

Tazarotene Induces Apoptosis in Human Basal Cell Carcinoma via Activation of Caspase-8/t-Bid and the Reactive Oxygen Species-Dependent Mitochondrial Pathway

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Previous studies suggest that tazarotene, a new member of the acetylenic class of RAR β/γ selective retinoids which is approved to treat a variety of skin diseases, exhibits an anti-proliferative effect in human basal cell carcinoma (BCC) by triggering caspase-dependent apoptosis. However, the detailed molecular mechanisms underlying the anti-tumor activity of tazarotene are poorly understood. This study aims at investigating the molecular mechanisms of tazarotene-induced apoptosis in human BCC cells. Our results are the first to demonstrate that tazarotene induces mitochondria-dependent cleavage of caspase-9 and -3 and PARP in BCC cells by producing reactive oxygen species (ROS) and activating caspase-8 through both ROS and death receptor signaling. These events are accompanied by a decrease in BCL-2 and BCL-x1 anti-apoptotic proteins as well as by survivin and XIAP, two IAP family members. Furthermore, our results presented for the first time that tazarotene triggers a convergence of the intrinsic and extrinsic apoptotic pathways via the caspase-8-truncated Bid signaling pathway. Collectively, these data provide insights into the molecular mechanisms underlying tazarotene-induced apoptosis in human BCC cells, suggesting that this compound is a potential anti-skin cancer drug.

Introduction

BASAL CELL CARCINOMA (BCC) is the most common type of skin cancer worldwide, and its incidence is increasing (Diepgen and Mahler, 2002; Kasper *et al.*, 2012). BCC is characterized by a non-aggressive behavior and is, therefore, a good candidate for treatment with traditional and experimental therapies (Wennberg, 2000). Surgical excision and cryosurgery are currently considered the standard treatments for BCC (Marghoob, 1997; Martinez and Otley, 2001). In addition, a number of treatment options are now available to patients for whom surgery is contraindicated. Topical immunotherapy, chemotherapy, and photodynamic therapy can be proposed for the treatment of select patients. The lesion location, lesion size, recurrence status, clinical type, patient's age, ease of treatment, and cost dictate which topical treatment is utilized

(Bastiaens *et al.*, 1998; Urošević and Dummer, 2002; Urošević *et al.*, 2003).

Numerous retinoids have exerted striking effects on proliferation, differentiation, and apoptosis in various cancers, including leukemia and non-melanoma skin cancers (Robert *et al.*, 2006; Lens and Medenica, 2008; Carr *et al.*, 2011). Tazarotene and tazarotenic acid, a free acid metabolite of tazarotene, are new members of the acetylenic class of RAR β/γ -selective retinoids that are approved to treat a variety of skin diseases, such as psoriasis, acne, and photoaging (Roeder *et al.*, 2004; Dando and Wellington, 2005; Tanghetti *et al.*, 2011). Recently, clinical studies have also indicated that long-term topical treatment with Tazarotene gel can cure one-fourth to one-half of human BCC cases without significant toxicity (Bianchi *et al.*, 2004; Orlandi *et al.*, 2004). Additional *in vitro* and *in vivo* studies have

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indicated that tazarotene exerts its anti-proliferative effects by triggering caspase-dependent apoptosis in BCC (Orlandi *et al.*, 2004). Although these findings suggest that tazarotene possesses anti-cancer activity in BCC cells, the details of the molecular mechanisms underlying the anti-tumor activity of tazarotene are poorly understood. Therefore, our present study aimed at elucidating the molecular mechanisms of tazarotene's anti-cancer activity in human BCC cells, focusing on the apoptotic death signaling pathway. We report that tazarotene induces apoptosis in BCC cells by producing reactive oxygen species (ROS) that activate caspase-8 at the death-inducing signaling complex. Activation of caspase-8 subsequently induces mitochondria-dependent activation of the caspase-9 and caspase-3 cascades. In addition, we found that tazarotene induces cell cycle arrest at the G0/G1 phase of the cell cycle. These events are concordant with decreased BCL-2 and BCL-xl anti-apoptotic proteins and down-regulation of the IAP family proteins survivin and XIAP.

Materials and Methods

Cell culture and reagents

The human BCC cell line (BCC-1/KMC) was a kind gift from Dr. L.C. Chiang at Kaohsiung Medical University (Yen *et al.*, 1996). The cells were grown in RPMI 1640 medium (GIBCO/Life Technologies) containing 10% fetal bovine serum (GIBCO/Life Technologies) and maintained at 37°C in a humidified incubator containing 5% CO₂. Dantrolene dimethyl sulfoxide (DMSO; Sigma-Aldrich Co.) was used to dissolve and dilute Tazarotene (Allergan) to produce a 100 mM stock solution.

Cell viability assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide (MTT) assay (Sigma-Aldrich Co.). Aliquots between 3×10^4 and 4×10^4 cells/well were dispensed into 24-well tissue culture plates in 1 mL of medium and then incubated with 0, 6.125, 12.5, 25, 50, and 100 μ M tazarotene (0%, 0.1% DMSO) for 12, 24, or 48 h. Then, 200 μ L of MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37°C. After removing the supernatant, color was developed by the addition of 600 μ L of DMSO to each well. The absorbance was read at 570 nm using a microplate reader (Sunrise Microplate Reader; TECAN).

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed using flow cytometry. BCC cells were seeded into 6-cm tissue culture dishes at a density of 3×10^5 cells/dish in 3 mL of medium and then incubated with 0, 25, 50, and 100 μ M tazarotene for 12, 24, and 48 h. At the indicated time point, cells were trypsinised, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol at -20°C overnight. The fixed cells were washed with PBS, stained in a solution containing RNase A (Sigma-Aldrich Co.) and propidium iodide (PI; Sigma-Aldrich Co.) for 30 min at room temperature, and analyzed by an Accuri™ C5 cytometer CAT. NO. 657214. (BD Biosciences). The percentage of cells in the sub-G1 (indicative of apoptosis), G0/G1, S, and G2/M phases was determined using Accuri™ C6 software version 1.0264.21 software.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay

Apoptosis was analyzed using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining method. TUNEL staining was performed using a commercially available kit (Guava TUNEL Kit; Millipore). In this assay, BCC cells were seeded into 6-cm tissue culture dishes at a density of 3×10^5 cells/dish in 3 mL of medium and then incubated with 0, 25, 50, and 100 μ M tazarotene for 24 h. After incubation, the cells were collected and washed with PBS. The cells were then fixed with 1% paraformaldehyde at 4°C for 60 min, washed twice with wash buffer, re-suspended in 70% ethanol, and incubated at -20°C for 2 h. Cells were collected by centrifugation and washed twice with rinsing buffer. Cells were stained with TUNEL reaction mixtures at 37°C for 60 min. After incubation, the cells were analyzed by flow cytometry (FACSCalibur; BD BioSciences).

Transient transfection and RNA interference

For Fas-associated death domain (FADD) siRNA delivery, 1×10^5 BCC cells were grown in six-well plates and were then transfected with 400 pmol of FADD siRNA (Santa Cruz) using Lipofectamine 2000 reagent. Cells were harvested at 24 h after transfection, and cell viability was assessed in the presence or absence of 100 μ M tazarotene for an additional 24 h using the MTT assay.

Measurement of ROS by flow cytometry

ROS generation was measured after staining BCC cells with 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA; Molecular Probes). BCC cells were seeded into 6-cm tissue culture dishes at a density of 3×10^5 cells/dish in 3 mL of medium. The medium was removed at 1 or 6 h after tazarotene treatment, and culture medium containing 5 μ M DCFDA was added under low-light conditions. The cells were incubated for 30 min at 37°C, and the level of ROS was analyzed by flow cytometry. To examine whether ROS participated in the tazarotene-induced reduction in cell viability, BCCs were grown overnight and then pre-treated with 5 mM *N*-acetylcysteine (NAC; Sigma-Aldrich) for 1 h. Cells were then treated with the 100 μ M tazarotene for 24 h. The treated cells were incubated with 5 mg/mL MTT for 4 h at 37°C. After removing the supernatant, color was developed by the addition of 600 μ L of DMSO to each well. The absorbance was read at 570 nm using a microplate reader.

Measurement of mitochondrial membrane potential

To measure the mitochondrial membrane potential (MMP), BCC cells were incubated with JC-1 (Invitrogen) and then assessed for red (J-aggregate) fluorescence. For this assay, BCC cells were seeded into 6-cm tissue culture dishes at a density of 3×10^5 cells/dish in 3 mL of medium and then incubated with 0, 25, 50, and 100 μ M tazarotene for 24 h. The treated cells were trypsinised, washed with PBS, and re-suspended with 1 mL of culture medium. Each treatment was stained with 2 μ L of JC-1 stock solution (final concentration 10 μ g/mL) for 10 min at 37°C. The cells were collected by centrifugation and washed with PBS. Each treatment group was then re-suspended in 1 mL of PBS and analyzed by flow cytometry (FACSCalibur; BD BioSciences).

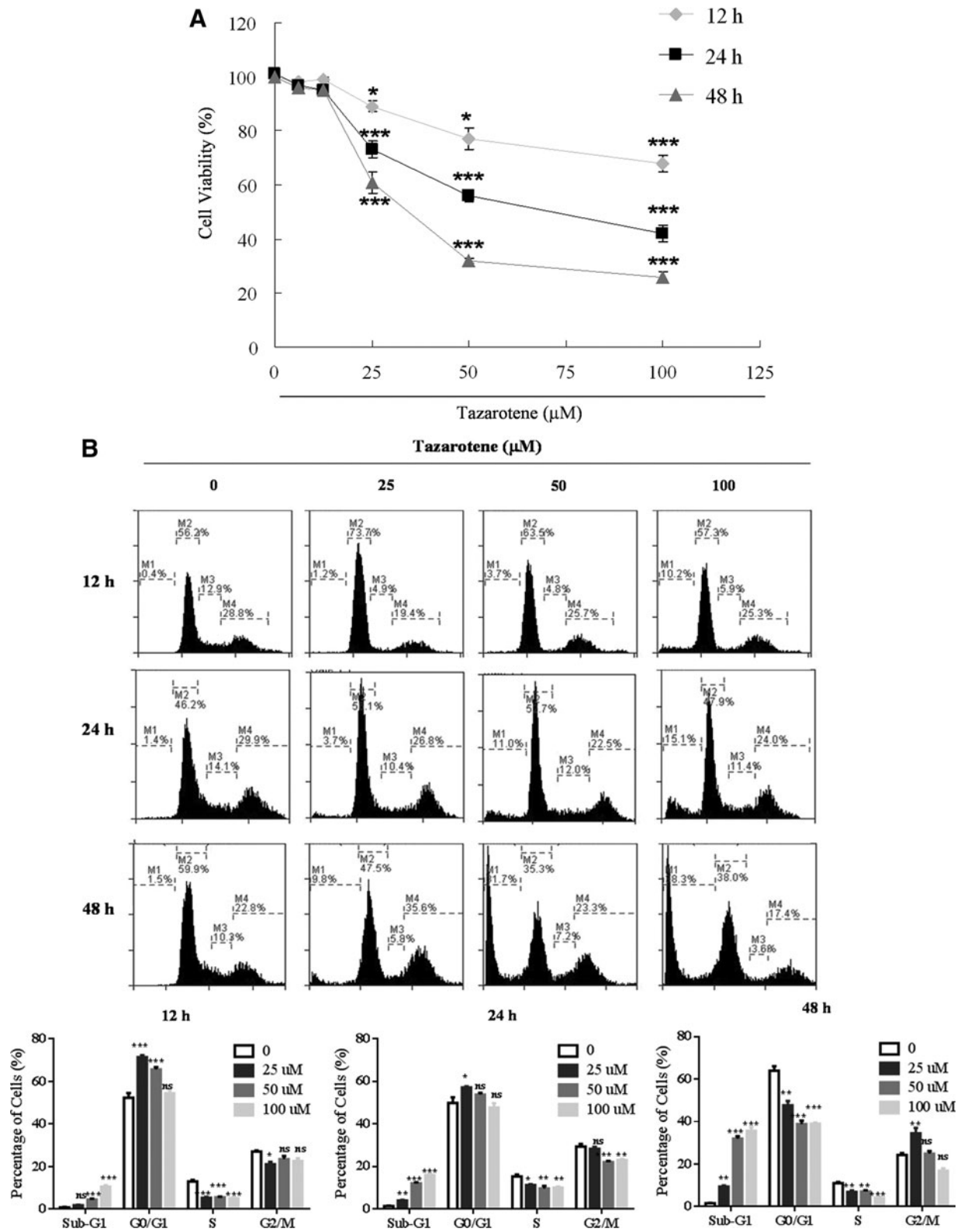


FIG. 1. Tazarotene-induced cytotoxicity and apoptosis in basal cell carcinoma (BCC) cells. (A) BCC cells were treated with various concentrations of tazarotene for 12, 24, or 48 h, and cell viability was measured with 3-(4,5-dimethylthiazolyl)-2, 5-diphenyltetrazoliumbromide assay as described in the “Materials and Methods” section. The data represent the mean \pm standard deviation (SD) from three wells. The data are representative of three independent experiments with similar results. (B) BCC cells were treated with various concentrations of tazarotene for 12, 24, or 48 h. Cells were trypsinized, propidium iodide stained, and analyzed by flow cytometry. The means \pm SD of the experimental triplicates are presented in the bar graph at the bottom. (C) BCC cells were treated with various concentrations of tazarotene for 24 h. The cells were then subjected to terminal deoxynucleotidyl transferase dUTP nick-end labeling staining and flow cytometric analysis. The means \pm SD of the experimental triplicates are presented in the bar graph at the bottom. ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the 0.1% dimethyl sulfoxide (DMSO)-treated group.

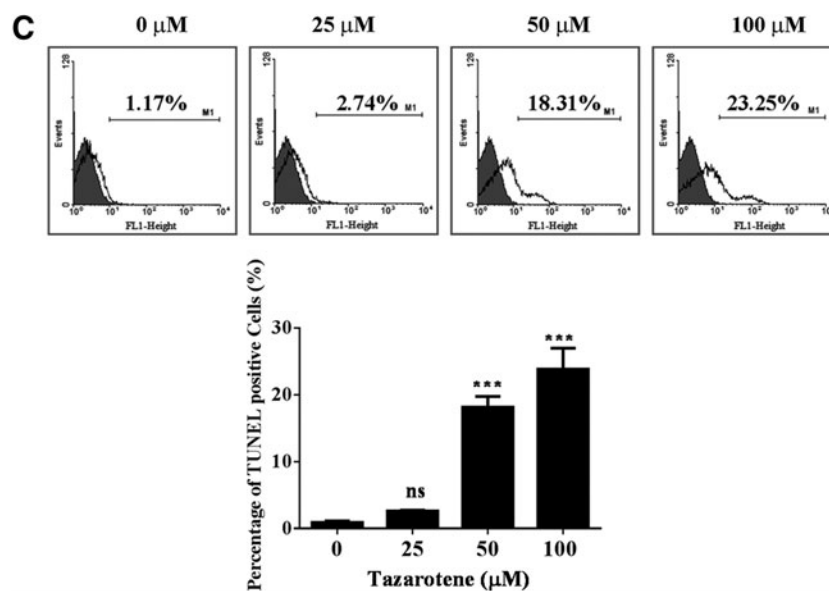


FIG. 1. (Continued).

Analysis of death receptors and ligands by flow cytometry

The BCC cells were seeded into 6-cm tissue culture dishes at 3×10^5 cells/dish in 3 mL of medium and then incubated with 0, 25, 50, and 100 μM tazarotene for 12 h. At the indicated time point, cells were trypsinised and washed with PBS. The treated cells were then stained with anti-DR4-FITC, anti-DR5-FITC, anti-Fas-FITC, anti-TRAIL-FITC, or anti-FasL-FITC (eBioscience) at 4°C for 30–45 min. The expression level of each molecule was determined by flow cytometry (FACSCalibur; BD BioSciences) and analyzed using WinMdi software (Scripps Research Institute).

In vitro assay for cytochrome c release

BCC cells were seeded into 10-cm tissue culture dishes at a density between 8×10^6 and 10×10^6 cells/dish in 8–10 mL of medium and then incubated with 0, 25, 50, and 100 μM tazarotene for 24 h. Cells were collected by centrifugation. Cytosolic fractions were isolated using the Mitochondria/Cytosol Fraction Kit (BioVision). The quality of the cytosolic fraction was estimated by Western blotting using an anti-cytochrome *c* antibody (BD Pharmingen).

Western blot analysis

The BCC cells were incubated with 0, 25, 50, and 100 μM tazarotene for 24 h, lysed in 2% sodium dodecyl sulfate (SDS; 10 mM ethylenediaminetetraacetic acid, 50 mM Tris base, 10% SDS, pH 8.0), and boiled at 100°C for 10 min. Protein concentrations were determined using the BCA Protein Assay Reagent (PIERCE). Proteins were separated by electrophoresis on a 12% or 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel and then transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% non-fat milk at room temperature and then incubated with primary antibodies against caspase-3, caspase-8, caspase-9, Bcl-2, Bcl-XL, Bak, Bax, Bid, truncated Bid (tBid), COX IV, XIAP,

cleaved PARP, and Survivin (Cell signaling) at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies (The Jackson Laboratory) for 2 h. The membranes were then incubated with the enhanced chemiluminescence system and developed using the LAS3000 system (Fuji-film). Densitometric analysis was performed with ImageJ software (National Institute of Health).

Caspase inhibitor assay

The BCC cells were seeded into 24-well tissue culture plates at between 3×10^4 and 4×10^4 cells/well. The cells were grown overnight and then pre-treated with inhibitors of caspase-3 (Ac-DEVD-CMK; Calbiochem), caspase-8 (Z-IETD-FMK; Calbiochem), and caspase-9 (Z-LEHD-FMK; Calbiochem) for 1 h. Cells were then treated with 100 μM tazarotene for 24 h. The treated cells were incubated with 5 mg/mL MTT for 4 h at 37°C. After removing the supernatant, color was developed by the addition of 600 μL of DMSO to each well. The absorbance was read at 570 nm using a microplate reader.

Statistical analysis

All statistically analyses were performed with the GraphPad Prism software package version 4.0. In addition, cell viability was evaluated by analysis of variance test between groups followed by Tukey's test to determine the significance of differences between pairs of groups.

Results

Tazarotene exerts potent cytotoxicity in BCC cells

To determine the effect of tazarotene on cell growth, human BCC cells were treated with various doses of tazarotene for 12, 24, or 48 h, and cell viability was measured using the MTT assay. As shown in Figure 1A, tazarotene significantly reduces BCC cell viability in a dose- and time-dependent manner.

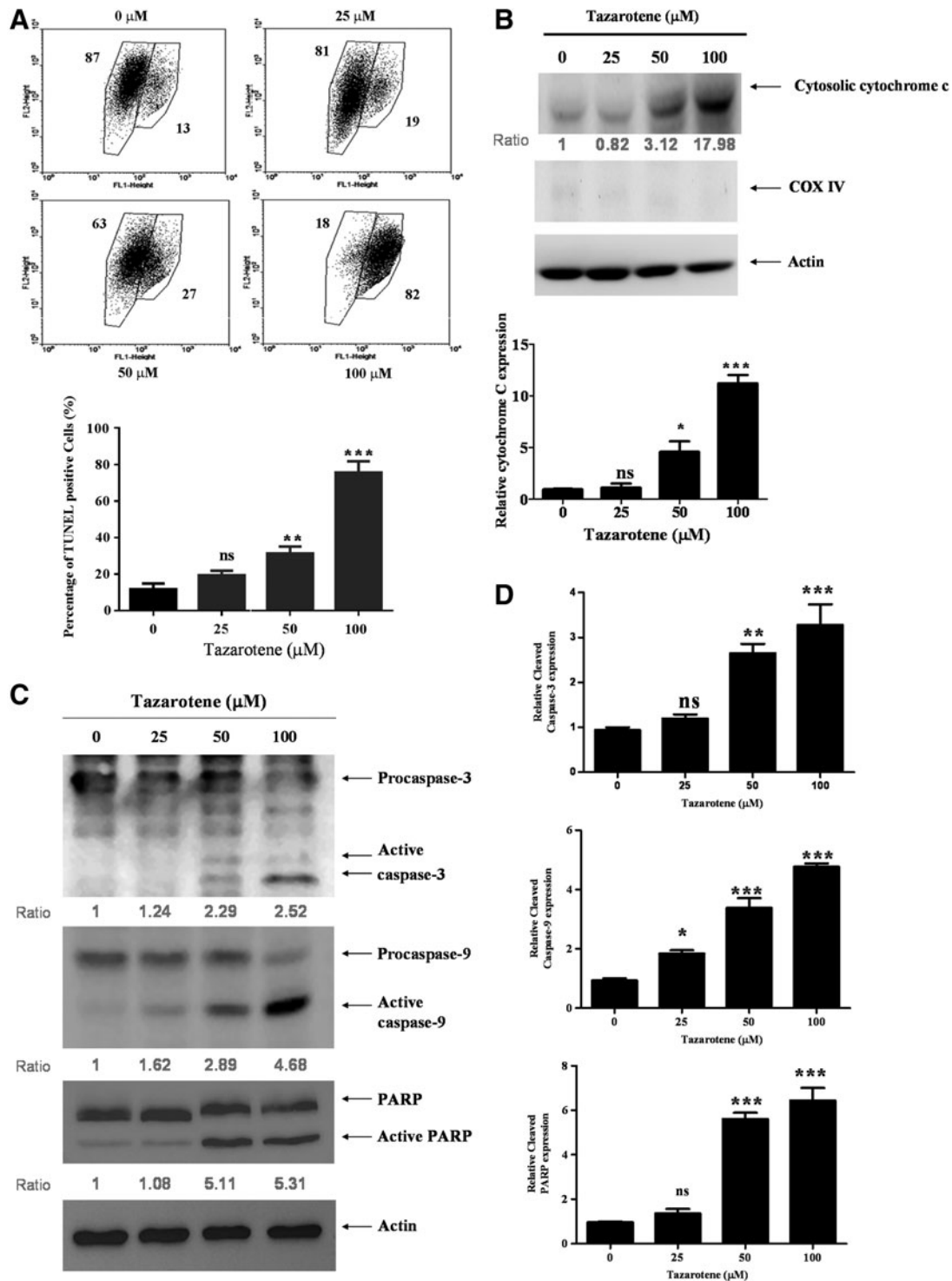


FIG. 2. Tazarotene triggered apoptotic events in BCC cells. (A) BCC cells were stained with JC-1 fluorescent dye, and mitochondrial membrane potential ($\Delta\psi_m$) was analyzed by flow cytometry. The means \pm SD of the experimental triplicates are presented in the bar graph at the bottom. (B) The cells were treated with indicated concentrations of tazarotene for 24 h. Cytosolic cell lysates were prepared, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with the indicated antibodies to detect cytochrome *c*. Cytochrome *c* oxidase subunit IV (COX IV) served as a mitochondrial marker. (C) Total cell lysates were prepared to detect the non-cleaved and cleaved forms of caspase-9, caspase-3, and PARP. (D) The bands were analyzed by ImageJ (National Institute of Health) and normalized to actin. The means \pm SD of the three independent experiments are presented in the bar graph. (E) The effect of caspase-3 and caspase-9 inhibitors on 100 μ M tazarotene-induced cell viability. The data represent the mean \pm SD from three wells. The data are representative of three independent experiments with similar results. (F) Total cell lysates were prepared and subjected to SDS-PAGE followed by Western blotting with antibodies to detect the expression of Bcl-2, Bcl-x1, Bax, Bak, XIAP, and Survivin. (G) The bands were analyzed by ImageJ and normalized to actin. The means \pm SD of the three independent experiments are presented in the bar graph. ^{ns} $p > 0.05$, ^{*} $p < 0.05$, ^{**} $p < 0.01$, and ^{***} $p < 0.001$, compared with the 0.1% DMSO-treated group. [#] $p < 0.05$, ^{###} $p < 0.001$ compared with the 100 μ M tazarotene-treated group.

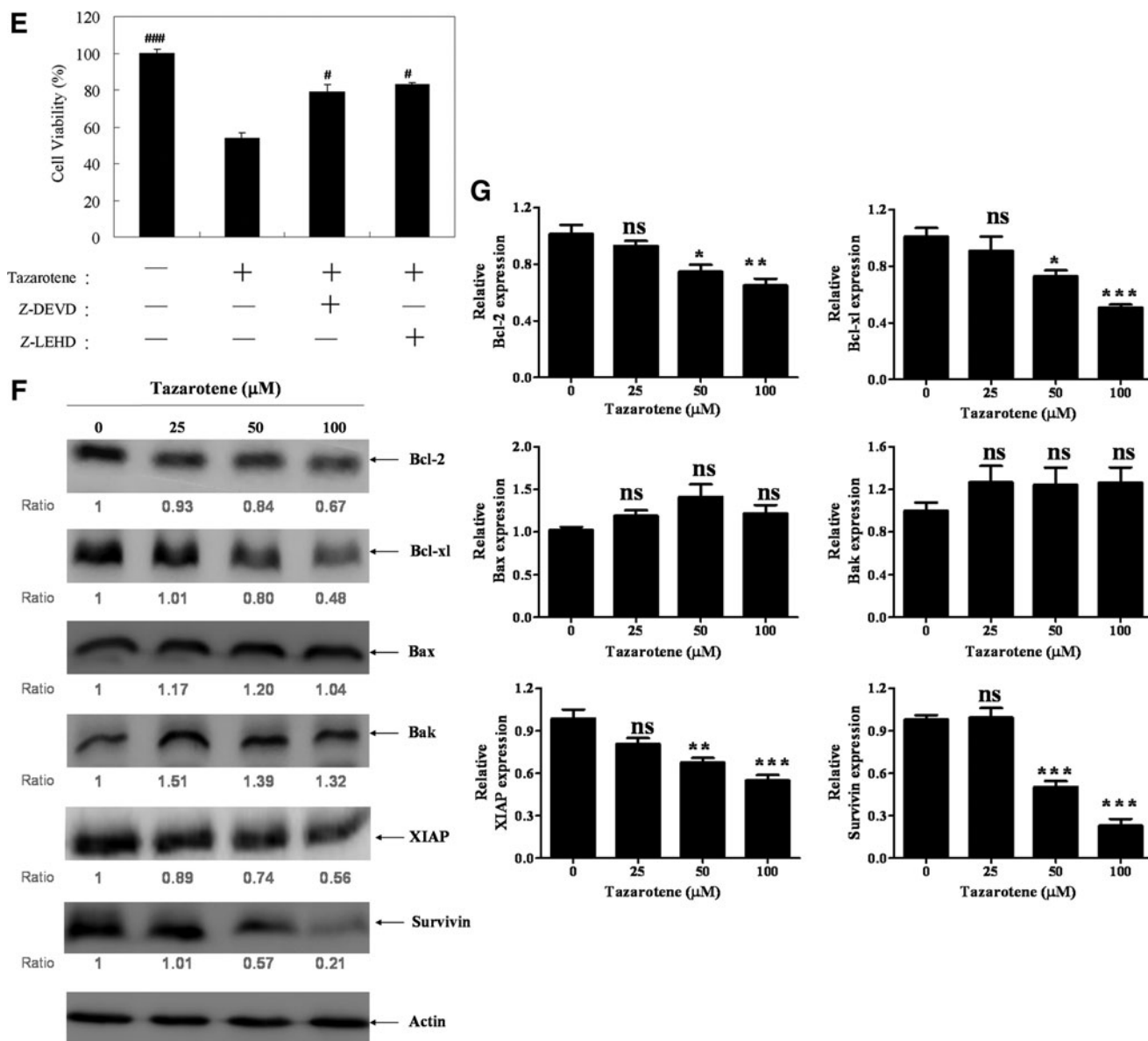


FIG. 2. (Continued).

Tazarotene-induced apoptosis in BCC cells

To determine whether tazarotene-induced growth inhibition occurs via cell cycle blockade, BCC cells were subjected to flow cytometric analysis. BCC cells were exposed to a series of different tazarotene concentrations for 12, 24, or 48 h. Figure 1B shows that 25 and 50 μM tazarotene treatment for 12 h and 25 μM tazarotene treatment for 24 h caused transient G0/G1 phase cell cycle arrest. In addition, the sub-G1 population, which is typically considered an apoptosis-related hypo-diploid DNA content peak, significantly increased in a dose-dependent manner at 12, 24, and 48 h after tazarotene treatment. To further confirm the observed tazarotene-induced apoptosis, we utilized flow cytometry to analyze TUNEL staining in cells treated with various concentrations of tazarotene for 24 h. As shown in Figure 1C, the percentage of TUNEL-positive BCC cells increased in a concentration-dependent manner. Taken to-

gether, these observations suggest that the anti-proliferative effect of tazarotene in BCC cells results, at least in part, from its ability to induce apoptosis.

Tazarotene alters MMP in BCC cells

Mitochondria play an essential role in apoptotic signaling given that both the intrinsic and extrinsic apoptotic pathways converge at the mitochondria to induce mitochondrial membrane permeabilization (MMP) (Wang, 2001; Danial and Korsmeyer, 2004). To examine whether the mitochondria-mediated apoptotic pathway is involved in tazarotene-induced apoptosis, we determined the effect of tazarotene on MMP ($\Delta\psi_m$) using flow cytometry to analyze staining with the fluorescent cationic dye JC-1. Loss of $\Delta\psi_m$ is an indicator of mitochondrial damage during apoptosis. After cells were treated with tazarotene, red fluorescence was detected in BCC cells in a concentration-dependent manner, suggesting

that tazarotene treatment results in a reduction in $\Delta\psi_m$ (Fig. 2A) and that mitochondrial dysfunction is involved in tazarotene-induced apoptosis.

Tazarotene-induced apoptosis involves the release of cytochrome c and caspase activation

Cytochrome *c* release from the mitochondria into the cytosol is an important event in the mitochondrial apoptotic

pathway (Boehning *et al.*, 2003). Mitochondrial dysfunction causes the activation of Apaf-1-associated caspase-9, which, subsequently, activates down-stream caspase-3 and induces the cleavage of PARP. In addition, Bcl-2 family proteins are frequently involved in mitochondrial dysfunction (Gross *et al.*, 1999). To elucidate the relationships among the apoptotic proteins mentioned earlier, BCC cells were exposed to tazarotene for 24 h, lysed, and assessed by Western blot analysis. Cytochrome *c* expression was assessed in the

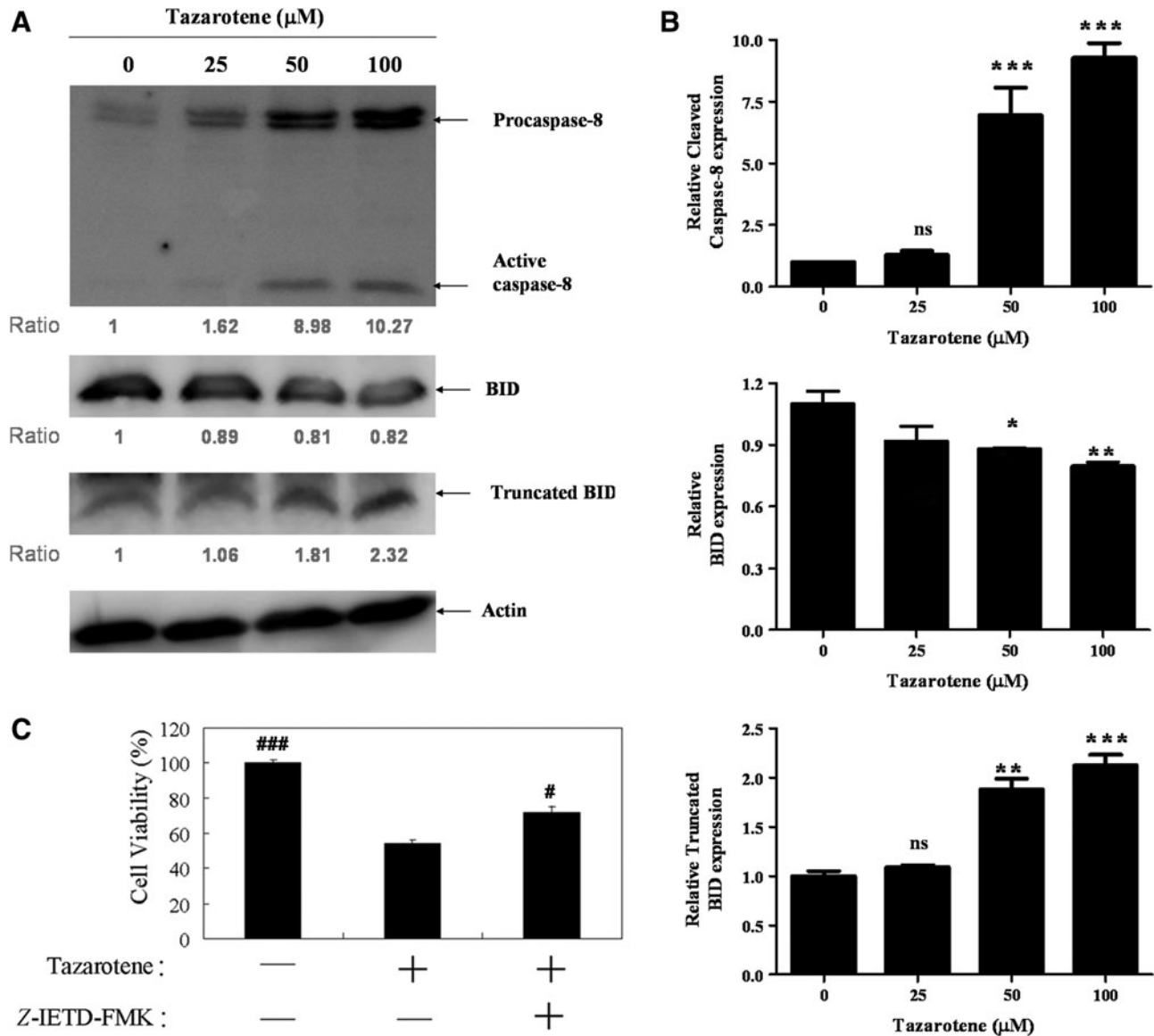


FIG. 3. Tazarotene-induced activation of the caspase-8-truncated Bid (tBid) cascade and the effects of the caspase-8 inhibitor Z-IETD-FMK in BCC cells. **(A)** The cells were treated with indicated concentrations of tazarotene for 24 h. Total cell lysates were prepared, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies to detect the non-cleaved and cleaved forms of caspase-8, Bid, and truncated Bid. **(B)** The bands were analyzed by ImageJ and normalized to actin. The means \pm SD of the three independent experiments are presented in the bar graph. ^{ns} $p > 0.05$, ^{*} $p < 0.05$, ^{**} $p < 0.01$, and ^{***} $p < 0.001$, compared with the 0.1% DMSO-treated group. **(C)** The reverse effect of the caspase-8 inhibitor on 100 μM tazarotene-induced reduction in cell viability. The data represent the means \pm SD from three wells. The data are representative of three independent experiments with similar results. **(D)** The sub-G1 population, **(E)** caspase and **(G)** Bid cleavage, and cytosolic cytochrome *c* expression are presented. **(F, H)** The bands were analyzed by ImageJ and normalized to actin. The means \pm SD of the three independent experiments are presented in the bar graph. [#] $p < 0.05$, ^{##} $p < 0.01$, and ^{###} $p < 0.001$, compared with the 100 μM tazarotene-treated group.

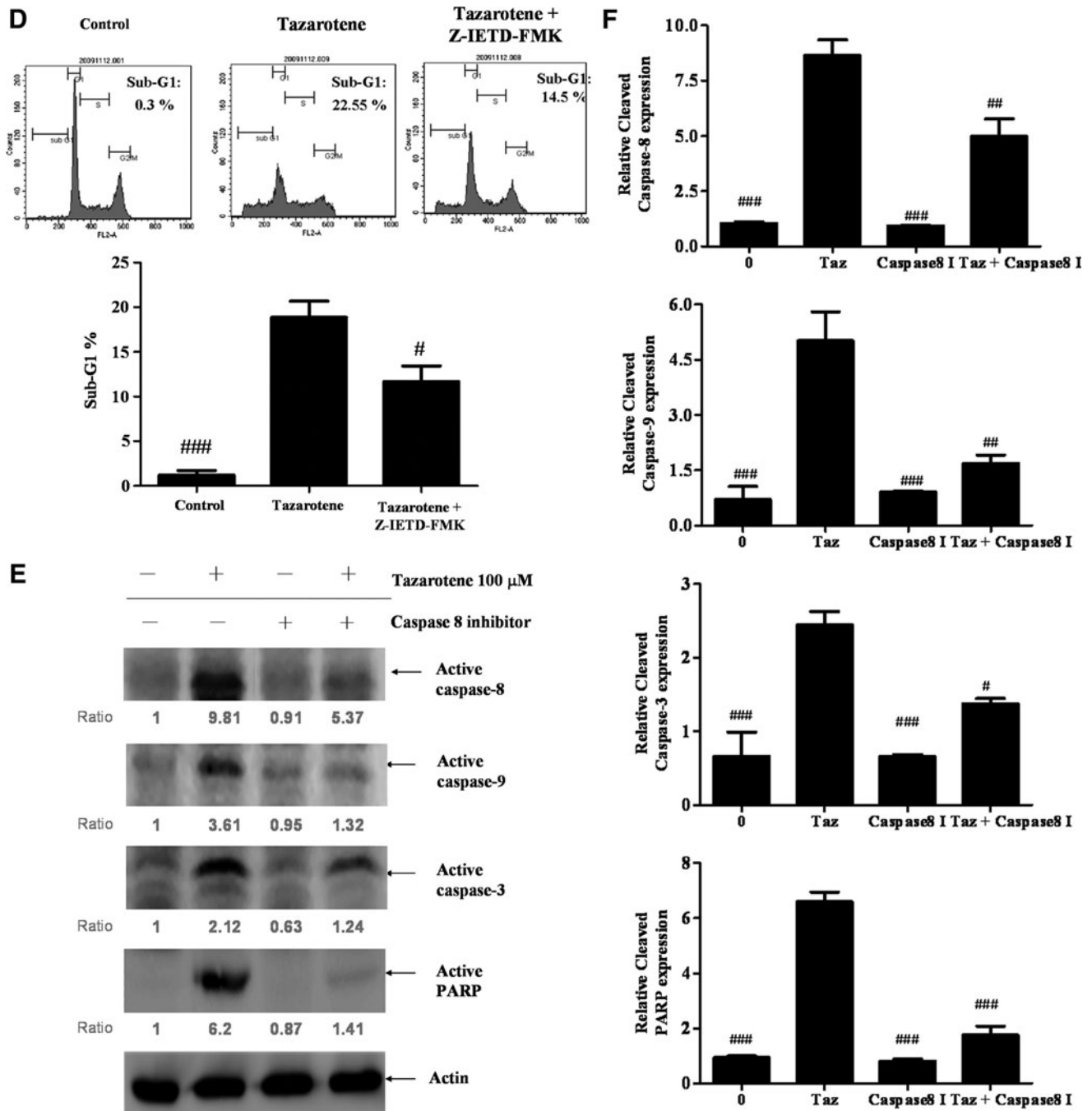


FIG. 3. (Continued).

cytosolic fractions, whereas the expression of caspase-3, caspase-9, and other apoptosis-related proteins was determined in whole cell lysates. As shown in Figure 2B, the data reveal a marked increase in cytosolic cytochrome *c* expression levels after 24 h of tazarotene treatment. As illustrated in Figure 2C and D, treatment with tazarotene induces the formation of the cleaved/activated forms of caspase-9, caspase-3, and PARP in BCC cells. Moreover, the treatment of BCC cells with a caspase-3 or caspase-9 inhibitor blocks tazarotene-induced decreases in cell viability, suggesting that the caspase-dependent pathway is involved (Fig. 2E).

Exposure to tazarotene also results in the downregulation of anti-apoptotic proteins Bcl-2 and Bcl-xl; however, tazarotene does not significantly increase the expression of apoptotic proteins Bax and Bak (Fig. 2F, G). On receiving a death signal, mitochondria release Smac/DIABLO into the cytoplasm. Smac/DIABLO binds to IAP family members and relieves IAP-mediated inhibition of caspase-9 and caspase-3, thus promoting apoptosis (Deveraux and Reed, 1999). Therefore, we also examined the expression of two IAP family proteins, survivin and XIAP, using Western blot analysis. As shown in Figure 2F and G, XIAP and survivin

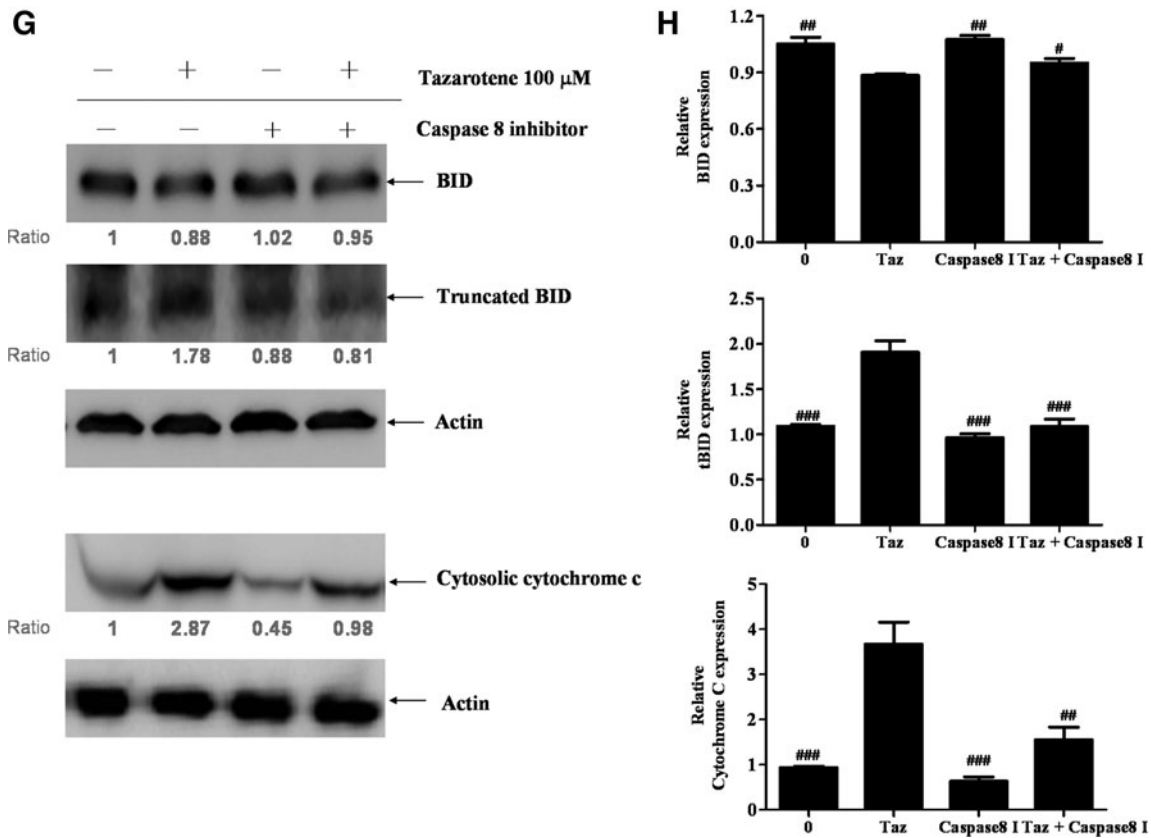


FIG. 3. (Continued).

protein expression is reduced on tazarotene treatment. Taken together, these findings suggest that treatment with tazarotene induces apoptosis primarily via the intrinsic mitochondrial pathway.

Activation of the caspase-8 pathway via tazarotene-induced apoptosis

Caspase-8 can activate caspase-3 directly through cleavage or indirectly by inducing the cleavage of Bid (23 kDa) into tBid, which subsequently triggers mitochondria-dependent activation of the caspase-9 and caspase-3 cascades (Li *et al.*, 1998). To determine whether tazarotene induces the activation of caspase-8, we treated BCC cells with various doses of tazarotene for 24 h. Our results indicate that tazarotene increases the expression of cleaved caspase-8 protein (Fig. 3A, B). In addition, Western blot analysis reveals that cleaved Bid expression increases after exposure to tazarotene (Fig. 3A, B).

Caspase-8 inhibition by Z-IETD-FMK blocks tazarotene-induced cleavage of Bid, caspase-3, and caspase-9 as well as cytochrome c release

To determine whether caspase-8 induces caspase-9 and caspase-3 activation in tazarotene-treated BCC cells, caspase-8 was inhibited with z-IETD-fmk, a selective caspase-8 inhibitor. Our results indicate that pre-treatment with z-IETD-fmk significantly rescues cell viability (Fig. 3C), inhibits

cell apoptosis (Fig. 3D), and prevents the cleavage of caspase-9, caspase-3, and PARP (Fig. 3E, F). tBid formation and cytochrome c release are also reduced when cells are pre-treated with z-IETD-fmk (Fig. 3G, H). Overall, these data indicate that caspase-8 plays an important role in tazarotene-induced apoptosis via the direct activation of effector caspase-8 and the indirect activation of the mitochondrial pathway via tBid.

Tazarotene induces the caspase-8 activation-dependent death receptor pathway

Caspase-8 cleavage can be induced through activation of death receptors, including TNF receptor-1 (TNF-R1), Fas (APO-1/CD95), TRAMP or death receptor-3 (DR3), and TRAIL receptor-1 and receptor-2 (TRAIL-R1/DR4, TRAIL-R2/DR5), via their respective ligands (Thorburn, 2004). Therefore, to explore whether a death receptor/ligand interaction was involved in tazarotene-induced caspase-8 activation and apoptosis, the expression levels of these factors on the surface were examined by flow cytometry after tazarotene treatment. As shown in Figure 4A and B, we observed that $\geq 25 \mu$ M tazarotene significantly increases Fas ligand (FASL), DR4, DR5, and TRAIL expression and $\geq 50 \mu$ M tazarotene significantly increases FAS expression after 12 h. Given that trimerization of the Fas and TRAIL receptors leads to the recruitment of the adaptor protein FADD and results in the activation of caspase-8 (Valmiki and Ramos, 2009), we further used FADD-targeted siRNA

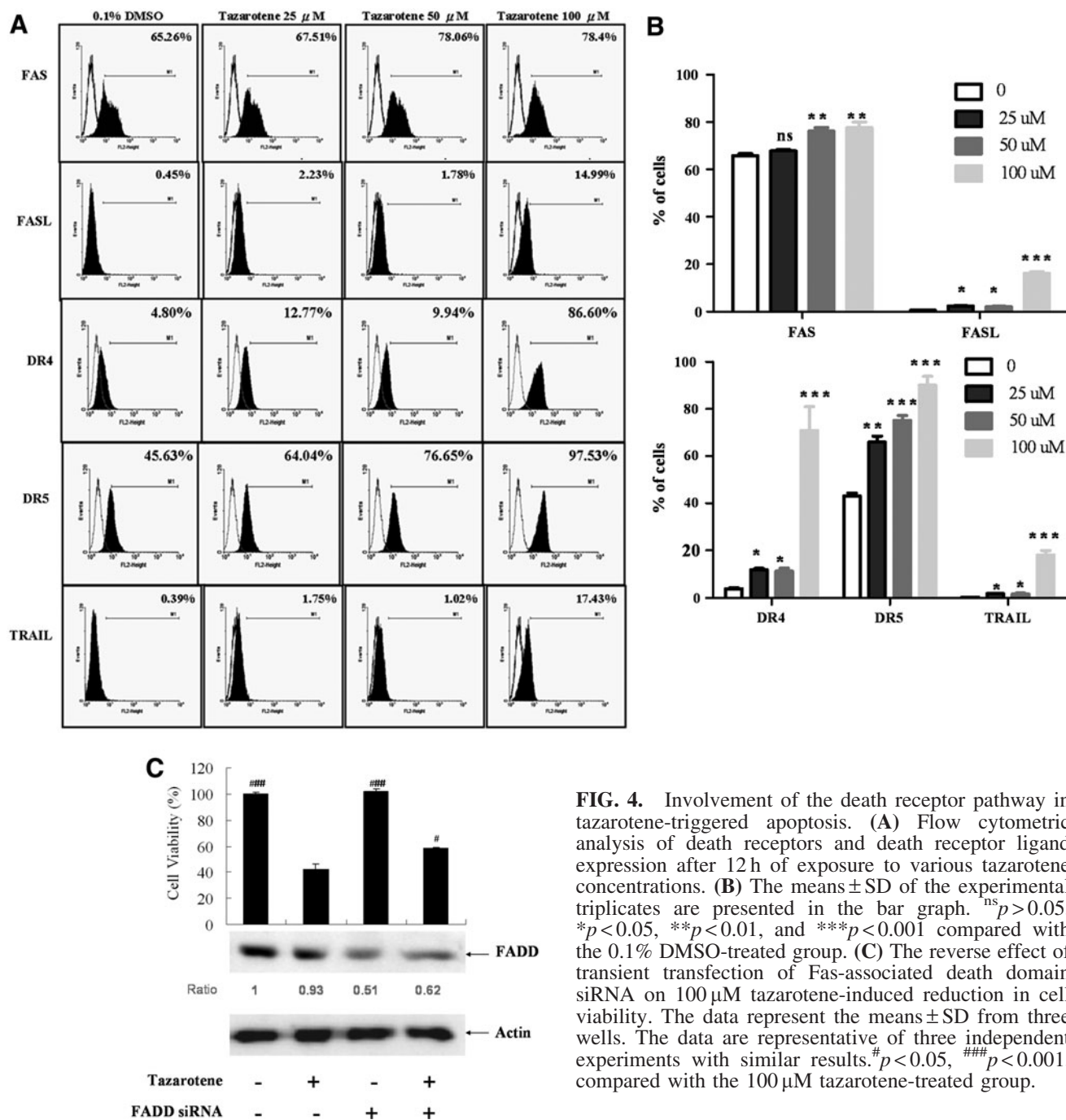


FIG. 4. Involvement of the death receptor pathway in tazarotene-triggered apoptosis. **(A)** Flow cytometric analysis of death receptors and death receptor ligand expression after 12 h of exposure to various tazarotene concentrations. **(B)** The means \pm SD of the experimental triplicates are presented in the bar graph. ^{ns} $p > 0.05$, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the 0.1% DMSO-treated group. **(C)** The reverse effect of transient transfection of Fas-associated death domain siRNA on 100 μ M tazarotene-induced reduction in cell viability. The data represent the means \pm SD from three wells. The data are representative of three independent experiments with similar results. $\#p < 0.05$, $###p < 0.001$, compared with the 100 μ M tazarotene-treated group.

to confirm the relationship between the death receptor pathway and tazarotene-induced apoptosis. Our results indicate that FADD silencing rescues cell viability (Fig. 4C), thereby suggesting that tazarotene-induced caspase-8 activation is partially dependent on death receptor signaling.

Tazarotene induces intracellular ROS

ROS are important players in apoptosis via the mitochondria-dependent pathway and caspase-8 activation (Boonstra and Post, 2004; Kim and Chung, 2007). However,

no report has indicated that tazarotene promotes the generation of ROS. Hence, we investigated the ability of tazarotene to induce ROS production using the fluorescent probe DCFDA. As shown in Figure 5A, DCFDA fluorescence intensity significantly increases in a dose-dependent manner in cells treated with tazarotene compared with untreated cells. In addition, 2 h of pre-treatment with the oxidant scavenger NAC suppresses tazarotene-mediated reduction in cell viability compared with cells treated with tazarotene alone (Fig. 5B). This result further suggests that tazarotene-induced cytotoxicity is associated with the generation of ROS in human BCC cells.

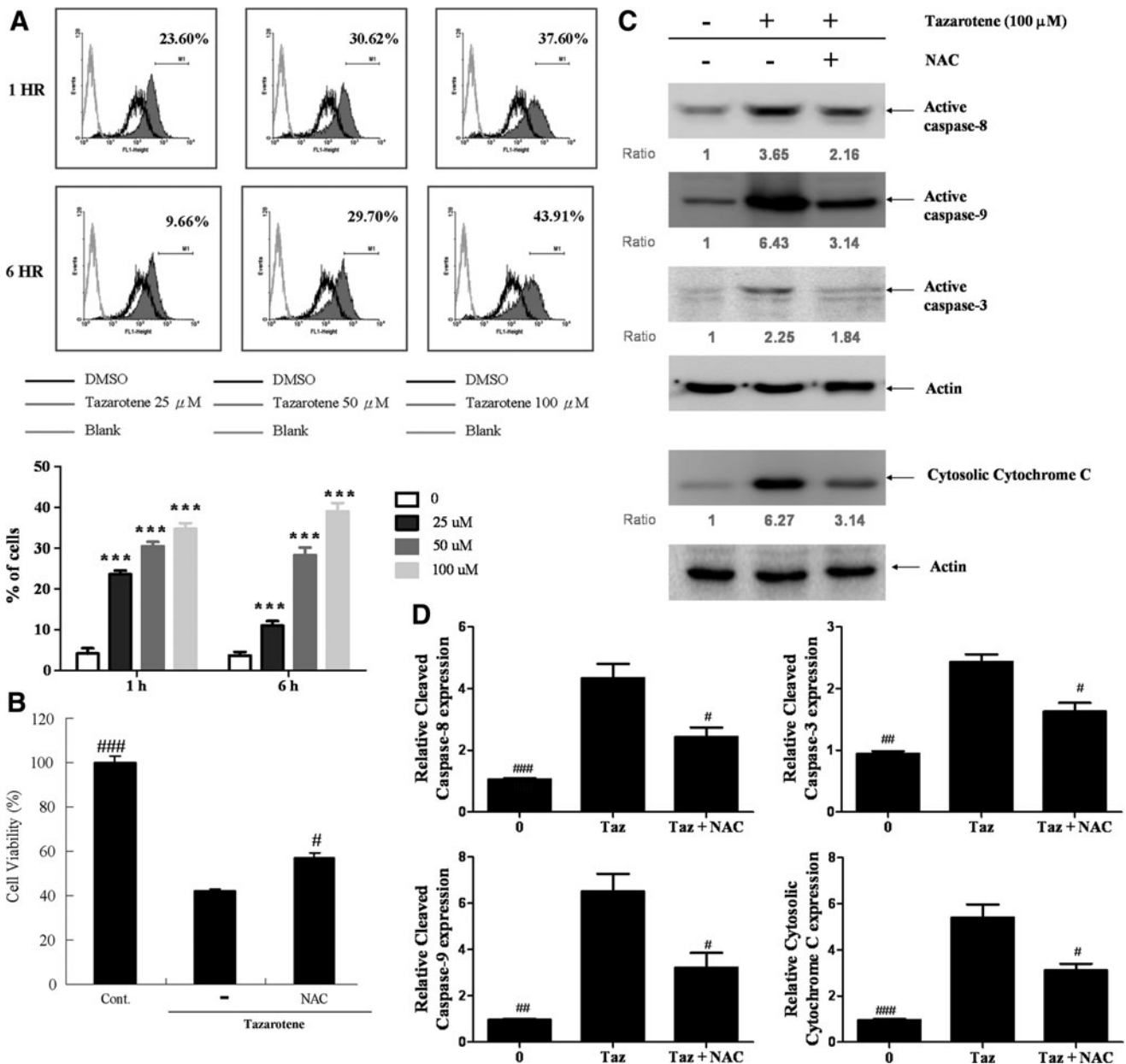


FIG. 5. The role of reactive oxygen species (ROS) in tazarotene-induced apoptosis and the reverse effect of oxidant scavengers. **(A)** BCC cells were exposed to various doses of tazarotene for 1 or 6 h, and ROS levels were analyzed by flow cytometry. The means \pm SD of the experimental triplicates are presented in the bar graph at the bottom. $***p < 0.001$, compared with the 0.1% DMSO-treated group. **(B)** Reversal of 100 μ M tazarotene-induced reduction in cell viability. The data represent the means \pm SD from three wells. The data are representative of three independent experiments with similar results. **(C)** Caspase activation and cytosolic cytochrome *c* expression induced by the oxidant scavenger *N*-acetylcysteine in BCC cells. **(D)** The bands were analyzed by ImageJ and normalized to actin. The means \pm SD of three independent experiments are presented in the bar graph. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$, compared with the 100 μ M tazarotene-treated group.

ROS are responsible for tazarotene-induced apoptotic cell death

To investigate the contribution of ROS to tazarotene-induced apoptosis and caspase activation, BCC cells were pre-treated with NAC. We observed that NAC partially suppresses tazarotene-induced cleavage of caspase-8, -3, and -9 as well as cytochrome *c* release (Fig. 5C, D). These

results suggest that ROS may be involved in tazarotene-induced BCC cell death and apoptosis.

Discussion

A clinical study suggests that topical treatment with tazarotene induces remission in BCC patients (Bianchi *et al.*, 2004), but the mechanism of tazarotene action was not

addressed. In the present study, we described the cellular and molecular events underlying the growth inhibitory effect of tazarotene in human BCC cells.

The disruption of MMP and cytochrome *c* release into the cytosol are early events in apoptotic signaling. The intrinsic and extrinsic apoptotic pathways converge at the mitochondria (Gross *et al.*, 1999; Boehning *et al.*, 2003; Wang, 2001; Danial and Korsmeyer, 2004; Sharma *et al.*, 2012), and anti-apoptotic members of the Bcl-2 family also regulate the mitochondrial apoptotic pathway (Ran *et al.*, 2005a; Wong and Puthalakath, 2008). With regard to the induction of the mitochondria-mediated apoptosis pathway, several mechanisms of action have been proposed for tazarotene, including increased Bax expression as reported in tazarotene-treated immortalized basal keratinocyte (C5N) cells (Orlandi *et al.*, 2004). In addition, tazarotene increases the number of A375 human melanoma cells expressing Bax and decreases the number of cells expressing Bcl-2 (Ran *et al.*, 2005b). In this study, our results indicate that the expression of anti-apoptotic Bcl-2 and Bcl-xl decreases in cell treated with tazarotene compared with the control group (Fig. 2F, G). However, the expression of the apoptotic proteins Bax and Bak is unaffected by tazarotene. The different effects of tazarotene on Bax expression is potentially explained by cell type differences.

Caspase-8 propagates the apoptotic signal by directly cleaving and activating downstream caspase-3 (Walczak and Krammer, 2000). However, no report has demonstrated that tazarotene induces caspase-8 activation. Our present study demonstrated, for the first time, that tazarotene could induce caspase-8 activation (Fig. 3A, B). In addition, caspase-8 inhibition significantly prevents apoptosis and caspase-3 cleavage (Fig. 3C, D). In addition, caspase-8 triggers Bid cleavage into tBid, which, subsequently, induces the release of cytochrome *c* from the mitochondria and caspase-9 activation (Li *et al.*, 1998). Our results indicate that tazarotene triggers the caspase-8–Bid cleavage pathway (Fig. 3A, B). In addition, tazarotene-induced cytochrome *c* release is reduced in the presence of a caspase-8 inhibitor (Fig. 3G, H), suggesting that caspase-8 acts as an upstream executor of the mitochondrial apoptotic pathway in tazarotene-treated cells.

In this study, our results indicate that the expression of the death receptor and its ligands, in particular FASL, DR4, DR5, and TRAIL, is upregulated in BCC cells on treatment with 25–100 μ M tazarotene; however, no statistically significant differences are observed between the 25 and 50 μ M tazarotene treatment groups (Fig. 4A, B), which do not demonstrate extensive caspase-8 activation. Thus, in addition to the death receptor signaling pathway, we hypothesize that additional apoptotic factors, such as endoplasmic reticulum stress (Rao *et al.*, 2004), Ca^{2+} influx (Chen *et al.*, 2005), p38, JNK, and mitogen-activated protein kinase (MAPK) (Iwaoka *et al.*, 2006; Cho *et al.*, 2013), are potentially involved in tazarotene-induced caspase-8 activation in BCCs.

In this study, we observed that tazarotene-induced ROS generation was evident as early as 1h after treatment. Moreover, NAC-mediated quenching of ROS generation confers partial protection against tazarotene-induced cell death (Fig. 5B) and apoptosis (Fig. 5C, D), suggesting that

tazarotene-induced ROS may act as molecules to trigger BCC cell death. However, the role of ROS in apoptosis remains controversial (Jacobson, 1996). In particular, Mochizuki *et al.* (2006) recently reported that ROS generated by Nox4 transmit cell survival signals through the AKT–ASK1 pathway in pancreatic cancer cells. Thus, further investigation is required to determine the mechanism(s) linking the possible source of ROS generation, such as the NADPH oxidase (Nox) family (Mochizuki *et al.*, 2006; Stasi *et al.*, 2010; Rousset *et al.*, 2013), in tazarotene-treated BCC cells.

In addition, the mechanism of ROS-induced caspase-8 activation remains unknown. ROS are implicated in the activation of MAPKs, and ASK1 is specifically required for the ROS-induced activation of JNK, p38, and apoptosis (Tobiume *et al.*, 2001). A previous study demonstrated that p38 MAPK-mediated cell death triggered by ROS is associated with caspase-8 activation (Iwaoka *et al.*, 2006). Thus, it is possible that ROS-induced caspase-8 activation after tazarotene treatment is mediated by MAPK activation. However, further investigation is required to elucidate the relationship between MAPK and caspase-8.

Cell cycle arrest is a common cause of cell growth inhibition and apoptosis (Pietenpol and Stewart, 2002). In this study, the proliferation of tazarotene-treated BCC cells was inhibited, and the cells were arrested in the G0/G1 phase of the cell cycle (Fig. 1B). Our results are consistent with previous studies in HL-60 human myeloblastic leukemia cells (Yen *et al.*, 2004) and Tca8113 human tongue squamous cell carcinoma cells (Ran *et al.*, 2005a, b) which demonstrated that tazarotene treatment inhibits proliferation by inducing G0/G1 phase arrest. Although the mechanism of tazarotene-induced G0/G1 arrest in BCC cells remains unclear and requires further study, a previous study demonstrated that tazarotene induces p73 expression (Papoutsaki *et al.*, 2004). Additional previous studies demonstrate that p73 induces G1 arrest (Zhu *et al.*, 1998; Ohtsuka *et al.*, 2003), prompting the hypothesis that p73 plays a role in tazarotene-induced BCC G0/G1 phase arrest.

IAPs suppress apoptosis by inhibiting activated effector caspases, such as caspase-3 and caspase-7. IAPs also inhibit cytochrome *c*-induced activation of caspase-9 (Deveraux and Reed, 1999). Furthermore, IAPs, such as survivin and XIAP, participate in the development of chemo-resistance in several cancer cells (Dubrez-Daloz *et al.*, 2008; Fulda, 2008). To our knowledge, no study describing the relationship between the IAP protein family and tazarotene action has been reported. Thus, we are the first to report that tazarotene can significantly decrease survivin and XIAP protein expression (Fig. 2F, G).

Given that our results indicate that neither caspase inhibitors FADD siRNA nor NAC completely reverse tazarotene-induced growth inhibition (Figs. 2E, 3C, 4C and 5B), other factors/molecules may also regulate the induction of cell death. Tazarotene, a new member of the acetylenic class of RAR β/γ -selective retinoids (Roeder *et al.*, 2004; Dando and Wellington, 2005; Tanghetti *et al.*, 2011), has been shown to induce a concentration-dependent increase in RAR-beta expression in the immortalized basal keratinocyte (C5N) tumor cell line

(Orlandi *et al.*, 2004). Our current results also demonstrate that tazarotene induces RAR-beta and RAR-alpha but not RAR gamma in BCC cells (data not shown). Further studies are required to examine whether a RAR β / α -dependent pathway participates in tazarotene-induced cell cycle arrest and apoptosis.

Tazarotene is now being tested in a clinical trial for skin diseases (Roeder *et al.*, 2004; Dando and Wellington, 2005; Tanghetti *et al.*, 2011). Many studies suggest that tazarotene can be used to treat skin cancers, including BCC (Bianchi *et al.*, 2004). Our present study is the first to demonstrate that tazarotene induces apoptotic cell death in BCC via caspase-8 activation and mitochondrial damage, which is accompanied by decreased levels of anti-apoptotic members of the Bcl-2 and IAP protein families. Our data also reveal that activated caspase-8 triggers mitochondrial apoptotic events by inducing Bid cleavage. Furthermore, our data suggest that ROS play a role in the activation of caspase-8 and the induction of cytochrome *c* release (Fig. 6). Taken together, these results provide insights into the molecular mechanisms underlying tazarotene-induced apoptosis in human BCC cells and suggest that this compound may be a promising candidate for cancer treatment.

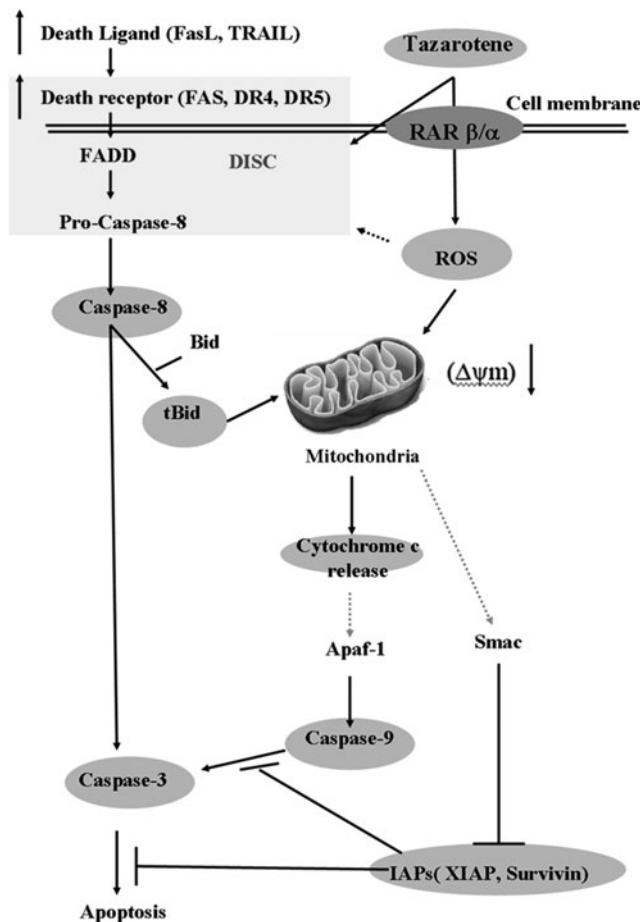


FIG. 6. A hypothetical model of the signaling pathways involved in tazarotene-mediated apoptosis in BCC cells.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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