Abstract. Glioblastoma is the most aggressive primary brain malignancy, and the efficacy of multimodality treatments remains unsatisfactory. Phenethyl isothiocyanate (PEITC), one member of the isothiocyanate family, was found to inhibit the migration and invasion of many types of human cancer cells. In our previous study, PEITC induced the apoptosis of human brain glioblastoma GBM 8401 cells through the extrinsic and intrinsic signaling pathways. In the present study, we first investigated the effects of PEITC on the migration and invasion of GBM 8401 cells. PEITC decreased the migration of GBM 8401 cells in a dose-dependent manner as determined from scratch wound healing and Transwell migration assays. The percentage of inhibition ranged from 46.89 to 15.75%, and from 27.80 to 7.31% after a 48-h treatment of PEITC as determined from the Transwell migration assay and invasion assay, respectively. The western blot analysis indicated that PEITC decreased the levels of proteins associated with migration and invasion, Ras, uPA, RhoA, GRB2, p-p38, p-JNK, p-ERK, p65, SOS1, MMP-2, MMP-9 and MMP-13, in a dose-dependent manner. Real-time PCR analyses revealed that PEITC reduced the mRNA levels of MMP-2, MMP-7, MMP-9 and RhoA in a dose- and time-dependent manner. PEITC exhibited potent anticancer activities through the inhibition of migration and invasion in the GBM 8401 cells. Our findings elucidate the possible molecular mechanisms and signaling pathways of the anti-metastatic effects of PEITC on human brain glioblastoma cells, and PEITC may be considered as a therapeutic agent.

Introduction

Glioblastoma is the most aggressive primary brain malignancy with a median survival rate of 14.6 months from diagnosis in unselected patients, even following maximal, feasible surgical resection, radiotherapy and standard adjuvant temozolomide (TMZ) therapy (1). Only 0.4-0.5% of all GBM patients with extracranial metastasis has been reported, which may be attributable to the extremely shortened survival of these patients (2). Combining radiotherapy and TMZ provides better survival outcomes of glioblastoma patients than radiotherapy alone (3). Survival and recurrence are significantly associated with the extent of resection and residual volume (4). Gross total resection associated with survival improvement is not always possible as the preservation of neurological functions is necessary. The efficacy of current multimodality treatments including surgery, radiotherapy, chemotherapy for this tumor remains unsatisfactory.

Phenethyl isothiocyanate (PEITC) is one of the most extensively studied isothiocyanates (5). PEITC can induce cell cycle arrest and apoptotic cell death in various tumor types (6-12). In our previous study, PEITC induced apoptosis through the extrinsic (death receptor) and intrinsic (mitochondrial) pathways, dysfunction of mitochondria and ROS-induced ER stress in GBM 8401 cells (13). PEITC displayed anti-metastatic
effects in vivo in a novel breast tumor metastasis model (14), and inhibited tumor migration and invasion via suppression of multiple signal transduction pathways in human colon cancer HT29 cells (15). Yet, there is no available literature concerning how PEITC affects the migration and invasion of human brain glioblastoma cells.

In the present study, we investigated the effects of PEITC on human brain glioblastoma cells in regards to migration and invasion through the signaling transduction pathways in GBM 8401 cells.

Materials and methods

Chemicals and reagents. PEITC, dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase, Tris-HCl, Triton X-100 and trypsin blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were purchased from Gibco-BRL/Invitrogen (Carlsbad, CA, USA). Matrigel invasion chambers were obtained from BD Biosciences (San Jose, CA, USA).

Cell culture. The GBM 8401 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75-cm² tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine and grown at 37°C under a humidified 5% CO₂ and 95% air at one atmosphere. The cells were subcultured with a solution of 0.25% trypsin and 0.02% EDTA. The medium was changed every 2 days (16).

Cell morphological changes and viability. GBM 8401 cells (1.6x10⁵ cells/well) on a 12-well plate were treated with 0, 0.5, 1, 2 and 4 µM PEITC, or 0 and 500 µM TMZ, and incubated for 0, 24 and 48 h. Cells in each well were examined, and representative images were captured at x200 magnification using a Nikon TE2000-U inverted microscope for morphological change examinations. After cells from each well were trypsinized and collected by centrifugation at 1500 rpm for 5 min, and washed twice with PBS, 5 µg/ml PI in PBS was added to determine the percentage of viable cells. Non-viable cells were stained by PI dye exclusion (indicative of an intact membrane) and displayed brighter fluorescence than the unstained (viable) cells. Cells were counted by flow cytometric analysis with FACS Calibur utilizing CellQuest software (Becton-Dickinson, San Jose, CA, USA) (17).

Scratch wound healing assay. GBM 8401 cells (1x10⁵ cells/well) were placed for 24 h in 6-well plates, and a wound at confluence was made with a pipette tip followed by washing with serum-free medium to remove cell debris. The cells were photographed under phase contrast microscopy (time=0) and then incubated in media with PEITC (0, 2 and 4 µM), or with TMZ (500 µM) at 37°C in 5% CO₂ and allowed to migrate into the wound area for up to 48 h. Cells were gently washed with phosphate-buffered saline (PBS). Images of the scratch wounds were quantified by ImageJ software. The migration inhibition rate = (original scratch width - new scratch width)/original scratch width x 100% (18).

Migration assay. GBM 8401 cells were cultured in serum-free RPMI-1640 medium containing 1% charcoal-stripped FBS for 48 h. The lower chamber of the Transwell filter was coated with 10 µg type IV collagen, and the lower chamber of each well was filled with RPMI-1640 supplemented with 1% charcoal-stripped FBS. The filter in the 6.5-mm Transwell was inserted in the 24-well plates, and the GBM 8401 cells (~3.2x10⁴ cells/filter) were placed on the filter. The cells were treated with 0, 2 and 4 µM PEITC and 500 µM TMZ for 48 h. Migrated cells were stained with 2% crystal violet and were then examined and photographed under a microscope (16,19).

Invasion assay. The same protocol was carried out as described in the migration assay except that cells were placed on a Matrigel-coated Transwell filter (Matrigel invasion chamber; BD Biosciences) and were then examined and photographed under a microscope (16,19).

Gelatin zymography assay. GBM 8401 cells (1.6x10⁵ cells/well) were plated on 12-well tissue culture plates and incubated with 0, 2 and 4 µM PEITC or 500 µM TMZ for 24 and 48 h. The conditioned medium was collected and separated by electrophoresis on 10% SDS-PAGE with 0.2% gelatin (Sigma-Aldrich Corp.). The gels were soaked in 2.5% Triton X-100 in dH₂O twice for a total of 60 min at 25°C at the end of the electrophoresis, and they were incubated in substrate buffer (50 mM Tris HCl, 5 mM CaCl₂, 0.02% NaN₃, and 1% Triton X-100, pH 8.0) at 37°C for 18 h. Bands related to the enzyme activity of MMP-2 were visualized by negative staining using 0.2% Coomassie blue in 50% methanol and 10% acetic acid (20). The bands were evaluated by Image J software.

Western blot assay. GBM 8401 cells (2.4x10⁶ cells/dish) were placed in a 10-cm dish, and 0, 2 and 4 µM PEITC or 500 µM TMZ were added to the cells. The cells were incubated for 48 h. The cells were collected and lysed in lysate buffer composed of 50 µM Tris (pH 8.0), 150 µM NaCl, 5 µM ethylenediaminetetraacetic acid and 0.5% NP-40 with protease inhibitor solution (Roche, Mannheim, Germany). The protein concentration from each treatment was determined using the Bio-Rad protein assay kit. Approximately 30 µg of protein from each sample was separated on a 10% sodium dodecyl sulfate-polyacrylamide electrophoretic gel (SDS-PAGE) and transferred to nitrocellulose membranes (GE Healthcare, Piscataway NJ, USA). The blot was soaked with blocking buffer, 5% non-fat dry milk in Tris-buffered saline containing Tween-20 (TBS-T) for 1 h at 25°C. They were incubated with the specific primary antibodies for matrix metalloproteinase (MMP)-2, MMP-9, Ras, urokinase-type plasminogen activator (uPA), Ras homolog gene family, member A (RhoA), growth factor receptor-bound protein 2 (GRB2), p38, phospho-Jun NH2-terminal kinase (p-JNK), p-extracellular-signal-regulated kinases (p-ERK), p65, Son of sevenless homolog 1 (SOS1), rho-associated coiled-coil-containing protein kinase 1 (Rock1) and MMP-13 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer at 4°C overnight. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies and detected by chemiluminescence (GE Healthcare) and autoradiography using BioMax LightFilm (Eastman Kodak, New Haven, CT, USA).
The relative protein amounts from each treatment were assessed by densitometry scanning of the X-ray film, and analyzed by Eagle Eye Image system (Stratagene, La Jolla, CA, USA).

**Real-time polymerase chain reaction (RT-PCR).** GBM 8401 cells (2.4x10^6 cells/dish) on 10-cm dish were treated with 0, 2 and 4 µM PEITC, or 500 µM TMZ, and incubated for 24 and 48 h. The cells from each sample were collected, and the total RNA was extracted using the Qiagen RNeasy Mini kit as previously described (16,22). According to the standard protocol of the supplier (Applied Biosystems), all RNA samples were reverse-transcribed for 30 min at 42˚C with High Capacity cDNA reverse transcription kit. Quantitative PCR conditions were: 2 min at 50˚C, 10 min at 95˚C, and 40 cycles of 15 sec at 95˚C, 1 min at 60˚C using 1 µl of the cDNA reverse-transcribed as described above, 2X SYBR-Green PCR Master Mix (Applied Biosystems) and 200 nM of the forward and reverse primers as shown in Table I. Each assay was processed using the Applied Biosystems 7300 Real-Time PCR system in triplicate, and fold-changes in expression were measured using the comparative CT method. The ratios of gene expression to that of GAPdh are presented.

**Statistical analysis.** Results are expressed as mean ± SD of 3 experiments. Differences between the PEITC-treated (experimental group) or the TMZ-treated (positive control group), and the vehicle control group were evaluated using the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant difference. P-values are indicated in the figure legends.

**Results**

**Effect of PEITC on cell morphological changes and the viability of GBM 8401 cells.** GBM 8401 cells were treated with 0, 0.5, 1, 2 and 4 µM PEITC or 500 µM TMZ for 24 and 48 h to determine the cytotoxic effects of PEITC. No marked morphological change in the GBM 8401 cells was induced by PEITC (Fig. 1A). Total percentages of viable cells were measured by flow cytometric assay. PEITC or TMZ did not decrease the percentage of viable GBM 8401 cells in a dose- and time-dependent manner (Fig. 1B). The total number of viable cells was not significantly decreased in the GBM 8401 cells following exposure to concentrations as high as 4 µM PEITC or 500 µM TMZ after a 24- and 48-h treatment. Consequently, concentrations of ≤4 µM PEITC or 500 µM TMZ were selected for use in subsequent experiments.

PEITC inhibits the migration of GBM 8401 cells. GBM 8401 cells were incubated with different concentrations of PEITC and 500 µM TMZ for 48 h to determine the effects of PEITC on cell migration. The scratch wound healing assay was performed, and the results are shown in Fig. 2. An apparent and gradual increase in cells in the wounded zone at different concentrations of PEITC was observed with light microscopy. The migration inhibition rates were 12.7, 42.4, 44.3 and 44.2% after cells were treated with 0, 2 and 4 µM PEITC or 500 µM TMZ for 24- and 48-h treatment. Consequently, concentrations of ≤4 µM PEITC or 500 µM TMZ were selected for use in subsequent experiments.

**Table I. Primer sequence used for real-time PCR.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>MMP-2</td>
<td>F: CCCCAGACAGTGATCTTGAC&lt;br&gt;R: GCTTGCGAGGGAAGAAGTTG</td>
</tr>
<tr>
<td>MMP-7</td>
<td>F: GGATGTAGCACTAGCTAGGATTAAC&lt;br&gt;R: AGGTGGATACATGACATGAG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>F: CGCTGGGCTTGAATGACCTCC&lt;br&gt;R: AGGTGGATACATGACATGAG</td>
</tr>
<tr>
<td>RhoA</td>
<td>F: TCAAGCCGAGGTCAACAAC&lt;br&gt;R: ACAGGCTGCATAGCAGAA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: ACACCCACTTCCACTTT&lt;br&gt;R: TAGCCAAATCTGTTGTCATA</td>
</tr>
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MMP, matrix metalloproteinase; RhoA, RAS homologue gene family member A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward primer; R, reverse primer.
Results from the Transwell migration assay indicated that PEITC significantly inhibited the migration of GBM 8401 cells at concentrations between 2 and 4 µM (Fig. 3), and the percentage of inhibition ranged from 46.89 to 15.75% when cells were incubated with PEITC for 48 h (Fig. 3B). These effects of PEITC on the migration of GBM 8401 cells as determined by the Transwell migration assay were also dose-dependent. TMZ also had an inhibitory effect on GBM 8401 cell migration at the concentration of 500 µM.

**PEITC decreases the enzyme activity of MMP-2 in GBM 8401 cells.** Gelatin zymography assay indicated that the enzyme activity of MMP-2 was reduced in a dose-dependent manner after GBM 8401 cells were treated with 2 and 4 µM PEITC for 24 and 48 h (Fig. 5). The enzyme activity of MMP-2 were also decreased after cells were treated with 500 µM TMZ for 24 and 48 h (Fig. 5).

**Discussion**

Several studies have investigated the effects of PEITC on human glioma cells (23,24). In our previous study, PEITC was found to induce the apoptosis of human brain glioblastoma...
cells (13). In the present study, the cell morphology of GBM 8401 cells was not significantly altered (Fig. 1A), and the cell viability was not significantly decreased following exposure to PEITC at a concentration as high as 4 µM, or 500 µM TMZ after a 24- and 48-h treatment (Fig. 1B). Thus, the concentrations of PEITC and TMZ for cell migration and invasion studies were determined. Based on the Transwell migration assay, PEITC significantly inhibited the migration of GBM 8401 cells at concentrations between 2 and 4 µM in a dose-dependent manner (Fig. 3), and the percentage of inhibition ranged from 46.89 to 15.75% when cells were incubated with PEITC for 48 h (Fig. 3B). Based on the invasion assay, PEITC also significantly inhibited the invasion of GBM 8401 cells at concentrations between 2 and 4 µM in a dose-dependent manner (Fig. 4), and the percentage of inhibition ranged from 46.89 to 15.75% when cells were incubated with PEITC for 48 h (Fig. 4B). Cells from the lower chamber were counted at x200 magnification. *P<0.05, significant difference between PEITC-treated or TMZ-treated groups and the control.

Figure 4. PEITC inhibits the migration of GBM 8401 cells. GBM 8401 cells (3.2x10^4 cells/filter) were treated with 0, 2 and 4 µM PEITC or 500 µM TMZ for 48 h. (A) Cells on the Transwell filter that penetrated through the Matrigel to the lower surface of the filter were stained with crystal violet, and were photographed under a light microscope at x200 magnification. (B) Cells from the lower chamber were counted at x200 magnification. *P<0.05, significant difference between PEITC-treated or TMZ-treated groups and the control.

Figure 5. Effects of PEITC on the enzyme activity of MMP-2 in GBM 8401 cells. Cells were treated with 0, 2 and 4 µM PEITC or 500 µM TMZ for 24 and 48 h. Cells were harvested for examination of MMP-2 activity following each treatment and MMP-2 activity was determined by gelatin zymography assay as described in Materials and methods.

Figure 6. Effects of PEITC on the levels of proteins associated with migration and invasion in GBM 8401 cells. Cells were treated with 0, 2 and 4 µM PEITC or 500 µM TMZ for 48 h. The proteins levels from each sample were determined by SDS-PAGE and western blotting. (A) Ras, uPA, Rho A, GRB2, (B) p-p38, p-JNK, p-ERK, p65 and (C) SOS1, Rock1, MMP-2, MMP-9 and MMP-13.
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27.80 to 7.31% after a 48-h treatment (Fig. 4B). The current standard chemotherapy, TMZ, also had inhibitory effects on the migration and invasion of GBM 8401 cells at the concentration of 500 µM.

MMPs, a family of zinc-dependent endopeptidases, play roles in brain development, synaptic plasticity and repair after injury to the pathogenesis of various brain disorders (25). MMP-mediated extracellular matrix (ECM) degradation promotes tumor invasion, progression and is involved in angiogenesis and metastasis. MMPs are able to degrade almost all known ECM components and play important roles in mediating glioblastoma tumor cell invasion (26). The levels of MMP-2, MMP-9 and membrane type 1 (MT1)-MMP expression in gliomas are higher than those in normal brain tissue. MMP-13 enzymatic activity was found to be critical to the highly invasive potential of cancer stem cells of human glioblastoma cell line U251 (27). The levels of MMP-7 expression are correlated with tumor aggressiveness and poor prognosis in solid tumors, but they are highly variable in patients with glioblastoma (27).

Cross-talk between the tumor and the surrounding stroma to regulate MMP-7 exists; the expression of MMP-7 in human U87 glioma cells is low in culture, but higher when the cells are implanted within the brain. In the present study, PEITC reduced the enzyme activity of MMP-2 in a dose- and time-dependent manner after GBM 8401 cells were treated with 2 and 4 µM PEITC for 24 and 48 h (Fig. 5). PEITC also decreased the protein levels of MMP-2, MMP-9 and MMP-13 (Fig. 6C) in a dose-dependent manner after cells were treated with 2 and 4 µM PEITC for 48 h. PEITC inhibited the mRNA levels of MMP-2 (Fig. 7A), MMP-7 (Fig. 7B), MMP-9 (Fig. 7C) in a dose- and time-dependent manner. Taken together, PEITC may inhibit the migration and invasion of GBM 8401 cells through reduction in the enzyme activity of MMP-2, the protein levels of MMP-2, MMP-9 and MMP-13, and the mRNA levels of MMP-2, MMP-7 and MMP-9.

uPA converts plasminogen to plasmin-activating MMPs, and GBM cell invasion may be enhanced by uPA-mediated
direct activation of MMP-9 (28). The enhanced invasive capacity of peritumoral cells in GBM requires simultaneous Rac and RhoA activation (29). Knockdown of GRB2, mediating receptor tyrosine kinase-induced activation of RAS and downstream signaling, can reduce invasive activity of breast cancer (30). Epidermal growth factor receptor (EGFR) vIII-mediated migration and transformation of U87MG (PTEN-mutant) glioblastoma cells was found to be downregulated by the effects of signal regulatory protein α1 (SIRPα1) on the activation loop of SHP-2/FAK/GRB2/SOS-1/MAPK (31). Knockdown of RhoA inhibited the expression of p-JNK and phospho-c-Jun (p-c-Jun), reduced MMP-2 activity and cell invasion in human glioma U251 cells under hypoxic conditions (32). The ROCK-dependent signaling pathway is involved in glioma migration, and antitumoral effects on glioma migration are executed by selective knockdown of either ROCK1 or ROCK2 (33). ROCK1 knockdown inhibits cell proliferation, while ROCK2 knockdown promotes it. In the present study, PEITC inhibited the protein levels of Ras, uPA, RhoA, GRB2 (Fig. 6A), p-p38, p-JNK, p-ERK, p65 (Fig. 6B) and SOS1 (Fig. 6C) in a dose-dependent manner after cells were treated with 2 and 4 μM PEITC for 48 h. PEITC decreased the mRNA levels of RhoA (Fig. 7D) in a dose- and time-dependent manner. Taken together, PEITC may inhibit the migration and invasion of GBM 8401 cells through the reduction in the protein levels of Ras, uPA, RhoA, GRB2, p-p38, p-JNK, p-ERK, p65, SOS1, Rock1 and the mRNA levels of RhoA.

In conclusion, our experiments indicated that PEITC has potent anticancer activities through the inhibition of the migration and invasion of GBM 8401 cells. PEITC decreased the expression levels of MMP-2, MMP-7, MMP-9, MMP-13, Ras, uPA, RhoA, GRB2, p-p38, p-JNK, p-ERK, p65 and SOS1 in GBM 8401 cells in vitro (Fig. 8). PEITC may have therapeutic potential, and our findings have elucidated the possible molecular mechanisms and signaling pathways of the anticancer properties of PEITC in regards to human brain glioblastoma cells.

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References


