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Research Article

Indole-3-carbinol enhances ultraviolet B-induced apoptosis by sensitizing human melanoma cells

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Abstract. Indole-3-carbinol (I3C) has been found to act against several types of cancer, while ultraviolet B (UVB) is known to induce the apoptosis of human melanoma cells. Here, we investigated whether I3C can sensitize G361 human melanoma cells to UVB-induced apoptosis. We examined the effects of combined I3C and UVB (I3C/UVB) at various dosages. I3C (200 μM)/UVB (50 mJ/cm^2) synergistically reduced melanoma cell viability, whereas I3C (200 μM) or UVB (50 mJ/cm^2), separately, had little effect on cell viability. DNA fragmentation assays indicated that I3C/UVB induced apoptosis. Further results show that I3C/UVB activates caspase-8, -3, and Bid and causes the cleavage of poly(ADP-ribose) polymerase. Moreover, I3C decreased the expression of the anti-apoptotic protein, Bcl-2, whereas UVB increased the translocation of Bax to mitochondria. Thus, an increased Bax/Bcl-2 ratio by I3C/UVB may result in melanoma apoptosis. In conclusion, our study demonstrated that I3C sensitizes human melanoma cells by down-regulating Bcl-2.

Keywords. Ultraviolet B, indole-3-carbinol, melanoma, apoptosis, cancer.

Introduction

Natural chemopreventives are gaining interest in cancer prevention. Dietary components, such as, indole-3-carbinol (I3C), isoflavones, curcumin, apigenin, and (–)-epigallocatechin-3-gallate (EGCG) inhibit the carcinogenic process, and thus reduce the risk of many types of cancer [1]. I3C is a major indole metabolite and is found in cruciferous vegetables (cabbage, broccoli, Brussels sprouts, and cauliflower), and has been found to inhibit cancer cell growth and to induce apoptosis in breast and prostate cancer cells [2]. In addition, I3C has been reported to prevent cervical and skin cancers in human papilloma virus type 16 (HPV16) transgenic mice [3]. Because I3C has been reported to have apoptotic effects in several human cancer cells [4, 5], we investigated its effects on malignant human melanoma cells.

Malignant melanoma is a type of skin cancer and is considered a refractory disease [6]. The incidence of melanoma is increasing and is associated with high mortality because of its rapid metastasis [7]. Usually, chemotherapy or radiotherapy is used to treat melanoma and, recently, combined chemo- and radiotherapy were proposed to have a synergistic effect: for example, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) combined with ionizing radiation [8], or tumor suppressor gene combined with ultraviolet B (UVB) [9, 10]. However, high doses of ionizing radiation or UV have adverse effects that lead to the destruction of normal cells and tissue.

Epidemiological evidence indicates that solar UV is a primary risk factor for melanoma [11] and, thus, UVB-induced DNA damage is generally believed to play a

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critical role in the pathogenesis of melanoma, because DNA damage is known to lead to mutations and carcinogenesis. To remove mutated cells, apoptotic pathways are also activated after UVB exposure, when DNA repair is not possible. Moreover, UVB-induced apoptosis involves a highly complex mechanism. Nuclear DNA damage is considered a major factor in UVB-induced apoptosis, although UVB has also been reported to be an activator of death receptors, such as CD95 (Fas) [12]. In addition, UVB generates reactive oxygen species (ROS), which also play important roles in the execution of apoptotic cell death [12]. Furthermore, we previously suggested that the UVB-induced apoptotic cell death of human melanocytes is the result of Bax elevation and redistribution [13].

In the present study, the effects of I3C on human melanoma cell death were tested in the presence of low-dose UVB, which does not induce apoptosis but may function as a carcinogen. To reduce possible side effects, low doses of I3C were used. Furthermore, we examined changes in the expressions of pro-apoptotic Bax and anti-apoptotic Bcl-2 levels after I3C/UVB treatment. In addition, we investigated the apoptotic mechanism involved with respect to caspase-8 and -3 activation and poly(ADP-ribose) polymerase (PARP) cleavage.

Material and methods

Materials. I3C was obtained from Sigma (St. Louis, Mo.) and DCFH-DA (2,7-dichlorofluorescein diacetate) was from Calbiochem (San Diego, Calif.). Antibody for cleaved caspase-3 (no. 9661) was purchased from Cell Signaling Technology (Beverly, Mass.) and antibodies recognizing caspase-8 (sc-7890), Bax (sc-526), Bcl-2 (sc-7382), and actin (I-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-Bid antibody (AF860) was obtained from R&D Systems (Minneapolis, Minn.), and anti-PARP antibody from BD Pharmingen (San Diego, Calif.).

Cell cultures

G361 (ATCC, Rockville, Md.) and SK-MEL-2 human melanoma cells were grown in RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin (10,000 U/ml and 10,000 µg/ml, respectively) in 5% CO_2 at 37 °C. Normal human fibroblasts and melanocytes were isolated from human foreskins obtained from child circumcisions. Isolated fibroblasts were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (10,000 U/ml and 10,000 µg/ml, respectively) in 5% CO_2 at 37 °C. Melanocytes were cultured in modified MCDB 153 (Sigma), supplemented with 5% FBS (Hyclone, Logan, Ut.), 13 µg/ml bovine pituitary extract (Gibco BRL, Gaithersburg, Md.), 10 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (Sigma), 5 µg/ml insulin (Sigma), 0.5 µg/ml transferrin (Sigma), 1 µg/ml tocopherol (Sigma), 0.5 µg/ml hydrocortisone (Sigma), 1 ng/ml human recombinant basic fibroblast growth factor (Sigma), and 1% penicillin-streptomycin (10,000 U/ml and 10,000 µg/ml, respectively) (Gibco BRL).

Detection of cell death, and microscopy. Cell viabilities were assessed using crystal violet assays [14]. After removing culture medium, cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature (RT) and then rinsed four times. The crystal violet retained by adherent cells was extracted with 95% ethanol, and absorbance was determined in lysates at 590 nm using an ELISA reader (TECAN, Salzburg, Austria). Before determining cell viabilities, cells were observed under a phase contrast microscope (Olympus Optical, Tokyo, Japan) and then photographed with a CoolSNAP$_{cf}$ digital video camera system (Roper Scientific, Tucson, Ariz.) supported by RS Image software (Roper Scientific Ariz.).

I3C and UVB treatments. To examine the synergistic effects of I3C/UVB, G361 cells (1×10^6 cells/well) were seeded into six-well plates. After serum starvation for 24 h, the cells were pretreated with phenol red-free RPMI containing 0.1% BSA and various concentrations of I3C (0–200 µM). Then, cells were irradiated with UVB (0–100 mJ/cm^2). BLE-IT158 Spectronics (Westbury, N.Y.) UV bulbs were used in combination with a Kodacel filter TA401/407 (Kodak, Rochester, N.Y.) to remove wavelengths of <290 nm (UVC). UV energy was measured using a Waldmann UV meter (model no. 585100; Waldmann, Villingen-Schwenningen, Germany). After irradiation, cells were cultured for another 24 h and cell viabilities were determined using crystal violet assays [14].

Generation of free radicals by I3C. The formation of free radicals was determined using DCFH-DA, which is oxidized by free radicals to dichlorofluorescein (DCF) [15]. To activate DCFH-DA, 350 µl of a 1 mM stock of DCFH-DA in ethanol was mixed with 1.75 ml of 0.01 N NaOH and allowed to stand for 20 min before adding 17.9 ml of 25 mM sodium phosphate buffer (pH 7.2). Reaction mixtures contained this prepared DCFH-DA solution and I3C (200 µM) and were irradiated with 0–300 mJ/cm^2 of UVB. DCF absorbances were measured at RT at 490 nm using an ELISA reader.

Detection of DNA fragmentation. After serum starvation for 24 h, G361 cells were treated with I3C and UVB at the dosages described above. Twenty-four hours later, cells were harvested. Then, DNAs were isolated using genomic DNA purification kits according to the manufacturer's recommendations (Promega, Madison, Wisc.). Ten micrograms of DNA from each sample was separated by

1.9% agarose gel electrophoresis and visualized by ethidium bromide staining.

Western blot analysis. Western blot analyses were performed as described previously [16]. Briefly, after serum starvation for 24 h, G361 cells were treated with the test substances for 24 h and lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were then saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1 : 1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using enhanced chemiluminescence plus kits (Amersham International, Little Chalfont, UK).

Confocal microscopy. After serum starvation for 24 h, glass cover slips containing G361 cells were stimulated with I3C (200 μM) and/or UVB (50 mJ/cm^2). After 30 min, cells were fixed for 10 min in 10% formaldehyde, incubated for 10 min in NH_4Cl, and then for 10 min in 0.01% Triton X-100. The cells were then incubated overnight at 4 °C with an antibody directed against Bcl-2 (sc-7382; Santa Cruz Biotechnology) at 1 : 100 in PBS. After three washes in PBS, cells were incubated with Bax antibody (sc-526; Santa Cruz Biotechnology) for 2 h. After washing, the cells were further incubated with FITC-labeled secondary antibody (Santa Cruz Biotechnology) at 1 : 100 in PBS for 2 h at room temperature, and then with rhodamine-labeled secondary antibody (Santa Cruz Biotechnology) at 1 : 100 in PBS. Specimens were analyzed by confocal microscopy (LSM510; Carl Zeiss, Jena, Germany).

Statistics. Differences between results were assessed for significance using Student's t test.

Results

The effects of I3C on melanoma cell viability. To explore the effects of I3C on cell viability, G361 human melanoma cells and normal human fibroblasts were treated with various doses (0–300 μM) of I3C, and cell death levels were measured by crystal violet assay. As shown in Figure 1a, treatment with I3C significantly increased G361 cell death in a dose-dependent manner. I3C at 200 μM proved to be slightly toxic and 300 μM was highly toxic to melanoma cells, whereas I3C did not affect normal human fibroblasts up to 300 μM (Fig. 1b). Based on these results, we used 200 μM as an experimental dose for further experiments.

The combined effects of I3C and UVB. To evaluate whether low doses of I3C and UVB work synergistically, G361 cells were treated with I3C (200 μM) for 30 min and UVB (0–100 mJ/cm^2). After incubation for 24 h, cell viabilities were measured. As shown in Figure 2a, UVB at < 50 mJ/cm^2 had little effect on cell viability, and UVB at > 75 mJ/cm^2 obviously reduced cell viability. However, even low doses (< 50 mJ/cm^2) of UVB markedly reduced cell viability, in the presence of I3C.

G361 cells were then treated with various concentrations of I3C (0–200 μM) for 30 min, and then irradiated with UVB (50 mJ/cm^2). Cell death was found to be considerably enhanced in an I3C-dose dependent manner, although I3C alone had only a mild effect (Fig. 2b).

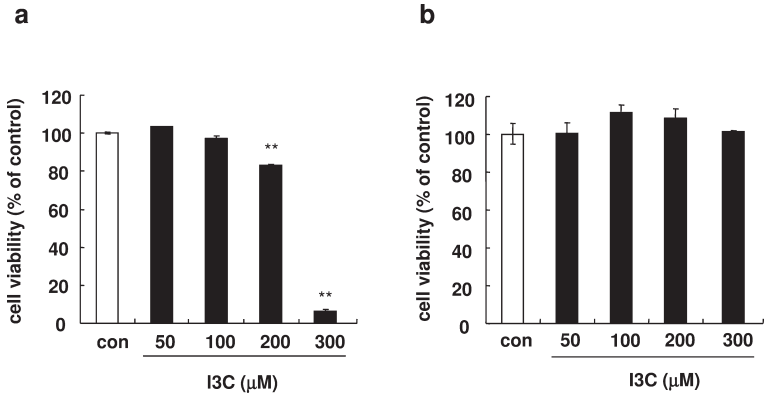

Figure 1. Effects of I3C on the viabilities of G361 cells and human fibroblasts. After serum starvation, G361 cells (*a*) and human fibroblasts (*b*) were treated with different concentrations (50, 100, 200, and 300 μM) of I3C for 24 h. Cell viabilities were measured using crystal violet assays. Data represent the means ± SD of triplicate assays expressed as percentages of the respective controls. Experiments were repeated at least twice, and representative results are shown. **$p < 0.01$ compared with the untreated control.

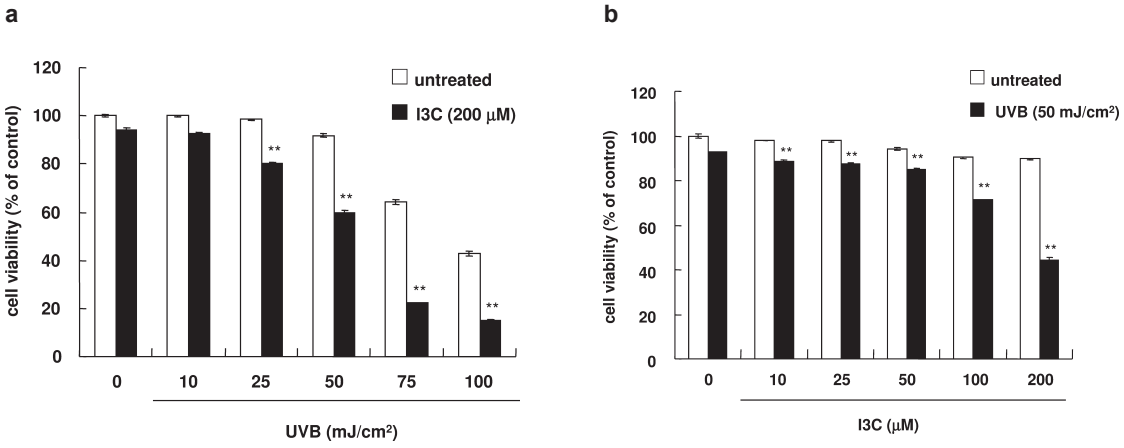

Figure 2. Effects of combined I3C/UVB treatment on cell viability. (*a*) After serum starvation, G361 cells were irradiated with different doses (10, 25, 50, 75, and 100 mJ/cm^2) of UVB in the absence (open columns) or in the presence (closed columns) of 200 μM I3C. (*b*) After serum starvation, G361 cells were treated with different concentrations (10, 25, 50, 100, and 200 μM) of I3C with (closed columns) or without (open columns) UVB (50 mJ/cm^2) treatment. Twenty-four hours later, cell viabilities were determined using crystal violet assays. Data represent the means ± SD of triplicate assays expressed as percentages of the respective controls. Experiments were repeated at least twice, and representative results are shown. **p < 0.01 compared with the I3C-treated control (*a*), **p < 0.01 compared with the UVB-treated control (*b*).

To further examine whether the observed effects were specific for cancer cells, G361 and SK-MEL-2 melanoma cells, normal human fibroblasts, and normal human melanocytes were treated with I3C/UVB. This experiment confirmed that the viabilities of G361 and SK-MEL-2 melanoma cells were reduced significantly by I3C (100 or 200 μM)/UVB (50 mJ/cm^2), whereas I3C/UVB had little effect on normal human fibroblasts or melanocytes (Fig. 3a–d). By phase contrast microscopy, the detached G361 cells corresponded with measured cell viabilities (Fig. 3e).

I3C/UVB produces free radicals, but they had little effect on cell viabilities. We hypothesized that I3C/UVB-induced cell death might be due to the production of free radicals if I3C is activated by UVB. To determine whether I3C is activated by UVB, we measured free radical generation when I3C (200 μM) was irradiated with UVB

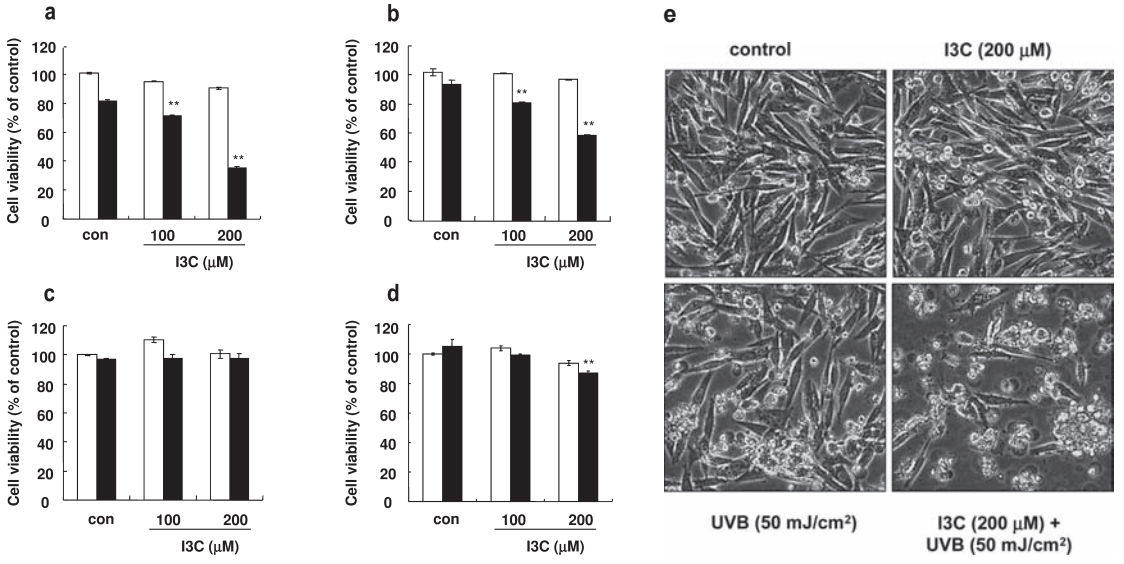

Figure 3. I3C/UVB-induced cell death in melanoma cells but not in normal human cells. G361 cells (*a*), SK-MEL-2 cells (*b*), normal human fibroblasts (*c*), normal human melanocytes (*d*). After serum starvation, cells were treated with I3C (100 or 200 μM) with (closed bars) or without (open bars) UVB (50 mJ/cm^2). Twenty-four hours later, cell viabilities were determined using crystal violet assays. Data represent means ± SD of triplicate assays expressed as percentages of the respective controls. Experiments were repeated at least twice independently, and representative results are shown. **p < 0.01 compared with the UVB-treated control. (*e*) Phase contrast pictures were taken using a digital video camera, as described in 'Materials and Methods'.

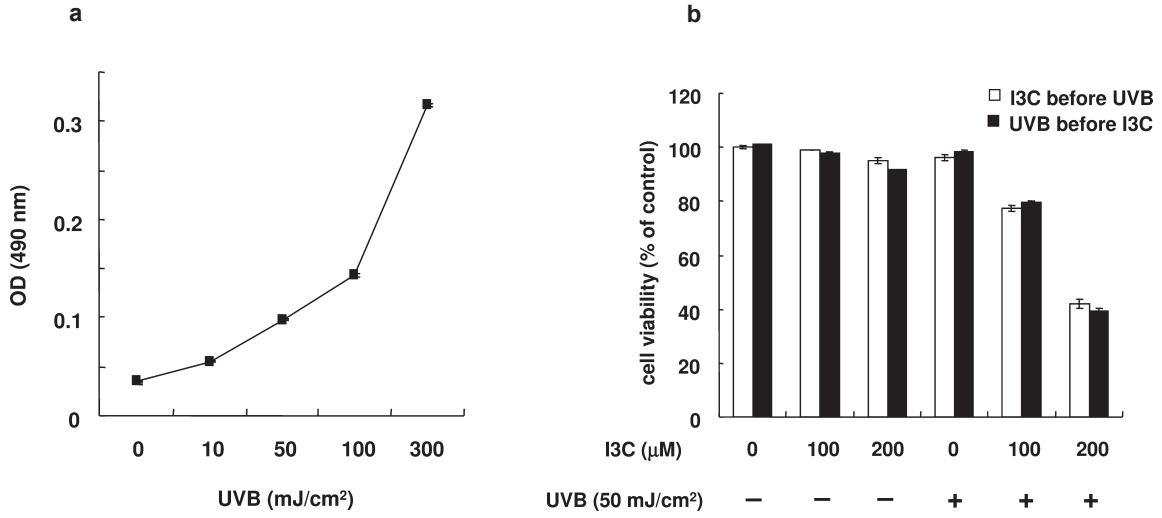

Figure 4. Cell death and free radical production by I3C/UVB. The production of free radicals was assessed using DCFH-DA, which is oxidized by free radicals to DCF, as described in 'Materials and methods'. (*a*) Increasing doses of UVB (10, 50, 100 and 300 mJ/cm^2) were added in the presence of 200 μM I3C. The values shown are the means ± SD of triplicate wells. (*b*) After serum starvation, G361 cells were pretreated with I3C (200 μM) for 30 min and then exposed to UVB (50 mJ/cm^2) (open bars, I3C before UVB). Alternatively, G361 cells were exposed to UVB (50 mJ/cm^2), and then treated with I3C (200 μM) (closed bars, UVB before I3C). Twenty-four hours later, cell viabilities were determined using crystal violet assays. Data represent the means ± SD of triplicate assays expressed as percentages of the respective controls. Experiments were repeated at least twice, and representative results are shown.

(0–300 mJ/cm^2) (Fig. 4a). I3C (200 μM) alone showed no presence of free radicals, but the irradiation of I3C solutions produced free radicals in a UVB dose-dependent manner.

To determine the involvement of free radicals in I3C/UVB-induced cell death, we performed two experiments. In one, G361 cells were pretreated with I3C before UVB exposure, whereas in the other, G361 cells were preirradiated and then treated with I3C. As shown in Figure 4b, these two experiments produced similar results.

I3C/UVB activated apoptotic pathways in G361 cells. The apoptotic process is known to involve DNA damage, caspase activation, and PARP cleavage. To characterize I3C/UVB-induced cell death, the presence of apoptotic features was determined using DNA fragmentation assays and Western blot analysis. In cells treated with I3C/UVB, a typical DNA ladder pattern was observed (Fig. 5a). In contrast, cells treated with I3C alone did not exhibit the ladder pattern, though DNA ladders were weakly detected in cells treated with UVB alone. As shown in Figure 5b, we also examined the proteolytic processings of caspase-8, Bid, caspase-3, and PARP, because caspases are known to become active when they are cleaved into fragments. Thus, we used anti-caspase-8 and anti-Bid antibodies directed against the precursor forms. The precursor forms of caspase-8 and Bid were much reduced 24 h after I3C (200 μM)/UVB (50 mJ/cm^2) treatment. In the case of caspase-3, we used an antibody directed against its cleaved form, and active caspase-3 was found to be considerably increased 24 h after I3C/UVB treatment. Caspase-3 is believed to be the most efficient PARP-cleaving caspase and, thus, we examined proteolytic PARP cleavage following I3C/UVB treatment, and found that 116-kDa full-length PARP was converted to the apoptotic 85-kDa fragment (Fig. 5b).

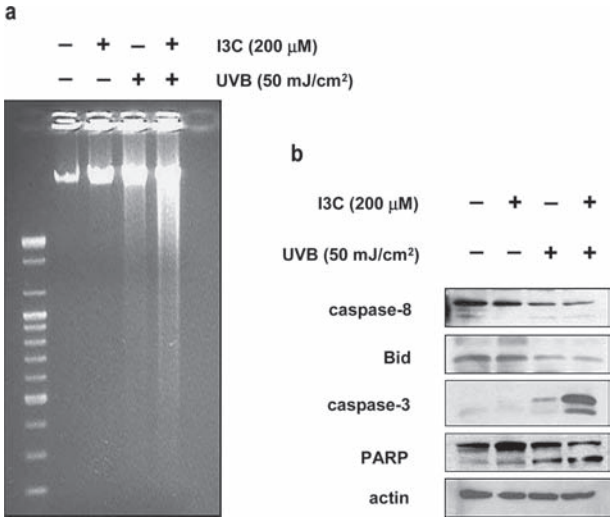

Figure 5. I3C/UVB treatment induced apoptosis via the activation of caspases and PARP cleavage. (*a*) Agarose gel electrophoresis showing DNA fragmentation of G361 cells treated with I3C (200 μM)/UVB (50 mJ/cm^2), as described in 'Materials and methods'. (*b*) Western blot analysis of caspase-8, Bid, caspase-3, and PARP after I3C (200 μM)/UVB (50 mJ/cm^2), as described in 'Materials and methods'. Equal protein loadings were confirmed using anti-actin antibody.

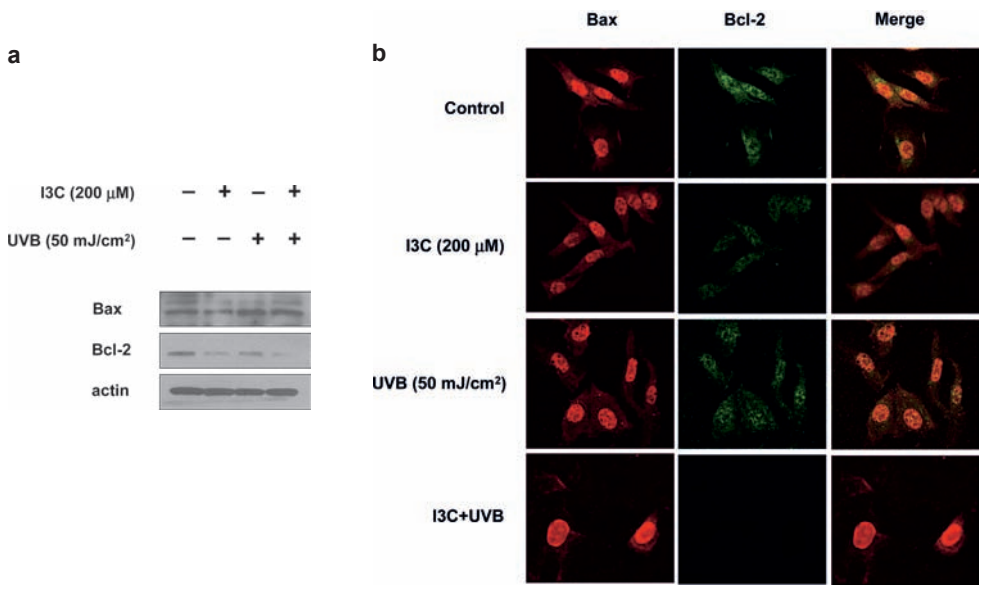

Figure 6. Changes in the expression of Bax and Bcl-2 induced by I3C and/or UVB. (*a*) Western blot analyses of Bax and Bcl-2 after I3C (200 μM)/UVB (50 mJ/cm^2), as described in 'Materials and methods'. Equal protein loadings were confirmed using anti-actin antibody. (*b*) After serum starvation, G361 cells were treated with I3C (200 μM)/UVB (50 mJ/cm^2) and 30 min later cells were incubated with anti-Bcl-2 and anti-Bax antibodies and stained with FITC- and rhodamine-conjugated secondary antibodies, as described in 'Materials and methods'. Fluorescence was detected by confocal microscopy.

I3C treatment decreased Bcl-2, and UVB-induced Bax redistribution. To further investigate the role of I3C on I3C/UVB-induced apoptosis, its effects on the expressions of Bax and Bcl-2 were studied by Western blotting. I3C clearly down-regulated Bcl-2 expression in G361 cells, but had little influence on Bax expression (Fig. 6a). On the other hand, UVB slightly increased Bax levels and decreased Bcl-2 levels. Thus, I3C/UVB treatment substantially increased the Bax/Bcl-2 ratio. These results were also confirmed by confocal microscopy and were consistent with Western blot data. Bcl-2 expression was also clearly down-regulated by I3C treatment (Fig. 6b). In addition, UVB was found to cause the cellular redistribution of Bax. These results suggest that I3C sensitizes melanoma cells by down-regulating anti-apoptotic protein Bcl-2, thus increasing the Bax/Bcl-2 ratio.

Discussion

I3C is a product of indole-3-acetic acid (IAA) oxidized by horseradish peroxidase (HRP) [17], and IAA oxidation by HRP has been reported to induce melanoma cell death [16, 18], and I3C has been reported to induce apoptosis in human breast, lung, and prostate cancer cell lines [19–21]. In addition, the efficacy of photodynamic cancer therapy was found to be enhanced by free radicals from IAA [22]. Therefore, we hypothesized that I3C may be transformed to potent cytotoxic radicals when activated by UVB. However, as shown in Figure 4b, direct free radical formation by the I3C/UVB combination was not the main mechanism underlying I3C/UVB-induced cell death because treatment with I3C after UVB had similar effects.

In the present study, we found that a sub-toxic dose of I3C acts synergistically with UVB, which suggests the possibility that low-dose I3C may prevent UVB-induced melanoma development. The enhancement of apoptotic signaling pathways represents an approach to the suppression of cancer development. DNA fragmentation, the activation of caspase-3, and the cleavage of PARP are commonly used to define typical apoptosis. In this study, we found that in the presence of I3C, UVB markedly enhanced DNA fragmentation, caspase-3 activation, and PARP cleavage, but I3C alone had little effect (Fig. 5b).

UV exposure often leads to the apoptosis of epidermal keratinocytes rather than melanocytes *in vivo*. Thus, the apoptosis of melanocytes after sun exposure is rarely observed [11], and high constitutive Bcl-2 levels are believed to be responsible for this resistance to UV radiation [23]. Although the precise mechanism of apoptosis needs to be further elucidated, the cellular ratio of anti-apoptotic Bcl-2 to its pro-apoptotic homologue Bax plays a key role in determining the susceptibility of cells to a death signal [24]. The Bax/Bcl-2 ratio has also been reported to determine the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis [25]. On the other hand, our confocal images show that Bax is localized in the nucleus (Fig. 6b). It is well known that Bax is involved in apoptosis via mitochondria. However,

Bax was reported to be localized in the cytoplasm and/or nucleus depending on cell lines, whereas Bcl-2 was localized only in the cytoplasm [26]. Furthermore, the formation of nuclear complexes between Bax and p53 has been found in human melanoma cell lines [27]. Thus, further study is needed to clarify the functional role of nuclear Bax in apoptosis. The present study demonstrates that I3C down-regulates Bcl-2 levels in human melanoma cells, and this down-regulation of Bcl-2 could increase melanoma cell sensitivity, although I3C alone at 200 μM is insufficient to induce apoptosis. Thus, we propose that I3C sensitizes melanoma cells to UVB by modulating the Bax/Bcl-2 ratio.

Furthermore, IC3 has been suggested to sensitize prostate cancer cells to TRAIL-induced apoptosis [28], and other chemotherapeutics have been found to act synergistically with UVB to induce melanoma cell apoptosis [10, 29]. For example, $p33^{ING1}$ (a tumor suppressor, ING1 isoform) and CP-31398 (a p53 stabilizer) were found to enhance the UVB-induced apoptosis of melanoma cells [9, 10].

The human body is continuously exposed to UV, which is known to be a major environmental risk factor for melanoma [11]. Because UV damages DNA and evokes mutation, cells containing damaged DNA should be either repaired or removed to avoid cancer formation. Based on our results, I3C can sensitize human melanoma cells to UVB-induced cell death, and thus, I3C in adequate doses could remove DNA-damaged cells before melanoma development.

In summary, the present study demonstrates that I3C at sub-toxic levels dramatically enhances UVB-induced apoptosis in human melanoma cells. Moreover, these synergistic effects were not observed in normal human fibroblasts or melanocytes. These results suggest that I3C could be used to suppress the development of malignant melanoma as induced by UV without affecting normal cells or tissues. Thus, I3C should be viewed as a means of preventing human melanoma caused by UVB.

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