Phenethyl Isothiocyanate-induced Apoptosis in p53-deficient PC-3 Human Prostate Cancer Cell Line Is Mediated by Extracellular Signal-regulated Kinases

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Abstract

Previous studies have suggested that p53 is required for apoptosis induction by phenethyl isothiocyanate (PEITC), which is a highly promising cancer chemopreventive agent. Here, we report that p53 is not required for PEITC-induced apoptosis in the PC-3 human prostate cancer cell line and that the PEITC-induced apoptosis is mediated by extracellular signal-regulated kinases (ERK1/2). Exposure of PC-3 cells to an apoptosis-inducing concentration of PEITC (10 μM) resulted in a rapid and sustained activation of ERK1/2 that was evident as early as 1 h after PEITC treatment and persisted for the duration of the experiment (24-h after PEITC exposure). The PEITC-mediated activation of ERK1/2 was associated with an increase in phosphorylation of its substrate Elk-1 at Ser383. The PEITC-induced activation of ERK1/2 as well as apoptosis was abolished in the presence of mitogen-activated protein/ERK kinase 1 (a kinase upstream of ERK1/2) inhibitor PD98059. Exposure of PC-3 cells to 10 μM PEITC also resulted in a time-dependent activation of p38 protein kinase that was associated with increased phosphorylation of activatin transcription factor 2 at Thr71. Even though the PEITC-induced activation of p38 protein kinase was abrogated in the presence of its specific inhibitor SB202190, inhibition of p38 protein kinase activation did not prevent PEITC-induced apoptosis. In contrast to previous reports in other cellular systems, c-Jun NH2-terminal kinases were not activated by PEITC treatment in PC-3 human prostate carcinoma cell line. In conclusion, the results of the present study indicate that p53 is not essential for PEITC-induced apoptosis and that the PEITC-induced apoptosis in PC-3 human prostate carcinoma cell line is mediated by ERKs. Thus, it seems reasonable to postulate that PEITC may be effective against tumors with normal as well as mutant p53.

Introduction

Dietary ITCs, such as PEITC, are present in rather large quantities in cruciferous vegetables including broccoli, cabbage, watercress, and so forth (1). ITCs are generated through myrosinase-catalyzed hydrolysis of corresponding glucosinolates (reviewed in Refs. 1, 2). Human epidemiological studies indicate a statistically significant inverse correlation between dietary intake of ITC-containing vegetables and the risk for prostate cancer (3, 4). The evidence for cancer-protective effect of ITCs also derives from animal studies (5–14). Chemopreventive activity for naturally occurring ITCs is unique to embryonic fibroblasts. The results of the present study clearly indicate that PEITC is a highly potent inhibitor of apoptosis in a p53-deficient cell line and that PEITC-induced apoptosis is mediated by ERKs.

Materials and Methods

Reagents. PEITC was purchased from Aldrich (Milwaukee, WI). F-12K Nutrient Mixture (Kaihn’s modification), PSN antibiotic mixture, and non-heat inactivated fetal bovine serum were from Life Technologies, Inc. (Grand Island, NY); sulforhodamine B and propidium iodide were from Sigma (St. Louis, MO); reagents for electrophoresis were from Bio-Rad (Richmond, CA); reagents for electrophoresis were from Bio-Rad (Richmond, CA); ApoAlert Annexin V-FITC apoptosis kit was from Clontech (Palo Alto, CA); and p44/42 (ERK1/2) and p38 MAPK assay kits were from Cell Signaling Technology (Beverly, MA). The MAPK inhibitors PD98059 and SB202190 were purchased from Calbiochem Technology (San Diego, CA). Antibodies against ERK1/2 were from BD Biosciences (San Diego, CA), whereas antibodies against p53, phospho-ERK1/2, p38, and phospho-p38 were from Santa Cruz Biotechnology (Santa Cruz, CA)

Cell Culture. Monolayer cultures of PC-3 cells, which were generously provided by Dr. Candace Johnson (University of Pittsburgh, Pittsburgh, PA),

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The abbreviations used are: ITC, isothiocyanate; PEITC, phenethyl isothiocyanate; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal regulated kinase; MEK, mitogen-activated protein/ERK kinase; MAPK, mitogen-activated protein kinase.

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were maintained in F-12K Nutrient Mixture supplemented with 7% (v/v) non-heat inactivated fetal bovine serum and PSN antibiotic mixture in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

**Sulforhodamine B Assay.** Effect of PEITC on survival of PC-3 cells was determined by a modified sulforhodamine B assay (22). Briefly, cells (2 × 10⁴ cells/well) were plated in 96-well microtiter plates and allowed to attach overnight. Medium was replaced with fresh complete medium containing desired concentrations of PEITC. Stock solution of PEITC was prepared in DMSO, and an equal volume of DMSO (final concentration <1%) was added to control wells. After a 24-h incubation at 37°C, a 10% (w/v) aqueous (100 µl) solution of ice-cold trichloroacetic acid was added to each well, and the incubation was continued for 1 h. Plates were washed with deionized water, allowed to air dry, and stained with 100 µl of 0.4% sulforhodamine B solution in 1% acetic acid for 15 min. Subsequently, plates were washed five times with 1% acetic acid to remove unbound dye, and the plates were allowed to air dry. A solution containing 10 mmol/liter Tris base (pH 10.5; 150 µl) was then added to each well, and the absorbance was measured at 570 nm using a Labsystems Multiskan plus plate reader.

**Determination of Apoptosis.** Apoptotic cells were quantified using ApoAlert Annexin V-FITC kit according to the manufacturer’s instructions. Briefly, PC-3 cells (10⁴) were exposed to desired concentration of PEITC in the absence or presence of PD98059 or SB202190 for 24 h at 37°C. Subsequently, both floating and adherent cells were collected. The floating cells were collected by centrifugation at 700 × g for 5 min, whereas adherent cells were harvested by trypsinization and collected by centrifugation at 700 × g for 5 min. Pooled cells were washed with the manufacturer-supplied 1 × binding buffer. Approximately 5 × 10⁶ cells were resuspended in 200 µl of manufacturer-supplied 1 × binding buffer, and mixed with 5 µl of Annexin V-FITC and 10 µl of propidium iodide. After 15 min of incubation in the dark, the cells were analyzed using a Coulter Epics XL flow cytometer.

**Western Blot Analysis, and p44/42 and p38 MAPK Assays.** PC-3 cells were treated with desired concentration of PEITC in the absence or presence of MAPK inhibitors for specified time intervals. For Western blotting, the cells were washed twice with ice-cold PBS, and lysed on ice for 40 min in a solution containing 50 mM Tris, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM Na₂VO₃, 12 mM β-glycerol phosphate, 10 mM NaF, 16 µg/ml benzamidine hydrochloride, 10 µg/ml phenanthroline, 150 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was centrifuged at 14,000 × g for 15 min, and the supernatant fraction was collected for Western blotting. Protein content in the supernatant fraction was determined by the method of Bradford (23). Supernatant fraction proteins were separated by SDS-PAGE according to the method of Laemmli (24). The proteins were transferred onto polyvinylidene fluoride membrane as described by Towbin et al. (25). After blocking for 1 h in 10% nonfat dry milk in Tris-buffered saline, the membrane was incubated with desired primary antibody for 1 h. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA) according to the manufacturer’s instructions. Each membrane was stripped and reprobed with antibodies against actin to correct for differences in protein loading. The p44/42 (ERK1/2) MAPK and p38 MAPK activities were determined using kits from Cell Signaling Technology (Beverly, MA) according to the manufacturer’s instructions.

**Results and Discussion**

As shown in Fig. 1, survival of PC-3 cells was reduced significantly by a 24-h exposure to PEITC in a concentration-dependent manner with an IC₅₀ of ~6 µM. To determine whether reduced survival of PC-3 cells in the presence of PEITC was associated with apoptosis induction, apoptotic cells were quantified in cultures of control (DMSO-treated) and PEITC-treated PC-3 cells using FITC-conjugated Annexin V kit. Annexin V is a calcium-dependent phospholipid binding protein with a high affinity for phosphatidylserine, which is a negatively charged membrane phospholipid located on the inner (cytoplasmic) surface of the plasma membrane of living cells (26). In cells undergoing apoptosis, phosphatidylserine is translocated to the outer side of the plasma membrane and becomes accessible for staining with Annexin V (27). Staining with propidium iodide, a nucleic acid binding dye that is impermeable in live cells, is indicative of necrosis. Fig. 1B depicts the percentage of apoptotic cells after a 24-h treatment of PC-3 cells to increasing concentrations of PEITC (2.5, 5, and 10 µM). As can be seen in Fig. 1B, PEITC treatment resulted in an increase in percentage of apoptotic cells in a concentration-dependent manner. In comparison with DMSO-treated control PC-3 cells where roughly 4% of the cells were apoptotic, the percentage of apoptotic cells was increased by about 1.8–7.9-fold on PEITC treatment. Previous studies have shown that the PC-3 cell line lacks normal p53 expression (28, 29), which was confirmed in the present study through Western blot analysis of PC-3 cell lysate using antibodies against p53 (data not shown). These results clearly indicated that p53 is not required for PEITC-induced apoptosis in PC-3 human prostate cancer cell line.

Previous studies have suggested that PEITC-induced apoptosis may be mediated by activation of JNK1/2 (18, 20). In contrast to these findings, we did not observe activation of JNK1 or JNK2 in PC-3 cell line exposed to 10 µM PEITC for 1, 4, 16, or 24 h (data not shown). Instead, we found that exposure of PC-3 cell line to 10 µM PEITC resulted in a rapid and sustained activation (phosphorylation) of ERK1/2 (Figs. 2A, middle panel). Activation of ERK1/2 was evident the experiment (24 h after PEITC treatment; Fig. 2, A and B). On the other hand, the expression of ERK1/2 protein was not altered by PEITC treatment (Fig. 2A, top panel). PEITC-mediated activation of ERKs was additionally confirmed by determining phosphorylation of one of its substrates (Elk-1). As shown in Fig. 2C, in comparison with control, the Ser383 phosphorylation of Elk-1 was increased by >25-fold after a 1-h exposure of PC-3 cells to 10 µM PEITC. Phosphorylation of Elk-1 was increased by ~50-fold relative to control at the 4-h time point (Fig. 2C).
induced apoptosis is mediated by ERKs. PD98059 (Fig. 3C). These results clearly indicated that PEITC-concentrations was more or less completely blocked in the presence of (30). Similar to ERKs, exposure of PC-3 cells to 10 M PEITC resulted in activation of p38 kinase that was evident at 4-h after treatment. The PEITC-mediated activation of p38 kinase persisted for the duration of the experiment (24-h after PEITC treatment). The expression of p38 kinase was not affected by PEITC (Fig. 4, A and B). Consistent with these findings, the PEITC-mediated activation of p38 protein kinase resulted in an increase in phosphorylation of its substrate ATF-2 at Thr71 (Fig. 4C).

Fig. 2. Effect of PEITC treatment on activation and expression (A and B), and kinase activity of ERKs (C) in PC-3 cells. For data in A, the cells were treated with 10 M PEITC for different time points. The control cells received an equal volume of DMSO. The cell lysates were prepared, and Western blotting was performed using antibodies against ERK1/2 and phospho-ERK1/2. B summarizes the PEITC-mediated activation of ERK1/2 relative to control after correction for differences in protein loading (reprobing the blots with antiaxin antibodies). Data are mean of two determinations. C shows the effect of PEITC treatment on kinase activity of ERKs as measured by phosphorylation of Elk-1 at Ser383. The kinase activity was determined using a kit from Cell Signaling Technology (Beverly, MA) according to the manufacturer’s instructions. Comparable data were obtained in duplicate determinations; bars, ± SD.

To experimentally verify the possible role of ERKs in PEITC-induced apoptosis, the effect of an inhibitor of MEK1 (an upstream kinase in ERK1/2 signaling pathway) on PEITC-induced apoptosis and ERK1/2 activation was determined. As shown in Fig. 3, A and B, the PEITC-mediated activation of ERKs was significantly abolished in the presence of MEK1 inhibitor PD98059 (compare Lanes 3 and 4 in Fig. 3A). Fig. 3C shows the effect of MEK1 inhibitor on PEITC-induced apoptosis. The percentage of apoptotic cells in cultures treated with DMSO (control) and 50 M MEK1 inhibitor alone were comparable. In comparison with DMSO-treated control cells, the percentage of apoptotic cells was significantly higher in cultures exposed to 5 or 10 M PEITC. The PEITC-induced apoptosis at both concentrations was more or less completely blocked in the presence of PD98059 (Fig. 3C). These results clearly indicated that PEITC-induced apoptosis is mediated by ERKs.

Studies using p38 kinase inhibitor SB202190 were carried out to determine whether activation of p38 kinase contributes to PEITC-induced apoptosis. As shown in Figs. 5, A and B, the PEITC-induced activation of p38 kinase was abolished in the presence of 25 M of the inhibitor. Interestingly, as shown in Fig. 5C, inhibiting p38 protein kinase with SB202190 did not affect the extent of apoptosis induced by 5 or 10 M PEITC.

Previous studies have shown that the mouse embryonic fibroblasts expressing wild-type p53 (p53 +/+ ) are highly sensitive to apoptosis induction by PEITC (21). The PEITC-induced apoptosis was observed after a 12-h exposure of p53 +/+ fibroblasts to 2.5–10 M concentrations (21). Interestingly, these investigators also showed that mouse embryonic fibroblasts from p53-deficient mice (p53 −/−) are resistant to PEITC-induced apoptosis suggesting that p53 may be required
for apoptosis induction by this dietary cancer-protective agent (21).

The main objective of the present study was to address the question of whether p53 dependence for apoptosis induction by PEITC is unique for the murine embryonic fibroblast system. The answer to this question has clinical implications, because tumor-suppressor p53 is mutated in roughly 50–55% of human cancers (31). We addressed the above question using a PC-3 human prostate cancer cell line that lacks normal p53 expression (28, 29). We found that the PC-3 cell line is highly sensitive to growth inhibition by PEITC. Our results also indicate that the reduced survival of PC-3 cells after exposure to PEITC is associated with apoptosis induction in a dose-dependent manner. Thus, it seems reasonable to conclude that p53 is not essential for apoptosis induction by PEITC in the PC-3 prostate cancer cell model as is the case for human leukemia cells (19).

The second goal of the present study was to elucidate the signal transduction mechanisms responsible for apoptosis induction by PEITC. Previous studies have shown that PEITC-induced apoptosis in HeLa, Jurkat, and 293 cells is associated with activation of JNKs (18). PEITC-mediated activation of JNKs has also been observed in human leukemia HL-60 cell line (20). The results of the present study indicate that PEITC-induced apoptosis in the PC-3 cell line is independent of JNK activation (data not shown). In mammalian cells, two other Ser/Thr MAP kinase cascades, ERKs and p38 protein kinase, are functional that are implicated in regulation of apoptosis in response to different stimuli (30). We found that the exposure of PC-3 cells to an apoptosis-inducing concentration of PEITC results in a rapid and sustained activation of both ERK1/2 and p38 protein kinases in a time-dependent manner. The activation of ERK1/2 was evident as early as 1 h after PEITC treatment, whereas maximal activation of p38 kinase was not observed until the 4-h time point. The PEITC-induced ERK1/2 activation as well as apoptosis was completely abolished by an inhibitor of MEK1, which is an upstream kinase in ERK1/2-signaling cascade. Interestingly, inhibition of p38 kinase did not affect PEITC-induced apoptosis even though the PEITC-mediated activation of p38 kinase was significantly inhibited. These results indicate that PEITC-induced apoptosis in PC-3 cells is mediated by ERKs but...
independent of p38 kinase. In a recent study, inhibition of benzof(a)pyrene-induced lung tumorgenesis in A/J mice by dietary N-acetyl cysteine conjugate of PEITC during the postinitiation phase was shown to be associated with activation of all three of the MAPKs (14). Thus, it seems reasonable to conclude that activation of MAPKs is an important event in chemopreventive activity of ITCs. However, the mechanism by which PEITC activates MAPKs remains to be established.

In conclusion, the present study demonstrates that: (a) PC-3 prostate cancer cell line is highly sensitive to growth inhibition by PEITC; (b) reduced survival of PC-3 cells after exposure to PEITC is associated with apoptosis induction; (c) PEITC can induce apoptosis in absence of normal p53; and (d) PEITC-induced apoptosis in the PC-3 cell line is mediated by activation of ERKs but not JNKs or p38 kinase.

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References

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