Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 17 (2006) 837–846
Journal of Nutritional Biochemistry

Phenylethyl isothiocyanate and its *N*-acetylcysteine conjugate suppress the metastasis of SK-Hep1 human hepatoma cells

Eun-Sun Hwang, Hyong Joo Lee*

Department of Food Science and Technology, School of Agricultural Biotechnology and Center for Agricultural Biomaterials, College of Agriculture and Life Sciences, Seoul National University, San 56-1, Shillim-dong, Gwanak-gu 151-921, Republic of Korea

Received 18 January 2006; received in revised form 15 February 2006; accepted 17 February 2006

Abstract

Phenylethyl isothiocyanate (PEITC), a hydrolysis compound of gluconasturtiin, is metabolized to *N*-acetylcysteine (NAC)-PEITC in the body after the consumption of cruciferous vegetables. We observed an inhibitory effect of PEITC and its metabolite NAC-PEITC on cancer cell proliferation, adhesion, invasion, migration and metastasis in SK-Hep1 human hepatoma cells. PEITC and NAC-PEITC suppressed SK-Hep1 cell proliferation in a dose-dependent manner, and exposure to 10 μM PEITC or NAC-PEITC reduced cell proliferation by 25% and 30%, respectively. NAC-PEITC inhibited cancer cell adhesion, invasion and migration to a similar or to an even larger degree than PEITC. The expression of matrix metalloproteinase (MMP) 2, MMP-9 and membrane type 1 matrix metalloproteinase (MT1-MMP) is a known risk factor for metastatic disease. Gelatin zymography analysis revealed a significant downregulation of MMP-2/MMP-9 protein expression in SK-Hep1 cells treated with 0.1–5 μM PEITC or NAC-PEITC. PEITC and NAC-PEITC treatment caused dose-dependent decreases in MMP-2/MMP-9 and MT1-MMP mRNA levels, as determined by reverse transcription polymerase chain reaction. PEITC and NAC-PEITC also increased the mRNA levels of tissue inhibitors of matrix metalloproteinase (TIMPs) 1 and 2. Our data suggest that this inhibition is mediated by downregulation of MMP and upregulation of TIMPs.

Keywords: Phenylethyl isothiocyanate; *N*-acetylcysteine; Adhesion; Invasion; Migration; Metastasis; Matrix metalloproteinase

1. Introduction

Glucosinolates are naturally occurring thioglucosides that are present in cruciferous vegetables such as broccoli, Brussels sprout, cabbage, cauliflower, turnip, radish and watercress [1]. Although glucosinolates do not appear to be bioactive, their enzymatic hydrolysis products, isothiocyanates (ITCs), appear to have anticarcinogenic activities in the liver, intestine, pancreas, mammary gland and lung [2–5]. Gluconasturtiin, one of the predominant glucosinolates in cruciferous vegetables, is hydrolyzed to yield phenylethyl isothiocyanate (PEITC) [6]. The absorption and metabolism of PEITC in humans involve glutathione conjugation followed by conversion, via the mercapturic acid pathway, to an *N*-acetylcysteine (NAC) conjugate that is excreted in the urine [7,8].

Naturally occurring PEITC has been reported to inhibit carcinogenesis induced by several carcinogens, such as 7,12-dimethylbenz[*a*]anthracene, benzo[*a*]pyrene, diethylnitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and methyl-benzylnitrosamine, particularly in the initiation period by influencing phase I and phase II enzyme activities [9–11]. These observations indicate that PEITC may represent a class of potential chemopreventive agents that plays an important role in modulating carcinogenesis.

Rat or human ingestion of cruciferous vegetables has been shown to lead to glutathione conjugation of PEITC and subsequent NAC-PEITC excretion in urine [8], and several researches have suggested that NAC–ITC conjugates are at least as effective as unconjugated ITCs [10–13]. The free and NAC-conjugated forms of PEITC and its NAC conjugate have previously been shown to inhibit cancer

Abbreviations: ITC, isothiocyanate; PEITC, phenylethyl isothiocyanate; NAC, *N*-acetylcysteine; MMP, matrix metalloproteinase; ECM, extracellular matrix; MT1-MMP, membrane type 1 matrix metalloproteinase; TIMP, tissue inhibitors of matrix metalloproteinase.

* Corresponding author. Tel.: +82 2 880 4853; fax: +82 2 873 5095.
E-mail address: leehyjo@snu.ac.kr (H.J. Lee).

0955-2863/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2006.02.004

cell growth and to induce phase II detoxification enzymes in culture [5,14,15]. Furthermore, NAC conjugates of PEITC and sulforaphane are reported to be effective not only in cell culture but also when administered to whole animals [10,11]. These results suggest that NAC-PEITC may also have chemopreventive activity.

Metastasis is the process through which malignant cells escape from the primary tumor and develop tumors at a secondary site via the blood or lymphatic vessels. It occurs as a complex multistep process involving cancer cell adhesion, invasion and migration [16]. These processes require the degradation of extracellular matrix (ECM) components by proteolytic enzymes [17]. Among various types of proteinases, matrix metalloproteinases (MMPs) are the principal ECM-degrading enzymes. Although no single gene has been identified as a major regulator of metastasis in all tumors, many animal studies have indicated a critical role for MMPs, including MMP-2 (gelatinase A) and MMP-9 (gelatinase B) [18]. In particular, MMP-2 and MMP-9 have the capacity to degrade type IV collagen — a major component of basement membranes. Degradation of basement membranes is considered to be essential in invasive growth and metastasis [18,19]. MMPs are also involved in cell differentiation, apoptosis, angiogenesis and cancer cell growth [20,21]. Therefore, inhibition of MMP activity is important for preventing early-stage carcinogenesis, particularly in tumor promotion.

Membrane type 1 matrix metalloproteinase (MT1-MMP) is a potent modulator of the pericellular environment through its proteolytic activity and promotes the migration, invasion and proliferation of tumor cells. MT1-MMP specially catalyzes pro-MMP-2 [22], and increased expression of MT1-MMP has previously been reported to correlate with an increasing grade of malignancy in gliomas and surgically resected cultured hepatocellular carcinoma tissues [23,24]. Most members of the MMP family are tightly regulated on several levels, with most being secreted from cells as proenzymes and subsequently undergoing proteolytic conversion to an active form. Unlike MMPs, MT-MMPs are membrane-bound, and studies have shown that they can be activated either intracellularly or on the cell surface [25,26]. In general, once activated, MMPs are inhibited by tissue inhibitors of matrix metalloproteinase (TIMPs). Studies have demonstrated that the regulation of pericellular proteolysis by MT-MMPs could provide an additional target for the inhibition of tumor cell invasion by inhibiting MMP activators.

In this study, we used gelatin zymography to examine the possible inhibitory activities of PEITC and NAC-PEITC toward MMP-2 and MMP-9 enzyme activities. We examined mRNA expression levels for MMP-2 and MMP-9 in SK-Hep1 cells. In addition, we investigated the influence of PEITC and NAC-PEITC on metastasis-related properties of SK-Hep1 cells, including cell viability, adhesion, invasion and migration.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and RNase A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin–EDTA and penicillin/streptomycin were from Gibco Life Technologies, Inc. (Paisley, UK), and fetal bovine serum (FBS) was obtained from Gemini Bio-Products (Calabasas, CA, USA). All solvents were from Fisher Scientific (Los Angeles, CA, USA) and were of analytical high-performance liquid chromatography grade. Cell culture supplies were purchased from Costar (Corning, Inc., Cypress, CA, USA) and Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). PEITC (99%) was purchased from Sigma. The NAC conjugate of PEITC was synthesized according to published methods, with some modifications [8].

2.2. Cell culture

SK-Hep1 human hepatocellular carcinoma cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). Cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin in a 37°C humidified incubator containing 5% CO_2 and 95% air.

2.3. Cell proliferation assay

SK-Hep1 cells (5×10^3/well) were cultured in 96-well plates at 37°C for 24 h. The medium was then removed and replaced with fresh FBS-free medium containing various amounts of PEITC or NAC-PEITC, and the cells were incubated for another 24–72 h. The medium was removed, and cell viability was determined using MTT assay. Briefly, MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and then filtered to sterilize the solution and to remove a small amount of insoluble residue. A mixture of 10 μl of the MTT stock solution and 90 μl of the medium was added to each well, and the plates were incubated for 4 h at 37°C. DMSO was then added to the wells and mixed by pipetting to dissolve the resulting dark blue formazan product. Optical densities for each well were read at 570 nm using a microplate reader (Molecular Devices, CA, USA).

2.4. Cell adhesion assay

Each well of a 24-well tissue culture plate was coated with 25 μg/well Matrigel and left to air-dry for 40 min. SK-Hep1 cells (5×10^4) suspended in DMEM containing 0.5% bovine serum albumin were then dispensed into each well. The plate was incubated in 5% CO_2 at 37°C for 1 h and then gently washed thrice with PBS to remove unattached cells. Attached cells were then stained with hematoxylin and eosin reagent and counted under a microscope (I×70; Olympus, Okaya, Japan). At least four independent experiments were performed.

Table 1
Sequences of PCR primers used in this study

Target gene	Primer	Sequence (5′ →3′)	Product size (bp)
MMP-2	Sense	GGC CCT GTC ACT CCT GAG AT	474
	Antisense	GGC ATC CAG GTT ATC GGG GA	
MMP-9	Sense	CGG AGC ACG GAG ACG GGT AT	573
	Antisense	TGA AGG GGA AGA CGC ACA GC	
MT1-MMP	Sense	TGG GTA GCG ATG AAG TCT TC	317
	Antisense	AGT AAA GCA GTC GCT TGG GT	
TIMP-1	Sense	GAT CCA GCG CCC AGA GAG ACA CC	677
	Antisense	TTC CAC TCC GGG CAG CAT T	
TIMP-2	Sense	GGC GTT TTG CAA TGC AGA TGT AG	500
	Antisense	CAC AGG AGC CGT CAC TTC TCT TG	
GAPDH	Sense	TGA AGG TCG GAG TCA ACG GAT TTG GT	983
	Antisense	CAT GTG GGC CAT GAG GTC CAC CAC	

2.5. Cell invasion assay

A Transwell system with a 6.5-mm-diameter polycarbonate filter membrane with 8-μm pores (Corning, Inc., Corning, NY, USA) was used to assess the rate of cell invasion. Matrigel (12.5 μg in 50 μl of PBS) was added to the filter to form a thin gel layer, and the filter was dried in a laminar hood overnight. The filter was then reconstituted with 100 μl of PBS at 37°C for 2 h.

SK-Hep1 cells at 90% confluency were harvested from culture wells using a cell dissociation solution. The cells (1×10^5) were suspended in 100 μl of serum-free medium with or without treatment compounds and then added to the upper chamber of the Transwell insert. The lower chamber was filled with 500 μl of the same medium as the upper chamber. After 24-h incubation at 37°C, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. Cells that had penetrated to the lower surface of the filter were stained with hematoxylin and counted under an Olympus I×70 microscope in 13 randomized fields at ×400 magnification. The assay was performed in at least four separate experiments.

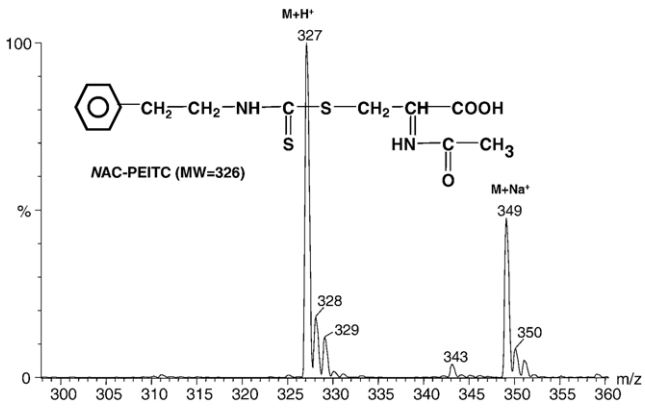

Fig. 1. Mass spectrum of synthetic NAC-PEITC.

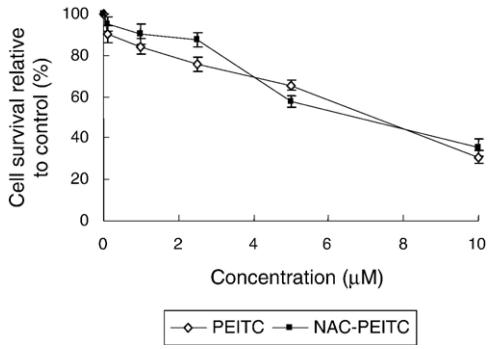

Fig. 2. Effect of PEITC and NAC-PEITC on SK-Hep1 cell proliferation, as measured by the MTT assay. Optical density was determined at 570 nm and is expressed as cell survival relative to control. Data represent the mean±S.D. from four independent experiments.

2.6. Wound migration assay

Cell motility was examined using wound migration assay. Cells were cultured in a six-well plate to 100% confluency and then pretreated with mitomycin C (25 μg/ml) for 30 min. An injury line was then made by drawing a plastic pipette tip across the center of the well to produce a clean 1-mm-wide wound area. The wells were rinsed with PBS, and the attached cells were allowed to migrate into the medium. An Olympus computer-based microscopic imaging system was used to measure wound healing at ×200 magnification. At the indicated time points, migration was assessed by quantification of the number of cells observed across the wound, as compared to the

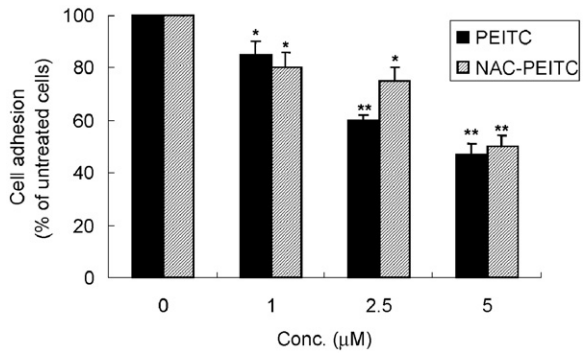

Fig. 3. Effect of PEITC and NAC-PEITC on cell adhesion with Matrigel. SK-Hep1 cells were exposed to different concentrations of PEITC or NAC-PEITC for 24 h in wells precoated with Matrigel. After 60-min incubation at 37°C, the percentage of adhering cells was counted under the microscope. Data were presented as the mean±S.D. of three separate experiments. The values marked as * and ** indicate significant differences with other treatments ($P<.05$).

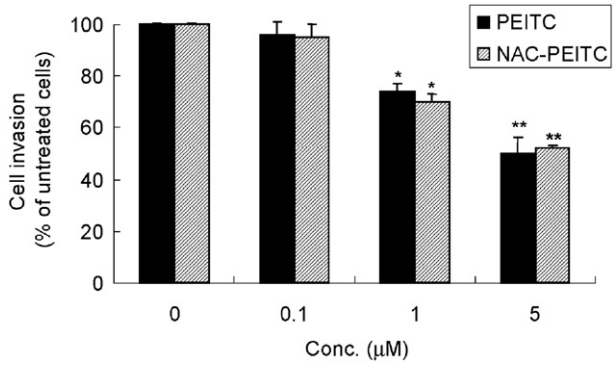

Fig. 4. Effects of PEITC and NAC-PEITC on SK-Hep1 cell invasion. The invasiveness of SK-Hep1 cells toward endothelial-cell-conditioned medium was measured using Transwell chambers with tissue-culture-treated filters with 8-μm pores. Each bar represents the mean±S.D. of three separate experiments. The values marked as * and ** indicate significant differences with other treatments ($P<.05$).

same frame at 0-h migration. At least five wound areas were measured per plate. The distances between the leading edge of the migrating cells and the edge of the wound were compared, and the migration rates of the treated cells were calculated as a percentage of the migration rates of untreated cells. At least four independent experiments were performed.

2.7. Gelatin zymography

Gelatinase activity was determined using gelatin zymography. Briefly, after SK-Hep1 cells had been incubated in serum-free DMEM for 48 h, the conditioned medium was collected and the protein level was determined. The protein-standardized conditioned medium was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on a 10% polyacrylamide gel containing 1 mg/ml gelatin. Electrophoresis was carried out at a constant voltage of 105 V.

After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 30 min to remove SDS. The gel was then incubated in a buffer containing 50 mM Tris–HCl (pH 7.6), 200 mM NaCl and 5 mM $CaCl_2$ for 18 h at 37°C to allow proteolysis to proceed. The gel was stained with 0.5% Coomassie brilliant blue for 1 h and then destained in water containing 10% glacial acetic acid and 30% methanol. Proteolysis was detected as a white zone in a dark field. The intensity of the bands was quantitated using ImageJ software (version 1.34; NIH, Bethesda, MD, USA).

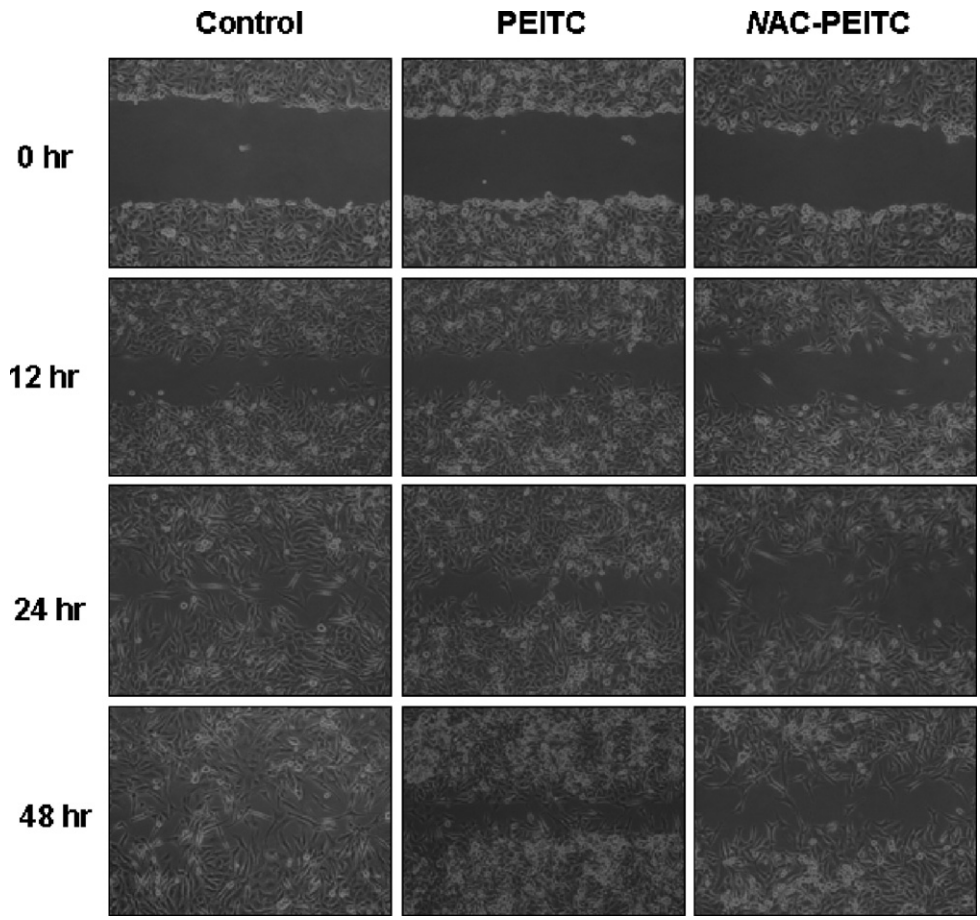

Fig. 5. Effects of PEITC and NAC-PEITC on wound healing migration of SK-Hep1 cells. A wound was introduced by scraping confluent cell layers with a pipette tip. Representative photographs of invading treated and untreated cells are presented.

2.8. Isolation of total RNA

Total RNA was isolated from 10^7 SK-Hep1 cells using TRIzol reagent (Life Technologies, Inc., Rockville, MD, USA). Homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. After the addition of 0.2 vol of chloroform, samples were shaken vigorously for 15 s, incubated for 2–3 min and centrifuged at 12,000×*g* for 15 min at 4°C. The total RNA remaining in the upper aqueous phase was precipitated by mixing with an equal volume of isopropanol. The mixtures were incubated for 10 min at 4°C and then centrifuged at 12,000×*g* for 10 min at 4°C. The total RNA pellet was washed with 70% ethanol, dried and dissolved in RNase-free water. The concentration and purity of total RNA were calculated based on optical density at 260 and 280 nm.

2.9. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

The primer sets used in this study are shown in Table 1. First-strand cDNA was synthesized with 1 μg of total RNA and 1 μM oligo(dT)$_{15}$ primer using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). A Taq PCR Master Mix kit (Qiagen) was then used to perform PCR with 0.5 μl of first-strand cDNA and 20 pmol of primers. The PCR protocol consisted of an initial denaturation step at 94°C for 3 min; 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min; and a final extension step at 72°C for 10 min.

Amplified PCR products were loaded into a 0.8% agarose gel. After ethidium bromide staining, the gel was photographed on a UV transilluminator using Polaroid film (Kodak, Needham, MA, USA). Band densities were determined using ImageJ 1.34 software.

2.10. Statistical analysis

All experiments were performed in triplicate. Mean standard deviation, mean square errors, analysis of variance, correlation and interaction of main effects were calculated using the Sigmastat 1.0 computer program 1.0 (Jandel Corp., San Rafael, CA, USA). Appropriate comparisons were made using the Student–Newman–Keuls test for multiple comparisons. $P<.05$ was considered statistically significant.

3. Results

3.1. Confirmation of synthetic NAC-PEITC

The presence of a synthetic product was confirmed by positive ion electrospray ionization tandem mass spectrometry (MS/MS; Quattro Micro, Manchester, UK). The electrospray ionization source had a capillary voltage of 4.2 kV, a source temperature of 120°C, a desolvation temperature of 240°C and a desolvation nitrogen gas flow rate of 650 l/h. Argon was used as collision gas at a pressure of 2.5 mbar. The synthesized NAC-PEITC exhibited a major peak in its mass spectrum at $m/z=327$, corresponding to a

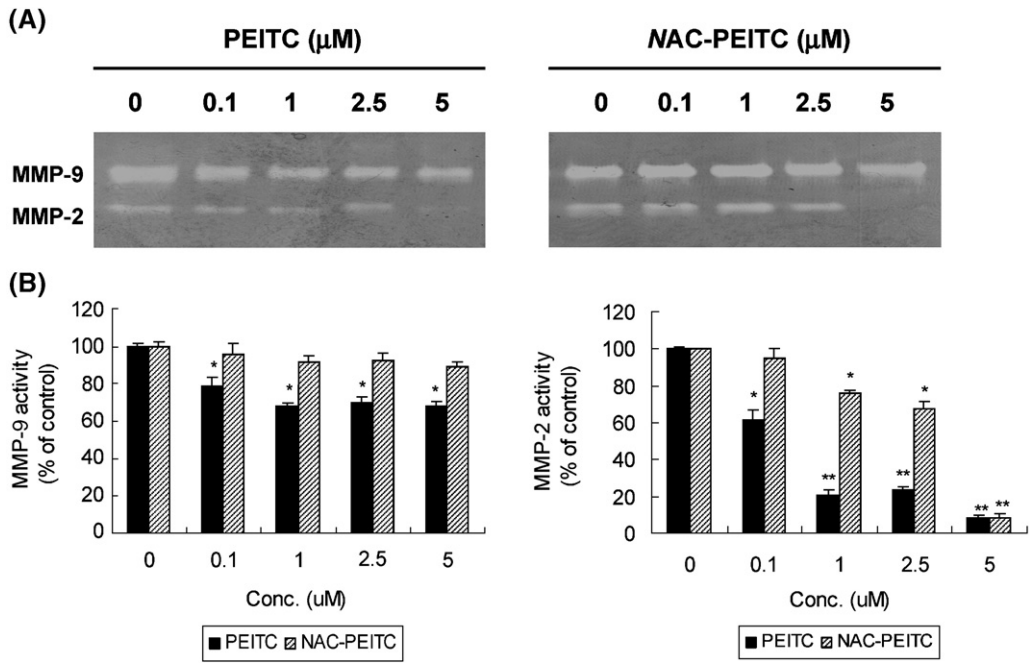

Fig. 6. Effects of PEITC and NAC-PEITC on MMP expression. MMP-2/MMP-9 protein expression was analyzed by gelatin zymography, which was performed on media conditioned by 2×10^5 SK-Hep1 cells treated with PEITC or NAC-PEITC for 48 h (A). Data shown are representative of three different experiments yielding essentially the same results. The enzyme activities of MMP-2 and MMP-9 in treated cells are expressed as a percentage of their activities in untreated cells (B). The values marked as * and ** indicate significant differences with other treatments ($P<.05$).

strong molecular ion (M^+) as the major peak at 263, which is the molecular weight (+1) of the NAC conjugate of PEITC. A distinct M^+Na^+ peak at $m/z=349$ was obtained due to sodium ion in the matrix (Fig. 1).

3.2. Effect of PEITC and NAC-PEITC on cell proliferation

The cytotoxicity of PEITC and NAC-PEITC toward SK-Hep1 human hepatoma cells was evaluated using the MTT assay. The effects of 0.1–10 μM PEITC or NAC-PEITC on cell growth after 24 h are shown in Fig. 2. After 24 h of incubation, PEITC inhibited cell proliferation in a dose-dependent fashion ($n=4$), with cell numbers significantly reduced by 9.6–69% compared to the control. NAC-PEITC also exhibited a dose-dependent inhibition of SK-Hep1 cell proliferation; after 24 h of incubation, cell numbers decreased by 42% and 65% at 5 and 10 μM NAC-PEITC, respectively.

3.3. Effect of PEITC and NAC-PEITC on adhesion

To investigate the effects of PEITC and NAC-PEITC on the adhesion of SK-Hep1 cells, adhesive cells were quantitated using an enzyme-linked immunosorbent assay reader. The incubation of SK-Hep1 cells with 1–5 μM PEITC or NAC-PEITC for 60 min significantly inhibited cell adhesion to the Matrigel-coated substrate in a concentration-dependent manner (Fig. 3). The magnitude of the decrease was in direct relationship to the concentrations of PEITC or NAC-PEITC treatment. Approximately 50% and 53% reductions were observed with 5 μM PEITC and NAC-PEITC, respectively.

3.4. Effect of PEITC and NAC-PEITC on invasion

We next examined the inhibitory effect of PEITC and NAC-PEITC on the ability of SK-Hep1 cells to invade a reconstituted ECM (Matrigel). Results were similar to those of the wound migration assay; PEITC and NAC-PEITC at 0.1–5 μM inhibited cell invasion in a dose-dependent manner. When the SK-Hep1 cells were grown on Matrigel, a significant reduction in the number of invasive cells was observed when the cells were treated with PEITC or NAC-PEITC for 18 h compared to the control (fresh medium alone). The levels of invasion were reduced to 50% and 52% of control levels at 5 μM PEITC and NAC-PEITC, respectively (Fig. 4). A significant reduction in invasion was not observed when the cells were treated with lower doses of PEITC or NAC-PEITC (0.1–2.5 μM) for only 6 h (data not shown).

3.5. Effect of PEITC and NAC-PEITC on migration

We assessed the effects of PEITC and NAC-PEITC on the migration of SK-Hep1 cells using wound migration

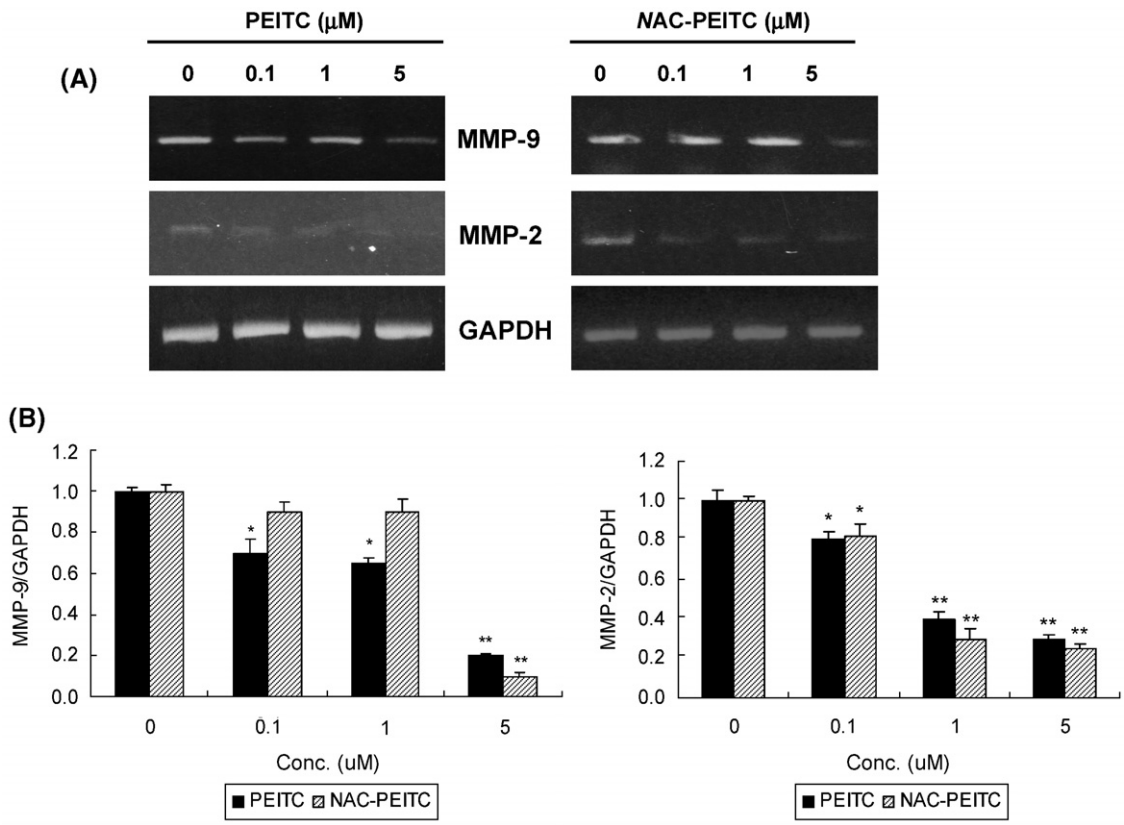

Fig. 7. Expression of MMP-2/MMP-9 mRNA in SK-Hep1 cells after treatment with various concentrations of PEITC and NAC-PEITC for 24 h, as measured by RT-PCR analysis. The experiment was repeated thrice with similar results. (A) PCR products were separated by agarose gel electrophoresis. (B) MMP-2/GAPDH and MMP-9/GAPDH PCR product ratios were determined by densitometry. The values marked as * and ** indicate significant differences with other treatments ($P<.05$).

assay. As shown in Fig. 5, cellular motility was controlled in a time-dependent manner by 5 μM PEITC at 24- and 48-h incubations. NAC-PEITC exhibited an inhibitory pattern similar to that of PEITC treatment.

3.6. Effect of PEITC and NAC-PEITC on MMP expression

To examine the possible antimetastatic mechanisms of PEITC and NAC-PEITC, we determined the effect of these compounds on the expression of MMP-2 and MMP-9. First, cultured conditioned media of SK-Hep1 cells were subjected to zymographic analysis. In the absence of treatment, SK-Hep1 cells constitutively secreted high levels of MMP-9 and relatively low levels of MMP-2. After 24 h, NAC-PEITC treatment at 0.1–5 μM did not show any inhibitory effect on MMP-9 activity, whereas PEITC treatment at 0.1–5 μM decreased MMP-9 expression by 21% and 30% with 1 and 5 μM PEITC, respectively (Fig. 6). Treatment with 1–5 μM PEITC did not show a dose–response relationship. Treatment with 5 μM PEITC or NAC-PEITC suppressed MMP-2 activity by 90% and 91%, respectively, and the inhibition was dose-dependent at 0.1–5 μM. Thus, in the SK-Hep1 cell system, MMP-2 was downregulated by PEITC and NAC-PEITC treatments. However, MMP-9 was not inhibited by NAC-PEITC even though PEITC suppressed MMP-9 activity compared to the control.

We also quantified MMP-9 mRNA expression in SK-Hep1 cells by RT-PCR with an internal standard [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] (Fig. 7). In the presence of PEITC and NAC-PEITC, MMP-9 and MMP-2 activities were reduced in a dose-dependent manner. MMP-9 expression was not potent at low concentrations (<1 μM) of PEITC and NAC-PEITC, but was highly inhibited at both 5 μM PEITC and NAC-PEITC by 80% and 90%, respectively, compared to the control. MMP-2 mRNA expression was more readily inhibited over all the doses used in this study, with 70% and 75% inhibition after 24 h of exposure to 5 μM PEITC and NAC-PEITC, respectively. For both MMP-2 and MMP-9, NAC-PEITC appeared to be a similarly potent inhibitor of mRNA expression due to PEITC.

3.7. Effects of PEITC and NAC-PEITC on TIMP and MT1-MMP expression

To further explore the modulation of the activation of pro-MMPs mediated by PEITC or NAC-PEITC, MT1-MMP and TIMP-1/TIMP-2 gene expression levels were determined. As shown in Fig. 8, both PEITC and NAC-PEITC suppressed MT1-MMP mRNA levels in a dose-dependent manner. Treatment with 5 μM PEITC or NAC-PEITC suppressed MT1-MMP activity by 68% and 81%, respectively. The effects of 24-h exposure to PEITC and NAC-PEITC on TIMP-1/TIMP-2 activities in SK-Hep1 cells are shown in Fig. 9. TIMP-1 mRNA was expressed in control, PEITC-treated and NAC-PEITC-treated cells, but there was no dose–response relationship in (1–5 μM) PEITC treatment. With 5 μM NAC-PEITC, TIMP-1 mRNA expression increased. TIMP-2 mRNA was expressed to a greater extent compared to TIMP-1, with 1.5- and 2.2-fold increases after 24-h

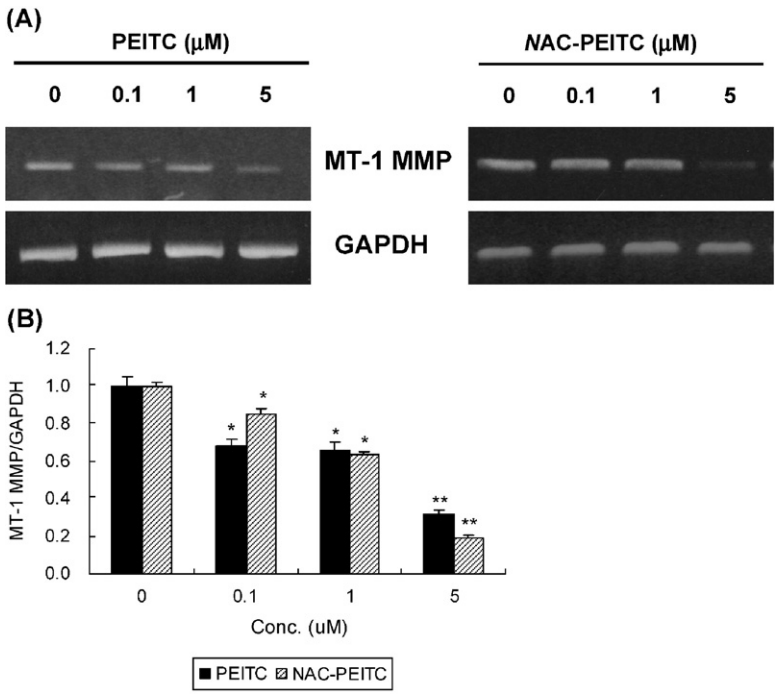

Fig. 8. Expression of MT1-MMP mRNA in SK-Hep1 cells after treatment with various concentrations of PEITC and NAC-PEITC for 24 h, as measured by RT-PCR analysis. The experiment was repeated thrice with similar results. (A) PCR products were separated by agarose gel electrophoresis. (B) MT1-MMP/GAPDH PCR product ratios were determined by densitometry. The values marked as * and ** indicate significant differences with other treatments ($P<.05$).

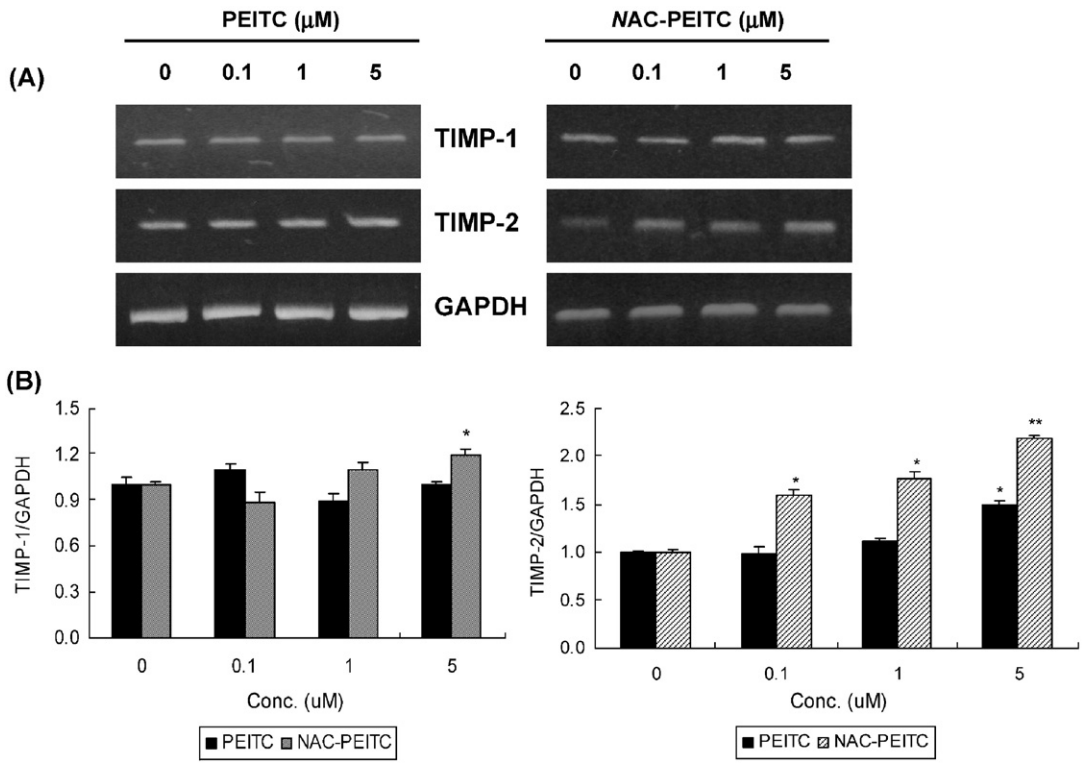

Fig. 9. Expression of TIMP-1/TIMP-2 mRNA in SK-Hep1 cells after treatment with various concentrations of PEITC and NAC-PEITC for 24 h, as measured by RT-PCR analysis. The experiment was repeated thrice with similar results. (A) PCR products were separated by agarose gel electrophoresis. (B) TIMP-1/GAPDH and TIMP-2/GAPDH PCR product ratios were determined by densitometry. The values marked as * and ** indicate significant differences with other treatments ($P<.05$).

exposure to 5 μM PEITC and NAC-PEITC, respectively. There were also significant correlations between mRNA expression and the protein expressions of MMP-2/MMP-9, MT1-MMP and TIMP-1/TIMP-2.

4. Discussion

Many in vitro and in vivo studies have indicated that the consumption of cruciferous vegetables protects against cancers, including prostate, colon and liver cancers, and that this might be due to the protective effects of ITCs [5,27,28]. In our previous study, we have determined the inhibitory effects of allylisothiocyanate (AITC) and its NAC conjugate on cell proliferation, expression of MMP, adhesion, invasion and migration in SK-Hep1 human hepatoma cells [29]. NAC-AITC inhibited cancer cell adhesion and invasion much more potently than AITC. NAC-AITC at 5 μM caused excellent inhibition of cell migration for 48 h. AITC and NAC-AITC suppressed MMP-2/MMP-9 activities in both protein and mRNA levels. The inhibition of MMP-2 mRNA expression was greater than that of MMP-9 mRNA, with 40% and 80% inhibition by 5 μM AITC and NAC-AITC, respectively. TIMP-1/TIMP-2 mRNA levels were expressed in control, AITC-treated and NAC-AITC-treated cells, but there was no dose–response relationship: TIMP-1/TIMP-2 activities were unaffected following treatment with AITC or NAC-AITC in our experiments. There were also no significant correlations between mRNA expression and the protein expressions of MMP-2/MMP-9 and TIMP-1/TIMP-2.

In this study, PEITC, another naturally occurring compound in cruciferous vegetables, and its major metabolite NAC-PEITC were examined for their antimetastatic activities. Several ITCs, such as AITC and sulforaphane, induced biologically relevant increases in the activities of phase II xenobiotic-metabolizing enzymes. Furthermore, some ITCs selectively modified the proliferation of precancerous cells, while their metabolites, such as NAC-AITC and NAC sulforaphane, modulated the growth of LNCaP prostate cancer cell growth and apoptosis [13,14].

Cell migration is a complex process involving many types of both intracellular and extracellular components and their associated signaling events. More specifically, cancer cell migration can be viewed as a process regulated by matrix-degrading proteinases, integrins and other cell adhesion molecules [30–32]. As migration is a critical event in cancer progression and especially metastasis, the inhibition of cell migration represents an attractive therapeutic agent.

Tumor invasion is defined as the penetration of tissue barriers, such as the basement membrane, by migrating cancerous cells [32,33]. During tumor progression, invasive

capacity is required at multiple steps. Tumor cells frequently invade surrounding tissues when the tumor starts to grow. Next, capillary endothelial cells invade the tumor and create tumor blood vessels. Thereafter, some tumor cells intravasate into the blood circulation for metastasis, whereas host immune cells invade the tumor. Finally, tumor cells arrest in distant organs, extravasate and migrate into new metastatic sites, and then start the invasive cycle again [32].

Metastasis is one of the most important factors in cancer prognosis and therapy. The development of novel antimetastatic drugs with low toxicity and high efficacy is an active area of cancer research. In this study, we found that PEITC and NAC-PEITC inhibit proliferation, adhesion, invasion and migration of SK-Hep1 human hepatoma cells. Migration of cancer cells through a coated membrane involves not only ECM degradation, but also the formation of adhesive interactions between cells and the matrix. Therefore, we used adhesion and invasion assays to demonstrate that PEITC and NAC-PEITC effectively inhibit the migration of SK-Hep1 human hepatoma cells. Decreased migration was correlated with decreased adhesion and invasion.

The expression of MMPs, in general, and of gelatinases, in particular, has been extensively studied in biopsies and resected materials from primary tumors and metastases of colon cancer patients. MMP-2 mRNA levels have been found to be overexpressed in carcinomas [34–36]. MMP-2 mRNA was more often present in tumor lesions than in normal colon tissues, whereas MMP-9 mRNA expression was not significantly different between tumors of the colon and healthy mucosa in the study by Masuda and Aoki [37]. MMPs are not only ECM-degrading proteases, they have other functions as well. They are involved in growth factor release, growth factor activation, immune surveillance and angiogenesis [21,38,39].

Giannelli et al. [40] investigated the expression of MT1-MMP, MMP-2 and TIMP-2 in the sera and tissues (primary and metastatic nodules) of hepatocellular carcinoma patients with and without metastasis whose clinical outcomes were followed over a 2-year period. In the results, they found that MT1-MMP expression was similar among primary nodule tissues of patients with and without metastasis. Serum and tissue levels of MMP-2 were not statistically different between patients with and without metastasis, but MMP-2 was concentrated at the invasive edge of metastatic tissues. In contrast, serum and tissue levels of TIMP-2 were significantly higher in hepatocellular carcinoma patients without metastasis than in those with metastasis. This situation was not only observed in primary hepatocellular carcinoma tissues, but also in metastatic nodules. These results indicate that an imbalance between the proteolytic activities of MMP-2 and TIMP-2 is responsible for the degradation of ECM components and plays a critical role in tumor invasion and metastasis.

In conclusion, PEITC and NAC-PEITC inhibited SK-Hep1 cell proliferation, invasion and metastasis by decreasing MMP-2/MMP-9 and MT1-MMP expression and by increasing TIMP-1/TIMP-2 expression. These data may represent the antimetastatic activities of PEITC and NAC-PEITC in SK-Hep1 human hepatoma cells.

Acknowledgments

This work was supported by Korea Research Foundation grants (KRF-2004-005-F00055 and KRF-2005-206-F00008) funded by the Korean Government (MOEHRD).

References

[1] Lampe JW, Peterson S. *Brassica*, biotransformation and cancer risk: genetic polymorphisms alter the preventive effects of cruciferous vegetables. J Nutr 2002;132:2991–4.

[2] Zhao B, Seow A, Lee EJ, Poh WT, Teh M, Eng P, et al. Dietary isothiocyanates, glutathione *S*-transferase-M1, -T1 polymorphisms and lung cancer risk among Chinese women in Singapore. Cancer Epidemiol Biomarkers Prev 2001;10:1063–7.

[3] Pham NA, Jacobberger JW, Schimmer AD, Cao P, Gronda M, Hedley DW. The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. Mol Cancer Ther 2004;3:1239–48.

[4] Jakubikova J, Bao Y, Sedlak J. Isothiocyanates induce cell cycle arrest, apoptosis and mitochondrial potential depolarization in HL-60 and multidrug-resistant cell lines. Anticancer Res 2005;25:3375–86.

[5] Hwang E, Jeffery EH. Induction of quinine reductase by sulforaphane and sulforaphane *N*-acetylcysteine conjugate in murine hepatoma cells. J Med Food 2005;8:198–203.

[6] Fenwick GR, Heaney RK. Glucosinolates and their breakdown products in cruciferous crops, foods and feedingstuffs. Food Chem 1983;11:249–71.

[7] Mennicke WH, Kral T, Krumbiegel G. Metabolism of some naturally occurring isothiocyanates in the rat. Xenobiotica 1983;13:203–7.

[8] Hwang E, Jeffery EH. Evaluation of urinary *N*-acetyl cysteinyl allyl isothiocyanate as a biomarker for intake and bioactivity of Brussels sprouts. Food Chem Toxicol 2003;41:1817–25.

[9] Futakuchi M, Hirose M, Miki T, Tanaka H, Ozaki M, Shirai T. Inhibition of DMBA-initiated rat mammary tumour development by 1-*O*-hexyl-2,3,5-trimethylhydroquinone, phenylethyl isothiocyanate, and novel synthetic ascorbic acid derivatives. Eur J Cancer Prev 1998;7:153–9.

[10] Hecht SS, Upadhyaya P, Wang M, Bliss RL, McIntee EJ, Kenney PM. Inhibition of lung tumorigenesis in A/J mice by *N*-acetyl-*S*-(*N*-2-phenethylthiocarbamoyl)-L-cysteine and *myo*-inositol, individually and in combination. Carcinogenesis 2002;23:1455–61.

[11] Conaway CC, Wang CX, Pittman B, Yang YM, Schwartz JE, Tian D, et al. Phenethyl isothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates inhibit malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice. Cancer Res 2005;65:8548–57.

[12] Conaway CC, Yang YM, Chung F-L. Isothiocyanates as cancer chemo-preventive agents: their biological activities and metabolism in rodents and humans. Curr Drug Metab 2002;3:233–55.

[13] Chiao JW, Chung F-L, Kancherla R, Ahmed T, Mittelman A, Conaway CC. Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. Int J Oncol 2002;20:631–6.

[14] Chiao JW, Chung F-L, Krzeminski J, Amin S, Ashad R, Ahmed T, et al. Modulation of growth of prostate cancer cells by the *N*-acetylcysteine conjugate of phenethyl isothiocyanate. Int J Oncol 2000;16:1215–9.

[15] Chiao JW, Wu H, Ramaswamy G, Conaway CC, Chung FL, Wang L, et al. Ingestion of an isothiocyanate metabolite from cruciferous vegetables inhibits growth of human prostate cancer cell xenografts by apoptosis and cell cycle arrest. Carcinogenesis 2004;25:1403–8.
[16] Price JT, Thompson EW. Mechanisms of tumour invasion and metastasis: emerging targets for therapy. Expert Opin Ther Targets 2002;6:217–33.
[17] Simpson-Haidaris PJ, Rybarczyk B. Tumors and fibrinogen. The role of fibrinogen as an extracellular matrix protein. Ann N Y Acad Sci 2001;936:406–12.
[18] Klein G, Vellenga E, Fraaije MW, Kamps WA, de Bont ES. The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. Crit Rev Oncol Hematol 2004;50:87–100.
[19] Zucker S, Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. Cancer Metastasis Rev 2004;23:101–17.
[20] Takada Y, Gillenwater A, Ichikawa H, Aggarwal BBSuberoylanilide hydroxamic acid potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis by suppressing nuclear factor-kappa B activation. J Biol Chem 2006;281:5612–22.
[21] Ii M, Yamamoto H, Adachi Y, Maruyama Y, Shinomura Y. Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Exp Biol Med 2006; 231:20–7.
[22] Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yanmamoto E, et al. Membrane type 1 matrix metalloproteinase expressed on the surface of invasive tumor cells. Nature 1994;370:61–5.
[23] Guo P, Imanishi Y, Cackowski FC, Jarzynka MJ, Tao HQ, Nishikawa R, et al. Up-regulation of angiopoietin-2, matrix metalloprotease-2, membrane type 1 metalloprotease, and laminin 5 gamma 2 correlates with the invasiveness of human glioma. Am J Pathol 2005;166:877–90.
[24] Ogasawara S, Yano H, Momosaki S, Nishida N, Takemoto Y, Kojiro S, et al. Expression of matrix metalloproteinases (MMPs) in cultured hepatocellular carcinoma (HCC) cells and surgically resected HCC tissues. Oncol Rep 2005;13:1043–8.
[25] Zhai Y, Hotary KB, Nan B, Bosch FX, Munoz N, Weiss SJ, et al. Expression of membrane type 1 matrix metalloproteinase is associated with cervical carcinoma progression and invasion. Cancer Res 2005;65:6543–50.
[26] Koyama S. Coordinate cell-surface expression of matrix metalloproteinases and their inhibitors on cancer-associated myofibroblasts from malignant ascites in patients with gastric carcinoma. J Cancer Res Clin Oncol 2005;131:809–14.
[27] Singh SV, Srivastava SK, Choi S, Lew KL, Antosiewicz J, Xiao D, et al. Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. J Biol Chem 2005;280: 19911–24.
[28] Traka M, Gasper AV, Smith JA, Hawkey CJ, Bao Y, Mithen RF. Transcriptome analysis of human colon Caco-2 cells exposed to sulforaphane. J Nutr 2005;135:1865–72.
[29] Hwang E, Lee HJ. Allyl isothiocyanate and its *N*-acetylcysteine conjugate suppress metastasis via inhibition of invasion, migration, and matrix metalloproteinase-2/-9 activities in SK-Hep1 human hepatoma cells. Exp Biol Med 2006 [in press].
[30] Chen X, Su Y, Fingleton B, Acuff H, Matrisian LM, Zent R, et al. Increased plasma MMP9 in integrin alpha1-null mice enhances lung metastasis of colon carcinoma cells. Int J Cancer 2005;116:52–61.
[31] Bjorklund M, Kojvunen E. Gelatinase-mediated migration and invasion of cancer cells. Biochim Biophys Acta 2005;1755:37–69.
[32] Matias-Roman S, Galvez BG, Genis L, Yanez-Mo M, de la Rosa G, Sanchez-Mateos P, et al. Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium. Blood 2005; 105:3956–64.
[33] Pirila E, Sharabi A, Salo T, Quaranta V, Tu H, Heljasvaara R, et al. Matrix metalloproteinases process the laminin-5 gamma 2-chain and regulate epithelial cell migration. Biochem Biophys Res Commun 2003;303:1012–7.
[34] Pellikainen JM, Ropponen KM, Kataja VV, Kellokoski JK, Eskelinen MJ, Kosma VM. Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in breast cancer with a special reference to activator protein-2, HER2, and prognosis. Clin Cancer Res 2004; 10:7621–8.
[35] Tang Y, Nakada MT, Kesavan P, McCabe F, Millar H, Rafferty P, et al. Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. Cancer Res 2005;65:3193–9.
[36] Choi J, Chio K, Benveniste EN, Hong YS, Lee JH, Kim J, et al. Bcl-2 promotes invasion and lung metastasis by inducing matrix metalloproteinase-2. Cancer Res 2005;65:5554–60.
[37] Masuda H, Aoki H. Host expression of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in normal colon tissue affects metastatic potential of colorectal cancer. Dis Colon Rectum 1999;42:393–7.
[38] Fiore E, Fusco C, Romero P, Stamenkovic I. Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell-mediated cytotoxicity. Oncogene 2002;21:5213–23.
[39] Liu S, Liang Y, Huang H, Wang L, Li Y, Li J, et al. ERK-dependent signaling pathway and transcriptional factor Ets-1 regulate matrix metalloproteinase-9 production in transforming growth factor-beta1 stimulated glomerular podocytes. Cell Physiol Biochem 2005;16:207–16.
[40] Giannelli G, Bergamini C, Marinosci F, Fransvea E, Quaranta M, Lupo L, et al. Clinical role of MMP-2/TIMP-2 imbalance in hepatocellular carcinoma. Int J Cancer 2002;97:425–31.