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Induction of apoptosis in a non-small cell human lung cancer cell line by isothiocyanates is associated with P53 and P21

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Abstract

This study was aimed at examining the effects of glucosinolate derivatives including phenylethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC), and indole-3-carbinol (I3C), on the induction of apoptosis in human non-small cell lung carcinoma A549 cells. The results indicated that all tested compounds inhibited the growth of A549 cells in a concentration-dependent manner. Flow cytometric analyses and annexin V staining showed that induction of apoptosis occurred at low concentrations of PEITC and BITC ($\leqslant$10 μM), and that necrosis occurred at higher concentrations of PEITC and BITC (25 μM); however, apoptosis was not the major pathway for the antiproliferative effects of I3C. Furthermore, Western blot analyses demonstrated that increased expression of P53 and P21 proteins, but not Bax protein, were associated with PEITC- and BITC-induced apoptosis.

Keywords: Isothiocyanate; Indole-3-carbinol; Apoptosis; Lung adenocarcinoma A549 cells; P53; P21

1. Introduction

As the leading cause of cancer deaths in most developed countries, lung cancer has garnered much attention. Clinically, lung cancer is classified into two groups, small cell and non-small cell lung cancer. The latter is more prevalent, accounting for almost 80% of lung cancers. Non-small cell lung cancer is composed of several subtypes, including lung adenocarcinoma, which is the most common lung cancer in the US. Traditionally, surgery, radiotherapy, and chemotherapy have been used to treat patients with lung cancer, but these treatments are usually accompanied by many side effects. Therefore, it is important to develop new approaches, such as apoptosis, for treating this type of lung cancer.

Apoptosis, or programmed cell death, is a common form of eukaryotic cell death. Apoptosis is a physiological cell suicide program that helps maintain homeostasis, in which cell death naturally occurs during tissue turnover (Samali et al., 1996; Staunton and Gaffney, 1998), but its aberrant activation and impairment may contribute to a number of diseases (Carson and Ribeiro, 1993; Thompson, 1995). For example, impaired apoptosis may be a significant factor in the etiology of cancer, so it has been suggested that therapeutics that increase the regulation of apoptosis may provide a new opportunity for the treatment of cancer (Kerr et al., 1994; Staunton and Gaffney, 1998). Cells undergoing apoptosis usually show several cellular changes, including formation of plasma membrane blebs, reduction in cell volume, chromatin condensation, and DNA fragmentation, but cells retain their membrane and organelle integrity. Apoptosis is regulated by various gene products including P53, Bcl, and Bax proteins (White, 1996; Staunton and Gaffney, 1998). In addition, several dietary phytochemicals that play significant roles in the

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anticarcinogenic process have been identified to trigger the apoptotic death of cancer cells (Hibasami et al., 1996; Huang et al., 1998; Moragoda et al., 2001).

Cruciferous vegetables consisting of cabbage, Napa cabbage, broccoli, and cauliflower and their bioactive components, glucosinolates, have been reported to be associated with lowered risks of cancers (Graham and Mettlin, 1979; Stoewsand, 1995; Verhoeven et al., 1996; Craig, 1997). The degradation products of glucosinolates such as indole-3-carbinol (I3C) and isothiocyanates, including β-phenylethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC), have been shown to possess anticarcinogenic activities (Zhang and Talalay, 1994; Grubbs et al., 1995), and to induce apoptosis in various cancer cell lines (Huang et al., 1998; Ge et al., 1999; Bonnesen et al., 2001; Yang et al., 2002; Nachshon-Kedmi et al., 2003). PEITC has been shown to inhibit tobacco smoke-induced lung tumors in mice (Witschi et al., 1998), but related studies are not available on lung cancer cells. Therefore, the purpose of this study was to explore the effects of the cruciferous vegetable derivatives, PEITC, BITC and I3C, on the induction of apoptosis in human non-small cell lung adenocarcinoma A549 cells. To understand the effects of these derivatives on cell growth, cell counts and cell proliferation were monitored. To identify the role of apoptosis in growth inhibitory effects, flow cytometric and annexin V staining analyses were performed. Apoptosis-associated P53, P21, and Bax proteins were also examined. The results obtained from this study may provide preclinical evidence on the potential use of compounds derived from cruciferous vegetables as cancer chemopreventive or chemotherapeutic agents.

2. Materials and methods

Chemicals and biochemicals. Indole-3-carbinol (I3C), β-phenylethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC), dimethyl sulfoxide (DMSO), and propidium iodide (PI) were obtained from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, trypsin, trypan blue, and sodium bicarbonate were purchased from GIBCO BRL (Grand Island, NY). Absolute ethanol was from Merck (Darmstadt, Germany). All other laboratory chemicals were of the highest quality available and were purchased from Sigma Chemical and USB (Cleveland, OH).

Cell culture. The human lung adenocarcinoma cell line A549 was obtained from the Culture Collection and Research Center in Taiwan (CCRC 60074). Cells were grown as monolayers in DMEM supplemented with 10% fetal bovine serum at 37 °C in a 95% air, 5% CO_2 atmosphere and routinely subcultured. PEITC and BITC were dissolved in absolute ethanol, and I3C was dissolved in DMSO; the concentrations of absolute ethanol and DMSO added to the media never exceeded 0.5% (v/v) and 0.2% (v/v), respectively.

Cell growth and cell proliferation assays. To evaluate the effects of PEITC, BITC, and I3C on the growth of cells, A549 cells (5×10^4/well) were plated in 6-well plates and cultivated in the presence of test compounds. Viable cells were estimated by the trypan blue dye exclusion method, and numbers of cells were counted with a Coulter counter.

Cell proliferation was examined with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay@ kit (Promega, Madison, WI). Basically, 10^3 cells/well were plated in 96-well plates, and were treated with test compounds for 3 days. The rate of proliferation was then determined by converting a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), to a colored formazan product by live cells as detected by a microplate reader at $OD_{492\ nm}$.

Flow cytometric analysis. To analyze the distribution of cellular DNA content, flow cytometric analysis was performed. After being treated with the test compounds for 24 h, cells were collected, suspended, and fixed with 75% ethanol on ice. Fixed cells were incubated with 3 μg/ml DNAase-free RNAase A in PBS for 30 min at 37 °C. After staining with PI (20 μg/ml), cells were analyzed by FACScan laser flow cytometry (Becton Dickenson, San Jose, CA) with excitation at 488 nm.

Annexin V staining assay. In the beginning of apoptosis, phosphatidylserine inside of the cell membrane is inverted to the outside. Therefore, binding of phosphatidyl serine to annexin V (green) and DNA to PI (red) can be used for the detecting apoptosis and its various stages. Annexin V staining was carried out with annexin V-FITC Apoptosis Detection kits (Oncogene, Boston, MA) following the manufacturer's instructions. In brief, media binding reagent and annexin V-fluorescein isothiocyanate were added to the cells for 15 min in the dark. Cells were treated with binding buffer and PI, and were then photographed under a fluorescent microscope.

SDS–PAGE and Western blot analyses. To determine whether P53, Bax, and P21 are associated with the induction of apoptosis, Western blot analysis was performed. Cell lysate was isolated by treating cells with lysis solution (150 mM NaCl, 1% Triton X-100, 10 mM Tris, pH 7.4, 5 mM EDTA, and 1 mM phenylmethyl-sulfonylfluoride), and the protein content was determined using a Bio-rad protein assay kit. After separation by 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE), the proteins were electroblotted onto a nitrocellulose membrane, and the blots were incubated with monoclonal antimouse P53 (DO-1), polyclonal Bax (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse monoclonal $P21^{WAF1/CIP1}$ antibodies (BD Transduction Lab,

Los Angeles, CA). Finally, the blot was treated with peroxidase-conjugated IgG (Santa Cruz Biotechnology), and specific bindings of these antibodies were detected using an enhanced chemiluminescent ECL Western detection system (Amersham Biosciences, Buckinghamshire, England).

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). One-way ANOVA followed by Fisher's test was used to determine the statistical differences among groups using SAS software version 6.12 (SAS Institute, Cary, NC). The significance of mean differences was based on a p value of <0.05.

3. Results

To examine the effects of cruciferous vegetable derivatives on the growth of A549 cells, cells were treated with various concentrations of PEITC, BITC, and I3C, and numbers of cells were determined after 3 and 5 days. Fig. 1 indicates that all three compounds inhibited the growth of A549 cells in concentration-dependent manners. PEITC and BITC, at the highest concentration (25 μM), completely suppressed the growth of cells. However, I3C, at 250 μM, produced 67% growth inhibition after 5 days of treatment. Results obtained from the MTS assay illustrate comparable dose–response patterns (Fig. 2), and the approximate IC_{50} values for PEITC, BITC, and I3C were 7.5, 3, and 200 μM, respectively.

To explore the mechanisms of PEITC, BITC, and I3C on the inhibition of cell growth, the DNA content of treated cells was determined by flow cytometric analysis. Fig. 3 indicates that the distribution of DNA contents in PEITC-treated cells differed from that of control cells after 24 h treatment. PEITC-treated cells showed a concentration-dependent increase in the number of cells with subdiploid DNA contents (sub G_1), i.e., apoptotic cells, which reached a maximum at 10 μM, and this change was accompanied by a decreased G_0/G_1 peak and an increased G_2/M peak. BITC produced similar results to those of PEITC (data not shown). By contrast,

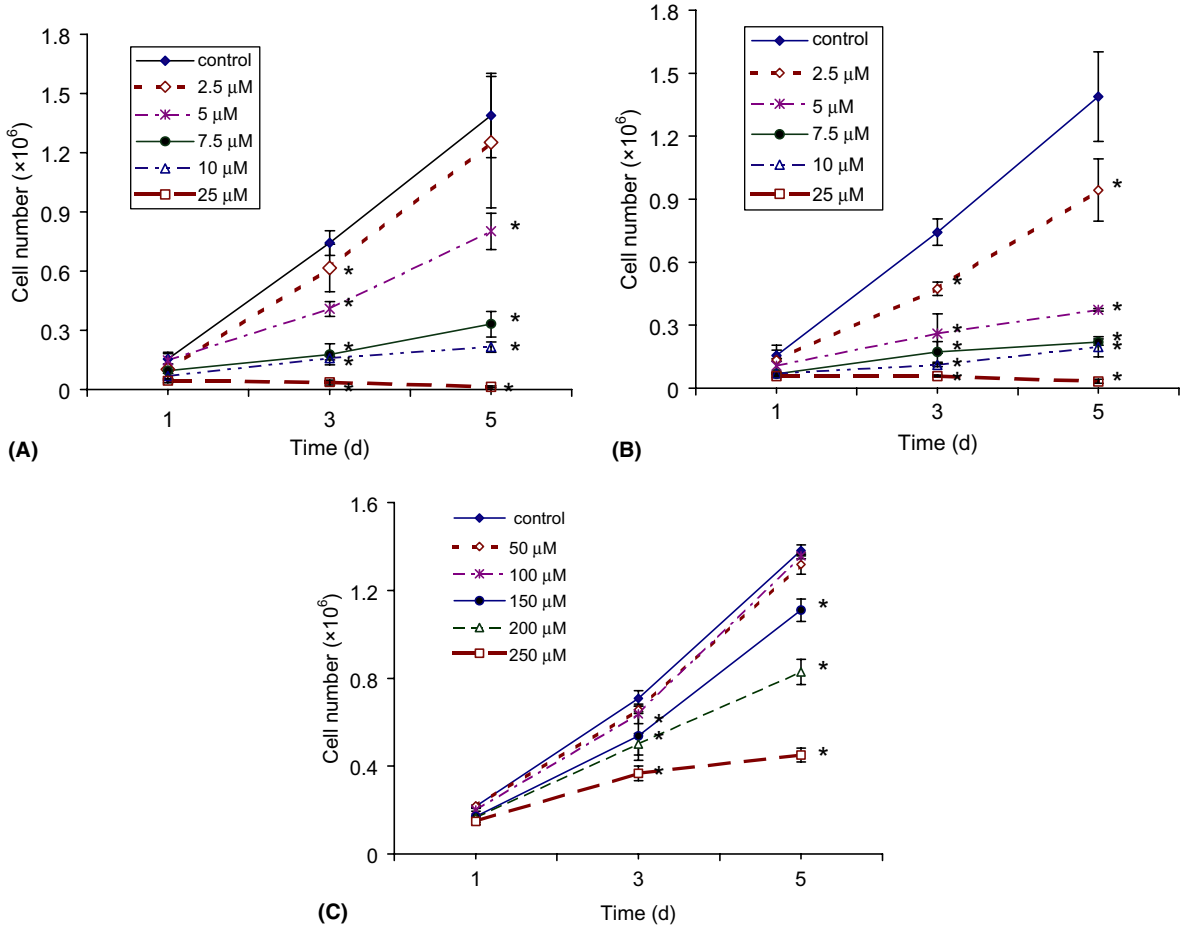

Fig. 1. Effects of PEITC, BITC, and I3C on the growth of A549 cells. Cells were treated with various concentrations of PEITC (A), BITC (B) and I3C (C), and then were collected and counted with a Coulter counter at the times indicated. Values represent the mean ± SD from three measurements. *$p < 0.05$ compared with the control at the same time point.

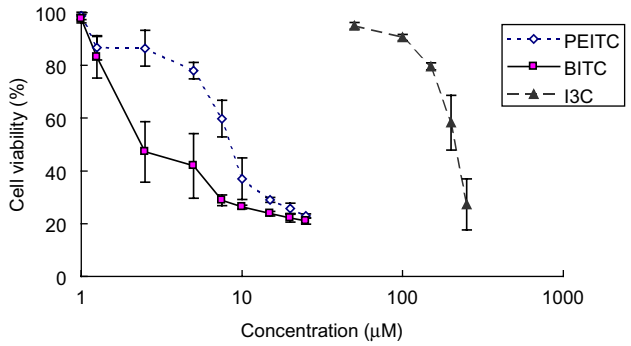

Fig. 2. Effects of PEITC, BITC, and I3C on the viability of A549 cells. Cells were treated with various concentrations of PEITC, BITC, and I3C for 72 h. Cell viability was then determined by an MTS assay kit. Values represent the mean ± SD from four measurements.

I3C (250 μM) did not affect the distribution of DNA content after 24- or 48-h treatments. A slight increase in the proportion of sub G_1 cells (from 0.6% to 1.3%) and an increased G_0/G_1 peak (from 79% to 90%) were observed in cells treated with I3C for 72 h (data not shown).

As a cell undergoes apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer membrane of the cell. Because annexin V binds to PS with high affinity, the apoptotic cells can be observed with fluorescent microscopy. Fig. 4 shows that 10 μM PEITC increased the numbers of annexin V-positive (green- or orange-colored) cells, indicating that the cells had undergone apoptosis. However, more PI-positive (red color) cells were present after treatment with 25 μM PEITC, suggesting that late apoptotic or necrotic cells are present at high concentrations of PEITC (Fig. 4C). Similar results were also observed in BITC-treated cells (data not shown). These results together with the results obtained from flow cytometry suggested that PEITC and BITC induce apoptosis in A549 cells at low concentrations (⩽10 μM), and necrosis at a higher concentration (25 μM).

A variety of proteins have been reported to be associated with the apoptosis pathway, so the effects of PEITC and BITC on various apoptosis-associated proteins were examined. Western blot analyses (Fig. 5) showed that PEITC enhanced the expression of P53 and $P21^{WAF1/CIP1}$ proteins in concentration dependent manners, and respectively produced 160% and 270% increases compared to that of the control at 10 μM. Although BITC also enhanced the expressions of P53 and $P21^{WAF1/CIP1}$ proteins, they were not concentration dependent, and showed a lesser effect on $P21^{WAF1/CIP1}$ expression compared to did PEITC. On the contrary, neither PEITC nor BITC affected the expression of cytosolic Bax protein.

4. Discussion

Different epidemiological studies have indicated that diet and cancers are closely associated (Cummings and Bingham, 1998; Labadarios and Parke, 1999). People who consume higher amount of fruits and vegetables have a lower risk of various types of cancers. Various studies demonstrate that fruits and vegetables contain natural occurring compounds that possess anticarcinogenic properties (Steinmetz and Potter, 1996; Craig,

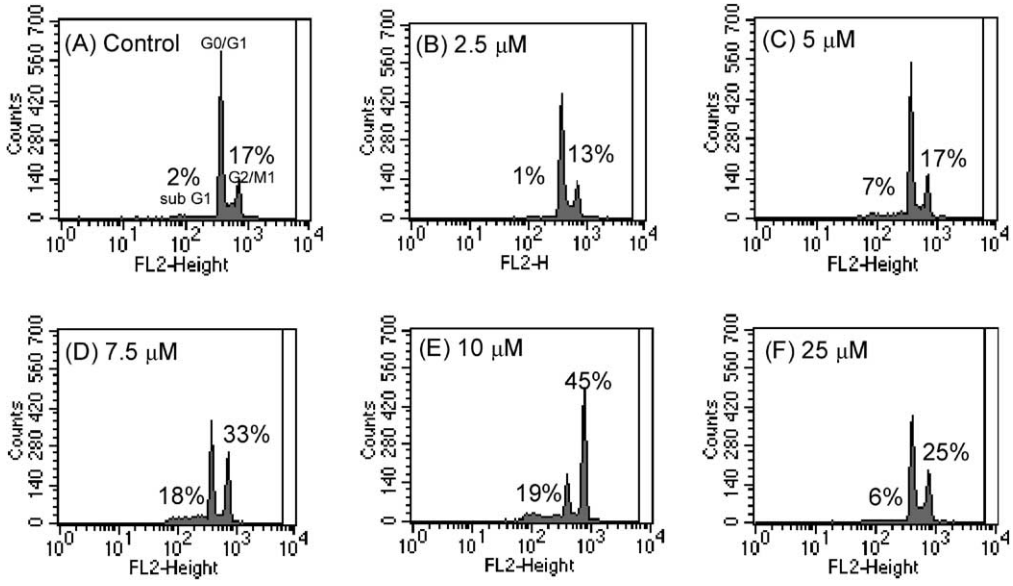

Fig. 3. Representative histograms of cytometric analysis after treatment of cells with PEITC. A549 cells were treated with various concentrations of PEITC for 24 h. Cells were then collected, fixed and stained with PI in the flow cytometric analysis for DNA content. Values represent the mean from four measurements.

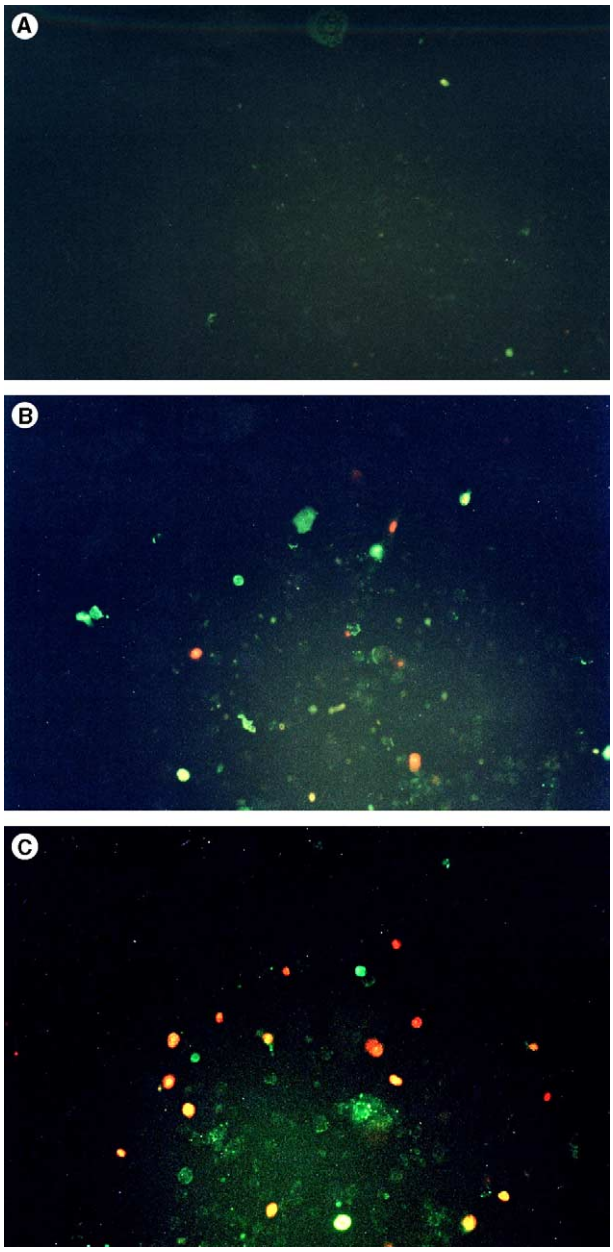

Fig. 4. Fluorescent microscopy of PEITC-induced morphological changes in A549 cells. Cells were treated with ethanol (A), 10 μM PEITC (B), or 25 μM PEITC (C) for 24 h. Cells were then stained with annexin V-FITC and PI and photographed under a fluorescence microscope. Green color indicates apoptotic cells at an early stage, green and red (orange) indicate cells at the middle or late stage of apoptosis, and red indicates necrotic cells. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

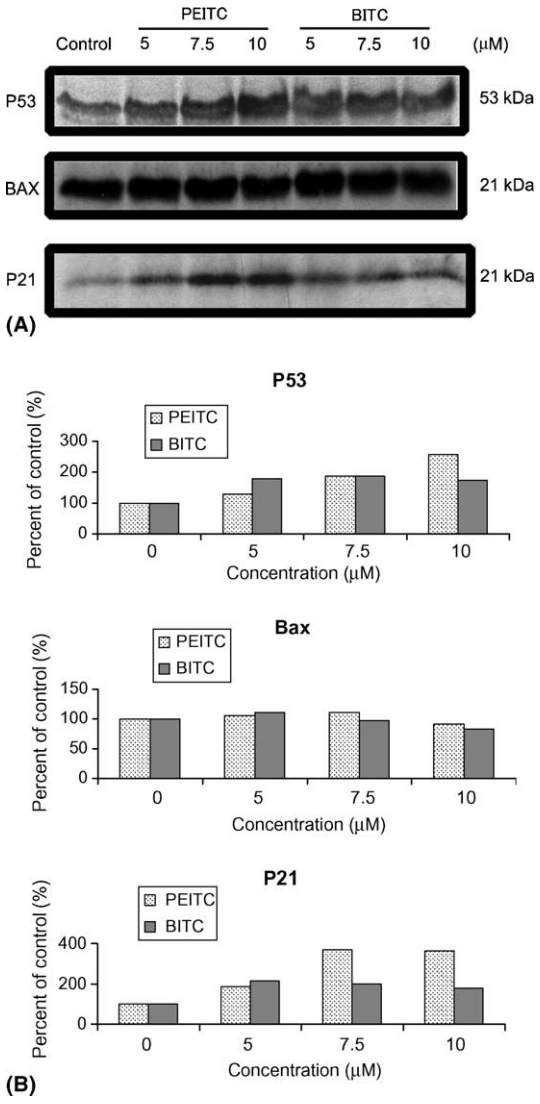

Fig. 5. Effects of various concentrations of PEITC or BITC on the expressions of P53, P21, and Bax proteins in A549 cells. (A) Cells were treated with various concentrations of PEITC or BITC, and the cytosolic protein was extracted after 24 h. 70 μg of cytosolic protein was separated by SDS–PAGE, and P53, P21, and Bax proteins were respectively detected. This experiment was repeated three times, and similar results were obtained. (B) Densitometric quantification of the proteins.

1997), such as glucosinolates in cruciferous vegetables (Stoewsand, 1995). Isothiocyanates and indoles are two major groups of autolytic breakdown products of glucosinolates, and both exhibit protective activities against cancers (Zhang and Talalay, 1994; McDanell et al., 1988). PEITC and I3C have been shown to modulate the activity of xenobiotic-metabolizing enzymes (Bradfield and Bjeldanes, 1984; Guo et al., 1992), to suppress lipopolysaccharide-induced nitric oxide production (Chen et al., 2003), and to induce apoptosis in cancer cells (Huang et al., 1998; Ge et al., 1999; Bonnesen et al., 2001; Chinni et al., 2001), pathways that have been suggested to be involved in the anticarcinogenic process. In the present study, for the first time, we have demonstrated that PEITC, BITC, and I3C, inhibit the growth of lung adenocarcinoma A549 cells. The isothiocyanate derivatives, PEITC and BITC, show higher potency in inhibiting proliferation by acting through induction of apoptosis at low concentrations (<10 μM)

and through necrosis at a high concentration (25 μM), whereas the indolic derivative I3C, with higher IC_{50} value (~200 μM), by acting through an apoptosis-independent pathway. Different dietary phytochemicals that play significant roles in the anticarcinogenic process have been identified to trigger the apoptotic death of human lung cancer cells, including curcumin (Pillai et al., 2004), kaempferol (Nguyen et al., 2003), and tea polyphenols (Suganuma et al., 1999), with IC_{50} values greater than 22 μM. Therefore, the results obtained from this study introduce new candidates of treatment for non-small cell lung cancers and provide the molecular mechanisms of PEITC and BITC in the induction of apoptosis in lung adenocarcinoma cells.

The antiproliferative effect of I3C in A549 cells was independent of the induction of apoptosis, although I3C, at concentrations of 50–250 μM, induces apoptosis in hormone-related cell lines including human breast cancer MCF-7 and human prostate cancer LNCaP cells (Ge et al., 1999; Nachshon-Kedmi et al., 2003). In addition, I3C shows observable toxicity to the human colon cell lines, LS-174 and Caco-2, only at high concentrations with IC_{50} values of 600 μM (Bonnesen et al., 2001); this suggests that hormone-responsive cells are more sensitive to I3C treatment. Because I3C is an estrogen receptor antagonist (Meng et al., 2000), and I3C-induced apoptosis in MCF-7 cells has been suggested to be estrogen receptor related (Ge et al., 1999), it is conceivable to explain that apoptosis in A549 cells not being induced by 250 μM I3C is due to its cell-type-dependence effect. On the other hand, mechanisms other than apoptosis may be involved in the growth inhibitory effect of I3C. Chinni et al. (2001) suggested that I3C induces G_1 cell cycle arrest in poorly differentiated prostate cancer PC-3 cells. Whether I3C inhibits the growth of A549 cells through induction of G_1 cell cycle arrest needs to be investigated further.

Both apoptosis and necrosis were associated with the antiproliferative effects of PEITC and BITC in A549 cells, in which apoptosis was mainly induced by low concentrations ($\leqslant$10 μM), whereas necrosis was essentially associated with higher concentrations (25 μM) of PEITC and BITC. These results are consistent with several other studies showing that PEITC and BITC (<20 μM) induce apoptosis in other cell lines, including human prostate cancer cells, human colon cancer cells, human HeLa cells, human leukemia Jurkat T-cells, mouse epidermal cells, and rat liver epithelial cells (Chen et al., 1998; Huang et al., 1998; Yu et al., 1998; Bonnesen et al., 2001; Nakamura et al., 2002). Meanwhile, some of these studies also showed that PEITC and/or BITC at concentrations greater than 30 or 50 μM induce necrosis in human HeLa cells, human leukemia Jurkat T-cells, and rat liver epithelial RL34 cells (Chen et al., 1998; Yu et al., 1998; Nakamura et al., 2002). Therefore, PEITC and BITC at concentrations below 10 μM are able to induce apoptosis in various cancer cell lines.

Several pieces of evidence have indicated that signals leading to activation of a variety of gene products, such as P53, P21 and Bcl-2 family proteins consisting of Bax, are important in the regulation and execution of apoptosis induced by various stimuli (Kerr et al., 1994). The induction of apoptosis by PEITC and BITC is associated with the increased expression of P53 suggesting the pivotal roles of P53 in this process, and this is consistent with the study by Huang et al. (1998) who demonstrated that apoptosis induced by PEITC occurs through a P53-dependent pathway in mouse epidermal C1 41 cells. P53 is a tumor suppressor protein and transcription factor that plays a substantial role in apoptosis. The increased expression of P53 may act through certain pathways, including mitochondrial cytochrome *c* release and Fas/APO1 signaling, to enhance apoptosis-downstream caspase enzymes to induce apoptosis (el Deiry, 1998; Shen and White, 2001). PEITC and BITC have been shown to upregulate caspase-3 and thus induce apoptosis in HeLa cells (Yu et al., 1998), so the increased P53 may act through increasing activities of caspases to induce apoptosis in A549 cells. P53 also plays an important role in cell cycle regulation. Studies have shown that P53 mediates apoptosis by a mechanism independent of that of cell cycle arrest (el Deiry, 1998; Shen and White, 2001). P21, a universal inhibitor of cyclin-dependent kinases (CDKs) (Xiong et al., 1993), is a P53-regulated protein. Upregulation of $P21^{WAF1/CIP1}$ by different chemopreventive agents is associated with G_2/M phase arrest in the cell cycle (Lian et al., 1998; Bilim et al., 2000). Because PEITC- and BITC-induced apoptosis in A549 cells is associated with increased expressions of P53 and $P21^{WAF1/CIP1}$, the mechanism of cell cycle arrest on the cell death signaling of PEITC and BITC cannot be ruled out. On the other hand, Xiao and Singh (2002) showed that P53 is not required for PEITC-induced apoptosis in human prostate cancer PC-3 cells. Because the expression of P21 can also be regulated by other pathways that are independent of P53 (Zeng and el Deiry, 1996; el Deiry, 1998), the increased expression of P21 might not be directly related to the increased expression of P53.

Functions of P53 are mainly regulated by phosphorylation. Different kinases including mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK1) have been suggested to be involved in the phosphorylation of P53 (Milczarek et al., 1997). Several pieces of evidence have demonstrated that PEITC and BITC induce apoptosis in various cell types through activation of JNK (Yu et al., 1996; Chen et al., 1998) or through suppression of JNK phosphatase activity (Chen et al., 2002). It is reasonable to postulate that the increased expression of P53 by PEITC and BITC may be mediated by the activation of JNK, and that

the increased P53 thus leads to increases in the activities of caspases and/or the expression of P21 to induce apoptosis or to cause cell cycle arrest. Therefore, treatment with PEITC and BITC may induce cell death in lung carcinoma cells, ultimately leading to the prevention of cancer.

Bax, another P53-regulated protein, is a member of the Bcl-2 family, and the ratio of Bax to Bcl-2 has been suggested to be a determinant of whether cells live or die (el Deiry, 1998; Shen and White, 2001), i.e., the Bax–Bax homodimer acts as an inducer of apoptosis, whereas the Bax–Bcl2 heterodimer acts as an inhibitor of apoptosis. Therefore, the existence of Bcl-2 and the balance between Bax and Bcl-2 may direct the process of apoptosis. Additionally, Bax dimers appear to control apoptosis at the level of mitochondrial cytochrome *c* release (el Deiry, 1998), so apoptosis induced by PEITC and BITC in A549 cells might not be related to the mitochondrial cytochrome *c* pathway. On the other hand, because the Bax protein undergoes subcellular redistribution during apoptosis (Hsu et al., 1997; Gross et al., 1998), the total amounts of Bax protein as shown in this study may not be enough to disclaim the independence of Bax in this process. Therefore, further experiments are required to confirm the role of Bax on the PEITC- and BITC-induced apoptosis in A549 cells.

In conclusion, we have demonstrated that the cruciferous vegetable isothiocyanate derivatives, PEITC and BITC, are able to inhibit the growth of A549 cells by inducing apoptosis at low concentrations and necrosis at high concentrations. The induction of apoptosis is associated with increased expression of P53 and P21 proteins. On the other hand, I3C, an indolic derivative of cruciferous vegetables, also possesses an antiproliferative effect in A549 cells, but not through induction of apoptosis. However, further experiments focusing on molecular mechanisms are needed to establish the role of the cruciferous derivatives, PEITC, BITC, and I3C, as chemopreventive or therapeutic agents against lung cancer.

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