Isothiocyanates inhibit the invasion and migration of C6 glioma cells by blocking FAK/JNK-mediated MMP-9 expression

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Abstract. Isothiocyanates (ITCs) derived from cruciferous vegetables, including benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and sulforaphane (SFN), exhibit preventative effects against various types of cancers. Yet, the inhibitory effects of ITCs on C6 glioma cell invasion and migration have not been reported. Thus, we aimed to analyze ITC-regulated MMP-9 activation, a crucial enzyme of cancer metastasis that degrades the extracellular matrix, in C6 glioma cells to investigate the inhibitory effects on cancer invasion and migration by ITCs. In the present study, we found that ITCs specifically suppressed PMA-induced MMP-9 secretion and protein expression. The inhibitory effects of ITCs on PMA-induced MMP-9 expression were found to be associated with the inhibition of MMP-9 transcription levels through suppression of nuclear translocation of NF-κB and activator protein-1 (AP-1). It was also confirmed that ITCs decreased MMP-9-mediated signaling such as FAK and JNK, whereas they had no effect on the phosphorylation of ERK and p38. Moreover, wound-healing and Transwell invasion assays showed that ITCs inhibited the migration and invasion of C6 glioma cells. These results suggest that ITCs could be potential agents for the prevention of C6 glioma cell migration and invasion by decreasing FAK/JNK-mediated MMP-9 expression.

Introduction

Gliomas are one of the most lethal types of cancer and are the most common types of brain tumor in the central nervous system (1). Although various therapeutic methods such as advanced surgical techniques, radiation treatment and new combined chemotherapy are performed, survival is low due to the extremely aggressive and invasive features of C6 glioma cells (2). Thus, the regulation of invasion and migration represents an important therapeutic target of cancer. Tumor cell invasion and migration is a complex multi-step process and essentially requires degradation of the extracellular matrix (ECM). The degradation enzymes of the ECM include the matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motif proteases, and serine proteases of the urokinase/plasmin-type (1). Among various degradation enzymes of the ECM, MMPs are associated with tumor spreading and poor prognosis (3).

MMPs have a signal peptide and a catalytic domain that contains the highly conserved zinc binding site (4). MMPs, such as collagenases, gelatinases, stromelysins and membrane-type matrix metalloproteinases (MT-MMPs), can degrade most ECM types. They have various roles in physiological and pathological processes, such as tissue development, remodeling, and inflammation in cancer. The overexpression of MMPs is correlated with metastasis and invasion of malignant cancer cells (5,6). In

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particular, MMP-9 and MMP-2 are the key enzymes involved in the degradation of type IV collagen and the ECM (7), and are mainly associated with tumor invasion, degradation of the blood-brain barrier, neuro-degenerative processes, and angiogenesis in gliomas (8,9). MMP-9 and MMP-2 have structural and catalytic similarities. However, MMP-9 can be increased by various agents, such as inflammatory cytokines, growth factor, and phorbol myristate acetate (PMA), whereas MMP-2 is continually expressed and is usually overexpressed in malignant tumors. Particularly, PMA induces inflammation and promotes tumor growth by increasing the invasion of various types of cancer cells through MMP-9 activation (10).

Furthermore, PMA regulates MMP-9 expression by regulating transcription factors including nuclear factor (NF)-κB and activator protein-1 (AP-1) via mitogen-activated protein kinase (MAPK) pathways (11,12). NF-κB and AP-1 are both important transcription factors associated with the modulation of cell migration and invasion (12). MAPK is modulated by the focal adhesion kinase (FAK) pathway in various types of cancers. FAK is a crucial target for the regulation of tumor invasion and metastasis (13). It also modulates cell motility and cell adhesion by transferring ECM signals from integrins to the intracellular compartment (14).

Natural dietary phytochemicals are found in fruits, spices, teas and vegetables. Among the various phytochemical compounds, isothiocyanates (ITCs) are isolated from cruciferous vegetables and are characterized as having a sulfur-containing N=C=S functional group (15). ITCs have shown biological and pharmacological activities in diseases, including chronic-degenerative diseases, which include cardiovascular diseases, neurodegeneration and diabetes (16,17). Additionally, ITCs, including benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) have preventative effects on various types of cancers (18). ITCs suppress myeloma, breast cancer, and pancreatic cancer by inhibiting MMP-9 through NF-κB (19,20). BITC was found to inhibit migration and invasion of human colon cancer HT29 cells through the MAPK signaling pathway (21). PEITC inhibits the migration and invasion of human gastric adenocarcinoma cells by suppressing the NF-κB signaling pathways (22). Moreover, SFN was found to sensitize TNFα-related apoptosis-inducing ligand-mediated apoptosis by downregulating extracellular signal-regulated kinase (ERK) in lung adenocarcinoma A549 cells (23).

Therefore, we hypothesized that the anti-metastatic activity of ITCs may function to modulate MMP-9 in C6 glioma cells. In this study, the effects of BITC, PEITC and SFN on PMA-induced MMP-9 expression were examined in C6 glioma cells. It was found that ITCs suppressed PMA-induced MMP-9 expression by inhibiting MMP-9 transcription levels by blocking FAK/JNK-mediated AP-1 and NF-κB activation. Additionally, suppression of C6 glioma cell invasion by ITCs was associated with inhibitory effects on MMP-9 expression.

Materials and methods

Cells and materials. The C6 rat glioma cell line was obtained from the American Type Culture Collection (ATCC; USA). The culture medium used in the experiments was Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific, Logan, UT, USA) containing 10% fetal bovine serum (FBS) and 1%

antibiotic-antimycotic. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. BITC, PEITC, SFN and N-methyl-β-phenethylamine (NMPEA) were obtained from Sigma (St. Louis, MO, USA).

Cell viability assay. C6 cells were seeded in a 96-well plate and allowed to attach for 24 h. Media were then discarded and replaced with 100 µl of new media containing various concentrations of ITCs and cultured for 24 h. 3-[4,5-Dimethylthiazol2-yl]-2,5-diphenyltetrazolium bromide (MTT; Roche Applied Science, Indianapolis, IN, USA) was added to each well. The amount of formazan deposits was quantified according to the supplier's protocol after 4 h of incubation with MTT reagent at 37°C in a 5% CO_2 incubator. The half maximal inhibitory concentrations (IC_{50}) were determined as the concentration of the test mixture that gave a 50% reduction in absorbance compared to that of the control.

Gelatin zymography assay. Zymography was performed using the procedure described by Lee *et al* with minor modification (24). C6 cells were seeded in 6-well culture plates and incubated until they reached 80% confluency. Fresh serum-free medium was then added with ITCs to each dish, and further cultured for 24 h. Conditioned medium, so obtained, was electrophoresed on polyacrylamide gels containing 0.1% (w/v) gelatin. Gels were washed at room temperature for 30 min with 2.5% Triton X-100 and then incubated at 37°C for 24 h in a buffer containing 10 mM $CaCl_2$, 0.01% NaN_3 and 50 mM Tris-HCl (pH 7.5). Gels were then stained with 0.2% Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA) and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field.

Quantification of intracellular reactive oxygen species (ROS). The intracellular concentration of ROS in C6 glioma cells was measured using an oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; Invitrogen Molecular Probes, Eugene, OR, USA). DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2',7'-dichlorodihydrofluorescein. This non-fluorescein analog gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent 2',7'-dichlorofluorescein level. C6 glioma cells were treated with ITCs and PMA for 12 h, after which the cells were incubated with 10 µM DCF-D at 37°C for 30 min. The fluorescence of 2',7'-dichlorofluorescein was detected in equivalent quantities of proteins using a multi-plate reader, VICTOR3 (excitation, 530 nm, emission, 485 nm; Perkin-Elmer, Waltham, MA, USA).

Western blot analysis. C6 cells were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 100 µM phenylmethylsulfonyl fluoride, 20 µM aprotinin and 20 µM leupeptin, adjusted to pH 8.0) at 4°C for 30 min, followed by centrifugation at 12,000 rpm for 10 min. In addition, to separate the proteins in cells buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 300 mM saccharose, 0.1% NP-10, 0.5 mM phenylmethylsulfonyl fluoride] was used. After incubation for 5 min on ice, the samples were centrifuged at 1,000 rpm at 4°C for 1 min and the pellet was separated. A separate pellet was dissolved
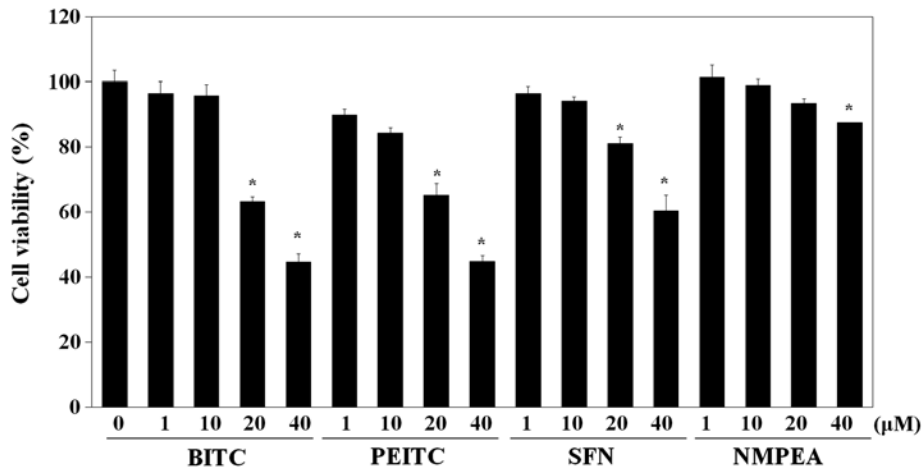
Figure 1. Effect of isothiocyanates on the viability of C6 glioma cells. C6 glioma cells were treated with the indicated concentrations of ITCs for 24 h. Cell viability was determined by MTT assay. Values represent the means ± SD of triplicate assays. *P<0.05 as compared to the untreated control. Results were analyzed using one-way ANOVA.

in buffer B [20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 100 mM NaCl$_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride]. After incubation for 15 min on ice, the samples were centrifuged at 1,000 rpm at 4°C for 5 min. Total protein concentration was determined using the Bradford assay (Bio-Rad). Total protein (30 μg) was separated on 6 to 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) using standard SDS-polyacrylamide gel electrophoresis procedure. The membranes were blocked in 5% skim milk in TBS-T for 1 h at room temperature. The membranes were then incubated with the primary antibody overnight at 4°C, washed three times with TBS-T, incubated with goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies for 1 h at room temperature and then washed with TBS-T three times. Signals were detected using enhanced chemiluminescence (ECL; Amersham Life Science Corporation, Arlington Heights, IL, USA) film and ChemiDOC XRS (Bio-Rad). Primary antibodies used in this study were MMP-9 and MMP-2 purchased from Millipore (Billerica, MA, USA). Phospho-ERK, phospho-JNK, phospho-p38, phospho-AKT, phospho-FAK, NF-κB, c-Jun, and c-Fos were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Reverse transcription-polymerase chain reaction. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. For RT-PCR, cDNA was synthesized from 1 μg of total RNA using Bioneer AccuPower PCR PreMix kit (Daejeon, Korea) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: MMP-9, 5'-AAACCT CCAACCTCACGGAC-3' (sense) and 5'-GAAAGGCGT GTGCCAGTAGA-3' (antisense); TIMP-1, 5'-CTGCAACTCG GACCTGGTTA-3' (sense) and 5'-GTGCACAAATCTGGAT TCCG-3' (antisense); and β-actin, 5'-ATGTGGATAAAGCCG TCAGTGG-3' (sense) and 5'-CTGGAGTGTCCATGGGAC AG-3' (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

Transwell invasion assay. Matrigel-coated filter inserts (8-μm pore size) that fit into 24-well migration chambers were obtained from Becton-Dickinson (Franklin Lakes, NJ, USA). Cells were then plated on the upper chamber. The lower chamber was filled with culture media containing various drugs. Cells in the chamber were incubated for 24 h at 37°C and cells that invaded the lower membrane surface were fixed with methanol and stained with hematoxylin and eosin. The cells that passed through the Matrigel and were located on the underside of the filter were counted. Random fields were counted by light microscopy (x400 magnification).

Wound-healing assay. This assay was performed using the procedure described by Lin et al with minor modification (25). Cells were seeded in 6-well plates and incubated until they reached 80% confluency. Monolayers were scratched with a 200-μl pipette tip to create a wound, and cells were then washed twice with serum-free culture media to remove floating cells. Media were then replaced with fresh serum-free media. Cells were subjected to the indicated treatment for 24 h, and cells were photographed at 24 h.

Statistical analysis. All *in vitro* results are representative of at least three independent experiments performed in triplicate. The significance of differences between the experimental and control values was analyzed by the Newman-Keuls test using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P-values of <0.05 were deemed to indicate a significant difference.

Results

ITCs inhibit PMA-induced MMP-9 expression and activity. Before investigating the anticancer pharmacological potential of ITCs on C6 glioma cells, the effect of ITCs on cell viability was first examined. As shown in Fig. 1, a cytotoxic effect on C6 glioma cells was exhibited by BITC (IC$_{50}$, 32.8 μM), whereas PEITC (IC$_{50}$, 34.3 μM) showed comparable cytotoxic activity and SFN (IC$_{50}$, 50.8 μM) exhibited relatively less cytotoxicity. NMPEA, which is a structural analog of PEITC
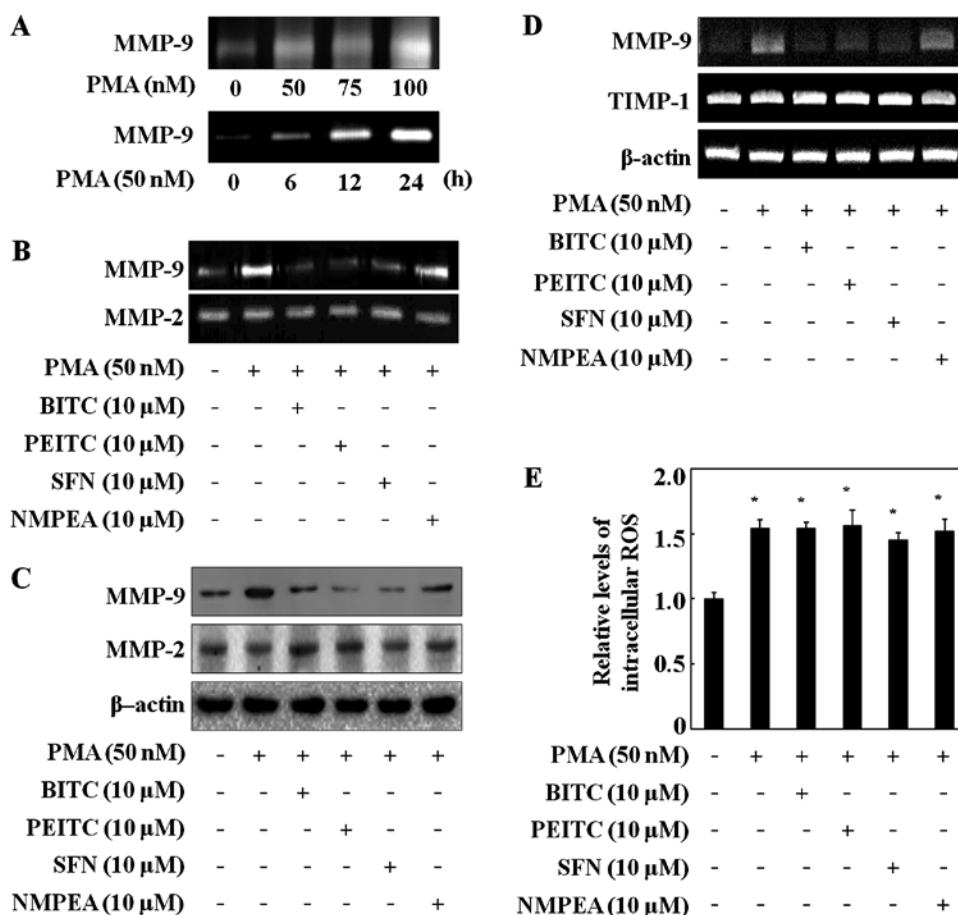
Figure 2. Isothiocyanates suppress PMA-induced MMP-9 activity and expression. (A) C6 glioma cells were treated with the indicated concentrations of PMA for 24 h (upper panel). C6 glioma cells were treated with 50 nM of PMA for the indicated times (lower panel). (B) C6 glioma cells were co-treated with 10 μM ITCs and 50 nM PMA. MMP-9 and MMP-2 activity was determined by gelatin zymography assay. (C) C6 glioma cells were co-treated with 10 μM ITCs and 50 nM PMA for 24 h. Expression levels of MMP-9 and MMP-2 were determined by western blot analysis. (D) mRNA levels of MMP-9 and TIMP-1 were analyzed by RT-PCR as described in Materials and methods. (E) C6 glioma cells were treated with PMA (50 nm) and 10 μM ITCs for 12 h, and then incubated with DCF-DA (10 μM) for 30 min. DCF fluorescence was detected using a multi-plate reader. Each value represents the mean ± SD of three independent experiments (*P<0.01 vs. controls).

without ITC functionality (26), was used as the negative control. NMPEA did not significantly affect the cell viability below a concentration of 20 μM. There was no obvious reduction in the cell viability of the C6 glioma cells after treatment with all ITCs drugs at doses <10 μM. Based on these results, a concentration of 10 μM of the ITCs was used in the following experiments. The MMP-9 secretion in the C6 glioma cells was induced by PMA in a dose-dependent manner and a time-dependent manner (Fig. 2A), whereas MMP-2 did not change (data not shown). In the following experiments, the C6 glioma cells were treated with 50 nM PMA for 24 h. Then the inhibitory effects of ITCs on MMP-9 and MMP-2 activity were determined via gelatin zymography assay. ITCs at a concentration of 10 μM decreased the MMP-9 activity in the C6 glioma cells but did not change MMP-2 activity (Fig. 2B).

To further confirm the influence of ITCs on MMP-9 expression, western blotting was performed. ITCs inhibited MMP-9 expression, whereas MMP-2 expression was not reduced in the C6 glioma cells (Fig. 2C). NMPEA had no effect on both MMP-9 activity and expression (Fig. 2B and C). ITCs also reduced the PMA-induced MMP-9 mRNA level in the C6 glioma cells, whereas NMPEA had no affect (Fig. 2D).

Moreover, since the activity of MMP-9 is regulated by the endogenous tissue inhibitor of metalloproteinase-1 (TIMP-1) (27), the expression level of TIMP-1 was measured via RT-PCR. ITCs did not alter TIMP-1 mRNA expression. These results suggest that the sulfur-containing functional group of the ITCs directly inhibited the MMP-9 transcription level, as NMPEA had no effect on PMA-induced C6 glioma cells. In addition, since ROS generation is known to trigger PMA-mediated induction of cell migration (28), the effects of ITCs on intracellular ROS concentrations were measured. The C6 glioma cells exposed to PMA had increased ROS levels compared with the untreated cells (Fig. 2E). However, ITCs did not affect the PMA-induced ROS generation in the C6 glioma cells, suggesting that the inhibitory effects of ITCs on MMP-9 were not related to ROS generation.

ITCs reduce FAK-dependent JNK phosphorylation in C6 glioma cells. MAPK is one of the pathways involved in the modulation of PMA-induced MMP-9 expression (29). To investigate the effects of ITCs on the MAPK expression associated with migration and invasion in C6 glioma cells, western blotting was performed. As shown in Fig. 3A, the ITCs suppressed the PMA-induced phosphorylation of JNK, but ERK and p38
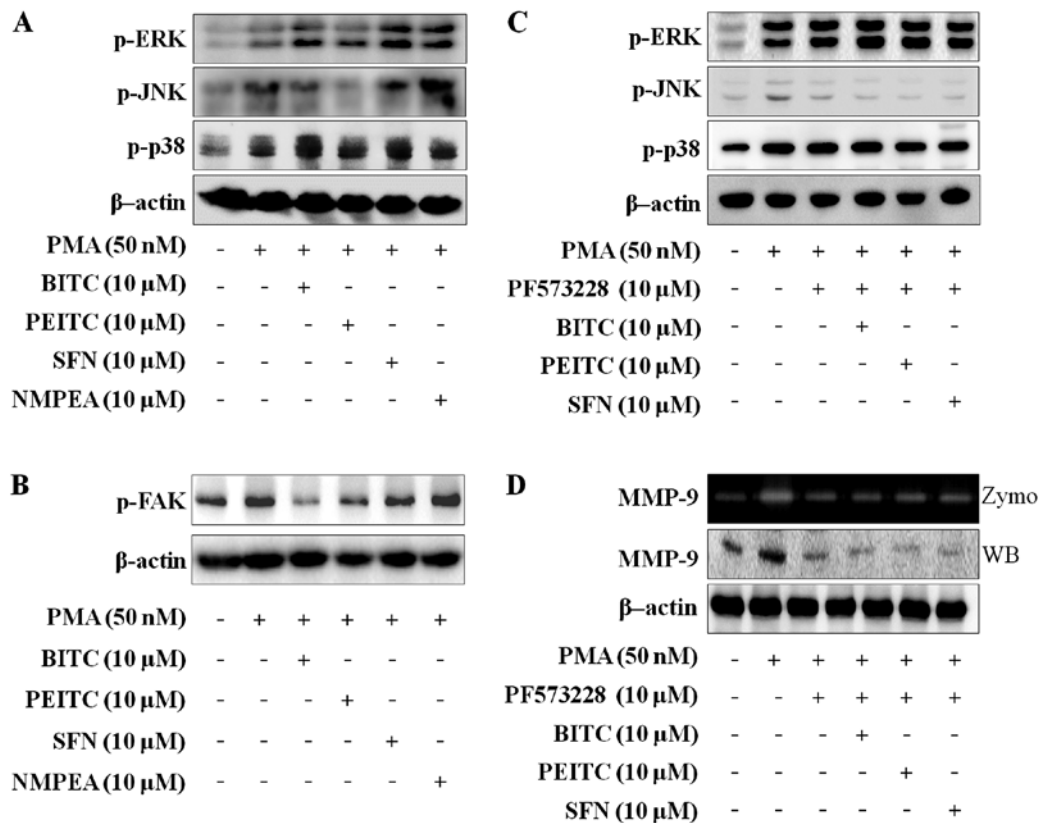
Figure 3. Isothiocyanates inhibit the PMA-induced phosphorylation of FAK and JNK in C6 glioma cells. (A) C6 glioma cells were pretreated with 10 μM ITCs and then induced by 50 nM PMA treatment for 15 min. Expression levels of phosphorylated ERK, JNK and p38 were analyzed by western blot analysis. (B) C6 glioma cells were pretreated with 10 μM ITCs for 24 h, followed by incubation with 50 nM PMA for 15 min. Phosphorylation of FAK was analyzed by western blot analysis. (C) C6 glioma cells were co-treated with 10 μM inhibitor of FAK (PF573228) and 10 μM ITCs for 24 h, followed by incubation with 50 nM PMA for 15 min. Phosphorylation of ERK, JNK and p38 was analyzed by western blot analysis. (D) C6 glioma cells were co-treated with 10 μM FAK inhibitor (PF573228) and 10 μM ITCs and then induced by 50 nM PMA treatment for 24 h. MMP-9 expression was determined by western blot analysis (lower band) and zymography (upper band). β-actin expression was used as a loading control.

were not changed. As the FAK/JNK and FAK/ERK signaling pathways control MMP secretion in carcinoma cells (30,31), it was determined whether the ITCs suppress PMA-induced phosphorylation of FAK in C6 glioma cells. ITCs with a concentration of 10 μM inhibited the PMA-induced phosphorylation of FAK (Fig. 3B), and NMPEA had no effect. To confirm that FAK modulates the PMA-induced MAPK pathways, C6 glioma cells were treated with 10 μM PF573228 (FAK inhibitor). The PMA-induced JNK phosphorylation was decreased by PF573228. However, PMA-induced phosphorylation of ERK and p38 were not changed (Fig. 3C). In addition, co-treatment with ITCs and PF573228 only blocked PMA-induced JNK phosphorylation in C6 glioma cells. We further analyzed the effect of PF573228 on MMP-9 expression and activity in PMA-induced C6 glioma cells. The MMP-9 expression and activity were blocked by PF573228 and co-treatment with ITCs and PF573228 (Fig. 3D). We suggest that ITCs suppress MMP-9 activity and expression via blocking FAK-dependent JNK phosphorylation.

ITCs decrease nuclear translocation of NF-κB and AP-1. NF-κB and AP-1 are both important transcription factors associated with the modulation of cell migration and invasion (12). As shown in Fig. 4A, PMA induced the nuclear translocation of the NF-κB subunit p65 and the AP-1 subunit c-fos. ITCs blocked the nuclear translocation of p65 and c-fos. It was

further confirmed that FAK regulates the nuclear translocation of p65 and c-fos using PF573228. The nuclear translocation of p65 and c-fos was blocked by PF573228, and co-treatment with ITCs and PF573228 (Fig. 4B). These data suggest that ITCs regulate the transcriptional activation of MMP-9 by reducing the PMA-induced nuclear translocation of the NF-κB subunit p65 and the AP-1 subunit c-fos.

ITCs inhibit the migration and invasion of C6 glioma cells. A wound-healing experiment was performed to evaluate the effect of ITCs on C6 glioma cell migration. C6 glioma cells were grown and then wounded by scraping. As illustrated in Fig. 5A and B, PMA induced the migration of the C6 glioma cells. BITC, PEITC and SFN at the concentration of 10 μM decreased C6 glioma cell migration by 88, 76 and 68% compared with PMA, respectively. To further determine the inhibitory effect of ITCs on invasion, C6 glioma cells were treated with 10 μM of ITCs, a Matrigel-based Transwell invasion assay was performed. BITC, PEITC and SFN reduced the C6 glioma cell invasion by 83, 70 and 61%, respectively (Fig. 5C and D). However, NMPEA did not affect PMA-induced cell migration and invasion, suggesting that ITCs suppressed the migration and invasion of C6 glioma cells by inhibiting MMP-9 expression and the sulfur-containing functional group plays an important role in the anti-metastatic effects of ITCs.
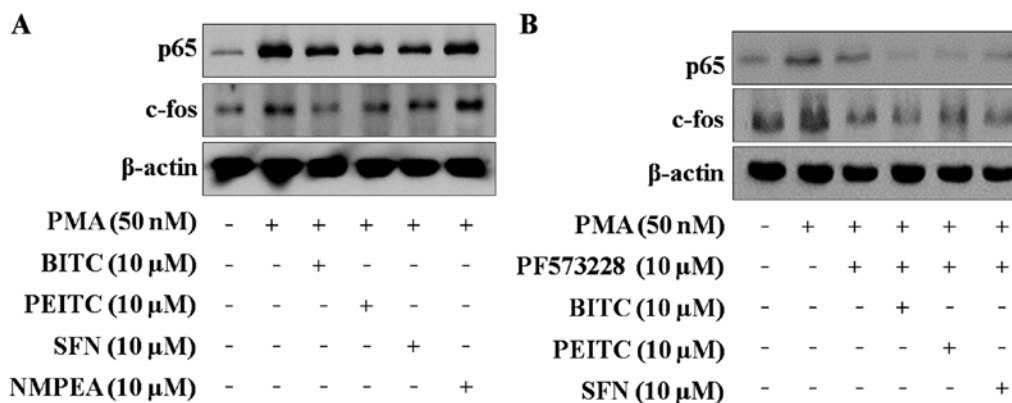
Figure 4. Isothiocyanates reduce the transcriptional activity of p65 and c-fos. (A) C6 glioma cells were pretreated with 10 μM ITCs for 24 h, followed by incubation with 50 nM PMA for 30 min. The nuclear extracts were examined for p65 and c-fos protein translocation by western blot analysis. (B) C6 glioma cells were co-treated with 10 μM inhibitor of FAK (PF573228) and 10 μM ITCs for 24 h, followed by incubation with 50 nM PMA for 30 min. The nuclear extracts were examined for p65 and c-fos protein translocation by western blot analysis.
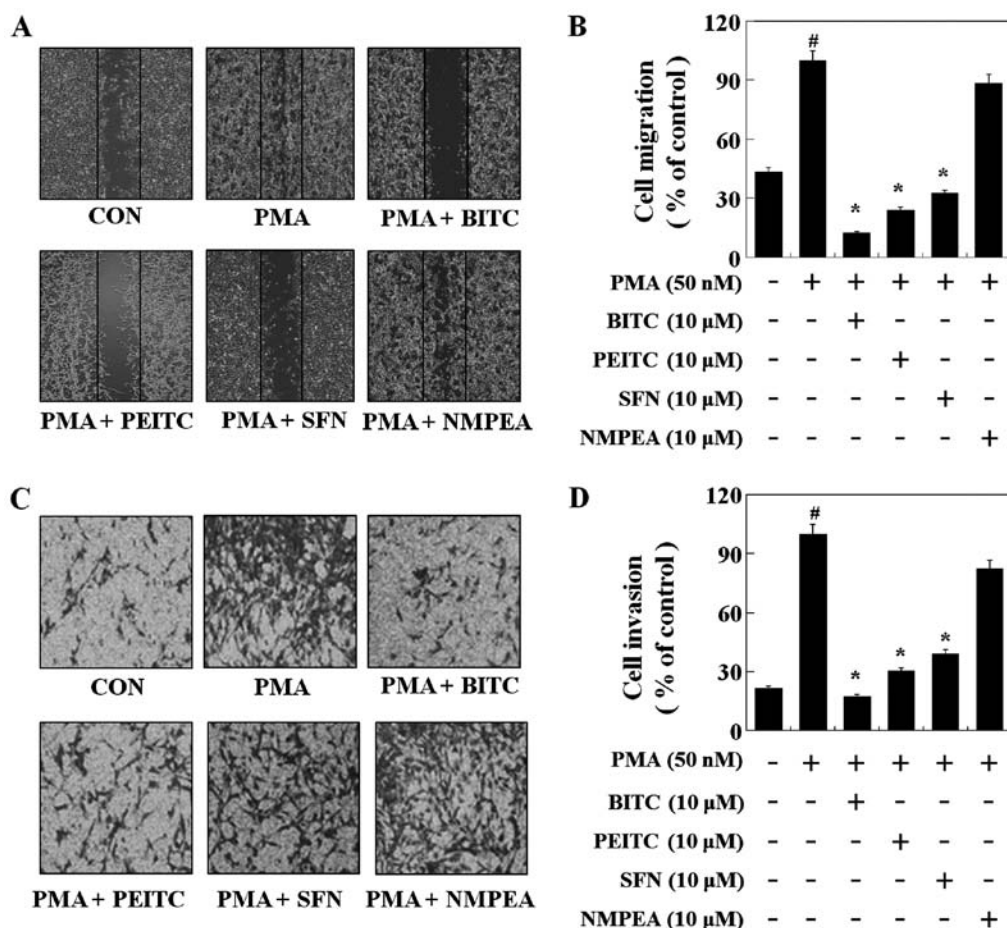

Figure 5. Isothiocyanates decrease the migration and invasion ability of C6 glioma cells. (A) Wound-healing assays were carried out using 10 μM ITCs in the presence of PMA in C6 glioma cells. After incubating for 24 h, migrated cells were photographed under a phase contrast microscope. (B) The migration activity of C6 glioma cells was determined as the number of treated cells divided by the control cell number. (C) Isothiocyanates suppress the invasion ability of C6 glioma cells. A Matrigel invasion assay was carried out with 10 μM BITC, PEITC and SFN in the presence of PMA (50 nM). After 24 h of incubation, the cells on the bottom side of the filter were fixed, stained and counted. (D) The invasion activity of C6 glioma cells was determined as the number of treated cells divided by the control cell number. Each value represents the mean ± SD of three independent experiments ($^\#$P<0.01 vs. controls; *P<0.05 vs. PMA-stimulated cells).

Discussion

ITCs, including BITC, PEITC and SFN, are natural phytochemicals. They have been reported to inhibit cancer

development, cardiovascular diseases, neurodegenerative diseases, and other chronic-degenerative pathologies (16,17). In detail, BITC was found to inhibit breast cancer stem cells (32). PEITC suppressed EGF-stimulated SAS human oral

squamous carcinoma cell invasion by targeting EGF receptor signaling (33). SFN was found to inhibit MMP-9-activated human brain microvascular endothelial cell migration and tubulogenesis (34). In addition, ITCs were found to induce the growth inhibition and apoptosis of human brain malignant glioma cells (35,36). Furthermore, our study confirmed that ITCs inhibit the migration and invasion of C6 glioma cells.

Gliomas are the most common brain tumors, which originate in glial cells. They are difficult to cure since extreme invasion recurs after surgical resection (1). Thus, the inhibitory effect on the invasiveness of these cancer cells is an important therapeutic target. Tumor metastasis is a multi-step process, that includes changes in cell-ECM interaction, separation of intercellular adhesion complexes, detachment of single cells from the solid tumor mass, degradation of the ECM, and tumor cell migration into the ECM. MMPs play a role in degrading all the components of the ECM (37) and are reported to be major proteinases involved in tumor growth, and associated with invasion and migration (38). MMP-9 and MMP-2 are involved in the invasion and migration of various types of cancers, including gastric cancer (39), hepatocellular carcinoma (40), and glioblastoma multiform (41). In our study, ITCs reduced the PMA-induced MMP-9 activity and expression but did not alter the MMP-2 expression in PMA-induced C6 glioma cells. ITCs also suppressed PMA-stimulated MMP-9 mRNA expression and did not affect the TIMP-1 mRNA expression. These results suggest that ITCs modulate PMA-induced MMP-9 activity by inhibiting MMP-9 transcription levels without altering TIMP-1. In addition, we found that the ITCs did not affect PMA-induced ROS generation, which is a potential inducer of cancer invasion and promotes apoptosis in cancer cells (28). It has been reported that PEITC induces ROS-mediated cancer cell death by inhibiting oxidative phosphorylation (42). SFN also induced the growth inhibition and apoptosis of neuroblastoma cells through an ROS-dependent pathway (43). Based on our results, however, non-cytotoxic concentrations of ITCs (10 μM) did not increase ROS generation. These results suggest that the inhibitory effects of ITCs on PMA-induced MMP-9 were not relevant to the apoptotic effects of ITCs.

The promoter of MMP-9 has *cis*-acting regulatory elements for transcription factors that contain the NF-κB site and the AP-1 site. The NF-κB transcription factor family consists of five proteins: c-Rel, p105/p50 (NF-κB1), p100/52 (NF-κB2), p65 (RelA) and RelB. AP-1 is a transcriptional activator composed of members of the Jun and Fos families (44). In this study, ITCs suppressed the PMA-induced translocation of p65 and c-fos, suggesting that ITCs decreased MMP-9 by inhibiting NF-κB and AP-1. Moreover, ITCs suppressed PMA-induced C6 glioma cell migration and invasion. These results showed that C6 glioma cell migration and invasion are inhibited through MMP-9 suppression.

A previous study showed that the MAPK pathway modulates MMP-9 expression by regulating transcription factors (45). MAPK is composed of ERK, JNK, and p38 and is found in various tumors, including the breast, and may play a crucial role in tumor metastasis and progression (46,47). In our study, ITCs suppressed the PMA-induced phosphorylation of JNK in the C6 glioma cells. In addition, FAK, a non-receptor kinase, was found to be overexpressed in several tumors and

regulates MMP-9 protein expression by regulating the MAPK pathway (48). It plays a major role in cell survival, proliferation, attachment, migration and invasion (49,50). ITCs decreased PMA-induced FAK phosphorylation in the C6 glioma cells. The FAK inhibitor (PF573228) decreased the JNK phosphorylation and MMP-9 protein expression in the C6 glioma cells. It also suppressed the transcription factors c-fos and p65. These results suggest that ITCs suppress MMP-9 expression by inhibiting the FAK/JNK pathway.

In conclusion, the present study showed that ITCs have partial antitumor effects by inhibiting migration and invasion through regulation of MMP-9 activation. We also found that the sulfur-containing functional group is important to the anti-metastatic effects of ITCs. Furthermore, it was demonstrated that the inhibitory effects of ITCs on PMA-induced MMP-9 protein expression are associated with the regulation of NF-κB and AP-1 by suppressing the FAK/JNK signaling pathway. These results suggest that ITCs are potential agents for the prevention of C6 glioma cell migration and invasion.

Acknowledgements

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