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PEITC inhibits cholangiocarcinoma via induction of mitochondrial dysfunction

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Abstract

Phenethyl isothiocyanate (PEITC), a natural compound found abundantly in cruciferous and other vegetables, has been shown to possess cancer chemopreventive activity. The purpose of this investigation was to examine the cytotoxic effect of PEITC in cholangiocarcinoma cells (CCA). Cholangiocarcinoma cells, KKU-100 and human liver Chang cells used for comparison in the study. Effects of PEITC on cell growth and induction of apoptosis was determined by fluorescent dye staining using acridine orange and ethidium bromide. Cultured cells were exposed to PEITC for 3, 12, 24 and 48 hours following assessment of cell viability and apoptotic cell death. PEITC can induce a large proportion of cells to undergo apoptosis in a dose-time dependent manners. The PEITC induced depletion of intracellular antioxidant GSH in the cell lines. Moreover, a rapid collapse of the mitochondrial transmembrane potential, as measured by JC-1 staining, was observed concurrently with an apparent apoptosis in both cells. Furthermore, Western blot analysis were used to examine the antioxidant and survival response related proteins. The results revealed that PEITC increased levels of Nrf2 and cyclin D1 in both cell lines, and Bax and Trx protein expression was up-regulated in KKU-100. The effect of PEITC on cell growth and apoptosis may contribute to cancer chemopreventive properties. In conclusion, our data lucidly evidence the chemopreventive merits of dietary phytochemical PEITC in suppression of cholangiocarcinoma.

Keywords: Phenylethyl isothiocyanate; Cholangiocarcinoma; Apoptosis; Cytotoxicity

Introduction

Cholangiocarcinoma (CCA) is a highly malignant adenocarcinoma originating from the cholangiocytes. The highest incidence of this cancer has been reported in Northeastern Thailand (1). Intrahepatic CCA is the most common type of CCA in Thailand and countries in Southeast Asian region. Infestation of *Opisthorchis viverrini* has been classified as a definite risk factor of the disease (2). Drug resistance in several cancers is often characterized by the increased activation of certain transcription factors, which provide the anti-apoptotic and pro-oncogenic signals.

Phenethyl isothiocyanate (PEITC), a hydrolysis product of a group of naturally occurring thioglucoside and glucosinolate compounds, found in cruciferous vegetables. PEITC has been extensively investigated for its chemopreventive activity against several cancers. The ability of PEITC to induce apoptosis has been suggested to be due to the disruption of mitochondrial redox status via the electrophilic isothiocyanate moiety (3). It seems probably that at low concentrations, PEITC causes mild oxidative stress, which consequently stimulates the cell to build up its antioxidant/cytoprotective defense contributing to chemopreventive effect. However, the relatively high concentrations result in

oxidative stress and irreversible changes in redox status, and mitochondrial dysfunction which leads to growth inhibition and apoptotic cell death in cancer cells (4).

The aim of this study was to examine the effects of PEITC on the induction of apoptotic cell death and mechanisms involving with were also investigated, including cellular redox status, mitochondrial energetics and adaptive antioxidant response of CCA cells.

Methods

In these experiments, cell cultures used in this study included KKU-100 and Chang liver cells. Cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 12.5 mM Hepes and 100 mg/ml gentamicin under 5% CO_2 in air at 37^o C. The media was renewed every 3 days, trypsinized with 0.25% trypsin-EDTA, and subcultured in the same media.

The cytotoxicity assay and induction of apoptosis was assessed by acridine orange/ethidium bromide (AO/EB) method. Cells were cultured at appropriate density and treated with PEITC at 1, 3, 10, 30 μM for 3, 12, 24 and 48 hours. Then cells were stained with AO/EB for fluorescence microscopic examination using an inverted microscope. The number of viable and apoptotic cells were counted.

To determine the pro-oxidant effect of PEITC, cultured cells were treated with 10 μM PEITC at 0, 3, 6, 12, 24 and 48 hours. Cells were washed and collected for assay of glutathione (5).

To examine the effect of PEITC on the mitochondrial transmembrane potential, cells were treated with 3, 10 and 30 μM PEITC for 1 or 3 hours, then the assay was performed using 5,5',6,'-tetrachloro- 1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining method (6). Fluorescent signals were examined by using a spectrofluorometer and fluorescence images were captured by fluorescence microscope. The intact mitochondria were stained with red fluorescence, while the depolarized mitochondria were stained with green fluorescence.

In Western blot analysis determined the level of Nrf2, cyclin D1, Bcl-xl, Bax and Trx expression, both cell lines were treated with PEITC at varied concentrations and cultured for 48 hours. Equivalent amounts of whole cell lysate proteins (40μg) were subjected to separation on 10% SDS-polyacrylamide gel electrophoresis and blotted on to a PVDF membrane. After blotting was carried out, the signals were visualized using ECL kit. The bands were determined intensities by Gel pro Analysis.

Results

KKU-100 and Chang cells were treated with varied concentrations (1, 3, 10 and 30 μM) of PEITC for various times (3, 12, 24 and 48 hours). PEITC induced growth suppression and apoptosis rapidly within 3 h and in a dose–dependent manner in both cell lines (Fig.1). The pro-oxidant effect of PEITC was assessed by assay of redox status in both cell lines. The total GSH levels in treated cells were declined rapidly in both cell lines. However, the levels were restored rapidly within 12 h in Chang cells. The cellular redox ratio is considered an indicative of redox balance, there was no change in KKU-100 but clearly suppressed in Chang cells, indicating that PEITC was more potent in induction of oxidative stress in Chang cells (Fig. 2). Depolarization of mitochondrial transmembrane potential often precedes an induction of apoptotic cell death. PEITC induced dissipation of mitochondrial transmembrane potential in both cell types, where JC-1 forms red fluorescent aggregates (JC-1-aggregates) in intact mitochondria and JC-1 monomers with green fluorescence in depolarized mitochondria (Fig.3). PEITC induced a rapid fall in the mitochondrial transmembrane potential at the concentration as low as 3 μM. It is apparent that Chang cells were shown to be more sensitive than KKU-100 cells by rapid change in mitochondrial transmembrane potential. Western blot analysis of antioxidant, proliferative and apoptotic-

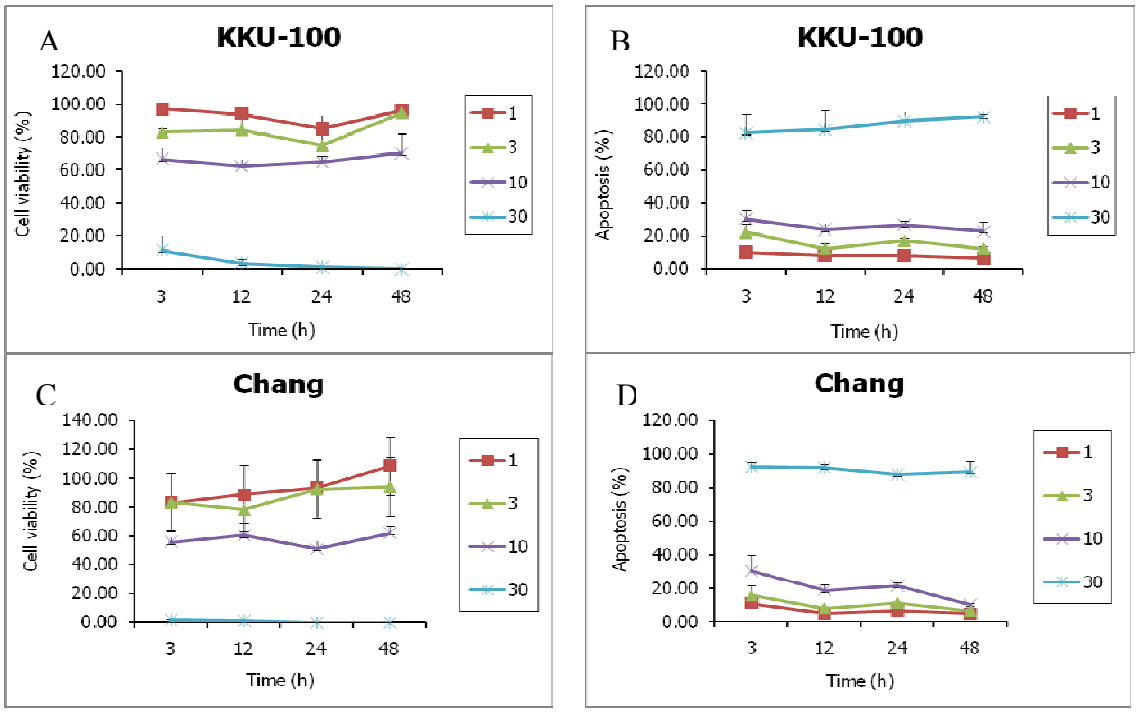

Figure 1 Cytotoxic effects of PEITC on KKU-100 and Chang cells. Cultured cells were incubated with varied concentrations of PEITC and at various time periods, cytotoxicity of the cells were examined by fluorescent staining method. (A, C) Percent viable cells and (B, D) apoptotic cells of KKU-100 and Chang cells, respectively, are showed. Data represent mean ± SE, each from 3 experiments.

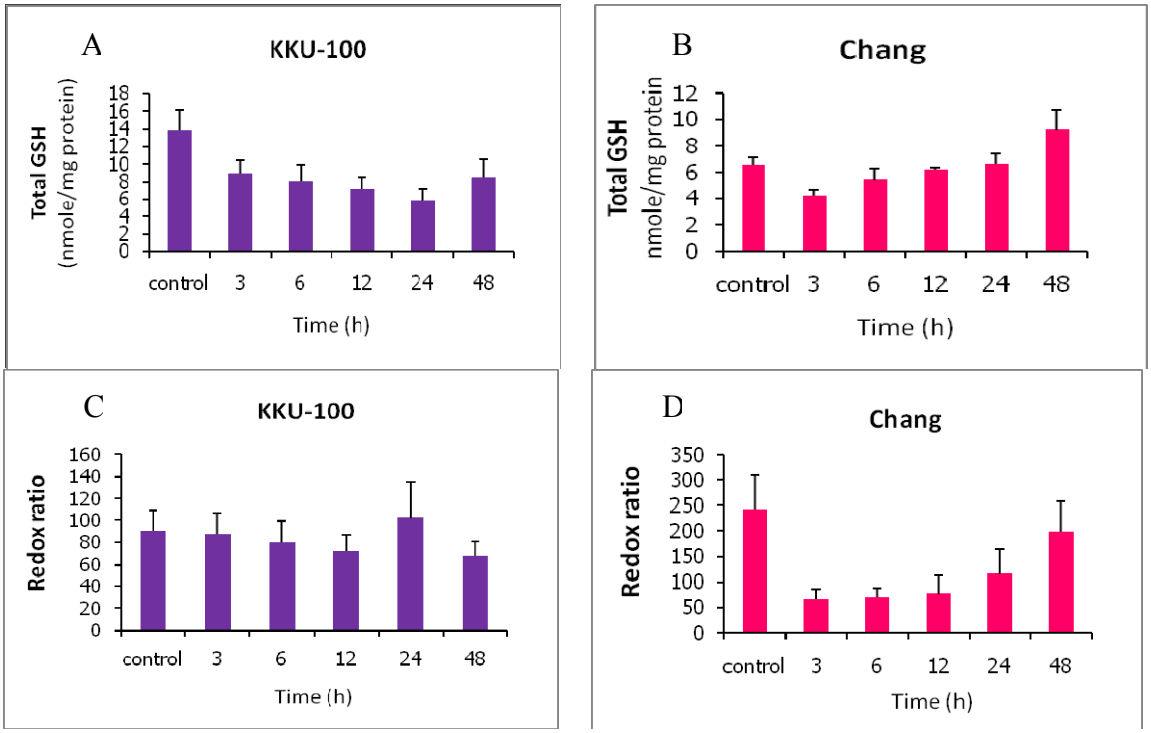

Figure 2 Effect of PEITC on redox status of KKU-100 and Chang cells. Cultured cells were incubated with 10 µM of PEITC and at various time periods redox status of cells were examined by glutathione assay. (A, B) Total intracellular glutathione (GSH) and (C, D) redox ratio (GSH/GSSG) of KKU-100 and Chang cells, respectively, are showed. Data represent mean ± SE, each from 3 experiments.

related proteins in CCA cells and Chang cells were analyzed after cells were treated for 48 hours. The results showed that PEITC increased level of cyclin D1 and Nrf2 in dose-dependent, whereas Trx and Bax were up-regulated in KKU-100 cells. However, there was no change in Bcl-XL in both cell lines (Fig.4).

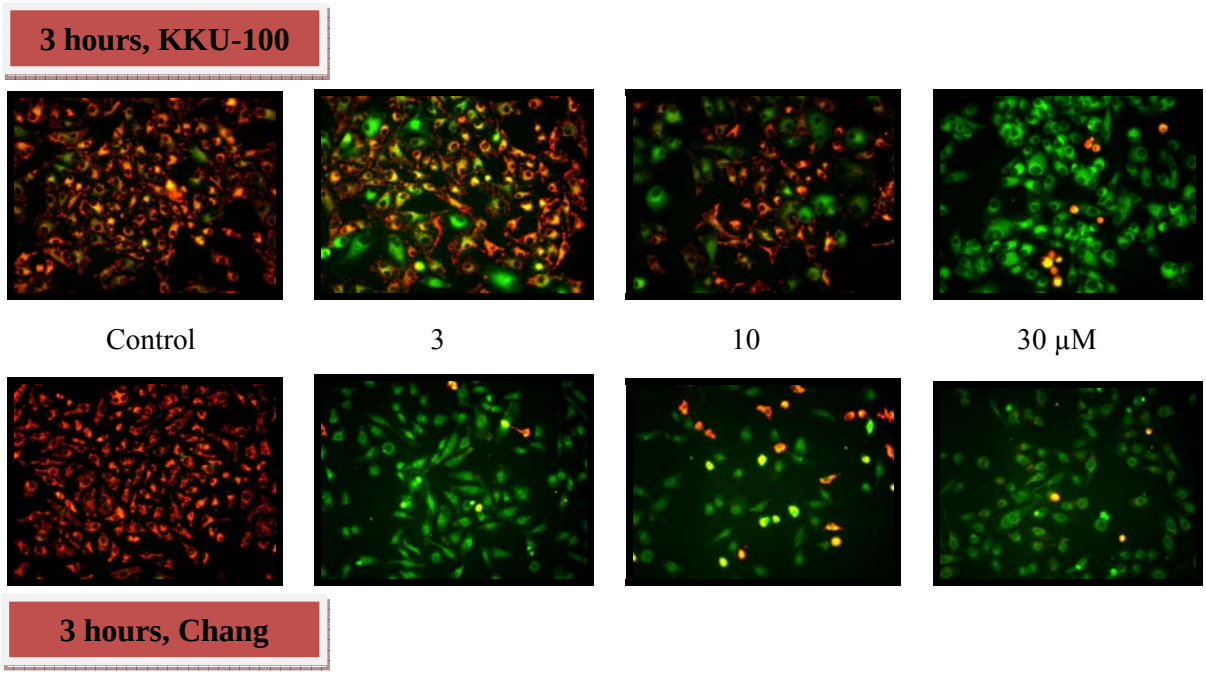

Figure 3 Effect of PEITC on mitochondrial transmembrane potential in KKU-100 and Chang cells. Cultured cells were treated with 3, 10 and 30 μM of PEITC for 3 h, and cells were stained with JC-1.

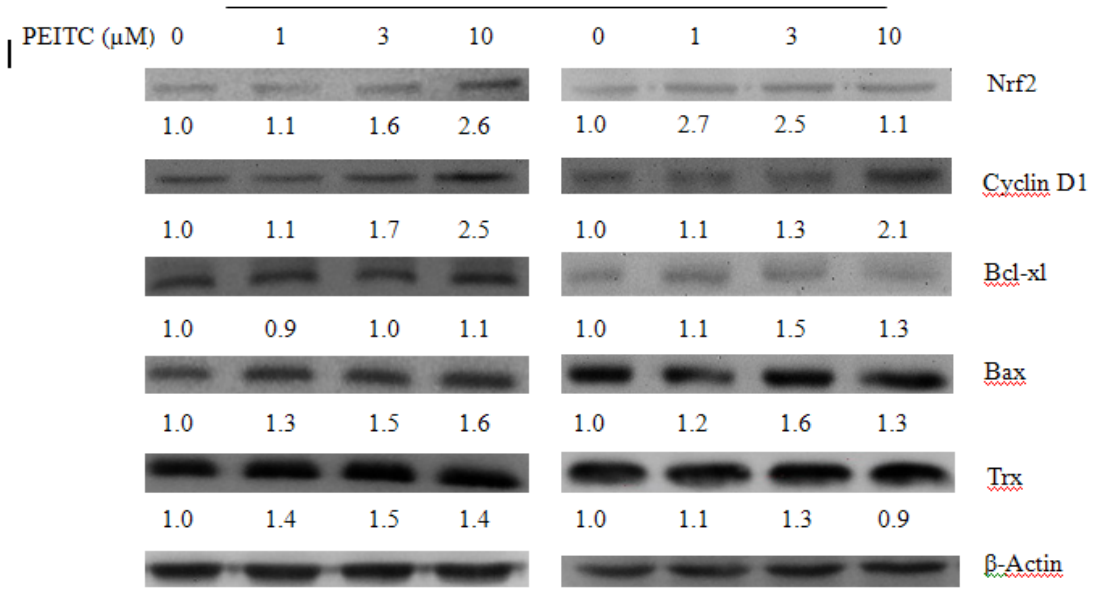

Figure 4 Western blot analysis of antioxidant (Nrf2, Trx), proliferative (cyclinD1) and Bcl-2 family proteins (Bcl-xl, Bax) proteins. KKU-100 and Chang cells were treated with PEITC at various concentrations for 48 hours. Representative pictures are showed and values denoted were mean from 3 experiments.

Discussion

Several dietary isothiocyanates from a variety of cruciferous vegetables such as *Brussica* species, are considered to be promising chemopreventive agents. PEITC is one of the most commonly investigated isothiocyanates (ITCs). In this study we have shown that treatment of CCA cells and normal liver cells with PEITC promotes rapid cell growth arrest and apoptosis in a comparable potency. The cytotoxicity was concurrently induced with rapid dissipation of mitochondrial transmembrane potential and decline of cellular GSH.

The redox capacity of the cells can be altered due to a depletion of reduced GSH, the important antioxidant defense mechanism in the cell. The rapid loss of cellular glutathione may be associated with cell death, where our finding is consistent with recent reports demonstrating association of reactive oxygen formation, loss of GSH and induction of apoptosis by PEITC in leukemic cells (7). Our data suggest that mechanism of PEITC-mediated an early growth inhibition of CCA involves the mitochondria. The loss of mitochondrial transmembrane potential can be detected with JC-1 as early as in the first hour indicating a rapid disruption of the inner membrane of mitochondria. This effect is similar to resveratrol which was shown to inhibit uveal melanoma tumor cells proliferation and stimulate apoptosis (8). Western blot analysis showed that treatment with PEITC increased Bax accumulation in KKU-100 cells. Severe oxidative stress induces translocation of Bax or Bak causing mitochondrial outer membrane permeabilization (9) and leading to cell apoptosis. This suggests PEITC induces cell killing via activation of Bax in KKU-100 cells.

Moreover, increased levels of Nrf2 were observed in both cell lines. Upregulation of Nrf2 mediated antioxidant and pro-survival pathway protecting cells from oxidative injury, however, activation of Nrf2 indeed protects cancer cells from chemotherapeutic agents (10). Trx, a Nrf2 down-stream regulated gene was slightly increased in KKU-100 cells, but unchanged in Chang cells. Trx is thought to have anti-apoptotic effects and identified as an interacting partner of the apoptosis-signaling kinase ASK-1 (11). Thus, up-regulation levels of Trx may be an adaptive responses to resist apoptosis. Cyclin D1 was increased with PEITC treatment. It has been shown that the cancer cells exert its survival through regulation of cell cycle and subsequent events lead to cell survival. In contrast, other investigator has reported that PEITC significantly suppressed the expression of cyclin D1 (12). PEITC did not inhibit Bcl-xl which is anti-apoptotic protein but other investigator has reported different observations showing that PEITC strongly inhibited Bcl-xl expression (13).

Thus, PEITC induced apoptosis in the CCA may be at least mediated via increased cellular stress, increased Bax expression and leading to inner mitochondrial membrane disruption. This is implied that other factors play role in modulating PEITC induced cell killing. Moreover, PEITC is seemed to inhibit KKU-100 and Chang cells in a non-selective manner. Thus, this may limit its utility in use in vivo.

Conclusion

PEITC can inhibit CCA proliferation and stimulate apoptosis. The mechanism of action may involve in activation of the mitochondrial apoptotic pathway via depletion of cellular GSH. In addition, increased level of Nrf2 and Trx may be the adaptive survival responses of cancer cells. An investigation of the key cellular signaling molecules as targets of the PEITC is warranted.

Acknowledgements

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