Phenethyl Isothiocyanate Inhibited Tumor Migration and Invasion via Suppressing Multiple Signal Transduction Pathways in Human Colon Cancer HT29 Cells

Kuang-Chi Lai,†‡ Shu-Chun Hsu,§ Chao-Lin Kuo,‖ Shu-Wan Ip,¶ Yuan-Man Hsu,* Hui-Ying Huang,* Shin-Hwar Wu,* and Jing-Gung Chung*,‡,§

†Department of Surgery, China Medical University Beigang Hospital, Yunlin, Taiwan, ‡School of Medicine, China Medical University, Taichung, Taiwan, §Department of Chemical Engineering, Hsining Institute of Technology, Taichung, Taiwan, ‖School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan, ¶Department of Nutrition, China Medical University, Taichung, Taiwan, *Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, ^Division of Critical Care Medicine, Department of Internal Medicine, Changhua Christian Hospital, Changhua, Taiwan, and ‡Department of Biotechnology, Asia University, Wufeng, Taichung, Taiwan

Phenethyl isothiocyanate (PEITC), one of the major compounds from dietary cruciferous vegetables, has been found to have antitumor properties and therefore could generate special interest for the development of chemopreventive and/or chemotherapeutic agent for human cancers. In the primary studies, we found that PEITC induced cytotoxic effect (decreased the percentage of viable cells) in human colon cancer HT29 cells. Here, in this study, we are the first to report the antimetastatic effect of PEITC in HT29 human colon cancer cells. The results show that PEITC exhibited an inhibitory effect on the abilities of adhesion, migration, and invasion by Boyden chamber assay. Western blotting examination indicated that PEITC exerted an inhibitory effect on the SOS-1, PKC, ERK1/2 and Rho A for causing the inhibition of cell proliferation in HT29 cells. Real-time PCR also showed that PEITC inhibited the gene expressions of MMP-2, -7, -9, FAK and Rho A after PEITC treatment for 48 h in HT29 cells. PEITC also inhibited the activities of AKT, ERK, JNK and PKC. Our results provide a new insight into the mechanisms and functions of PEITC which inhibit migration and invasion of HT29 human colon cancer cells. These results suggest that molecular targeting of NF-κB led to the inhibition of MMP-2, -7, -9 and it might be a useful strategy for the inhibition of migration and invasion on human colon cancer.

KEYWORDS: PEITC; migration; invasion; MMP-2; MMP-9; human colon cancer HT29 cells

INTRODUCTION

Colon cancer is the second leading cause of human cancer death in the US (1). In Taiwan, about 18.5 persons per 100 thousand die annually from colon cancer, based on reports from the People’s Health Bureau of Taiwan in year 2008. Currently, the treatment of colon cancer includes surgery, radiation, chemotherapy, or combination of radiotherapy with chemotherapy. However, the mortality in colon cancer patients remains high. Epidemiologic studies have demonstrated that dietary intake of cruciferous vegetables may decrease the risk of various types of malignancies (2) including colon cancer (3). The anticarcinogenic effect of cruciferous vegetables is attributed to organic isothiocyanates (ITCs) in edible cruciferous vegetables including broccoli (2). Phenethyl ITC (PEITC) is one of the ITC family of compounds which exhibits cancer chemopreventive activity (4). ITCs inhibit cancer formation including lung, esophagus, mammary gland, liver, small intestine and bladder (5).

It was reported that PEITC induces apoptosis in HT-29 cells in a time and dose-dependent manner via the mitochondria caspase cascade, and the activation of JNK (6). PEITC was shown to inhibit cytochrome P450 (CYP) enzymes and to induce phase II detoxification enzymes (7). Furthermore, PEITC was shown to inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary neoplasia in rats and mice (8, 9) and azoxymethane-induced colon aberrant crypt festi formation in rats (10). However, there is no available information to address the effects of PEITC on invasion and migration of cancer cells.

It is well-documented that invasion and migration are fundamental properties of malignant cancer cells. The formation of metastatic nodules of colon cancer involves multiprocess cascades such as cell adhesion, migration, and proteolysis of the extracellular matrix, and plays an important role in the metastatic process of cancer cells.
In Vitro Wound Healing Assay. and photographed under a light microscope at atmosphere with 95% air and 5% CO2 at 37. The lower chamber. Cells were incubated for 24 or 48 h in a humidified RPMI-1640 medium for 24 h before being trypsinized and resuspended in colon cancer cells. Therefore, in the present study, we focused on the effect of PEITC on the migration and invasion of HT29 human colon cancer cells in vitro. MATERIALS AND METHODS

Chemicals and Reagents. Phenethyl isothiocyanates (PEITC), dimethyl sulfoxide (DMSO), propidium iodide, potassium phosphates, Triton X-100 and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 medium, t-glutamine, fetal bovine serum, penicillin-streptomycin, and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA). Primary antibodies used for Western analysis were obtained as follows: antibodies for P3K, PKC, Ras, GRB2, SOS1, P-ERK, ERK1/2, MMP-2, MMP-9, Rho A, FAK, iNOS, COX-2 and NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and diluted in cell culture medium before use.

HT29 Cell Line. The HT29 cells were colon cancer cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in 88% RPMI-1640 medium with 1.5 mM t-glutamine supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY), and 2% penicillin-streptomycin (100 units/mL penicillin and 100 μg/mL streptomycin) and were cultured in a humidified atmosphere of 5% CO2 and 95% air at 37 °C.

Effects of PEITC on the Percentage of Viable HT29 Cells. The HT29 cells (2 × 10^5 cells/well) were placed in 12-well plates and incubated at 37 °C for 24 h before each well was treated with 0, 0.01, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 μM PEITC for 24 h. 0.5% DMSO (solvent) was used through the whole study. Cells were harvested by centrifugation before being used for determining cell viability, and the flow cytometric protocol was used, as previously described (17, 18).

Wound Healing Assay. HT-29 cells were grown on 6-well plates dish plates to 100% confluent monolayer and then scratched to form a 100 μm “wound” using sterile pipet tips. The cells were then cultured in the presence or absence of PEITC (0.01, 0.25 μM) in serum-free media for 24 h. The images were recorded at 24 and 48 h after scratch using an Olympus photomicroscope (19).

In Vitro Migration Assay. The migration of HT29 cells was also measured by chemotactic directional migration which was determined using a 24-well Transwell insert briefly. 8 μm pore filters (Millipore, MA) were coated with 30 μg of type I collagen (Millipore, MA) for 1 h. The HT29 cells (10^5 cells/0.4 mL of RPMI-1640) were plated in the upper chamber with or without PEITC (0.01 or 0.25 μM) and allowed to undergo migration for 24/48 h. In the upper chamber, the nonmigrated cells were removed with a cotton swab and the filters were stained with 2% crystal violet. Migrated cells adherent to the underside of the filter were counted and photographed under a light microscope at ×200 (20, 21). Each treatment was assayed twice, and three independent experiments were performed.

In Vitro Invasion Assay. The invasion of HT29 cells was measured using Matrigel-coated Transwell cell culture chambers (8 μm pore size) as described previously (21, 22). Cells were maintained in serum-free RPMI-1640 medium for 24 h before being trypsinized and resuspended in serum-free medium and placed in the upper chamber of the Transwell insert (5 × 10^5 cells/well) and then treated with 0.5% DMSO or PEITC (0.01, 0.25 μM). RPMI-1640 medium containing 10% FBS was placed in the lower chamber. Cells were incubated for 24 or 48 h in a humidified atmosphere with 95% air and 5% CO2 at 37 °C.

Table 1. The DNA Sequence Was Evaluated Using the Primer Expression

<table>
<thead>
<tr>
<th>primer name</th>
<th>primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>homo MMP-2-F</td>
<td>CCCCCAGACAGGTGATCTTGAC</td>
</tr>
<tr>
<td>homo MMP-2-R</td>
<td>GCCGTTGCGAGGAGAAAGTG</td>
</tr>
<tr>
<td>homo MMP-7-F</td>
<td>GGAGTTGACAGTCTGAGGATACTA</td>
</tr>
<tr>
<td>homo MMP-7-R</td>
<td>AGGTGGTGACATCAGTGGATTAGG</td>
</tr>
<tr>
<td>homo FAK-F</td>
<td>TGAATGGAACCTCGCAGTCA</td>
</tr>
<tr>
<td>homo FAK-R</td>
<td>TCCGGTCGCTCTGTTC</td>
</tr>
<tr>
<td>homo RhoA-F</td>
<td>TCAAGCGCAGGAGTCACAAAC</td>
</tr>
<tr>
<td>homo RhoA-R</td>
<td>ACAGGCTGGCATAGCAAG</td>
</tr>
<tr>
<td>homo ROCK1-F</td>
<td>ATGAGTATTTCCCTACCTATCCATTTC</td>
</tr>
<tr>
<td>homo ROCK1-R</td>
<td>TACACTGAGCTTGGACAGATCAG</td>
</tr>
<tr>
<td>homo GAPDH-F</td>
<td>ACCACACTCTCCTCCACCTT</td>
</tr>
<tr>
<td>homo GAPDH-R</td>
<td>TACGGAATTTGCTGTACATC</td>
</tr>
</tbody>
</table>

*RNA samples were reverse-transcribed for 30 min at 42 °C with High Capacity cDNA Reverse Transcription Kit according to the standard protocol of the supplier (Applied Biosystems). Each assay was run on an Applied Biosystems 7300 Real-Time PCR system at least twice to ensure reproducibility.

with 4% formaldehyde in PBS and stained with 2% crystal violet in 2% ethanol. The noninvasive cells in the upper chamber were removed by wiping with a cotton swab. The cells in the lower surface of the filter which penetrated through the Matrigel were counted under a light microscope at ×200.

Western Blotting Analysis. HT29 cells were cultured in 6-well tissue culture plates and grown for 24 h. PEITC was added to cells at a final concentration of 2.5 μM, while DMSO (solvent) alone was added to control cells. Cells were incubated with PEITC in 90% RPMI-1640 medium with 1% FBS at 37 °C for 0, 6, 12, 24, and 48 h. The cells were then harvested and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100. The cells were sonicated and centrifuged at 13000g for 10 min at 4 °C to remove cell debris. The supernatant was collected and total protein concentration of each sample was determined using a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin (BSA) as the standard. SDS gel electrophoresis and Western blotting were conducted as described previously (23, 24). Western blotting was performed to determine effects of PEITC on protein levels of P3K, PKC, Ras, GRB2, SOS1, P-ERK, ERK1/2, MMP-2, MMP-9, Rho A, FAK, iNOS, COX-2 and NF-κB.

Real-Time PCR of MMP-2, -7, and -9, FAK and RhoA. HT29 cells were cultured in 6-well plates and grown for 24 h. PEITC was added to cells in each well for a final concentration of 2.5 μM for 24 h. Cells were then harvested and total RNA was extracted using the Qiagen RNeasy Mini Kit as described previously (21, 25). RNA samples were reverse-transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit for 30 min according to the protocol of the supplier (Applied Biosystems). Quantitative PCR conditions were as follows: 2 min at 50 °C, 10 min at 95 °C; and 40 cycles of 15 s 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 forward and reverse primers as shown in Table 1. Applied Biosystems 7300 Real-Time PCR system was used for each assay in triplicate, and expression fold-changes were derived using the comparative Cq method.
RESULTS

Effects of PEITC on the Percentage of Viable WEHI-3 Cells. To verify the effect of PEITC on cell viability, HT29 cells were exposed to different concentrations of PEITC for 24 h, and cells were examined under microscope. They then were collected for propidium iodine staining for viability analysis. The results are present in Figure 1 and indicated that a significant loss of viability was detected at 2.5, 1, and 5 μM PEITC in a dose-dependent manner (Figure 1). Cell viability by PEITC was further confirmed by trypan blue dye exclusion method. Based on the decreased percentage of viable HT29 cells following PEITC treatment, we investigated the functional effects of PEITC on cell migration and invasion.

Effects of PEITC on Migration and Invasion of HT29 Cells. HT29 cells have an ability to migrate a 24-well Transwell insert. Treatment of PEITC for 24 and 48 h exhibited significant inhibition of cell migration in a dose-dependent manner. PEITC also inhibited cancer cell migration in the wound healing test at concentrations of 0.01 and 0.25 μM (Figure 2A). Results from migration assay are shown in Figures 2B and 2C, which indicate that PEITC had a significant inhibitory effect on cell migration at concentrations between 0.01 and 0.25 μM. Data in Figure 2C

Figure 1. Effect of PEITC on cell viability in human colon cancer HT29 cells. HT29 cells were incubated with various concentrations (0, 0.01, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 μM) for 24 h. Cells were directly photographed (×200) and then were harvested and stained with PI; then the percentage of viable HT29 cells was determined as described in Materials and Methods. A: Percentage of viable cells. B: The migration of cells. Data represents mean ± SD of three experiments. *p < 0.05 compared with the untreated control (dose 0).

Figure 2. Effect of PEITC on migration and invasion of HT29 cells. HT29 cells were treated with various concentrations (0, 0.01, and 0.25 μM) of PEITC for 24 and 48 h. (A) Cell motility was determined by wound healing assay after PEITC treatment for 24 and 48 h. (B) Cell migration was measured in a Boyden chamber for 12 and 24 h with polycarbonate filters (pore size, 8 μm). (D) Cell invasion was measured in a Boyden chamber for 12 and 24 h; polycarbonate filters (pore size, 8 μm) were precoated with Matrigel. Migration (C) and invasion (E) ability of HT29 cells were quantified by counting the number of cells that invaded the underside of the porous polycarbonate membrane under microscopy and represent the average of three experiments. *p < 0.05 compared with the untreated control (dose 0). Scale bar, 40 μm.
204 indicate that the inhibition was at 57–64% and 61–69% when 205 cells were incubated with PEITC for 24 and 48 h treatment, re- 206 spectively. HT29 cells have an ability to invade through Matrigel- 207 coated Transwell cell culture chambers. Treatment of PEITC for 208 24 and 48 h exhibited the significant inhibition of cell invasion in a 209 dose-dependent manner. Results from invasion assay are shown 210 in Figures 2D and 2E. Figure 2D shows that HT29 cells moved 211 from the upper chamber to the lower chamber in the absence 212 of PEITC (control group). However, the penetration of the EHS- 213 coated filter by HT29 cells was inhibited in the presence of 214 PEITC. The percentage inhibition at 0.01 was 18–58%, and at 215 0.25 μM inhibition, it ranged from 29 to 54% (Figure 2E). 216

Effects of PEITC on Levels of Proteins Associated with Migra- 217 tion and Invasion in HT29 Cells. Results from Western blotting 218 assay are shown in Figures 3A–D, which indicates that PEITC 219 reduced levels of PERK, FAK, ERK1/2 and JNK (Figure 3A), 220 GRB2, Rho A, ROCK1, SOS1, p135 and PKC (Figure 3B), iNOS, 221 NF-κB p65, COX-2 and uPA (Figure 3C), and MMP-2 and MMP-7 222 (Figure 3D), but increased protein levels of MEEK3 (Figure 3A) 223 in examined HT29 cells. These effects may lead to the inhibition 224 of migration and invasion of HT29 cells. Effects of PEITC on MMP-2, MMP-7, MMP-9, FAK and Rho A mRNA Expressions in HT29 Cells. To further investigate whether 225 or not PEITC affected migration- and invasion-associated gene 226 expression in HT29 cells, cells were treated with PEITC (2.5 μM) 227 for 0, 24, and 48 h. Total RNA was isolated from control and 228 PEITC treatment, and gene expressions were examined by real- 229 time PCR. The results are shown in Figure 4, and they indicate 230 that the expression levels of MMP-2, MMP-7 and MMP-9 were 231 inhibited during PEITC treatment for 48 h but only MMP-7 was 232 inhibited in 24 h treatment (Figure 4A). However, FAK and Rho 233 A mRNA were decreased at 48 h treatment, but it did not show in 234 24 h treatment of PEITC (Figure 4B). Effects of PEITC on AKT, ERK, JNK and PKC Activities. Results from Western blotting indicate that PEITC decreases the 237 protein levels of AKT, ERP, JNK and PKC, and we further investigated whether PEITC also affected the activities of AKT, 239 ERK, JNK and PKC; the results are shown in Figure 5A–D.
Figure 5 indicated that PEITC inhibited the activities of AKT, ERK, JNK and PKC in a dose-dependent manner. However, the initial inhibition concentrations were different such as AKT from 15.63 to 100 μM, ERK from 62.5 to 100 μM, JNK from 7.81 to 100 μM, and PKCα from 3.71 to 100 μM of PEITC.

**DISCUSSION**

Tumor invasion requires degradation of basement membranes, proteolysis of ECM, pseudopodial extension, and cell migration (28). A number of proteolytic enzymes, including MMPs and serine proteinases, are involved in these tumor host interactions, such as degradation of underlying basement membrane. Of these basement membrane degrading enzymes, MMPs, especially activated forms of MMP-2 and MMP-9, are thought to play an important role in its degradation because of their ability to cleave the type IV collagen. MMPs are produced by cancer cells or through the induction of surrounding stromal cells. Several studies indicate that inhibition of MMP expressions or enzyme activities can be used as early targets for preventing cancer metastasis (29–31). It is well-known that cell migration is a multicomplex process which provides many molecular targets for the development of therapeutic agents to inhibit cancer metastasis.

Although PEITC was reported to possess anticancer potential against several cancer cell lines (6–9), the role of PEITC against the migration and invasion of HT29 cells and associated protein levels and gene expressions is still unclear. Our results showed that...
PEITC induced cytotoxicity and inhibited the migration and invasion in HT29 cells and that these effects are dose dependent (Figure 1). Furthermore, we found that PEITC decreased the migration and invasion associated protein levels such as PI3K, ERK1/2, JNK, p38 (Figure 3A), Ras, GrB2, Rho A, ROCK1, SOS1, PI3K and PKC (Figure 3B, iNOS, NF-κB p65, COX-2 and uPA (Figure 3C) MMP-2, MMP-7 and MMP-9 (Figure 3D) cells. PEITC also inhibited the activities of AKT, ERK, JNK and PKC. Our results also showed that PEITC suppressed MMP-9 gene expression via suppressing the PKC/ MAPK and PI3K/AKT/NF-κB cascades with consequent suppression of colony formation, tumor migration and invasion by human colon cancer HT29 cells. The activities of MMP-2 and u-PA have been shown to play a critical role in degrading the basement membrane in cancer invasion and migration. We also found that PEITC tremendously reduced MMP-2 activity in a dose-dependent manner, whereas uPA activity was also inhibited by PEITC (data not shown).

It was reported that MMP-2 overexpressed in highly metastatic tumors, and that MMP-9 can be stimulated by TNF-α (32) or a growth factor such as VEGF, EGF and TGF-β (33–35), or Ras oncogene (36, 37) through activation of different intracellular signaling pathways. It was also reported that the activation of PKC led to the translocation of the protein to membranes and led to control the expression of MMP-9 through modulating the activation of transcription factors such as AP-1, NF-κB or Sp-1 through MAPK and PI3K signaling pathways (38–40).

It was reported that the activation of NF-κB is involved in the induction of the MMP-9 gene associated with the invasion and metastasis of tumor cells by different agents including TPA, growth factors such as EGF, VEGF, platelet-derived growth factor, transforming growth factor-b, and inflammatory cytokines (32, 41). Therefore, in the present study, the regulation of NF-κB, and the downstream of the PI3K/Akt and MAPK (ERK1/2, p38 and JNK) pathways, might be involved in PEITC suppressed MMP-9 expression and invasion in HT29 cells. We also found that HT29 cells were treated with PEITC which led to decrease the protein levels of PI3K, Akt, MMP-2 and MMP-9. It was reported that PI3K activation leads to activate the downstream main target Akt which plays various important roles in regulating cellular growth, differentiation, adhesion, the inflammatory reaction, and invasion (33, 42). We also found that PEITC decreased the JNK and PKC levels (Figure 5). It was reported that resveratrol suppresses MMP-9 expression in TPA-induced human Ca Ski cells by blocking JNK and PKCα signal transduction (43). So far, there is no report to show the receptor in cells for PEITC. However, there may be other possible mechanisms in which PEITC penetrates cells, probably to compete with coenzymes or ATP to inhibit the activity of PKC.

Other factors also play an important role in migration and invasion such as 52-kDa uPA which plays a major role in the decomposition of basement membranes. This enzyme is highly expressed in solid tumors. It was reported that the activation of the uPA/uPAR/plasmin proteolytic network has been shown to play key roles in tumor invasion and dissemination of various malignancies (44, 45). The presence of uPA in tumor tissues has been proposed as a potential prognostic factor, and the levels of uPA and uPAR expression serve as prognostic markers in various malignancies. However, high levels of expression are often associated with a poor prognosis (46). We then examined whether PEITC blocks the expressions of MMP-2, -7, and -9 and uPA which are closely associated with tumor invasion, and the results confirmed this hypothesis.

The present study provides proof that, through a molecular mechanism, PEITC promotes a strong anti-invasive and antimigration effect through downregulation of PKC and then blocking MAPK and PI3K/Akt signaling pathways, NF-κB, and uPA which then led to the inhibition of MMP-2 and MMP-9 (Figure 6). Therefore, we conclude that PEITC may have a potential for inhibiting the migration and invasion of human colon cancer in future.

ABBREVIATIONS USED

ERK, extracellular signal-regulated kinases; FAK, focal adhesion kinase; JNK, c-Jun NH2-terminal kinase; MMPs, matrix metalloproteinase; NF-κB, nuclear factor kappaB; PEITC, phenethyl isothiocyanate; PKC, protein kinase C; RhoA, ras homolog gene family, member A; GRB2, growth factor receptor-bound protein 2; Cox-2, cyclooxygenase-2; INOS, inducible nitric oxide synthase; PI3K, phosphoinositide 3-kinases; SOS1, son of sevenless homologue 1; AP-1, activator protein 1; MAPK, mitogen-activated protein kinase.

LITERATURE CITED

(10) Zhang, Y.; Kensler, T. W.; Cho, C. G.; Posner, G. H.; Talalay, P. Anticarcinogenic activities of sulforaphane and structurally related...


530 Received for review June 21, 2010. Revised manuscript received August 31, 2010. Accepted September 5, 2010. This work was supported by Grant CMU98-ASIA-10 from China Medical University, Taichung, Taiwan.