhibitor PD98059 and the JNK inhibitor SP600125 (Fig. 6B). c-Jun is a major component of the AP-1 complex in many cells. In addition, c-Jun is a moderately labile protein, subject to polyubiquitination on multiple lysine residues and phosphorylation, leading to increased transcriptional activity. By using EMSA in our present study, we found that GDNF induces a time-dependent increase in AP-1 DNA binding activity (Fig. 6C). Furthermore, GDNF-enhanced glioma cell migration was inhibited by the AP-1 inhibitors tanshinone IIA and curcumin (Fig. 6D). In addition, PD98059 and SP600125 reduced GDNF-increased AP-1 promoter activity. In conclusion, GDNF increased MEK/ERK and JNK activation, resulting in increased MMP-13 expression via AP-1 activation.

These results indicate that GDNF directs the migration of glioma cells through the activation of Ret, GPRα1 and GPRα2, and via up-regulation of MMP-13 expression. Moreover, GDNF also increased MEK/ERK and JNK activation, and increased MMP-13 expression via AP-1 activation, thereby contributing to tumor migration (Fig. 7).

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