Bax Translocation to Mitochondria Is an Important Event in Inducing Apoptotic Cell Death by Indole-3-Carbinol (I3C) Treatment of Breast Cancer Cells

Fazlul H. Sarkar, K. M. Wahidur Rahman and Yiwei Li

Department of Pathology, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201

ABSTRACT Indole-3-carbinol (I3C), a natural component of Brassica vegetables, has been found to be a promising cancer preventive agent. However, the precise molecular mechanism(s) by which I3C exerts its inhibitory effects on cancer cells has not been fully elucidated. We investigated the molecular mechanism of action of I3C during apoptotic processes in breast epithelial cells. Nontumorigenic and tumorigenic breast epithelial cells were exposed to I3C, and growth inhibition, apoptosis and expression of genes involved in apoptotic processes were measured. Translocation of Bax to the mitochondria was accessed by confocal imaging. Mitochondrial potential and cytochrome c release also were measured. We found that I3C inhibited the growth of breast cancer cells and induced apoptosis in these cells, concomitant with upregulation of Bax, and downregulation of Bcl-2. I3C induced translocation of Bax to the mitochondria in both tumorigenic and nontumorigenic cells, but concomitant loss of mitochondrial potential, release of cytochrome c and induction of apoptosis were observed only in cancer cells. In conclusion, I3C exerts its effects by regulating cell cycle and by altering the expression of genes involved in apoptotic pathway. The translocation of Bax to the mitochondria alone is not sufficient during I3C-induced apoptosis. Translocation of Bax followed by mitochondrial depolarization and cytochrome c release is necessary, which may be responsible for selective induction of apoptosis in cancer cells, supporting the potential preventive and/or therapeutic benefit of I3C against cancers.


KEY WORDS: • Bax translocation • apoptosis • I3C

Indole-3-carbinol (I3C), a compound found in high concentrations in Brassica vegetables, has been known to possess anticarcinogenic effects in experimental animals (1,2). The interest in I3C, as a cancer chemopreventive agent, has increased significantly in the past 10 y. Most in vivo and in vitro studies on I3C have shown the inhibitory effects of I3C on cancer cells (1–4). Administration of I3C to mice has been shown to reduce the incidence of spontaneous mammary tumor formation (5) and I3C also has been shown to greatly decrease the incidence of mammary tumor in mice (6). One mechanism by which I3C may inhibit carcinogenesis is through the induction of enzymes, such as cytochrome P-450-dependent monoxygenases, glutathione S-transferases (GST) or epoxide hydrolases (EH), which metabolize carcinogens to more polar and excretable forms (4,5,7,8). The studies from our laboratories and others have shown that I3C inhibits cell growth, arrests cell cycle at the G1 checkpoint and induces apoptosis in breast, prostate and other cancer cells (9–15). However, the precise molecular mechanism(s) by which I3C exerts its inhibitory effects on cancer cells has not been fully elucidated.

There are several important molecules involved in the apoptotic process. The Bcl-2 family (e.g., Bax, Bcl-2, Bcl-XL, etc.) has been reported to play a major role in determining whether cells will undergo apoptosis (16). Bax induces apoptosis, whereas Bcl-2 and Bcl-XL inhibit apoptosis (16–19). It has been reported that the ratio of Bax:Bcl-2 (16,20), rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis in cancer cells. Moreover, the subcellular location of Bax protein has been found to be important in the apoptotic processes. Bax is generally sequestered in the cytosol and removed from the cytosol into mitochondria with large aggregates during apoptosis (21–23). Bax translocation into mitochondria targets the mitochondrial intermembrane contact sites and releases cytochrome c (24,25). Bax is then packaged into large aggregates on mitochondria. The release of cytochrome c from the mitochondria cleaves and activates caspase-3 and caspase-9, resulting in the sequence of apoptotic processes (22–24).

Breast cancer is the most common cancer in women and remains the second leading cause of cancer-related female...
deaths in the U.S. (26). Thus, there is a tremendous need for the development of mechanism-based and targeted preventive and therapeutic strategies for breast cancer. The facts that I3C inhibits the growth of cancer cells and induces apoptosis may make it a potent agent against breast cancer. In the present investigation, we have examined the effect of I3C on breast cancer cells and the molecular changes associated with I3C-induced apoptotic processes. We found that Bax translocation from cytosol into mitochondria, mitochondrial depolarization and release of cytochrome c might be one of the molecular mechanism(s) by which I3C induces apoptosis and inhibits breast cancer growth. However, translocation of Bax into mitochondria is not sufficient for the induction of apoptotic processes as demonstrated by our studies.

**MATERIALS AND METHODS**

**Cell lines and cell culture**

The human breast epithelial cell lines, MCF10A (nontumorigenic) and MCF10CA1a (cancer cells) were obtained from the Karmanos Cancer Institute in Detroit, MI. MCF10A is a spontaneously immortalized human breast epithelial cell line that was derived without viral or chemical intervention from immortal diploid human breast epithelial cells containing wild-type p53. MCF10CA1a (CA1a) cells were cancer cells and derived from MCF10AneoT model system. MCF10AneoT is a cell line derived from MCF10A by transfecting mutated H-ras oncogene and has a transformed phenotype. It is a transplantable, xenograft model of human proliferative breast disease with proven neoplastic potential and commonly considered a pre-malignant human breast epithelial cell line. MCF10CA1a cells were cultured in DMEM/F-12 (1:1) media (Invitrogen, Carlsbad, CA) supplemented with 5% horse serum (Invitrogen), 2 mmol/L L-glutamine, 1% penicillin and streptomycin in a 5% CO2 atmosphere at 37°C. MCF10A cells were cultured in the above media plus 1 mg/L insulin, 0.1 mg/L cholera toxin, 0.5 mg/L hydrocortisone (Sigma, St. Louis, MO), 0.5 mg/L fungzone and 0.02 mg/L EGF (Invitrogen).

**Cell growth inhibition**

The MCF10A and CA1a cells were seeded at a density of 5 x 10^4 well in a six-well culture dish. After 24 h, the cells were treated with 15, 30, 60 or 100 μmol/L of I3C (Sigma) or 0.1% DMSO (vehicle control). These concentrations were chosen based on previously published reports showing the effect of I3C on breast cancer cells. Cells treated with I3C or DMSO for 1–3 days were harvested by trypsinization, stained with 0.4% trypan blue, and viable cells were counted by using a hemocytometer.

**Flow cytometry for detecting apoptosis**

7-Amino actinomycin D (7AAD) staining and flow cytometry was conducted to detect and quantify apoptosis. Cells treated with 60 or 100 μmol/L I3C for 24, 48, 72 h or with DMSO for 72 h (as control) were subjected to this analysis. Briefly, 7AAD (Calbiochem-Novabiochem, La Jolla, CA) was dissolved in acetone and diluted in PBS to a concentration of 200 μg/mL. A total of 100 μL of 7AAD solution was added to 10^6 cells suspended in 1 mL of PBS and mixed thoroughly. Cells were stained for 20 min at 4°C while protected from light and pelleted by centrifugation. The cells were resuspended in 500 μL of 1% PBS-BSA solution. Unstained fixed cells were used as negative control. Samples were analyzed on a FACScan (Becton, Dickinson, CA) within 30 min of fixation. Data on 10,000 and 20,000 cells were acquired and processed using Lyssys II software (Becton). Scattergrams were generated by combining forward light scatter with 7-AAD fluorescence, and regions were drawn around clear-cut populations having negative, dim and bright fluorescence. The frequency of cells with low, medium and high 7-AAD fluorescence was assessed. The purity and enrichment of the sorted populations were then calculated.

**Western blot analysis**

MCF10A and CA1a cells were plated and cultured in complete medium and allowed to attach for 24 h before the addition of 60 or 100 μmol/L of I3C and incubated for 24, 48 and 72 h. Control cells were incubated in the medium containing 0.1% DMSO for the same time points. After incubation, the cells were lysed in 62.5 mmol/L Tris-HCl and 2% SDS. Protein concentration was then measured using BCA protein assay (Pierce). Cell extracts were subjected to 12% SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane. Membranes were incubated with monoclonal Bcl-2 (1:500, Oncogene, Cambridge, MA), Bax (1:500, Trevigen, Gaithersburg, MD), and rabbit polyclonal β-actin (1:5000, Sigma) antibodies, washed with TTBS and incubated with secondary antibodies conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce).

**Measurement of loss of mitochondrial potential by JC-1 staining**

The 5 x 10^4 cells were plated on glass cover slips in each well of a six-well plate. The cells were treated with 30, 60 or 100 μmol/L of I3C for 6, 12, 24, 48 and 72 h. After treatment, the cells were fixed in ice-cold 100% methanol for 10 min and washed in PBS three times. The nonspecific binding was blocked with 0.2% BSA in PBS for 45 min. The cells were incubated with Bax antibody (1 mg/L, Trevigen) in PBS-0.1% saponin solution for 2 h and washed with PBS-0.1% saponin solution three times. After washing, the cells were incubated with secondary fluorescent goat anti-mouse antibody (Alexa Fluor 488, 1 mg/L, Molecular Probes, Eugene, OR) in PBS-0.1% saponin and 5% serum for 1 h and washed thereafter with PBS-0.1% saponin. They were next fixed with 100% methanol and washed three times with PBS before being mounted in antifade solution (Molecular Probes) on slides, and the fluorescence images were captured with Zeiss Laser Scanning Inverted Confocal Microscope System 310 with a 63 x/1.2 oil immersion objective.
fraction (for Bax) isolated from control and I3C treated cells were loaded on a 12% SDS-PAGE gel and then, following a standard Western blot procedure, the membranes were probed with cytochrome c antibody (1:100, Clontech), Bax antibody (1 μg/mL, Trevigen) or COX-4 antibody (1:500, Clontech).

Densitometric analysis

Autoradiograms of Western blots were scanned with Gel Doc 1000 image scanner (Bio-Rad, Hercules, CA). The bidimensional optical density (O.D.) of Bcl-2, Bax, cytochrome c and actin proteins on the films were quantified and analyzed with Molecular Analyst software (Bio-Rad). The ratio of Bax/Bcl-2 (total), cytochrome c/β-actin (cytosolic) and Bax/COX-4 (mitochondrial) were calculated by standardizing the ratios of each control to the unit value.

RESULTS

Cell growth inhibition

The effect of I3C on the growth of MCF10A and CA1a cells are depicted in Figure 1. The treatment of MCF10A and CA1a cells with 15, 30, 60 and 100 μmol/L of I3C for 24–72 h resulted in dose- and time-dependent inhibition of cell proliferation. There appeared to be more pronounced growth inhibition by low concentration of I3C in CA1a cells than in MCF10A cells. The degree of cell growth inhibition was lower in MCF10A nontumorigenic cell lines with 60 and 100 μmol/L of I3C as compared to CA1a cells (Fig. 1). The proliferation of CA1a cells was significantly inhibited by 100 μmol/L of I3C treatment for 48 and 72 h as compared to MCF10A cells. This inhibition of cell proliferation could be due to cell cycle arrest or induction of apoptotic cell death induced by I3C. Therefore, we investigated whether I3C could induce apoptosis in these cells.

Induction of apoptosis

The apoptosis was measured in both cell lines treated with 60 and 100 μmol/L of I3C by Western blot analysis of PARP. The PARP cleavage analysis showed that the full size PARP (116 KD) was cleaved to yield an 85 KD fragment after treatment with I3C in both cell lines (Fig. 2). However, the cleaved fragment was much more pronounced in CA1a cells as compared to MCF10A cells in accordance with the data of cell growth inhibition. To quantify the apoptotic cells induced by I3C, flow cytometric analysis with 7-amino actinomycin D (7-AAD) staining was conducted. The results showed an increased number of apoptotic cells (~84% apoptotic cells, Fig. 3) in CA1a cells treated with 60 μmol/L I3C for 72 h as compared to untreated control (16% apoptotic cells). In contrast, apoptotic cells were increased only twofold in MCF10A cells with 72 h of I3C treatment (Fig. 3), suggesting that the degree of apoptotic cell death is much more
pronounced in breast cancer cells as compared to non-tumorigenic breast epithelial cells. To explore the molecular mechanism(s) by which I3C induces apoptosis, we further investigated the alterations in the expression of selected genes, which are involved in the complex processes of apoptotic cell death.

**Expression of Bax and Bcl-2**

The effects of I3C on Bcl-2 and Bax expression in MCF10A and CA1a cells were studied by Western blot analysis. The expression of Bcl-2 in I3C treated CA1a cells was found to be significantly downregulated (Fig. 4). No significant changes in the expression of Bcl-2 were found in I3C treated MCF10A cells. Optical density measurement showed a significant increase in the ratio of Bax to Bcl-2 protein expression in CA1a cells treated with 60 or 100 μmol/L I3C for 48 h (Fig. 4), and no such induction was observed in MCF10A cells, which is likely due to no changes in Bcl-2 protein expression. These results suggest that I3C can inhibit the anti-apoptotic activity of Bcl-2, and that increased ratio of Bax/Bcl-2 may contribute to the induction of apoptotic processes in I3C-treated breast cancer cells. Therefore, we focused our investigation on mitochondrial translocation of Bax, which is known to play important roles in the regulation of apoptotic processes.

**Mitochondrial translocation of Bax**

Treatment of cells with 30, 60 and 100 μmol/L of I3C for 6, 12, 24, 48 and 72 h resulted in a cellular redistribution of Bax as shown by immunostaining and confocal imaging. Bax had a diffuse pattern in untreated cells, and became a more punctate pattern in MCF10A and CA1a cells within 6 and 12 h after the treatment with 60 μmol/L of I3C (Fig. 5). The punctate staining of Bax clearly suggested its mitochondrial translocation after I3C treatment, providing strong evidence that the translocation of Bax to mitochondria in I3C treated cells may lead to the induction of apoptotic processes. However, the question remains why I3C treated MCF10A cells do not show significant apoptosis, even though Bax appears to be translocated to the mitochondria. It is well known that the mitochondrial translocation of Bax leads to the loss of mitochondrial potential. Hence, we sought to investigate whether I3C treatment could result in the loss of mitochondrial potential in these cells.

**Loss of mitochondrial potential**

JC-1 staining was conducted to demonstrate changes in the mitochondrial potential. The dye, JC-1, is taken up by hyperpolarized mitochondria within the cell, but in depolarized mitochondria it remains within the cytosol. The mitochondrial population with varying potential could be detected by flow cytometric analysis of JC-1 stained cells due to different energetic states of mitochondria. There was a significant (p = 0.01) loss of mitochondrial potential that occurred at 6 h after 60 or 100 μmol/L of I3C treatment of CA1a cells (Fig. 6). In contrast, there was no significant loss of mitochondrial potential when MCF10A cells were treated with 60 or 100 μmol/L of I3C. However, these results do not fully explain why I3C treated MCF10A cells do not show significant apoptosis, even though Bax appears to be translocated to the mitochondria.
Cytochrome c release induced by I3C

Cytochrome c release was significantly (*: p = 0.04) increased in CA1a cells within 6 h of treatment with I3C (Fig. 7). In contrast, MCF10A cells treated with I3C failed to release cytochrome c in the cytoplasm (Fig. 7), providing further direct evidence for the differential effects of I3C on apoptotic processes in tumorigenic and nontumorigenic breast epithelial cells. In addition, we found significantly increased levels of mitochondria-associated Bax (*: p < 0.05) in CA1a cells treated with 60 or 100 µmol/L of I3C for 6 h as compared to MCF10A cells when corrected for cytochrome c oxidase (COX-4) levels (Fig. 7).

DISCUSSION

There are growing in vitro and in vivo evidences that support the anticancer activity of I3C (1,2,4,27). We previously reported that I3C inhibited the cell growth of breast (MDA-MB-435) and prostate (PC3) cancer cells with cell cycle arrest in G1 phase (9–11). We also found that I3C induced apoptosis in MDA-MB-435 and PC3 cells with upregulation of Bax and p21WAF1, and downregulation of Bcl-2 (9–11). In this study, we observed that I3C inhibited cell proliferation more significantly in CA1a breast cancer cells than in MCF10A nontumorigenic breast epithelial cells. Similarly, we found that I3C induced much more pronounced apoptosis in CA1a cells than in MCF10A cells, suggesting that I3C selectively exerts its inhibitory effects on breast cancer cells. Moreover, the analysis of gene expression in protein level also revealed that I3C altered the expression of genes critically involved in apoptotic processes in breast cancer cells. The major apoptotic signal transduction cascades associated with programmed cell death include the proteins of Bcl-2 family. The members of this group of proteins either promote cell survival (e.g., Bcl-2 and Bcl-xL) or induce programmed cell death (e.g., Bax) (16,18). In this study, significant downregulation of Bcl-2 was observed in CA1a breast cancer cells treated with I3C, suggesting that I3C regulated the expression of Bcl-2 family more selectively in breast cancer cells as compared to nontumorigenic breast epithelial cells. Because Bcl-2 is thought to inhibit apoptosis by forming heterodimers with Bax, the downregulation of Bcl-2 causes the increased ratio of Bax/Bcl-2. This may induce apoptosis in cancer cells. Thus, downregulation of Bcl-2 may be one of the mechanism(s) by which I3C induces apoptosis in cancer cells.

Bax translocation from cytosol into mitochondria has been known as a critical event that occurs during apoptotic processes (21,22). In this study, we observed translocation of Bax from cytosol into mitochondria in both MCF10A nontumorigenic and MCF10CA1a cancer cells treated with I3C. However, no significant apoptosis was observed in MCF10A nontumorigenic cells treated with I3C, suggesting that Bax translocation from cytosol into mitochondria alone is not sufficient to induce apoptotic cell death. It has been reported that the translocation of Bax from cytosol into mitochondria targets the mitochondrial intermembrane contact sites, causing the mitochondrial permeability transition, loss of mitochondrial potential, release of cytochrome c, subsequent activation of caspases and DNA fragmentation, resulting in apoptotic cell death (22–25). We found that I3C failed to induce the loss of mitochondrial potential and the release of cytochrome c in MCF10A nontumorigenic cells, even though Bax was translocated from cytosol into mitochondria upon I3C treatment, suggesting that I3C-induced loss of mitochondrial potential is a more important and direct event for the release of cytochrome c and induction of apoptosis in cancer cells. The fact that I3C selectively induces the loss of mitochondrial potential, the release of cytochrome c, and apoptosis in CA1a breast cancer cells rather than MCF10A nontumorigenic cells, makes I3C an ideal agent for preventive and/or therapeutic purposes against breast cancer.
Ak and NF-κB pathways are two important cell-signaling pathways that are related to the Bcl-2 family and apoptotic processes. Ak exerts its antiapoptotic effects through several downstream targets, including the proapoptotic Bcl-2 family member Bad, Forkhead transcription factors and the cyclc AMP response element-binding protein (CREB) (28–30). It has been shown that Ak inhibits a conformational change in Bax protein and prevents its translocation into mitochondria, thus inhibiting the disruption of the mitochondrial inner membrane potential, caspase-3 activation, and apoptosis (31,32), and that the inactivation of Ak will facilitate the translocation of Bax from cytosol to mitochondria. It has been reported that NF-κB exerts its antiapoptotic function in these cells through the control of the Bcl-2 family of proteins (33,34). The studies from our laboratory have shown that I3C inhibits Ak kinase activity and NF-κB activity in I3C treated prostate cancer cells (9). Thus, the Bax translocation from cytosol to mitochondria in breast cancer cells treated with I3C may be induced through the inhibition of the Ak pathway. The induction of apoptosis in I3C treated breast cancer cells also may be mediated by the inhibition of NF-κB pathway and the regulation of the Bcl-2 family of protein. Further investigations are needed to establish the relationship between the Ak pathway and Bax translocation in our system.

In conclusion, our data provide some important evidences in favor of I3C-induced apoptosis in breast cancer cells that are mediated through regulation of the Bcl-2 protein family, induction of Bax translocation, loss of mitochondrial potential and release of cytochrome c. This may be the molecular mechanisms by which I3C inhibits cell growth and induces apoptosis in breast cancer cells. However, more studies are needed to address the differences on the effects of I3C between breast cancer cells and nontumorigenic breast epithelial cells, and to delineate the molecular mechanism of action of I3C against breast cancer. Finally, we hope that the tools of genomics and proteomics will play an important role in facilitating such discoveries.

LITERATURE CITED