Essential Role of p53 in Phenethyl Isothiocyanate-induced Apoptosis

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Abstract

Phenethyl isothiocyanate (PEITC) is a natural product that is among the most effective cancer chemopreventive agents known. Mechanistic studies indicate that the chemopreventive activity of PEITC is associated with its favorable modification of carcinogen metabolism and its induction of apoptosis. Here, we found that PEITC blocks tumor promoter (12-O-tetradecanoylphorbol-13-acetate or epidermal growth factor)-induced cell transformation in mouse epidermal JB6 cells, and this inhibitory activity on cell transformation is correlated with induction of apoptosis. Most importantly, apoptosis induction by PEITC occurs through a p53-dependent pathway. This was demonstrated not only by results that PEITC induction of p53 protein expression and p53-dependent transactivation but also by PEITC-induced apoptosis in p53+/+ cells but not in p53−/− cells. In contrast, PEITC induced apoptosis in cells with both normal or deficient sphingomyelinase activity. Our results demonstrate for the first time that p53 elevation is required for PEITC-induced apoptosis, which may be involved in its cancer chemopreventive activity.

Introduction

p53 is one of the classical tumor suppressor genes that interferes with cell transformation events (1, 2). p53 also plays a critical role in cell cycle control and induction of apoptosis (3, 4). It is elevated in response to genotoxic agents, such as ionizing radiation, UV light, or chemicals (5, 6).

Apoptosis, or programmed cell death, has been characterized as a fundamental cellular mechanism that occurs under a range of physiological and pathological conditions (5–9). It plays an essential role as a protective mechanism against neoplastic development in the organism by eliminating genetically damaged or excess cells that have been induced improperly to divide by a mitotic stimulus (10, 11). Growing evidence from both in vitro and in vivo studies demonstrates that suppression of apoptosis is involved in tumor promotion by chemical agents. Inhibition of apoptosis by phenobarbital, peroxisome proliferators, cyproterone acetate, and dichloroacetate acid may be involved in their effect on liver carcinogenesis (12–14). The tumor promoter TPA3 inhibited apoptosis induced in cultures of C3H-10T1/2 cells by exposure to ionizing radiation, β-radiation, or acute serum deprivation (15). Therefore, suppression of apoptosis may be a feature of tumor promotion by chemical carcinogens.

PEITC, which occurs as a conjugate in certain cruciferous vegetables, has been extensively investigated for its chemopreventive activity against cancer in rats and mice (16–22). It has effectively inhibited chemically induced lung, forestomach, and esophageal tumorigenesis (16–21, 23). It has been hypothesized that the inhibitory action of PEITC is due to competitive inhibition and inactivation of enzymes, such as cytochromes P450, involved in the bioactivation of carcinogens (23–26). The chemopreventive properties of PEITC and other structurally related isothiocyanates are also associated with the induction of phase II detoxifying enzymes, including glutathione S-transferases, quinone reductase, epoxide hydrolase, and UDP-glucuronosyltransferases (22, 23–26).

Recently, it was reported that PEITC induces activation of JNK activity and that this activation is associated with its apoptosis induction (27, 28). Here, we demonstrate that PEITC also induces p53 transactivation in a dose- and time-dependent fashion and that this plays a crucial role in PEITC-induced apoptosis and its antitumor promotion effects in JB6 C1 41 cells.

Materials and Methods

Reagents. PEITC was obtained from Aldrich Chemical Co. (Milwaukee, WI), and protein G plus protein A agarose, monoclonal mouse IgG against p53 (Ab1), and polyclonal rabbit IgG against p53 (Ab10) were from Oncogene Research Products. TPA was purchased from Sigma Chemical Co.; EGF was from Collaborative Research; luciferase assay substrate was from Promega; and FBS, Eagle’s MEM, DMEM, RPMI 1640, and BME were from BioWhittaker.

Cell Culture. JB6 P+ (tumor promoter-sensitive) mouse epidermal cell line C1 41 and its stable FG13 luciferase reporter plasmid transfects, C1 41 p53 cells, were cultured in monolayers at 37°C and 5% CO2 using Eagle’s MEM containing 5% FCS, 2 mM L-glutamine, and 25 µg/ml gentamicin (29, 30). EBV-transformed normal human lymphoblast CY cells and Niemann-Pick disease MS1418 lymphoblasts (31) were maintained in a mixture of RPMI 1640 and DMEM (1:1, v/v) containing 15% FBS, 2 mM L-glutamine, and 25 mg/ml gentamicin. Normal mouse embryo fibroblasts (p53+/+) and p53−/− deficient embryo fibroblasts (p53−/−) were cultured in DMEM with 10% FBS, 2 mM L-glutamine, and 25 mg/ml gentamicin (32).

Anchorage-independent Transformation Assay. JB6 C1 41 cells (1 × 104) were exposed to TPA (10 ng/ml) or EGF (10 ng/ml) with or without different concentrations of PEITC in 1 ml of 0.33% BME agar containing 10% FBS over 3.5 ml of 0.5% BME agar medium containing 10% FBS. The cultures were maintained in a 37°C/5% CO2 incubator for 14–21 days, and the colonies were scored by the methods described (29, 33).

Assay for p53-dependent Transcription Activity. Confluent monolayers of C1 41 p53 cells were trypsinized, and 8 × 104 viable cells suspended in 100 µl of 5% FBS MEM were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO2. Twelve to 24 h later, cells were starved by culturing them in 0.1% FBS MEM for 12 h. The cells were exposed to different concentrations of PEITC for 53 induction for 24 h. The cells were extracted with lysis buffer, and luciferase activity was measured using a luminometer (Monolight 2010). The results are expressed as relative p53-dependent transcription activity (30).

Immunoprecipitation Assay. The level of p53 protein after PEITC treatment was measured by Western blot for immunoprecipitation with specific antibodies against p53. Briefly, 80% confluent JB6 C1 41 cells cultured in 5% FBS MEM were treated with PEITC (10 µM) for different times. The cells were then lysed on ice for 1 h in the lysis buffer and spun at 14,000 rpm for 5 min. The lysates were immunoprecipitated using p53 antibodies (Ab1) and protein G plus protein A agarose. The beads were washed, and the p53 protein was eluted.

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3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PEITC, phenethyl isothiocyanate; JNK, c-Jun NH2-terminal kinase; EGF, epidermal growth factor; FBS, fetal bovine serum; BME, basal medium Eagle’s; SMase, sphingomyelinase; NNNK, 4-(methyltritosaminio)-1(3-pyridyl)-1-butanone.

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was selectively measured by Western immunoblotting using a chemiluminescence detection system and specific p53 antibodies (Ab10).

DNA Fragmentation Assay. Cells were treated with different concentrations of PEITC for 24 h. All of the cells were harvested by centrifugation and lysed with a lysis buffer (5 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.5% Triton X-100) on ice for 45 min. Fragmented DNA in the supernatant of a 14,000 rpm centrifugation (30 min at 4°C) was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and once with chloroform and precipitated by ethanol and salt. The DNA pellet was washed once with 70% ethanol and resuspended in Tris-EDTA buffer (pH 8.0) with 100 ng/ml RNase A, ranging from 2.5 to 10 μM (Fig. 1).

Results

Inhibition of TPA- and EGF-induced Cell Transformation by PEITC. We used the JB6 Cl 41 cell line, a well-characterized late-stage tumor promotion cell culture model, to investigate the effects of PEITC. As shown in Fig. 1, PEITC inhibits tumor promoter (TPA or EGF)-induced cell transformation in a dose-dependent manner, ranging from 2.5 to 10 μM (Fig. 1).

Induction of Apoptosis in JB6 Cl 41 Cells by PEITC. Inhibition of apoptosis is one of the mechanisms of tumor formation and chemopreventive agents may act through the induction of apoptosis to block the carcinogenesis process (14, 15, 34, 35). We, therefore, hypothesized that the induction of apoptosis may be involved in the anticarcinogenic effect of PEITC. To test this, we treated JB6 Cl 41 cells with different concentrations of PEITC. The results from DNA fragmentation assays show that treatment of cells with PEITC induced apoptosis in the Cl 41 cells in the same dose range at which it inhibited cell transformation (Fig. 2). These results support our notion that the cancer-preventive effect of PEITC may occur partially through the induction of apoptosis. During this study, apoptosis induction by PEITC was also reported in HeLa cells and HT 1080 cells by others (27).

No Difference in Apoptosis Induction between a Cell Line with Wild-Type SMase and a SMase-deficient Cell Line. A cell will undergo apoptosis as a result of information received from its environment and interpreted in the context of internal information (36). Although the intracellular signals involved in induction of apoptosis are often involved in promotion of proliferation or differentiation in other cellular contexts, some pathways appear to be of particular significance in the control of cell apoptosis. It is known that ceramide and p53 are of particular importance in the induction of apoptosis in many instances (36–38). Ceramide and/or SMase activity is required for many agents such as tumor necrosis factor-α, IFN, or γ-irradiation to induce apoptosis (11–13, 31, 38). To assess the role of ceramide/SMase in PEITC-induced apoptosis, we used a SMase-deficient lymphoblast cell line, MS1418, and a normal lymphoblast control cell line, JY (31). The results show that PEITC induced apoptosis in both cell lines in the same dose ranges (Fig. 3). These data rule out the involvement of ceramide/SMase in PEITC-induced apoptosis.

Treatment of JB6 Cells with PEITC Results in p53 Transactivation and Expression. Normal p53 function is thought to be crucial in the induction of apoptosis in human and murine cells following DNA damage (39, 40). This notion was further supported by the findings that p53 is the most commonly mutated tumor suppressor gene and that lack of p53 expression or function is associated with an increased risk of tumor formation (41–43). Apoptosis of thymocytes and intestinal crypt cells following irradiation was almost completely blocked in p53-deficient mice (40), and incidence of spontaneous tumors in p53+/− mice was high and occurred rapidly (44). To investigate the possible role of p53 in the induction of apoptosis by PEITC, we analyzed the influence of PEITC on p53-dependent transcription activity and p53 protein expression in PG13-lucIFRase JB6 cell transfectants (30). We found that PEITC markedly activates the p53-dependent transcription activity in a dose-dependent manner (Fig. 4A). The dose ranges of PEITC for transactivating p53 activity are consistent with those for their induction of apoptosis and inhibition of
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Chinese cabbage (16–23). Here, we investigated the effect of PEITC in a mouse epidermal tumor promotion cell culture model. The results show that PEITC blocks tumor promoter (EGF or TPA)-induced cell transformation and induces cell apoptosis at the same dose, ranging from 9 to 24 h after the cells were exposed to PEITC (Fig. 4B). Treatment of cells with PEITC also led to an elevated level of p53 protein (Fig. 4C). These results indicate that induction of p53 protein by PEITC is at least partially responsible for increasing p53-dependent transcriptional activity, suggesting that increased p53 protein and p53-dependent transcriptional activity may be associated with PEITC-induced apoptosis. We also noticed that induction folds of p53 protein appear to be greater than the effect of PEITC on p53-dependent transcription activity. This may be due to other mechanisms, such as p53 phosphorylation, and cofactors are also involved in regulation of transcription factor activity.

Activation of p53 Is Required for PEITC-induced Apoptosis. Our results suggested the possible role of p53 transactivation in PEITC-induced apoptosis. To obtain direct evidence for the involvement of p53 in PEITC-induced apoptosis, we used two fibroblast cell lines (p53 +/+, p53 −/−) that were derived from mouse embryos that either contained wild-type p53 (p53 +/+) or were p53-deficient (p53 −/−), as reported previously (32). The results show that p53 −/− fibroblasts failed to respond to PEITC with apoptosis induction, whereas apoptosis was observed in p53 +/+ cells treated with PEITC (Fig. 5). These results provide direct definitive evidence for the requirement of p53 in PEITC-induced apoptosis.

Discussion

Many classes of cancer chemopreventive agents, including naturally occurring and pharmaceutical compounds, are being studied for efficacy in vivo and in vitro. Among the most extensively investigated of them is PEITC, which occurs naturally as a glucosinolate in a variety of cruciferous vegetables, such as watercress, turnips, and...
Apoptosis is a crucial element in the behavior of mammalian cells in many different situations. It is believed that apoptosis is responsible for the deletion of unwanted cells during organ and tissue development and pathologically induced tissue damage as well as excess cells that have improperly been induced to divide by a mitotic stimulus (10, 11). In addition, apoptosis of individual cells may represent a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells (10, 11). A cell will undergo apoptosis as a result of signals received from its environment, interpreted in the context of internal signals. External signals trigger apoptosis through a signal transduction pathway, which may involve the stimulation of the receptor, the activation of protein kinase/phosphatase cascades, and the release of second messengers to up-regulate or suppress the transcription of specific genes (50). Although the great variety of external signals that can control apoptosis means that many signaling pathways can be involved, p53 and ceramide are the two most extensively investigated pathways (36–38). Ceramide and/or SMase activity is required for many agents such as tumor necrosis factor-α, IFN, and γ-irradiation to induce apoptosis (10, 11, 38). Normal p53 function was shown to be crucial in the induction of apoptosis in human and murine cells following DNA damage (50). This notion was supported by the findings that p53 mutations occur in 50–55% of all human cancers (50) and that these mutations are strongly selected for p53 proteins that fail to bind to DNA in a sequence-specific fashion. Lack of p53 expression or function is associated with an increased risk of tumor formation (41–43). Transgenic mice expressing the SV40 large T antigen develop choroid plexus papillomas during the first 3 months of life (51). The tumor growth is slowed considerably by p53-mediated apoptosis when a T antigen mutant is used that cannot inactivate p53 function in these cells (51). When this mutant is expressed in p53 −/− mice, the rapid tumor growth resumes with less apoptosis (52). Apoptosis of intestinal crypt cells following irradiation was almost completely blocked in p53-deficient mice (40). Furthermore, incidence of spontaneous tumors in p53 −/− mice was high and occurred rapidly (44). Hence, p53-mediated apoptosis clearly is an important part of the tumor suppressor phenotype and subsequent p53 deficiency may permit a population of genetically damaged cells to escape the normal process of apoptotic deletion. Here, we found that PEITC markedly activates p53-dependent transcription activity in a dose-dependent manner. It was known that p53 was activated in response to DNA damage. The activation of p53 may be due to DNA damage and chromosomal aberrations caused by PEITC (53). We also demonstrated that PEITC induced p53 protein expression and transactivation in the same dose range as that which inhibited cell transformation and induced apoptosis in the same cell line. These results suggest that increased p53 protein and p53-dependent transcriptional activity may be associated with PEITC-induced apoptosis and antitumor activity of PEITC. The strongest evidence for the role of p53 in PEITC-induced apoptosis was provided by the study using cell lines with wild-type p53 (p53 +/+ ) or p53 deficiency (p53 −/− ). Apoptosis induction by PEITC was only observed in p53 +/+ cells, not in p53 −/− cells, whereas apoptosis was seen in both the SMase-deficient cell line and the cell line with wild-type SMase.

In summary, we demonstrate here that PEITC induces apoptosis in the same concentration range at which it inhibits cell transformation. PEITC-induced apoptosis occurs through a p53-dependent, SMase-independent pathway. We suggest that the induction of apoptosis mediated by p53 is a possible explanation for the antitumor promotion effect of PEITC in the JB6 cell model.
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References

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