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Indole-3-carbinol and ultraviolet B induce apoptosis of human melanoma cells via down-regulation of MITF

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We investigated the mechanism of indole-3-carbinol (I3C)/ultraviolet B (UVB)-induced apoptosis using SK-MEL-2 and SK-MEL-5 human melanoma cells. I3C/UVB significantly reduced the viability of SK-MEL-2 cells, whereas it had little influence on SK-MEL-5 cells. Correspondingly, cell cycle analysis showed that I3C/UVB induced a clear increase in the sub-G_0/G_1 phase in SK-MEL-2 cells. Furthermore, I3C/UVB activated caspase-9, caspase-8, caspase-3, and Bid and caused the cleavage of poly(ADP-ribose) polymerase (PARP) in SK-MEL-2 cells. In contrast, I3C/UVB showed no effects on the apoptotic signaling pathways in SK-MEL-5 cells. Moreover, we found that I3C down-regulated the microphthalmia-associated transcription factor (MITF) in SK-MEL-2 cells, but not in SK-MEL-5 cells. Next, to investigate the involvement of MITF in I3C/UVB-induced apoptosis, MITF silencing was conducted using small interfering RNA (siRNA) for MITF in SK-MEL-5 cells. Interestingly, I3C/UVB dramatically decreased the viability of MITF-down-regulated SK-MEL-5 cells. These results indicate that MITF plays a critical role in melanoma cell survival.

1. Introduction

Normal melanocytes synthesize melanin pigments and protect the body against the damage caused by solar ultraviolet (UV) radiation. Melanoma, which develops from normal melanocytes, is known to increase in response to excessive UV exposure (Gandini et al. 2005). Because of the increasing incidence of melanoma with frequent metastases and high mortality, it has emerged as an increasing health problem (Chin et al. 2006). For treatment of melanoma, a combination of several methods including surgery, chemotherapy, and radiotherapy have been attempted. Because melanoma is highly resistant to chemotherapy and radiotherapy (Soengas and Lowe 2003), more specific and effective combined therapies are needed (Chin et al. 2006). Indole-3-carbinol (I3C) is a natural phytochemical component found in cruciferous vegetables that has been found to exhibit potent anticancer activities in several cancer cell lines, including lung, liver, colon, cervical, endometrial, prostate, and breast cancer (Aggarwal and Ichikawa 2005; Verhoeven et al. 1997). I3C has also been shown to inhibit cancer cell proliferation by causing G1 cell cycle arrest in human breast and prostate cancer cells (Cram et al. 2001; Zhang et al. 2003). Moreover, it has been reported that I3C caused apoptosis and caspase-dependent poly(ADP-ribose) polymerase (PARP) cleavage in breast and prostate cancer cells (Chinni et al. 2001; Ge et al. 1999). We also reported that I3C enhances UVB-induced apoptosis by sensitizing human melanoma cells (Kim et al. 2006). Consequently, the results presented here suggest that I3C is a potent chemopreventive and chemotherapeutic substance with anticancer activities.

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper (bHLH-ZIP) transcription factor that acts as an essential regulator of melanocyte lineage development (Hodgkinson et al. 1993). MITF up-regulates the expression of tyrosinase, tyrosinase-related protein 1 (TRP1), and tyrosinase-related protein 2 (TRP2), which control melanocyte differentiation and pigmentation (Steingrimsson et al. 2004; Vachtenheim et al. 2001). Thus, mutations of MITF cause a pigmentary disorder, Waardenburg syndrome type IIA, in humans (Tassabehji et al. 1994). Conversely, it has been reported that MITF plays an important role as a lineage survival oncogene amplified in malignant melanoma (Garraway et al. 2005; Levy et al. 2006; Polakis 2000). Thus, MITF is a sensitive and specific melanocyte marker in the diagnosis of melanoma (Hodgkinson et al. 1993; Salti et al. 2000). Furthermore, MITF is known to regulate Bcl-2 expression, which plays a critical role in protecting melanocytes from apoptosis (Du et al. 2004; McGill et al. 2002).

In the present study, we investigated the effects of I3C/UVB on melanoma cell death using SK-MEL-2 and SK-MEL-5 human melanoma cells. Interestingly, one cell line was sensitive to I3C/UVB treatment, whereas the other was not. To elucidate the difference between cell lines, we examined cell cycle distribution, caspases activation, and apoptotic pathways as well as the expression of Bax, Bcl-2, and MITF after I3C/UVB treatment.

2. Investigations and results

2.1. Effects of I3C/UVB combination on melanoma cell viability

To examine the combined effects of I3C and UVB on the cell viability of human melanoma cells, SK-MEL-2 and SK-MEL-5 cells were treated with various concentrations of I3C (0–500 μM) and then irradiated with UVB (50 mJ/cm^2). Cell

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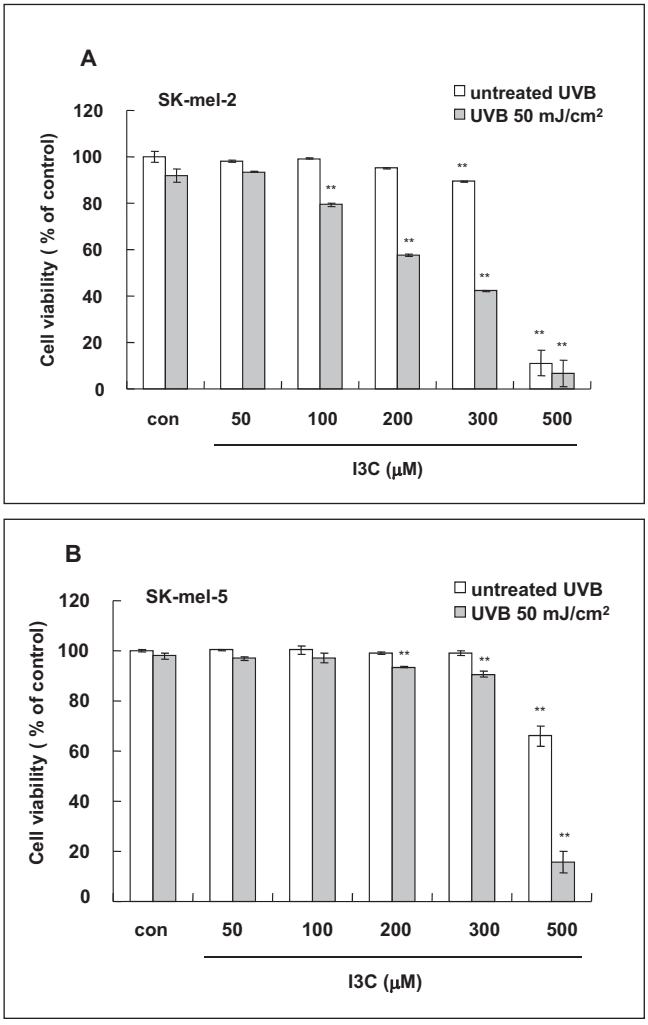

Fig. 1: Effects of I3C/UVB on the viabilities of SK-MEL-2 and SK-MEL-5 cells. After serum starvation, SK-MEL-2 cells (A) and SK-MEL-5 cells (B) were treated with various concentrations (50–500 μM) of I3C in the absence and presence of UVB irradiation (50 mJ/cm^2). After 24 h, cell viability was measured by crystal violet assays. Data represent the means ± S.D. of triplicate assays expressed as percentages of the control. All experiments were repeated at least twice independently, and representative results are shown. **P<0.01 compared to the untreated control

viability was measured using a crystal violet assay 24 h after I3C and/or UVB treatment. As shown in Fig. 1, 300 μM I3C alone was shown to be slightly toxic against both SK-MEL-2 and SK-MEL-5 cells. However, I3C/UVB caused significant death of SK-MEL-2 cells in an I3C-dose-dependent manner (Fig. 1A), whereas I3C/UVB had little effect on SK-MEL-5 cell viability up to 300 μM (Fig. 1B). These results show that I3C/UVB synergistically reduced melanoma cell viability and that I3C/UVB was more effective against SK-MEL-2 cells than SK-MEL-5 cells.

2.2. I3C/UVB-mediated cell death due to apoptosis

Previously, we reported that I3C (200 μM)/UVB (50 mJ/cm^2) synergistically reduced melanoma cell viability, and that I3C/UVB combination resulted in apoptosis of G361 human melanoma cells (Kim et al. 2004). Therefore, we investigated I3C/UVB-induced cell death to determine if it is due to apoptosis. Based on Fig. 1A and B, cells were treated with a low dose of I3C (200 μM) and irradiated with UVB (50 mJ/cm^2), after which the cell cycle was measured by flow cytometric analysis. Our results showed that I3C/UVB combination significantly increased the sub-G_0/G_1 phases in SK-MEL-2 cells. However, I3C/UVB did not affect the cell cycle in SK-MEL-5 cells (Fig. 2). These results showed that I3C/UVB induced apoptotic cell death in SK-MEL-2 cells, but not in SK-MEL-5 cells.

2.3. I3C/UVB activated apoptotic pathways and down-regulated MITF and Bcl-2 in SK-MEL-2 cells

The apoptotic pathway is known to involve caspase activation and PARP cleavage. Thus, we examined I3C/UVB to determine if it could activate the proteolytic processing of caspase-9, -8, -3, Bid, and PARP (Fig. 3A). Because caspases are known to become active when they are cleaved into fragments, we used anti-caspase-9, -8, -3, and anti-Bid antibodies directed against the precursor forms. After I3C (200 μM)/UVB (50 mJ/cm^2) treatment for 24 h, the precursor forms of caspase-9, -8, -3, and Bid were clearly reduced in SK-MEL-2 cells. However, SK-MEL-5 cells did not show any changes. We further examined PARP cleavage after I3C/UVB treatment and found that 116-kDa full-length PARP was converted into the apoptotic 85-kDa fragment in SK-MEL-2 cells, but not in SK-MEL-5 cells (Fig. 3A).

To further examine the I3C/UVB-mediated apoptosis, its effects on the expression of Bax and Bcl-2 were studied by Western blotting. Our results showed that I3C/UVB clearly suppressed Bcl-2 expression in SK-MEL-2 cells, but not in SK-MEL-5 cells. In addition, no effect on Bax expression was observed (Fig. 3B). Bcl-2 has been reported to be an MITF-dependent transcriptional target (McGill et al. 2002); therefore, we determined the MITF level after I3C/UVB treatment. MITF was found to be strongly reduced by I3C/UVB in SK-MEL-2 cells, whereas it was not changed in SK-MEL-5 cells (Fig. 3B). These findings suggest that I3C/UVB induces apoptosis of SK-MEL-2 cells by MITF and subsequent Bcl-2 down-regulation.

2.4. MITF knockdown was responsible for I3C/UVB-induced apoptosis

We hypothesized that I3C/UVB-induced apoptosis could be due to down-regulated MITF in SK-MEL-2 cells. To assess the effect of MITF, SK-MEL-5 cells were transfected with MITF siRNA. Transfection with MITF siRNA decreased protein expression of MITF in SK-MEL-5 cells (Fig. 4A). Moreover, the Bcl-2 level was also down-regulated in MITF siRNA-transfected SK-MEL-5 cells (Fig. 4A). Next, we examined the SK-MEL-5 cell viability. I3C/UVB did not decrease SK-MEL-5 cell viability. However, I3C/UVB did significantly reduce the viability of MITF siRNA-transfected SK-MEL-5 cells.

3. Discussion

The incidence of melanoma has risen in recent years and conventional therapies have shown that it has a limited effect against melanoma. Thus, recent advances suggest that melanoma should be treated by combined therapy (Tsao et al. 2004). Recently, we reported that the I3C/UVB combination induces apoptosis of G361 human melanoma cells, suggesting that it could be used as a new melanoma therapy (Kim et al. 2006). Cellular stress stimuli including chemotherapeutic agents, irradiation, and oxidative stress have been found to trigger apoptotic cell death (Degterev et al. 2003). Apoptotic signaling pathways involve cell cycle arrest, caspase activation, and PARP cleavage, which are potential targets for cancer therapy (Degterev et al. 2003). I3C has been reported to induce cell death through G1 cell cycle arrest, caspase-3 activation, mitochondrial uptake of pro-apoptotic Bax, decreased anti-apoptotic Bcl-2, and increased

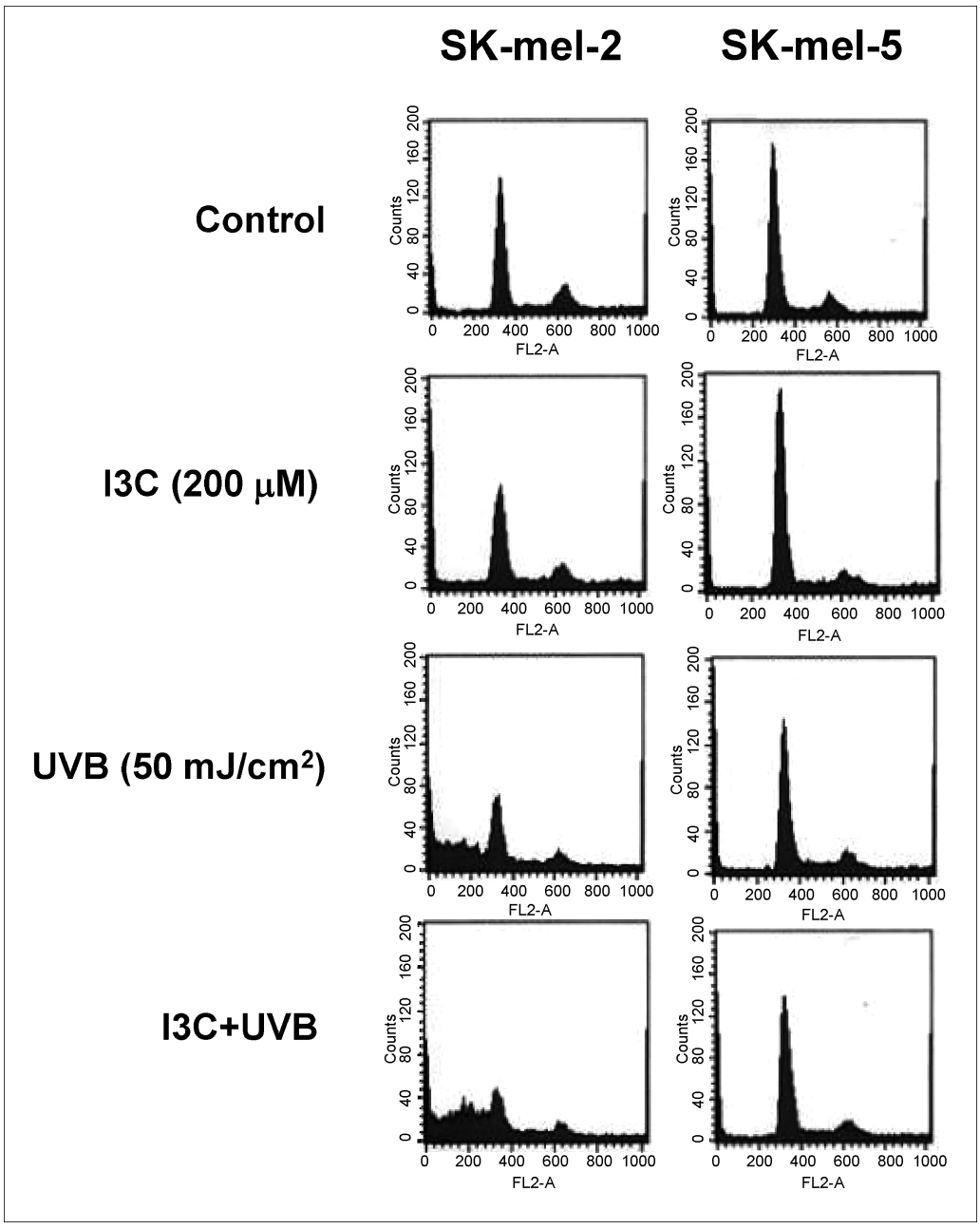

Fig. 2: I3C/UVB increased apoptosis in SK-MEL-2 cells, but not in SK-MEL-5 cells. After serum starvation, SK-MEL-2 cells and SK-MEL-5 cells were treated with I3C (200 μM) and/or UVB (50 mJ/cm^2). After 24 h, cell cycle analysis was performed by flow cytometric analysis as described in the Experimental section

Bax/Bcl-2 ratios in various human cancer cells (Chinni et al. 2001; Cram et al. 2001; Ge et al. 1999; Zhang et al. 2003).

In the present study, we found that I3C/UVB enhances apoptosis in SK-MEL-2 cells. Treatment with I3C (200 μM) alone was not sufficient to cause apoptosis, but induced SK-MEL-2 cell apoptosis synergistically with UVB (Fig. 1A). However, the same condition of I3C/UVB did not lead to apoptosis or activation of apoptotic pathways in SK-MEL-5 cells. Thus, this study was conducted to determine why human SK-MEL-5 cells were resistant to I3C/UVB.

It has been reported that UVB has little effect on the viability of melanocytes because melanocytes maintain sufficient levels of Bcl-2 after UVB irradiation (Kim et al. 2000; Plettenberg et al. 1995). The Bax/Bcl-2 ratio was reported to determine the susceptibility of melanoma cells to death signals (Adams and Cory 2007). In the present study, we demonstrated that I3C/UVB decreases the expression of Bcl-2, but that it does not increase the expression of Bax in SK-MEL-2 cells. Interestingly, it was found that I3C/UVB also decreases the expression of MITF in parallel with Bcl-2 levels in SK-MEL-2 cells, whereas MITF levels were not changed in SK-MEL-5 cells (Fig. 3B). Bcl-2 was found to be a direct transcription target of MITF, and MITF was reported to modulate endogenous Bcl-2 expression in melanocytes and melanoma cells (McGill et al. 2002). Furthermore, it was reported that MITF and Bcl-2 expression were increased by melanoma oncogenic growth factors such as NGF and SCF (Zhai et al. 1996).

Therefore, we evaluated I3C/UVB to determine if it induced apoptosis by down-regulating MITF and subsequently Bcl-2. To accomplish this, MITF knock-down was conducted in SK-MEL-5 cells. The results revealed that I3C/UVB reduces cell viability in MITF down-regulated SK-MEL-5 cells (Fig. 4B). Moreover, MITF is known to be a substrate for processed proteolytic degradation by caspase-3 upon TRAIL-induced apoptosis, indicating that the cleavage of MITF is deeply involved in melanocyte and melanoma cell apoptosis (Larribere et al. 2005). However, in

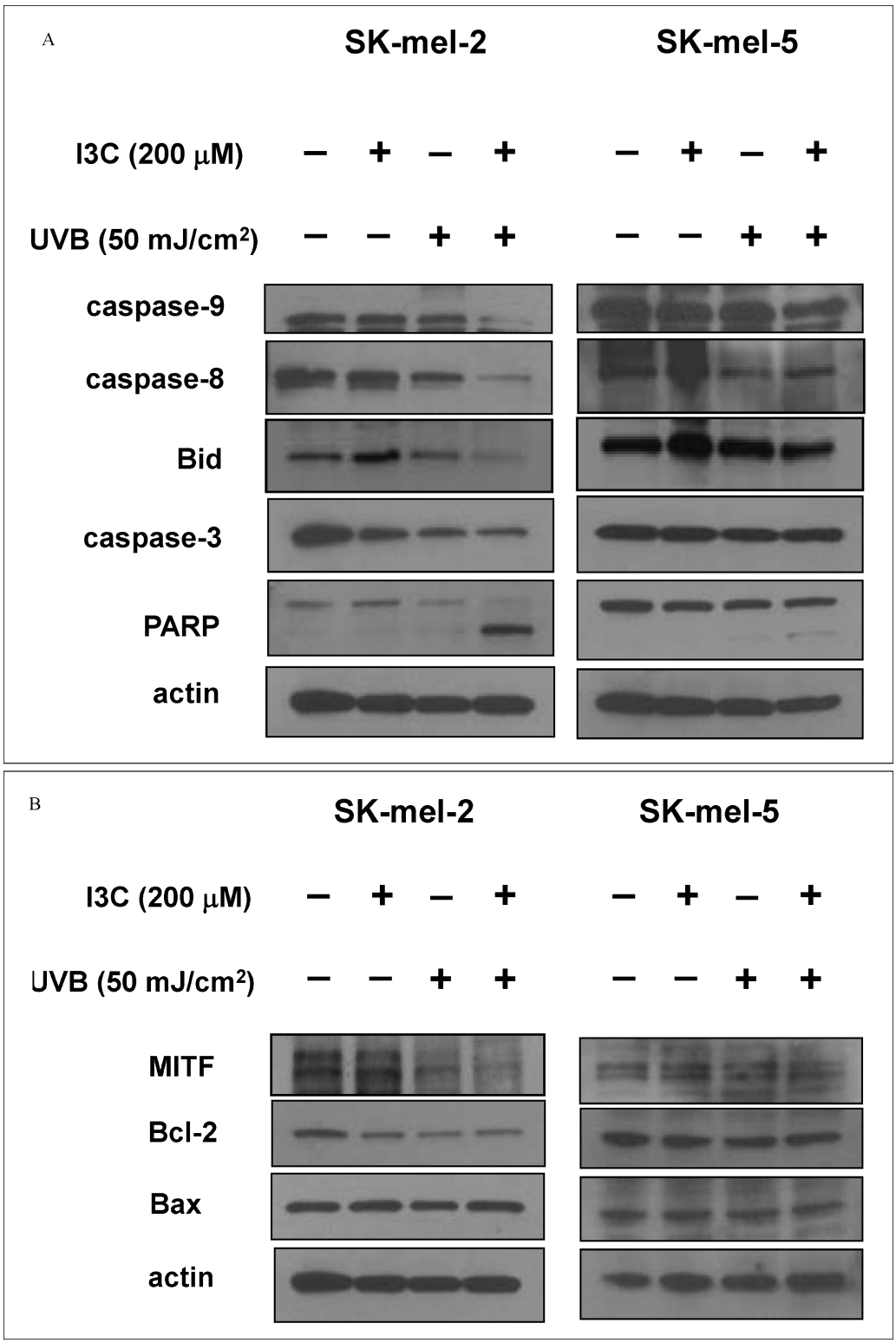

Fig. 3: I3C/UVB activated apoptotic pathways and decreased MITF in SK-MEL-2 cells, but not in SK-MEL-5 cells. After serum starvation, SK-MEL-2 and SK-MEL-5 cells were treated with I3C (200 μM) in the absence and the presence of UVB irradiation (50 mJ/cm^2). (A) Cell lysates were analyzed by Western blot analysis using antibodies against caspase-9, -8, -3, Bid, and PARP after I3C and/or UVB as described in the Experimental section. (B) Western blot analysis was performed for MITF, Bax, and Bcl-2 after I3C and/or UVB as described in the Experimental section. Equal protein loadings were confirmed using anti-actin antibody

the present study, MITF siRNA treatment alone did not affect cell viability (Fig 4B), indicating that MITF knock-down itself is not sufficient to induce apoptosis. In addition, Bcl-2 over-expression could not rescue melanoma clonogenic growth in the case of MITF disruption, indicating that MITF may have other target genes beside Bcl-2 (Du et al. 2004; Loercher et al. 2005; McGill et al. 2002). Thus, MITF and Bcl-2 disruption may provide a potent anti-melanoma therapy against I3C/UVB-resistant melanoma cells.

In conclusion, the results of the present study demonstrate that I3C/UVB enhances human melanoma cell apoptosis when MITF and subsequent Bcl-2 are downregulated. These results suggest that MITF and Bcl-2 disruption can be potential targets for the development of anti-melanoma treatment.

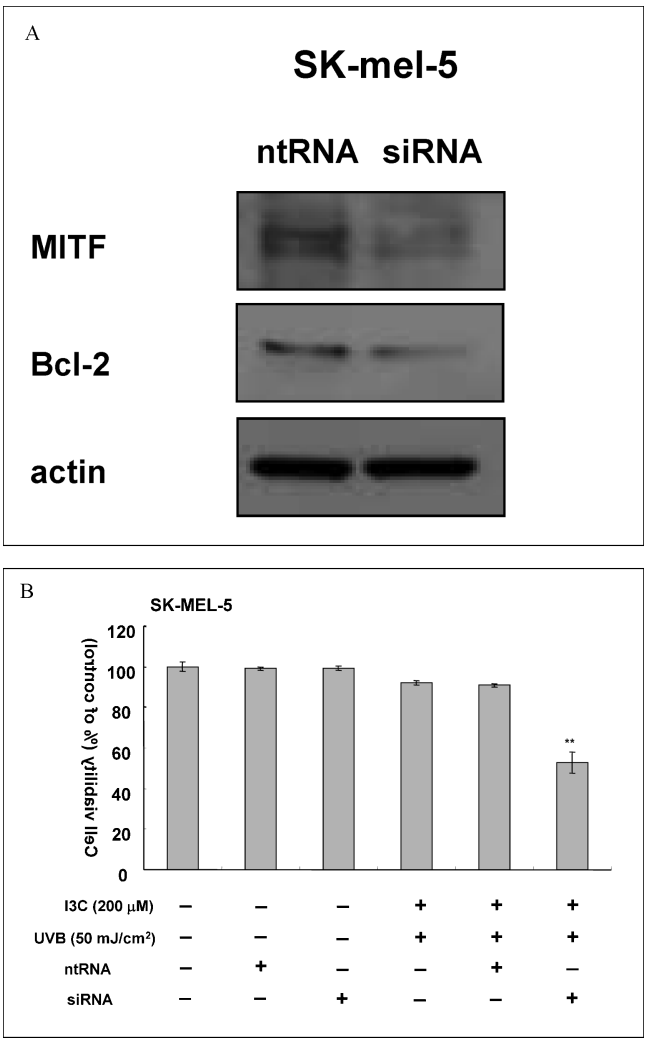

Fig. 4: MITF knockdown enhanced melanoma apoptosis by I3C/UVB. SK-MEL-5 cells were transfected with MITF siRNA or non-targeting RNA (ntRNA) as described in the Materials and Methods. (A) After 48 h of transfection, cells were harvested and lysates were prepared for Western blot analysis using anti-MITF and anti-Bcl-2 antibodies. Equal protein loadings were confirmed using anti-actin antibody. (B) After 48 h of transfection, cells were treated with I3C (200 μM) and/or UVB (50 mJ/cm^2). After 24 h, cell viability was assessed by crystal violet assay. Data represent the means ± S.D. of triplicate assays expressed as percentages of the control. Experiments were repeated at least twice independently, and representative results are shown. ** $P < 0.01$ compared to the untreated control

4. Experimental

4.1. Materials

Indole-3-carbinol was purchased from Sigma Aldrich (St. Louis, MO). Antibodies recognizing caspase-3 (sc-7272), caspase-8 (sc-7890), caspase-9 (sc-8355), Bax (sc-526), Bcl-2 (sc-7382), and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Bid antibody (AF860) was obtained from R&D Systems (Minneapolis, Minn.), anti-PARP antibody was purchased from BD Pharmingen (San Diego, CA), and anti-MITF antibody (Ab-3, C5 + D5, MS-773-P0) was obtained from NeoMarkers (Fremont, CA).

4.2. Cell cultures

The human melanoma cell lines SK-MEL-2 and SK-MEL-5 were obtained from the Korean Cell Line Bank. The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin-streptomycin (10,000 U/ml and 10,000 μg/ml, respectively) under 5% CO_2 at 37 °C.

4.3. Detection of cell death

Cell viabilities were assessed using crystal violet assays. After removing the culture media, cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at RT and then rinsed four times in distilled water. The crystal violet retained by adherent cells was extracted with 95% ethanol, and the absorbance was determined in lysates at 590 nm using an ELISA reader (TECAN, Salzburg, Austria).

4.4. I3C and UVB treatments

To examine the synergistic effects of I3C/UVB, cells (4×10^4 cells/well) were seeded into 24-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 (0–500 μM). Next, the cells were irradiated with UVB (50 mJ/cm^2) using a UVB source (BLE-1T158, Spectronics Corp., Westbury, NY). A Kodacel filter (TA401/407. Kodak, Rochester, NY) was used to eliminate light wavelengths of less than 290 nm (UVC). UV energy was measured using a Waldmann UV meter (model No. 585100; Waldmann Co., Villingen-Schwenningen, Germany). After irradiation, cells were cultured for another 24 h and the cell viabilities were determined using crystal violet assays.

4.5. Cell cycle analysis

After serum starvation, the cells were treated with I3C/UVB for 24 h, the culture supernatant was collected for floating dying and apoptotic cells and the adherent cells were harvested by trypsinization. Cell fractions were collected and washed with ice cold phosphate-buffered saline (PBS) and then resuspended in 2 ml of ethanol. Following incubation at 4 °C for 1 h, the ethanol was removed. Next, 100 μl of RNase solution (10 mg/ml) was added to each test tube. The tubes were then re-incubated at room temperature for 30 min, after which 500 μl of analysis solution (37 mM EDTA and 0.1% Triton X-100 in PBS) and 100 μl of propidium iodide solution (400 μg/ml) were added. Samples were stored in the dark at 4 °C and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). The proportion of cells in the sub-G_0/G_1 phases with low DNA content were regarded as apoptotic cells.

4.6. Western blot analysis

After serum starvation for 24 h, the cells were treated with I3C/UVB for 24 h and then 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 were 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).

4.7. Small interfering RNA (siRNA) and transfection

siRNA targeting the specific sequence 5'-GUG GUA GAA AGG UAC UGC UUU ACC U-3' was selected to down-regulate MITF expression. A non-targeting RNA (ntRNA) sequence 5'-GUG AUG AGA UGG AAU CAU UUC GCC U-3' was used as control siRNA. MITF-siRNA and ntRNA were synthesized at Invitrogen (Carlsbad, CA). For MITF-siRNA and ntRNA transfections, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions. The transfection was conducted when the cells reached approximately 50% confluence in 6-well plates, and the transfected cells were used for Western blot analysis and crystal violet assay.

4.8. Statistics

The statistical significance of the differences between groups was assessed by analysis of variance (ANOVA), followed by Student's *t*-test. *P* values < 0.05 were considered significant.

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