Betulinic Acid-induced Programmed Cell Death in Human Melanoma Cells Involves Mitogen-activated Protein Kinase Activation[1]

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ABSTRACT

Betulinic acid, a naturally occurring triterpene found in the bark of the white birch tree, has been demonstrated to induce programmed cell death with melanoma and certain neuroectodermal tumor cells. We demonstrate currently that treatment of cultured UISO-Mel-1 (human melanoma cells) with betulinic acid leads to the activation of p38 and stress activated protein kinase/c-Jun NH_2-terminal kinase [widely accepted proapoptotic mitogen-activated protein kinases (MAPKs)] with no change in the phosphorylation of extracellular signal-regulated kinases (antiapoptotic MAPK). Moreover, these results support a link between the MAPKs and reactive oxygen species (ROS). As demonstrated previously, cells treated with betulinic acid generate ROS. Preincubation of cells with antioxidants blocks the process of programmed cell death, and prevents the phosphorylation of p38 and stress activated protein kinase/c-Jun NH_2-terminal kinase. These data suggest that ROS act upstream of the MAPKs in the signaling pathway of betulinic acid. In addition to mediating these responses, treatment of cells with betulinic acid resulted in a gradual depolarization of mitochondrial membrane potential, a phenomenon established to contribute to the induction of programmed cell death. Interestingly, p38 was capable of partially modulating this perturbation, and investigations of mitochondria-associated apoptotic events indicate no involvement of known caspases. These data provide additional insight in regard to the mechanism by which betulinic acid induces programmed cell death in cultured human melanoma cells, and it likely that similar responses contribute to the antitumor effect mediated with human melanoma carried in athymic mice.

INTRODUCTION

In 1995, betulinic acid was reported as a selective inhibitor of human melanoma (1), and this compound is currently undergoing development with assistance from the Rapid Access to Intervention in Development (RAID) program of the National Cancer Institute.[4] Unlike some other natural product antitumor agents, sourcing is not a problem because betulin is a major component of the bark of the white birch tree, and this can readily be converted to betulinic acid (2, 3). Subsequent studies revealed that cytotoxic responses can be mediated with other cell types (4–8), but a key observation remains the significant antitumor response with human melanoma carried in athymic mice (1) under experimental conditions yielding little or no toxicity. Accordingly, a question of great interest concerns the mechanism of action facilitated by betulinic acid, and human melanoma represents an excellent model system for studying this response. As reported previously, betulinic acid is capable of modulating apoptosis (1, 4), and mitochondria have been a focus of attention (9). Whereas it is clear that mitochondria are integrally involved in the process of programmed cell death, our objective was to identify more specific cellular targets to explain specificity.

MAPKs[5] are known to be triggered early in the apoptotic process. MAPKs are a family of serine/threonine protein kinases capable of phosphorylating numerous cytoplasmic and nuclear targets (10). They are responsive to diverse extracellular stimuli that regulate important cellular processes such as cell survival and adaptation. MAPK activity is tightly controlled through three hierarchies composed of MAPK kinase kinases that phosphorylate and thereby activate MAPK kinases. Activated MAPK kinases then phosphorylate and activate MAPKs. There are at least four distinct subgroups of MAPKs, of which the p38 MAPKs, the JNKs (also known as SAPs) and the ERKs have been extensively described. Generally, p38 and SAP/JNK are involved in the induction of apoptosis, whereas ERK has been implicated as a factor involved in cell survival (11).

Using human melanoma cells in culture, we demonstrate currently that treatment with betulinic acid activates p38 and SAP/JNK early in the programmed cell death process. Simultaneously, ERK is not activated. The signaling sequence involves the generation of ROS upstream of the MAPK response, and mitochondrial membrane alterations are partially modulated by the MAPKs. Furthermore, programmed cell death occurs independently of several key caspases, namely caspases-3, -8, and -9. Thus, our present findings strongly support the involvement of MAPKs in the apoptosis-like cell death signaling pathway induced by betulinic acid and may help define the antitumor response mediated by this substance.

MATERIALS AND METHODS

Cell Culture. UISO-Mel-1 cells, originally derived from the lymph node of a melanoma patient with metastatic disease at the University of Illinois (1, 12), were maintained in minimum essential medium (with Hanks' salts) medium (Invitrogen-Life Technologies, Inc., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biological, Norcross, GA) and 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 25 ng/ml amphotericin B, in a 5% CO_2 atmosphere at 37°C.

Chemicals and Antibodies. Betulinic acid was obtained from Indofine Chemicals (Somerville, NJ). z-VAD-FMK (a highly specific and irreversible inhibitor of caspases-1, -3, -4, and -7), SB203580 (a highly specific and potent inhibitor of p38 MAPK), and SP600125 (a selective and potent inhibitor of JNK) were obtained from Calbiochem (La Jolla, CA). Anti-phospho-p38, -phospho-JNK, -phospho-ERK, -p38, -SAP/JNK, and -ERK antibodies were from Cell Signaling Technology (Beverly, MA). Anticaspase-3, -8, -9 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). GSH, NAC, and VE were purchased from Sigma (St. Louis, MO).

Western Blotting Analysis. After treatments, cells were washed three times with ice-cold PBS and lysed in 500 μl of buffer [10 mM Tris base, 50 mM NaCl, 30 mM Na PP_I, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 5 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (complete mixture; Roche Molecular Biochemicals, Indianapolis, IN), and 0.5% Triton X-100 (pH 7.05)]. Cells were scraped from the plates and homogenized by passing through a 23-gauge needle three times. Lysates were kept at 0–4°C for 30 min and vigorously vortexed before centrifugation at 12,500 $\times$ g for 15 min at 4°C. Total protein (30 μg), as determined by a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), was resolved on 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes using a semi-dry blotting system (Fisher-Scientific, Springfield, NJ). The membranes were blocked with 5% BSA in TBST [20 mM Tris-HCl (pH 7.6), 8.2 g/liter NaCl, and 0.1% Tween 20] for 1 h at room temperature before incubation with primary antibody diluted with 3% BSA in TBST at 4°C overnight. The membranes were washed three times with TBST and incubated with secondary antibody conjugated with horseradish peroxidase (1:5000 dilution in 3% BSA in TBST; Jackson ImmunoRe-search Laboratories, West Grove, PA) at room temperature for 1 h. Proteins were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech Inc., Piscataway, NJ) after three washes in TBST.

Annexin V-FITC Flow Cytometric Analysis. After treatment with betulinic acid, floating and adherent cells were pooled and harvested. The cells were washed twice with cold PBS before resuspension in 1 $\times$ binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$]. Annexin V-FITC (7.5 μl; BD PharMingen, San Diego, CA) was added to the cell suspension, which was gently vortexed and incubated for 25 min. PI (2 μl of 50 μg/ml stock solution; Clontech, Palo Alto, CA) was added immediately before flow cytometric analysis (FACScan; Becton Dickinson, San Jose, CA). PI was used to distinguish cells that had lost membrane integrity.

Mitochondrial Membrane Potential Analysis. Fifteen min before collection of cells, 40 nM $DiOC_6$ (Molecular Probe, Eugene, OR) was added to the cells. Detached cells were pooled with cells obtained by treatment with trypsin and centrifuged at 500 $\times$ g for 8 min at 4°C. The cell pellets were washed once with PBS before resuspending in 500 μl PBS containing 40 nM $DiOC_6$. Fluorescence intensities of 200 μl of cell suspension in PBS containing $DiOC_6$ were analyzed by flow cytometry (FAC-Scan) with excitation and emission settings of 484 and 500 nm, respectively. Momentarily before flow cytometric analysis, 2 μl of PI (50 μg/ml) were added to gate out dead cells.

Intracellular ROS Measurement. UISO-Mel-1 cells were seeded in 12-well plates (2 $\times$ 10^5 cells/well) for 2 days before treatment with 8 μg/ml betulinic acid for various periods of time in a buffer containing 20 mM HEPES and HBSS (pH 7.4). To detect ROS generation, the oxidation-sensitive fluorescent dye, DCFH-DA, available from Sigma, was added to the wells (5 μg/ml) at the end of the various incubation periods. Untreated cells received DCFH-DA alone. After a 1-h incubation with DCFH-DA at 37°C, fluorescence was determined at 485 nm excitation and 530 nm emission, with a fluorescence spectrophotometer (PerSeptive Biosystems, Inc., Framingham, MA).

Caspase-3 Activity Analysis. After UISO-Mel-1 cells were incubated with 8 μg/ml of betulinic acid for various periods of time, the cells were washed twice with ice-cold PBS and lysed in buffer [50 mM Tris (pH 7.4), 50 mM β-glycerophosphate, 15 mM $MgCl_2$, 15 mM EGTA, 100 μM phenylmethylsulfonylfluoride, 1 mM DTT, and 150 μg/ml digitonin]. The lysates were homogenized by passing through a 23-gauge needle three times, and incubated at 0–4°C for 30 min. Then, the homogenates were centrifuged at 12,500 $\times$ g for 20 min at 4°C to collect the total protein. Total protein (10 μg), as determined with the Bio-Rad Protein Assay, was incubated with 200 μM acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin in 50 μl of protease assay buffer containing 100 mM HEPES (pH 7.5), 10% sucrose, 10 mM DTT, and 0.1% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate. After incubation at 37°C for 2 h, fluorogenic activity was measured with excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (PerSeptive Biosystems, Inc.). One unit of enzymatic activity for caspases was defined as the release of 1 pmol of amino-4-methylcoumarin per min at 37°C.
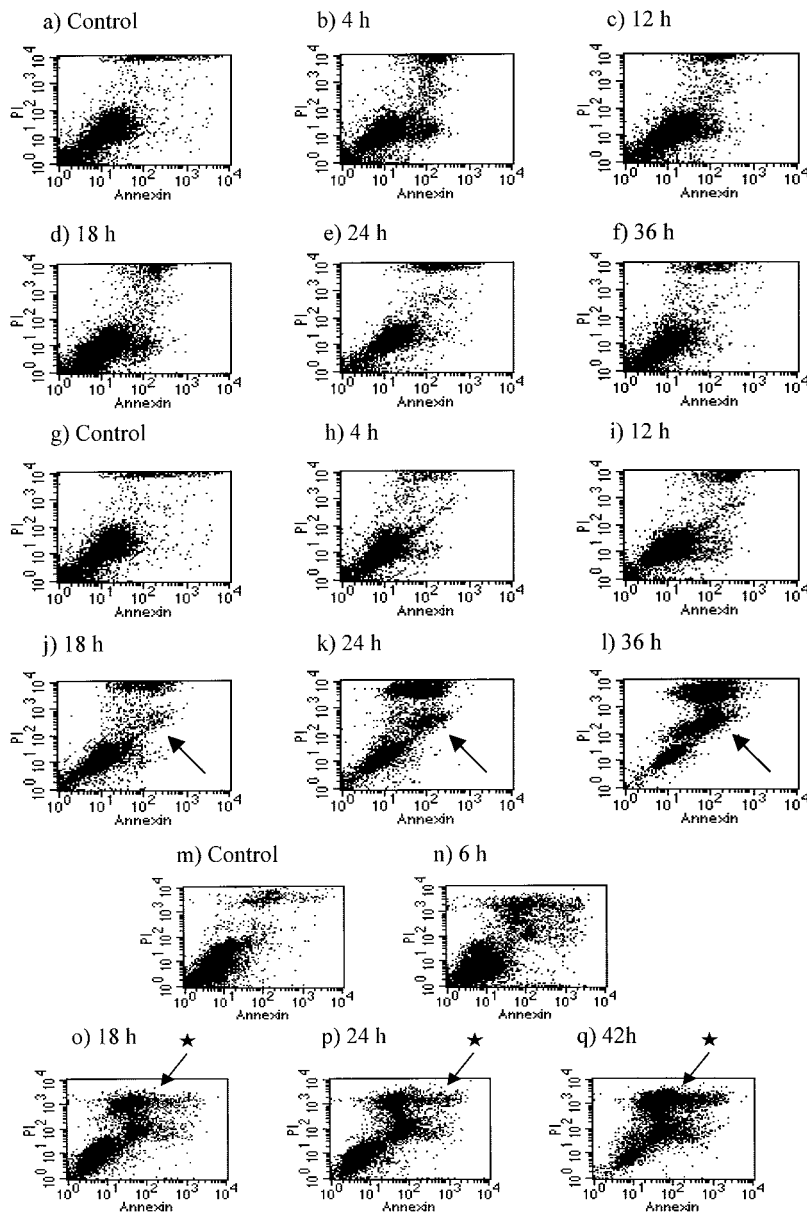
Fig. 1 Annexin V-FITC flow cytometry plots illustrating the dose and time course for apoptosis induction. UISO-Mel-1 cells treated with 4 (*a–f*) or 8 μg/ml (*g–l*) betulinic acid. *Arrows* point to the apoptotic population of cells. *m–q* show UISO-Mel-1 cells treated with 8 μg/ml betulinic acid for increasing periods of time. *Arrows* (*with stars*) point to increasing necrotic cell population.
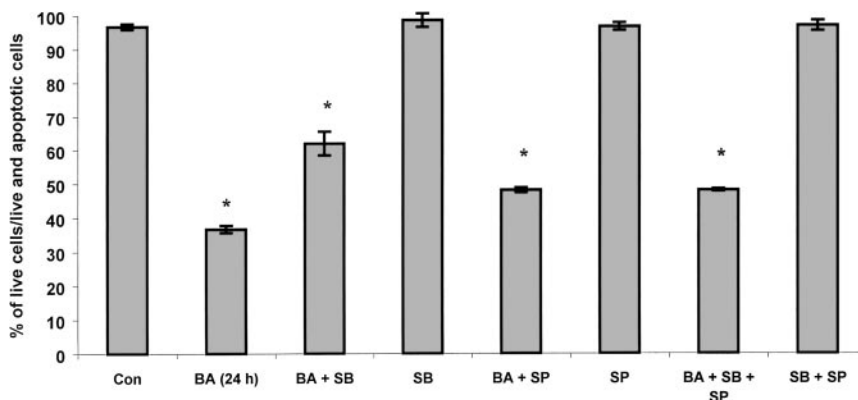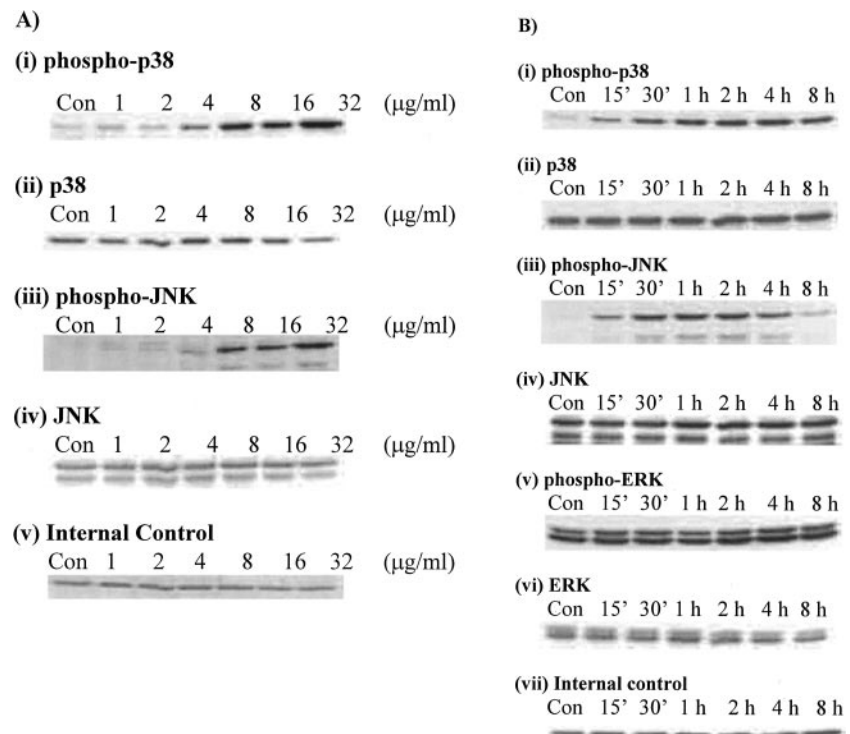
RESULTS

Detection of Programmed Cell Death Induction after Treatment with Betulinic Acid. Two doses of betulinic acid (4 and 8 μg/ml) were added to UISO-Mel-1 cells for various time periods ranging up to 36 h (Fig. 1, *a–l*). Flow cytometric analysis using annexin V-FITC indicated the lack of programmed cell death induction using 4 μg/ml betulinic acid. However, using 8 μg/ml betulinic acid, an apoptotic-like population of cells was observed (Fig. 1, *j–l*, marked by arrows), beginning at 18 h of incubation. When betulinic acid (8 μg/ml) treatment was continued for 42 h, the cells appeared necrotic, as demonstrated by the level of PI staining (Fig. 1, *o–q*).

Proapoptotic MAPK Protein Phosphorylation. A dose-dependent study in which UISO-Mel-1 cells were incubated with betulinic acid for 4 h demonstrated the phosphoryl-

ation of MAPK proteins (Fig. 2*A*), namely p38 and SAP/JNK, involved in programmed cell death. Western blots using anti-p38 and anti-SAP/JNK antibodies confirmed that the alterations involved only the phosphorylation states of the above-mentioned proteins, because there were no significant changes in the endogenous protein levels. A separate set of experiments was performed to determine the time course of p38 and SAP/JNK protein phosphorylation. A concentration of 8 μg/ml was selected on the basis of annexin V-FITC flow cytometric analysis. As shown in Fig. 2*B*, both p38 and SAP/JNK protein phosphorylation began after 15 min of incubation. The extent of protein phosphorylation increased over time. Whereas the level of p38 phosphorylation reached a plateau, SAP/JNK protein phosphorylation decreased after 2 h, indicating a transient phosphorylation pattern. On the other hand, in the same study, the

A)

(i) phospho-p38

Con 1 2 4 8 16 32 (μg/ml)

(ii) p38

Con 1 2 4 8 16 32 (μg/ml)

(iii) phospho-JNK

Con 1 2 4 8 16 32 (μg/ml)

(iv) JNK

Con 1 2 4 8 16 32 (μg/ml)

(v) Internal Control

Con 1 2 4 8 16 32 (μg/ml)

B)

(i) phospho-p38

Con 15' 30' 1 h 2 h 4 h 8 h

(ii) p38

Con 15' 30' 1 h 2 h 4 h 8 h

(iii) phospho-JNK

Con 15' 30' 1 h 2 h 4 h 8 h

(iv) JNK

Con 15' 30' 1 h 2 h 4 h 8 h

(v) phospho-ERK

Con 15' 30' 1 h 2 h 4 h 8 h

(vi) ERK

Con 15' 30' 1 h 2 h 4 h 8 h

(vii) Internal control

Con 15' 30' 1 h 2 h 4 h 8 h

Fig. 2 Western blots displaying MAPK protein phosphorylations in UISO-Mel-1 treated with betulinic acid. *A,* results exhibiting the effects of various concentrations of betulinic acid (0–32 μg/ml) using a 4-h incubation period. Anti-p38 and -SAP/JNK antibodies demonstrate that the total protein levels were unchanged. *B,* time course (0–8 h) data revealing phosphorylation pattern changes in UISO-Mel-1 cells treated with 8 μg/ml betulinic acid. The change in phosphorylation is additionally supported by the fact that endogenous protein levels of p38, SAP/JNK, and ERK remained unaltered.

Fig. 3 Analysis of annexin V-FITC flow cytometry results demonstrating the partial protection against apoptosis induced by betulinic acid (8 μg/ml for 24 h) in UISO-Mel-1 cells preincubated with 10 μM SB203580 (p38-specific inhibitor), 10 μM SP600125 (JNK-specific inhibitor), or both SB203580 and SP600125 together. The different MAPK inhibitors themselves had no effect on the cells. ∗, statistically significant differences relative to the control based on Student's *t* test; $P < 0.01$. *BA,* betulinic acid; *SB,* SB203580; *SP,* SP600125; *bars,* ±SD.
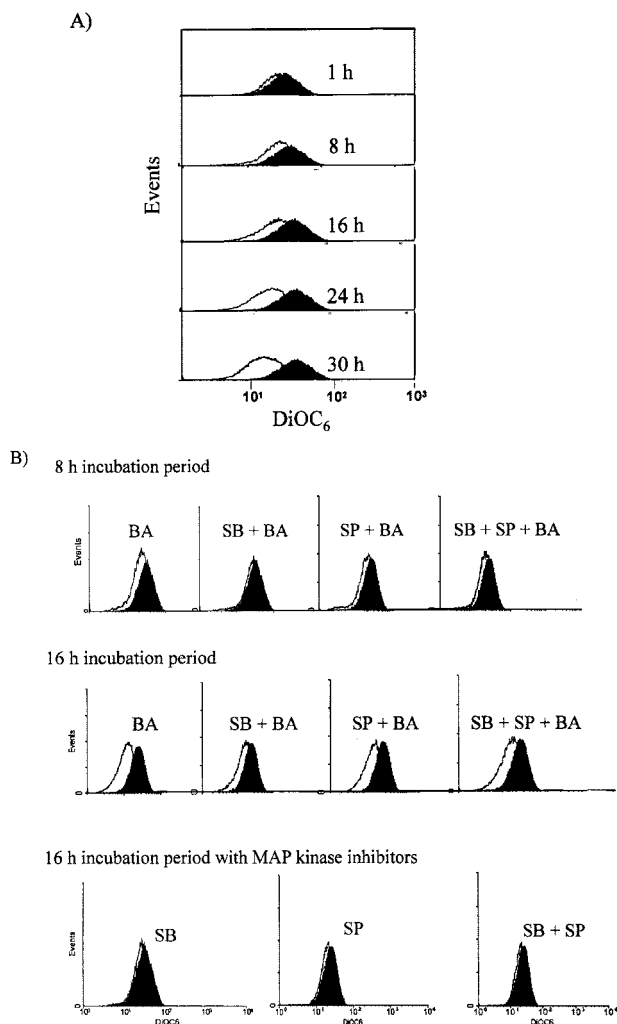
phosphorylation pattern of ERK, an antiapoptotic MAPK family member, did not change. The quantities of p38, SAP/JNK, and ERK proteins did not change over time (Fig. 2).

Partial Involvement of the p38 Pathway in Betulinic Acid-induced Apoptosis. Having determined the involvement of p38 and SAP/JNK proteins in betulinic acid-induced programmed cell death, we investigated the level of control exerted by these proteins in the process. Using either 10 μM SB203580, a p38-specific inhibitor, or 10 μM SP600125, a SAP/JNK-specific inhibitor, or both substances simultaneously, a set of experiments using annexin V-FITC flow cytometry was conducted. UISO-Mel-1 cells were incubated with the MAPK inhibitors for 2 h before the addition of 8 μg/ml betulinic acid for 24 h, in the attempt to protect these cells from betulinic

acid-induced programmed cell death. Although SB203580 failed to completely block the induction of programmed cell death, as seen by the annexin V-FITC flow cytometric analysis (Fig. 3), a higher percentage of living cells was observed, relative to the betulinic acid-treated samples. In comparison with SB203580, SP600125 protected the cells to a lesser degree.

Changes in Mitochondrial Membrane Potential. DiOC$_6$ is a dye used to measure mitochondrial membrane potential. Live cells contain mitochondria capable of retaining the dye, whereas dead or apoptotic cells lose this ability because of the loss of mitochondrial membrane integrity and, thus, exhibit an extrusion of the dye. Because mitochondria are known to be involved in the process of programmed cell death, we investigated changes in mitochondrial membrane potential with betu-

A)

B) 8 h incubation period

16 h incubation period

16 h incubation period with MAP kinase inhibitors

Fig. 4 $DiOC_6$ dye uptake was used to assess changes in mitochondrial membrane potential during apoptosis. *A*, time course showing mitochondrial membrane depolarization (left shift of peaks compared with control peaks, illustrated as *filled histograms*) of UISO-Mel-1 cells treated with 8 μg/ml betulinic acid. *B*, effects on mitochondrial membrane potential mediated by MAPK inhibitors (10 μM SB203580 and/or 10 μM SP600125) incubated with UISO-Mel-1 cells before treatment with 8 μg/ml betulinic acid (8 and 16 h). The inhibitors themselves did not significantly affect mitochondrial membrane potential.

linic acid-treated UISO-Mel-1 cells. A left shift of peaks of mitochondrial dye uptake, a phenomenon also known as the depolarization of the mitochondria membrane, was observed over time, indicating a gradual loss of $DiOC_6$ within the mitochondria of cells treated with betulinic acid (Fig. 4A). SB203580 (10 μM) was able to block depolarization of the mitochondrial membrane, but only for a period of 8 h (Fig. 4B). After 16 h of incubation, SB203580 no longer protected against mitochondrial perturbation induced by betulinic acid. A weaker protective effect was provided by 10 μM SP600125 (Fig. 4B).

Intracellular ROS Generation. The generation of ROS was monitored using an oxidation-sensitive fluorescent dye, DCFH-DA. DCFH-DA is a nonfluorescent cell-permeant compound. Once inside the cell, this substance is cleaved by endog-
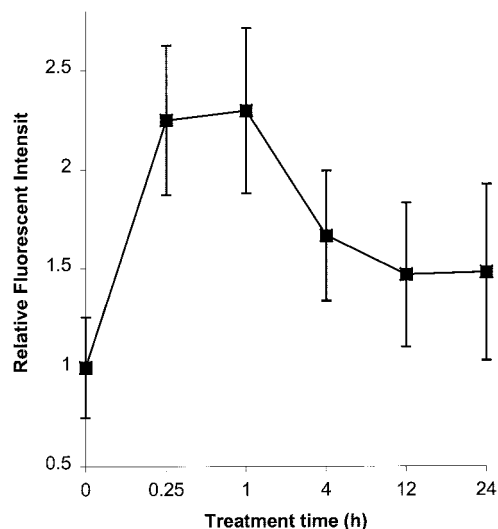

Fig. 5 Detection of the dye DCFH-DA using fluorescence spectroscopy (PerSeptive Biosystems, Inc.) to measure the generation of ROS. After the addition of 8 μg/ml betulinic acid to UISO-Mel-1 cells, a maximal burst of ROS was observed at time periods <1 h. ROS continued to be released throughout the experimental period of 24 h. All time points were statistically significant compared with 0 h using Student's *t* test, $P < 0.05$; *bars,* ±SD.

enous esterases, and the resulting product is trapped within the cell. The de-esterified product is converted to the fluorescent compound 2′,7′-dichlorofluorescein on exposure to ROS. As demonstrated in Fig. 5, maximal response was observed between 0.25 and 1 h, with a gradual decrease to 1.5-fold difference compared with the control after 24 h.

Antioxidants Prevent Apoptosis Induction. Antioxidants are commonly used to antagonize the effects of ROS or to protect against programmed cell death (13). A number of antioxidants were selected to investigate the importance of the ROS in the process of programmed cell death mediated by betulinic acid. Preincubation of UISO-Mel-1 cells with 30 mM GSH, 1 mM VE, or 30 mM NAC, before the addition of 8 μg/ml betulinic acid, significantly protected the cells from programmed cell death, as demonstrated by annexin V-FITC flow cytometric analysis (Fig. 6). GSH and VE provided greater protection against programmed cell death than NAC. The antioxidants by themselves did not exert any notable effect on the cells.

Antioxidants Alter MAPK Phosphorylation. The results described above suggest a prominent role played by ROS in betulinic acid-induced programmed cell death. We then investigated the temporal sequence of this class of molecule in the signaling pathway. Treatment of UISO-Mel-1 cells with GSH or VE before treatment with betulinic acid caused a decrease in p38 phosphorylation, in comparison with cells treated with betulinic acid alone (Fig. 7). This change in the p38 phosphorylation pattern correlated with ERK phosphorylation, *i.e.,* the dephosphorylation of ERK increased as the level of p38 phosphorylation increased. All three of the antioxidants decreased SAP/JNK phosphorylation. Expression of the respective unphosphorylated MAPKs was maintained at a constant level throughout the experiment (Fig. 7).
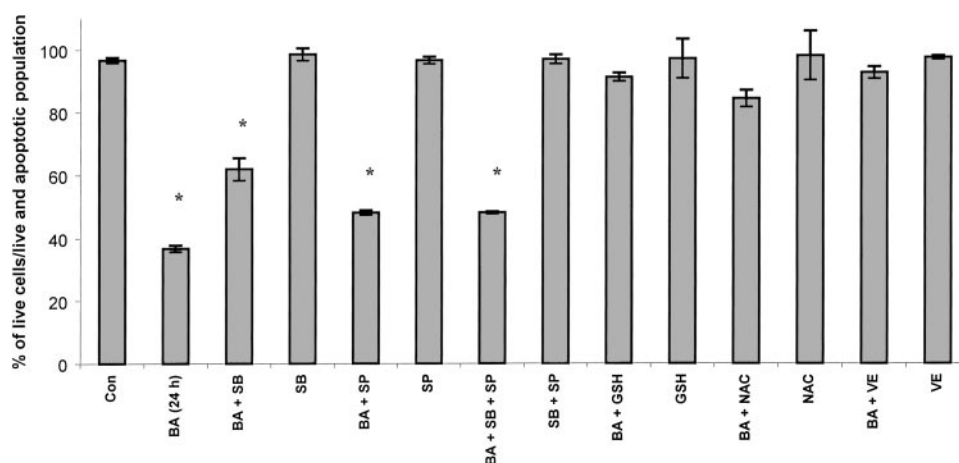
Fig. 6 Analysis of annexin V-FITC flow cytometry results exemplifying the different levels of protective effect afforded by various MAPK inhibitors (10 μM SB203580 and/or 10 μM SP600125) and antioxidants (30 mM GSH, 30 mM NAC, or 1 mM VE). UISO-Mel-1 cells were first incubated with the indicated test substance for 1 h followed by treatment with 8 μg/ml betulinic acid for 24 h. The test substances themselves did not exert any effect on the cells. *, statistically significant differences relative to the control sample based on Student's *t* test, $P < 0.01$; *bars*, ±SD. *BA*, betulinic acid; *SB*, SB203580; *SP*, SP600125.
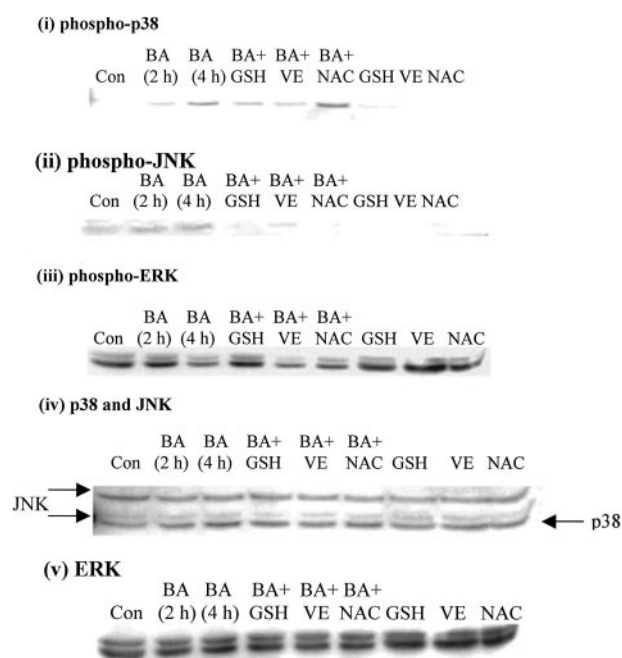
Fig. 7 Western blots showing the effects of various antioxidants on MAPK phosphorylation. GSH (30 mM), VE (1 mM), or NAC (30 mM) were preincubated with UISO-Mel-1 cells for 1 h before treatment with 8 μg/ml betulinic acid (*BA*) for 3 h. The membranes were stripped and reprobed with anti-p38, -JNK, and -ERK antibodies to show that the endogenous protein levels were constant. Only one isoform of the phosphorylated JNK protein was detectable. A possible explanation is a much lower expression of the other isoform, as indicated by the Western blot using unphosphorylated JNK antibody.

Lack of Caspase-3 Activity. Caspases work in a sequential fashion, beginning with initiator caspases and ending with the activation of effector caspases that are responsible for typical apoptotic cell morphology. Caspase-3 has been deemed a downstream effector caspase, and many of the biochemical and morphological changes associated with apoptosis are because of caspase-3 activation (14). Caspase-3 activity can be monitored through the cleavage of a specific substrate with the amino acid sequence DEVD. Using this method, we were not able to detect significant caspase-3 activity in UISO-Mel-1 cells incubated with betulinic acid (8 μg/ml) for time periods ranging up to 30 h (Fig. 8*A*).

No Involvement of Known Caspases. There are two possibilities for the lack of caspase-3 activity: absence of caspase-3 protein or caspase-3 protein remains uncleaved. To examine these possibilities, we performed Western blots using anticaspase-3 antibody. As a result, caspase-3 was detected (Fig. 8*B, panel i*), but this protease was not cleaved, indicating that it remained in an inactive state. Two initiator caspases, caspases-8 and -9, known to activate caspase-3, also exhibited a similar state of inactivation (Fig. 8*B, panels ii* and *iii*). The lack of involvement of known caspases was also verified using annexin V-FITC flow cytometric analysis with 25 μM of the broad-spectrum caspase inhibitor, z-VAD-FMK. Addition of z-VAD before treatment with betulinic acid had no effect on the distribution of cells (Fig. 8*C*).

DISCUSSION

As noted in previous studies (1, 4–7), treatment of cells with betulinic acid leads to programmed cell death. Consistent with these data, based on annexin V-FITC flow cytometric analysis of UISO-Mel-1 cells treated with betulinic acid at a concentration of 8 μg/ml, programmed cell death was observed after ~18 h of treatment. However, with longer treatment periods (>24 h), necrosis was the dominant form of cell death. Therefore, to study the events leading to programmed cell death, experiments were normally restricted to 24-h treatment periods. Our data analysis differs slightly from convention, because these adherent cells require a trypsinization step for flow cytometric analysis. Trypsinization is known to disrupt cell membranes and
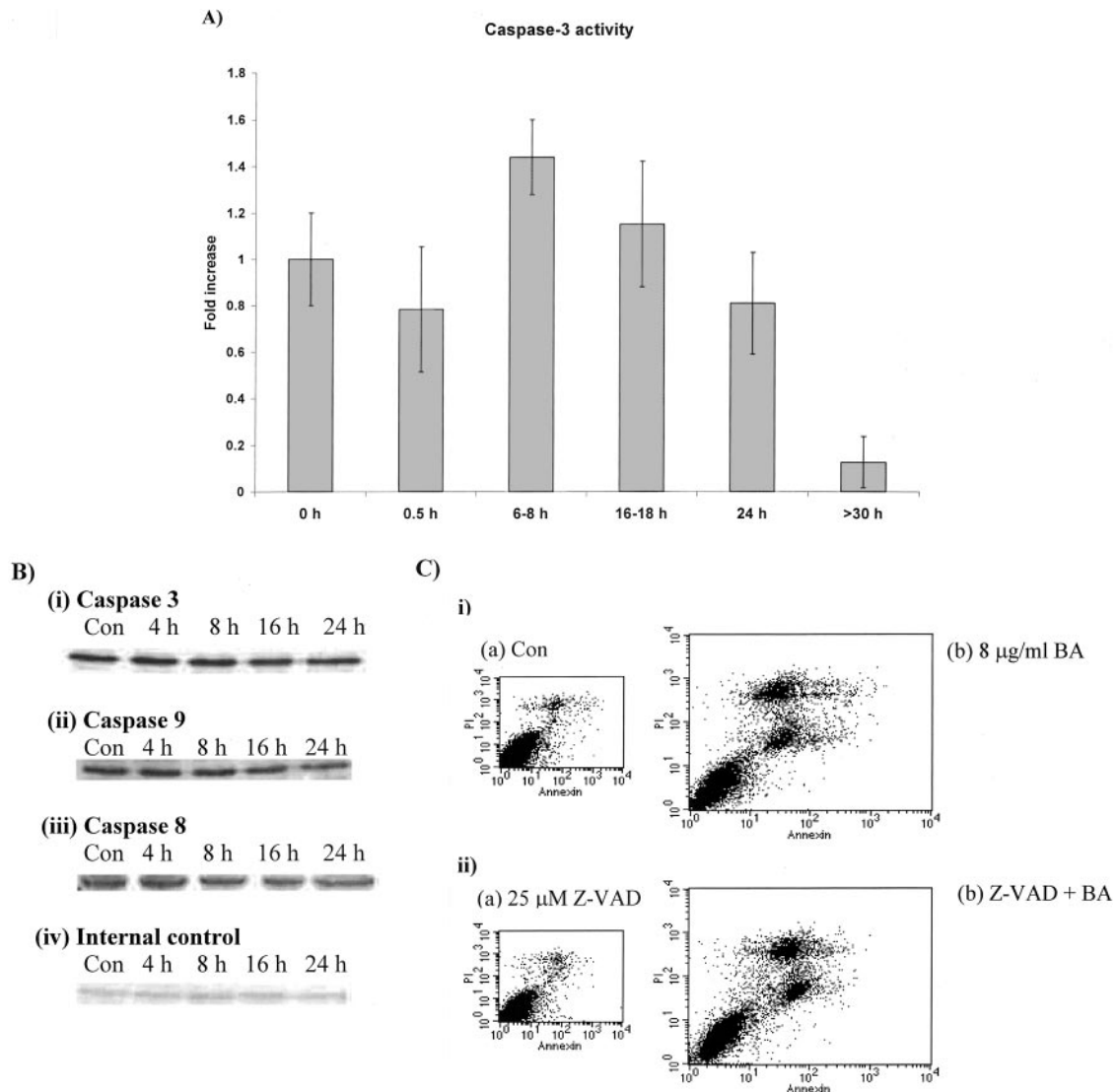
Fig. 8 Analysis of caspase activity in UISO-Mel-1 cells. *A,* determination of caspase-3 activity using fluorogenic caspase-3 substrate acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (200 μM) incubated with 10 μg of protein lysates, analyzed by fluorescence spectroscopy (PerSeptive Biosystems, Inc.). Etoposide, which is known to activate caspase-3 in HeLa cells, was incubated with HeLa cells for 24 h as a positive control for the assay (data not shown). The fold increase of caspase activity observed was not statistically significant, except the decrease at >30 h, at which time programmed cell death had already occurred (Student's *t* test, *P* < 0.05); *bars,* ± SD. *B,* Western blots used to analyze caspase activation in UISO-Mel-1 cells treated with 8 μg/ml betulinic acid over a period of 30 h. Antibodies against caspases-3, -8, and -9 were used; no differences were observed during the course of the experiment. *C,* Annexin V-FITC flow cytometric plots of UISO-Mel-1 cells preincubated with the pan-caspase inhibitor, z-VAD (25 μM). Similar results were obtainable in the presence or absence of the inhibitor (*larger panels, right side*) and samples containing z-VAD alone were comparable with control samples (*smaller panels, left side*). *BA,* betulinic acid.

affect PI staining. Thus, we distinguished apoptotic-like and necrotic cell populations not solely by the intensity of annexin V staining, but also by taking into account the extent of PI staining. The apoptotic-like cell population was considered to exhibit low PI staining with annexin V staining, whereas the necrotic cell population exhibited high PI staining with annexin V staining. As a result, these data sets were sufficient to effectively design studies for the investigation of signaling mechanisms responsible for betulinic acid-induced programmed cell death.

As indicated clearly by Western blot analyses, p38 and SAP/JNK were involved in the signaling pathway mediated by betulinic acid with cultured UISO-Mel-1 cells. This mechanistic relationship was established based on changes in the phosphorylation states of the respective MAPKs as a result of betulinic acid treatment. A large body of evidence interrelates activation of p38 and SAP/JNK pathways with apoptosis (15, 16). Mediation of a greater effect may be expected by p38 relative to SAP/JNK, because its phosphorylation pattern increased over time and remained constant until 8 h, whereas that of SAP/JNK was transient, increasing from 0.25 until 2 h, and beginning to decrease after ~4 h. The fact that these proapoptotic MAPKs are phosphorylated and activated within a short period of time

after betulinic acid treatment is consistent with reports documenting early involvement of the MAPK pathway in apoptosis. Induction of apoptosis was additionally supported by the lack of any significant change in the phosphorylation patterns of pro-survival (antiapoptotic) proteins such as ERK (11).

Having established the involvement of p38 and SAP/JNK in the programmed cell death pathway, the level of control exerted by these proteins was investigated. First, specific inhibitors were preincubated with the cells before the addition of betulinic acid. As indicated by flow cytometric analysis using annexin V-FITC, 10 μM SB203580, the p38-specific inhibitor, did not completely abrogate the programmed cell death response, but the response was reduced. These data suggest that p38 was involved in the cell death program but was not the sole determinant. In the same set of experiments, the role of SAP/JNK was examined using 10 μM SP600125, a specific inhibitor of this kinase. Inhibition was observed, but consistent with earlier Western blots revealing transient activation of SAP/JNK protein, SP600125 displayed weaker protection against betulinic acid-induced programmed cell death, relative to SB203580. Thus, it appears that pathways in addition to MAPKs are required to trigger a complete manifestation of the cell death program.

Previous studies have indicated that mitochondria may serve as direct targets for betulinic acid (9, 17). These organelles are implicated frequently in programmed cell death because the release of mitochondrial proteins into the cytosol triggers several relevant pathways (18–20). To release such proteins, the mitochondrial membrane must undergo specific changes that allow the passage of proapoptotic proteins (21). Such alterations can be detected using various dyes, based on the principle that intact and disrupted mitochondria exhibit differential patterns of dye uptake. Using the mitochondrial dye, DiOC$_6$, a gradual depolarization of mitochondrial membrane potential was noted over a period of 30 h with betulinic acid treatment. Thus, the mitochondrial membrane is indeed compromised as the cells undergo programmed cell death. Interestingly, this pattern of membrane depolarization could be blocked by preincubation with SB203580 before betulinic acid treatment, for a period up to 8 h. However, with longer treatment periods (*e.g.,* 16 h), preincubation with SB203580 did not prevent membrane depolarization. Because MAPK activation occurs relatively early in this model system, it is possible that late-stage programmed cell death events begin to unfurl after 8 h, and mitochondria are subsequently altered by such events. Under these circumstances, SB203580, being p38-specific, is not capable of protecting the mitochondria. On the other hand, SP600125, a JNK-specific inhibitor, was less capable of reducing mitochondrial membrane changes, even for 8 h. This provides additional support for the notion that among the different MAPKs, p38 plays a more prominent role in programmed cell death, although it is not the sole determining factor in the entire process. If p38 acts upstream of the mitochondrial response, and betulinic acid is capable of activating p38, the mitochondria would not serve as direct targets for betulinic acid. Nonetheless, based on our data and previous reports (9), it is clear that the mitochondria are central components of programmed cell death pathways (22–25).

ROS are one class of agents known to attack the mitochondria, and apoptosis-inducing drugs sometimes generate these species (26, 27). Thus, we investigated the potential role of ROS in betulinic acid-induced programmed cell death. Generation of these species was detected by 15 min after addition of betulinic acid to cultured melanoma cells. This response was dependent on a cellular event, because addition of betulinic acid alone to the medium resulted in no effect. Assuming these ROS are truly signaling molecules involved in programmed cell death, then quenchers of ROS such as antioxidants should prevent programmed cell death. Therefore, reduced GSH, NAC, and VE were evaluated for potential to antagonize the deleterious effects of ROS. As illustrated by annexin V-FITC flow cytometric analysis, programmed cell death was greatly reduced by the simultaneous addition of antioxidants, particularly GSH or VE. MAPK inhibitors afforded lesser protection. In this model system, these data suggest that ROS are largely responsible for the regulation of programmed cell death. The mechanism by which ROS are generated is unknown, but possibilities include mitochondria, the endoplasmic reticulum, or various enzymes such as lipooxygenase, cyclooxygenase, and monoamine oxidases (13, 28, 29). Notably, the mitochondria are regarded as a major site for the generation of ROS. In this sense, although ROS may initiate programmed cell death, mitochondria can be viewed as direct targets of betulinic acid.

Another factor worthy of consideration is that ROS have been linked to MAPK activation (30–33). Thus, we examined changes in MAPK phosphorylation patterns in cells preincubated with antioxidants. Consistent with the data obtained by annexin V-FITC flow cytometry, wherein GSH and VE provided significant levels of protection against betulinic acid-induced programmed cell death, these antioxidants significantly reduce the extent of p38 phosphorylation. In contrast, whereas p38 phosphorylation was diminishing, ERK phosphorylation was increasing. These results support the view that p38 and ERK act in opposition in the balance of cell survival and death. In addition, all three of the antioxidants reduced SAP/JNK phosphorylation, relative to samples treated with betulinic acid alone.

We also examined caspases, because their activity is well established in the process of apoptosis (25). Caspases cleave numerous proteins, the consequences of which have been established as hallmarks of apoptosis, such as DNA laddering, membrane blebbing, and loss of overall cell shape (34). Caspases work in a cascade, with initiator caspases activating effector caspases. In initial studies, we assessed the activity level of one of the most downstream effector caspases, caspase-3. However, caspase-3 activity was not observed, indicating that this factor does not play a causative role in betulinic acid-mediated apoptosis. In addition, the protein levels and cleavage patterns of upstream initiator caspases, caspases-8 and -9, were examined. Caspases-8 and -9 usually reside within cells as inactive zymogens in their proforms. After an apoptotic stimulus, their prodomains are removed, and they become active proteases (14, 34). A decrease in the level of native structure and an increase in the cleavage product suggests activation. However, when cells were treated with betulinic acid, levels of caspases-8 and -9 remained unchanged, and no detectable cleavage products were observed. Because this lack of caspase involvement is uncommon, annexin V-FITC flow cytometry studies were conducted in which the

broad-spectrum caspase inhibitor z-VAD-FMK was included. Consistent with the results described above, there was no observable difference between the cells treated with betulinic acid alone, and those treated with z-VAD and betulinic acid. Thus, although it is possible that other caspases are involved in betulinic acid-induced programmed cell death, caspases-3, -8, and -9 do not appear to participate in the signaling pathway.

To date, apoptosis is the most well-defined cell death program. Whereas classic apoptotic mechanisms involving caspases have been extensively studied, additional programmed cell death signaling mechanisms are beginning to emerge, including well-documented caspase-independent controlled death mechanisms (35–37). In addition, several proteins released from the mitochondria have been discovered to lead to apoptosis by themselves, in the absence of caspases (38–43). These studies provide strong evidence that programmed cell death can be mediated by caspase-independent mechanisms. Furthermore, rather than viewing apoptosis and necrosis simply as two unrelated events, these two phenomena may be considered as the ends of a continuum. Programmed cell death can take many forms, and given a systematic sequence of events that constitutes a pathway, it seems reasonable to view the process mediated by betulinic acid as being closer to the apoptosis end of the continuum rather than at the necrotic boundary.

The studies described herein are the first to demonstrate the role of MAPK proteins in programmed cell death signaling induced by betulinic acid. It appears that the MAPK pathway is activated in response to the generation of ROS. Clearly, however, additional factors are involved in the overall pathway of programmed cell death, and it will be important to determine how various cellular pathways interrelate and facilitate this overall process. These data should be useful for enhancing the development of betulinic acid as a clinical entity, or evaluating derivatives, prodrugs, or other chemical species that may function by these mechanisms.

ACKNOWLEDGMENTS

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