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Effects and mechanisms of aloe-emodin on cell death in human lung squamous cell carcinoma

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Abstract

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone) is an active component from the root and rhizome of *Rheum palmatum*. The study investigated the effects and mechanisms of aloe-emodin-induced cell death in human lung squamous cell carcinoma cell line CH27. Aloe-emodin (40 μM)-induced CH27 cell apoptosis was confirmed by DNA fragmentation (DNA ladders and sub-G_1 formation). Aloe-emodin-induced apoptosis of CH27 cells involved modulation of the expression of Bcl-2 family proteins, such as $BclX_L$, Bag-1, and Bak, and was associated with the translocation of Bak and Bax from cytosolic to particulate fractions. Aloe-emodin-treated CH27 cells had an increased relative abundance of cytochrome *c* in the cytosolic fraction. Results demonstrated that the activation of caspase-3, caspase-8, and caspase-9 is an important determinant of apoptotic death induced by aloe-emodin. These results suggest that aloe-emodin induces CH27 cell death by the Bax and Fas death pathway.

Keywords: Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone); Human lung squamous cell carcinoma cell line CH27; Apoptosis; Bcl-2 family protein; Cytochrome *c*; Caspase

1. Introduction

Aloe-emodin, an active component of the root and rhizome of *Rheum palmatum* L. (Polygonaceae) (Yang et al., 1999a,b), was found to have antitumor, genotoxicity, antioxidation, and antibacterial effects (Malterud et al., 1993; Muller et al., 1996; Wang et al., 1998; Hatano et al., 1999; Muller and Stopper, 1999). Pecere et al. (2000) have reported that aloe-emodin has specific antineuroectodermal tumor activity. However, the molecular mechanisms underlying the biological effects of aloe-emodin remain unknown.

Apoptosis is a major form of cell death characterized by a series of stereotypic molecular features, such as expression and translocation of Bcl-2 family proteins, release of cytochrome *c*, and activation of caspases. Cytochrome *c*, which is usually present in the mitochondrial intermembrane space, is released into the cytosol following the induction of apoptosis by many different stimuli including Fas, tumor necrosis factor (TNF), and chemotherapeutic agents (Liu et al., 1996; Kluck et al., 1997; Reed, 1997). Caspases, a family of cysteine proteases, play a critical role during apoptosis. There are at least two major mechanisms by which a caspase cascade resulting in the activation of effector caspases (caspase-3, -6, and -7) may be initiated by the most apical caspase, one involving caspase-8 and the other involving caspase-9 (Zou et al., 1997; Srinivasula et al., 1998). Therefore, two typical apoptosis pathways, receptor (Fas)-mediated (involving caspase-8) and chemical-induced (involving caspase-9) apoptosis, have been suggested (Deveraux et al., 1999; Sun et al., 1999). The Bcl-2 family proteins, such as Bcl-2, $Bcl\text{-}X_L$, Bak, and Bax, are the best-characterized regulators of apoptosis (Evan and Littlewood, 1998; Nomura et al., 1999). Many reports have indicated that the release of mitochondrial cytochrome *c* and the activation of caspase-3

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are blocked by anti-apoptosis members of the Bcl-2 family, such as Bcl-2 and Bcl-X_L, and promoted by proapoptotic members, such as Bak and Bax (Kluck et al., 1997; Yang et al., 1997; Jurgensmeier et al., 1998).

The major purpose of this study was to investigate whether aloe-emodin induced cancer cell death. Since the mechanisms of aloe-emodin are unclear, this study also investigated the mechanisms of aloe-emodin-induced cell death in human lung squamous cell carcinoma cell line CH27. The results of the present study suggest that aloe-emodin induces CH27 cell death by the Bax and Fas death pathway. In other words, the pathway of aloe-emodin-induced CH27 cell apoptosis might involve not only chemical-induced but also receptor-mediated apoptosis.

2. Materials and methods

2.1. Materials

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone), antipain, aprotinin, dithiothreitol, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), leupeptin, pepstatin, phenylmethylsulfonyl fluoride, propidium iodide, and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma (St. Louis, MO); anti-mouse and anti-rabbit immunoglobulin G peroxidase-conjugated secondary antibodies were purchased from Amersham (Buckinghamshire). Antibodies to various proteins were obtained from the following sources: caspase-3 and δ protein kinase C were obtained from Transduction Laboratory (Lexington, KY); Bag-1, Bax, Bcl-2, and Bcl-X_L were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Bak, caspase-8, cytochrome *c*, and poly(ADP-ribose)polymerase were purchased from PharMingen (San Diego, CA); caspase-9 was purchased from Oncogene Research Products (Boston, MA). Enhanced chemiluminescent (Renaissance) detection reagent was obtained from NEN Life Science Products (Boston, MA).

2.2. Cell culture

The human lung squamous carcinoma cell line CH27 was provided by Taichung Veterans General Hospital, Taiwan. The cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml stretomycin), and 2 mM glutamine at 37 °C in a humidified atmosphere comprising 95% air and 5% CO_2. When CH27 cells were treated with aloe-emodin, culture medium containing 1% fetal bovine serum was used. All data presented in this report are from at least three independent experiments showing the same pattern of expression.

2.3. Cell viability assay

Cells were seeded at a density of 1×10^5 cells/well onto 12-well plates 24 h before being treated with drugs. Drugs were added to the medium, at various indicated times and concentrations. The control cultures were treated with 0.1% DMSO (dimethylsulfoxide). After incubation, cells were washed with PBS (phosphate-buffered saline). The number of viable cells was determined by staining cells with Trypan blue. One part of 0.2% Trypan blue dissolved in PBS was added to one part of the cell suspension, and the number of unstained (viable) cells was counted.

2.4. DNA fragmentation assay

DNA fragmentation was assayed as previously described (Hsu et al., 1999). Adherent and floating cells were collected and lysed in 400 μl of ice-cold lysis buffer (containing 50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.3% Triton X-100), incubated on ice for 30 min, and then centrifuged. RNase A (100 μg/ml) was added to the supernatant, which was then incubated at 50 °C for 30 min, followed by the addition of 200 μg/ml proteinase K and further incubation at 37 °C for 1 h. Fragmented DNA was extracted with phenol/chloroform and precipitated at −20 °C with ethanol/sodium acetate. The DNA fragments were electrophoresed on a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide.

2.5. Flow cytometry analysis

The percentage of hypodiploid cells was determined as described previously (Hsu et al., 1999). Briefly, 2×10^6 cells were trypsinized, washed twice with PBS, and fixed in 80% ethanol. Fixed cells were washed with PBS, incubated with 100 μg/ml RNase for 30 min at 37 °C, stained with propidium iodide (50 μg/ml), and analyzed on a FACScan flow cytometer (Becton Dickinson Instruments). The percentage of cells that had undergone apoptosis was assessed to be the ratio of the fluorescent area smaller than the G_0–G_1 peak to the total area of fluorescence. The average of the results from at least three samples of cells for each experimental condition is presented.

2.6. Total protein preparation

Protein was extracted by a modification of the method of Hsu et al. (1999). Adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were resuspended in modified RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium

fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml antipain) for 30 min at 4 °C. Lysates were clarified by centrifugation at 100,000 × *g* for 30 min at 4 °C and the resulting supernatant was collected, aliquoted (50 μg/tube), and stored at −80 °C until assay. The protein concentrations were estimated with the Bradford method (Bradford, 1976).

2.7. Preparation of subcellular fractions

Cell fractionation was performed as described previously (Jun et al., 1999) with some modifications. Briefly, adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were frozen at −80 °C, thawed at 4 °C, and resuspended in cytosol extraction buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml antipain) for 20 min at 4 °C until > 95% of the cells were Trypan blue positive. Lysates were clarified by centrifugation at 100,000 × *g* for 30 min at 4 °C. The resulting supernatant was collected as the 'cytosolic' fraction, aliquoted (10 μg/tube for cytochrome *c*; 50 μg/tube for Bak and Bax), and stored at −80 °C until assay. The pellet was resuspended in modified RIPA buffer for 30 min at 4 °C and centrifuged as before. The supernatant was collected as the 'particulate' fraction, aliquoted (50 μg/tube), and stored at −80 °C until assay. The particulate fraction includes membrane-organelle proteins and nuclear-associated proteins.

2.8. Western blot analysis

Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8%, 10%, 11%, 12%, and 15% gels. The SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris, pH 9.0–9.4, 40 mM glycine, 0.375% SDS, 20% methanol) and electrotransferred to Immobilon-P Transfer Membranes. The blot was blocked with a solution containing 5% nonfat dry milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl) with 0.05% Tween 20 (TBST) for 1 h, washed, and incubated with antibodies to Bag-1 (1:500), Bak (0.2 μg/ml), Bax (1:500), Bcl-2 (1:500), Bcl-X_L (1:500), caspase-3 (1:1000), caspase-8 (1:1000), caspase-9 (0.1 μg/ml), cytochrome *c* (1:500), poly(ADP-ribose) polymerase (1:2000), δ protein kinase C (1:500), and β-actin (1:5000). Secondary antibody consisted of a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (for Bag-1, Bak, Bax, Bcl-2, Bcl-X_L, caspase-8, and cytochrome *c*) or horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (for caspase-3, caspase-9, poly(ADP-ribose)polymerase, δ protein kinase C, and β-actin). The enhanced chemiluminescent (Renaissance) detection system was used for immunoblot protein detection.

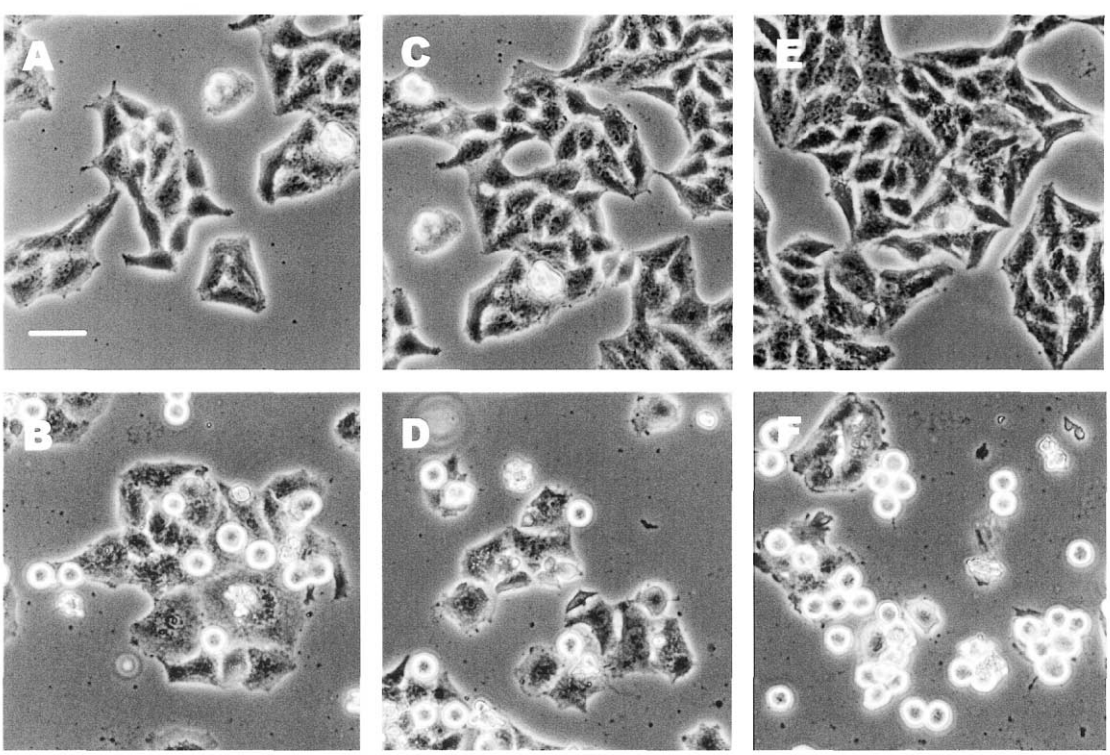

Fig. 1. Changes in CH27 cell morphology during aloe-emodin-induced apoptotic cell death. Shown are phase-contrast views of CH27 cells cultured for 18 (A and B), 36 (C and D), and 72 h (E and F) under control conditions (A, C, and E) or in the presence of 40 μM aloe-emodin (B, D, and F). Bar = 50 μm.

3. Results

3.1. Morphological alterations by aloe-emodin in CH27 cells

The phenotypic characteristics of aloe-emodin-treated CH27 cells were evaluated by microscopic inspection of overall morphology. When cells were treated with 40 μM aloe-emodin for 18 h, cells formed many apoptotic bodies and eventually rounded (Fig. 1B). Treatment with aloe-emodin for 36 h resulted in cell death and left cellular wreckage on the bottom of the Petri dish (Fig. 1D). Following 72 h aloe-emodin exposure, cell death was more extensive and fewer adherent cells remained (Fig. 1F). In contrast, cells incubated in control medium were well spread, with a flattened morphology (Fig. 1A,C and E). To demonstrate whether the floating cells were dead, the detached cells were collected and incubated with fresh medium in the absence of aloe-emodin for 24 h. No cells were observed on the bottom of the Petri dish (data not shown), suggesting that aloe-emodin could induce the death of CH27 cells.

3.2. Aloe-emodin induces CH27 cell death in a dose- and time-dependent manner

This study determined the effect of aloe-emodin on cell viability by Trypan blue dye exclusion. Viable cells were counted at 24, 36, 48, and 72 h after the addition of control medium or various concentrations (1, 3, 10, 30, and 50 μM) of aloe-emodin. As shown in Fig. 2, 72 h of continuous exposure to various concentrations of aloe-emodin resulted in time- and dose-dependent decreases in cell number relative to control cultures. IC50 value, the aloe-emodin concentration lethal to 50% of the CH27 cells, was about 25 μM. The aloe-emodin-induced CH27 cell death was significant at 50 μM aloe-emodin at all times studied. Therefore, 40 μM aloe-emodin was chosen for further experiments.

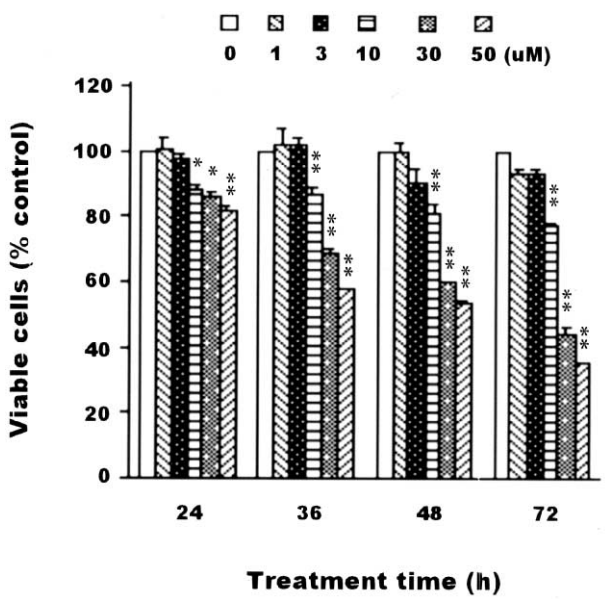

Fig. 2. Effect of aloe-emodin on cell death in CH27 cells. Cells were cultured for 24 h before drug treatment in 12-well plates. Cells were treated without (0.1% DMSO) or with aloe-emodin (1, 3, 10, 30, and 50 μM) in the presence of 1% serum at 37 °C for different times (24, 36, 48, and 72 h), and cells were washed and counted for Trypan blue exclusion with a hemocytometer. All results are expressed as the mean percentage of control ±S.D. of triplicate determinations from three independent experiments. Asterisks indicate values significantly different from control values ($^{*}P < 0.05$, $^{**}P < 0.01$).

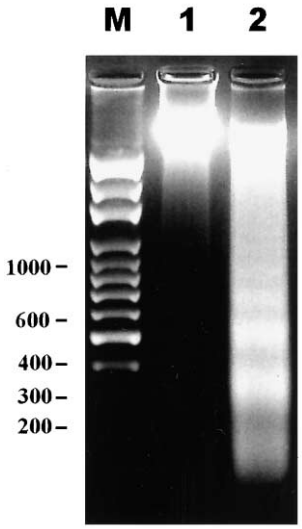

Fig. 3. Aloe-emodin-induced internucleosomal DNA fragmentation in CH27 cell nucleus. CH27 cells were incubated with 0.1% DMSO (lane 1) or 40 μM aloe-emodin (lane 2) for 24 h in 1% serum-containing medium. Total DNA was extracted from cells and separated by electrophoresis on 1.5% agarose gel. The molecular weight marker lane (lane M) represents DNA base pairs. Results are representative of three independent experiments.

3.3. Aloe-emodin induces apoptosis of CH27 cells

To obtain further support for the induction of apoptosis by aloe-emodin in CH27 cells, in situ DNA fragmentation analysis and flow cytometry analysis were performed. Treatment with 40 μM aloe-emodin for 24 h resulted in internucleosomal DNA fragmentation, evidenced by the formation of a DNA ladder on agarose gels (Fig. 3), a hallmark of cells undergoing apoptosis. No DNA ladders were detected in the sample from control cells. Apoptosis was also confirmed by the appearance of a sub-G_1 peak of DNA content by flow cytometry, suggesting the presence of cells with fragmented DNA. A sub-G_1 peak (the fluorescent area smaller than the G_0–G_1 peak/the total area of fluorescence × 100%, 10 μM aloe-emodin: 5.0 ± 0.3%; 40 μM aloe-emodin: 24.3 ± 1.2%) was detected following 24 h of exposure to 10 or 40 μM aloe-emodin. These data suggest that aloe-emodin induced CH27 cell death by apoptosis.

3.4. Aloe-emodin-induced CH27 cell death is irreversible

In order to determine whether the induction of cell death by aloe-emodin is reversible or irreversible, CH27 cells were treated with 10 or 40 μM aloe-emodin in the presence of 1% serum for 1, 2, 4, 8, 16, 24, 48, and 72 h, and then washed free of the reagents. These cells were then incubated with fresh serum-containing medium for 72 h. The viable cells were then measured by Trypan blue dye exclusion and the fraction of viable cells was calculated by defining the number of cells not treated with aloe-emodin as 100%. When 10 μM aloe-emodin was washed out after 1, 2, and 4 h of treatment, the cells started to grow and the growth rate was similar to that of the untreated cells. However, the fraction of viable cells decreased compared with that of untreated cells after cells were treated with 10 μM aloe-emodin for 4 h (Fig. 4). After cells were treated with 40 μM aloe-emodin for the indicated time intervals, the number of viable cells significantly decreased with time compared with that of untreated cells (Fig. 4). CH27 cells were also treated with 1, 3, and 5 μM aloe-emodin for 72 h, washed, and incubated with fresh medium for 72 h. Cells treated with 1 and 3 μM aloe-emodin recovered, but those treated with 5 μM did not (data not shown). The results indicate that aloe-emodin (40 μM)-induced CH27 cell death is irreversible.

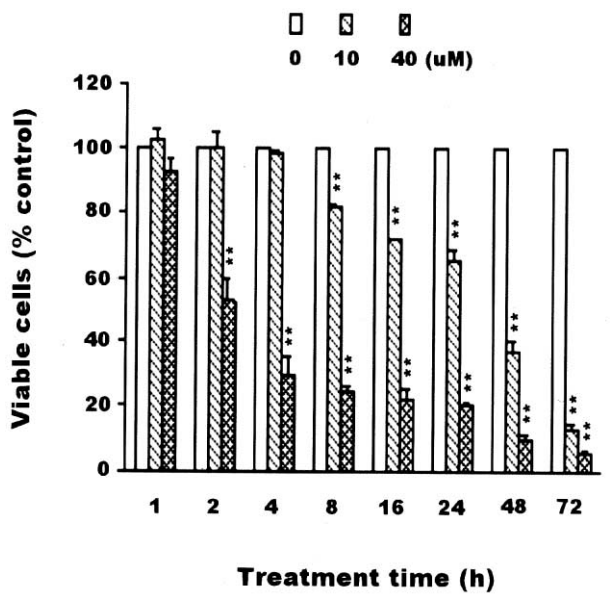

Fig. 4. Induction of cell death by aloe-emodin is irreversible. Cells were treated with vehicle alone or with 10 or 40 μM aloe-emodin in the presence of 1% serum for 1, 2, 4, 8, 16, 24, 48, and 72 h, and then washed. After that, the cells were incubated with fresh medium without aloe-emodin for 3 days. The viable cells were measured by Trypan blue dye exclusion, and the fraction of viable cells was calculated by defining the number of viable cells without treatment with aloe-emodin as 100%. The results are the means ± S.D. of three independent experiments. $^{**}P < 0.01$ compared to the control values.

Fig. 5. Effects of aloe-emodin on the expression of death-related proteins. The effect of aloe-emodin (40 μM) on death-related proteins was detected by Western blot analysis in CH27 cells. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1% serum for 2, 4, 8, 16, and 24 h. Cell lysates were analyzed by 12% (Bcl-2, Bcl-X_L, Bag-1, Bax, and Bak) or 15% (cytochrome *c*, Cyt c) SDS-PAGE and probed with primary antibodies as described in Materials and methods. Results are representative of three independent experiments.

3.5. Effect of aloe-emodin on the expression of Bcl-2 family proteins

To elucidate whether the expression of these cell death-related proteins is involved in aloe-emodin-induced apoptosis, this study examined by Western blotting techniques, the regulation of Bcl-2, Bcl-X_L, Bag-1, Bax, and Bak levels during aloe-emodin-mediated apoptosis. Exposure of CH27 cells to 40 μM aloe-emodin resulted in increases in Bak levels after 2 h of treatment (Fig. 5). Up to 24 h, there were no changes in Bcl-2 and Bax protein levels (Fig. 5). However, the level of Bcl-X_L was significantly decreased at 2 and 16 h; the level of Bag-1 was also decreased significantly after 8 h of treatment with aloe-emodin (Fig. 5). The detection of β-actin was used as an internal control. The study also investigated the distribu-

tion of Bak and Bax in cytosolic and particulate fractions. Bak and Bax were clearly detected in cytosolic and particulate fractions. After the incubation of CH27 cells with 40 μM aloe-emodin for 2 h, there was extensive translocation of Bak and Bax from the cytosolic fraction to the particulate fraction (Fig. 6). These data show that the expression of Bcl-2 family proteins was consistent with the onset of apoptosis in this cell line.

3.6. Effect of aloe-emodin on the release of cytochrome c in CH27 cells

Since cytochrome *c* released from mitochondria is controlled by members of the Bcl-2 family of apoptosis regulators, this study characterized the effect of aloe-emodin on the release of cytochrome *c*. Western blotting analysis of the cytosolic fraction of aloe-emodin (40 μM)-treated CH27 cells revealed increases in the relative abundance of cytochrome *c* at the indicated time intervals (Fig. 5).

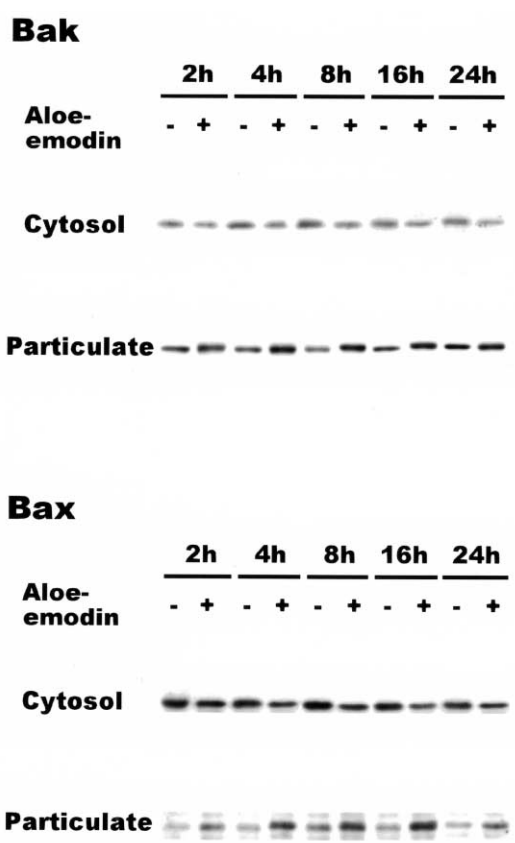

Fig. 6. Western blot showing the effects of 40 μM aloe-emodin on Bak and Bax translocation in CH27 cells. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1% serum for 2, 4, 8, 16, and 24 h. Cytosolic and particulate fractions were analyzed by 12% SDS-PAGE and probed with primary antibodies as described in Materials and methods. Results are representative of three independent experiments.

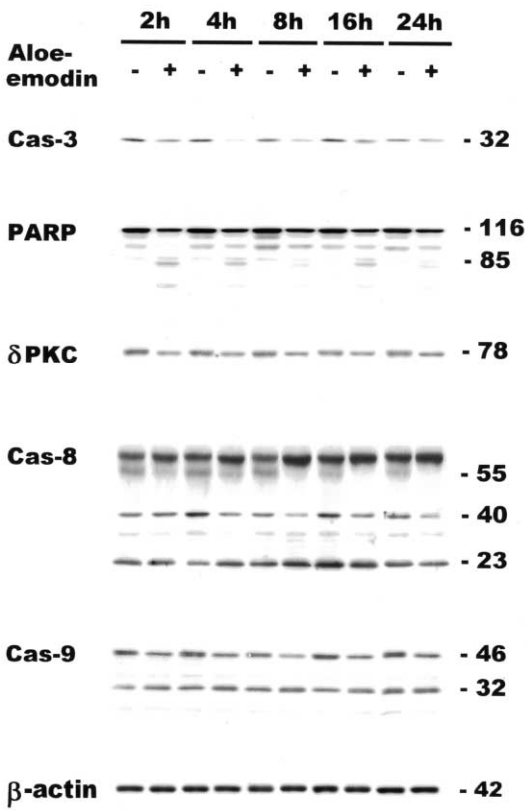

Fig. 7. Effects of aloe-emodin on the expression of caspase-3, -8, and -9. The effect of aloe-emodin (40 μM) on caspase-3 (Cas-3), -8 (Cas-8), and -9 (Cas-9) was detected by Western blot analysis in CH27 cells. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1% serum for 2, 4, 8, 16, and 24 h. Cell lysates were analyzed by 8% (poly(ADP-ribose)polymerase, PARP), 10% (δ protein kinase C, δPKC), 11% (caspase-8 and caspase-9), and 12% (caspase-3) SDS-PAGE, and then probed with primary antibodies as described in Materials and methods. Results are representative of three independent experiments.

3.7. Effect of aloe-emodin on the caspase-3, -8, and -9 in CH27 cells

To investigate the pathway of aloe-emodin-induced CH27 cell death, the activation of caspase-3, -8, and -9 was detected. Caspases are a family of cysteine proteases that are activated during apoptosis. Once activated, caspases cleave and activate downstream caspases. The levels of the proform of caspase-3 (32 kDa) and caspase-9 (46 kDa) were significantly decreased at all times after aloe-emodin (40 μM) treatment (Fig. 7), and that of caspase-8 (55 kDa) was decreased after 2 h (Fig. 7). In control cells, a low level of processing of caspase-8 (23 kDa) and caspase-9 (32 kDa) was observed; this may reflect basal caspase activity. Proteolysis of caspase substrates provides a marker of apoptosis in general and of caspase activity in particular. To further determine whether caspases were activated in aloe-emodin-treated CH27 cells, Western blot analysis of the caspase substrates δ protein kinase C and poly(ADP-ribose)polymerase was performed. Both

poly(ADP-ribose)polymerase (116 kDa) and δ protein kinase C (80 kDa) were processed to their predicted caspase cleavage products of 85 and 40 kDa, respectively, during aloe-emodin treatment 16 h (Fig. 7).

4. Discussion

Rheum palmatum L. has been used in Chinese medicine for a long time. Previous treatments of diseases with herbs were empirical more than theoretical. Therefore, clarification of the mechanisms of action of the components of herbs may be important for developing their applications. Aloe-emodin, an active component of the root and rhizome of *Rheum palmatum* L. (Yang et al., 1999a,b), has been demonstrated to possess antileukemic and antineuroectodermal tumor activity (Kupchan and Karim, 1976; Pecere et al., 2000). However, the mechanism of the aloe-emodin-produced anticancer effects remains unknown.

Apoptosis is a major form of cell death and is essential for normal development and for the maintenance of homeostasis. In addition, current antineoplastic therapies, chemotherapy and radiation therapy, are likely to be affected by the apoptotic tendency of cells; thus this process has obvious therapeutic implications (Green et al., 1994). During apoptosis, certain characteristic morphological events, such as nuclear condensation, nuclear fragmentation, and cell shrinkage, and biochemical events, such as DNA fragmentation, occur (Hsu et al., 1999; Shinoura et al., 1999). In this study, aloe-emodin-induced CH27 cell DNA fragmentation (DNA ladders and sub-G_1 formation) and cell death (Trypan blue dye exclusion) were observed. Based on the above results, aloe-emodin-induced CH27 cell death was indicative of typical apoptosis. Furthermore, once cell death was triggered by aloe-emodin, it was irreversible.

In the present study, we characterized the regulation of antiapoptotic (Bcl-2, Bcl-X_L) and proapoptotic (Bak, Bax) proteins of the Bcl-2 family. Aloe-emodin-induced apoptosis of CH27 cells did not change the levels of Bcl-2 and Bax proteins, but appeared to be associated with the decreased expression of cellular Bag-1 proteins. Some investigators have suggested that Bag-1 is a novel anti-apoptosis protein, and its loss may be involved in cell death (Yang et al., 1999a,b; Hayashi et al., 2000). Previous reports demonstrated that the Bcl-2 protein level was not changed by cytotoxic drugs (Chen and Chuang, 1999; Henkels and Turchi, 1999). It has also been suggested that Bcl-2 is mainly located in the mitochondria, where it forms heterodimers with Bax, followed by the release of cytochrome *c* and activation of caspase-3 (Jia et al., 1999; Von-Harsdorf et al., 1999). Exposure of CH27 cells to 40 μM aloe-emodin resulted in increases in Bak levels in the present study. This result is consistent with previous observations in which Bax and Bak overexpression induced cell apoptosis due to a variety of stimuli, including chemotherapeutic agents such as etoposide and paclitaxel (Ibrado et al., 1997; Jones et al., 1998; Pastorino et al., 1998; Nomura et al., 1999). In this study, the translocation of Bak and Bax from the cytosolic to the particulate fraction was observed after treatment with 40 μM aloe-emodin. Thus, Bak and Bax exert their proapoptotic activity by translocation from the cytoplasm to the mitochondria. This result is consistent with previous observations in which Bak and Bax translocation was found to be involved in cell apoptosis (Cosulich et al., 1997; Shimizu et al., 1999; Eilon et al., 2000).

Many reports have shown that Bak and Bax directly target the mitochondrial outer membrane channel and allow cytochrome *c* to pass through this channel (Cosulich et al., 1997; Shimizu et al., 1999; Eilon et al., 2000). This study has shown that aloe-emodin (40 μM)-treated CH27 cells have increased levels of cytochrome *c* in the cytosolic fraction. The time point of the release of cytochrome *c* is consistent with the time point of the translocation of Bak and Bax. This indicated that the translocation of Bak and Bax induced by aloe-emodin could be associated with the release of cytochrome *c* from mitochondria into the cytosol.

Recently, some studies have shown that mitochondria release cytochrome *c* into the cytosol during apoptosis and that this promotes the activation of caspase (Li et al., 1997; Reed, 1997; Zou et al., 1997). Caspases have been proposed as "initiator" caspases, such as caspase-8 (Fas pathway) and caspase-9 (Bax death pathway), that either directly or indirectly activate "effector" caspases, such as caspase-3, -6, and -7 (Fraser and Evan, 1996; Sun et al., 1999). Therefore, caspase-8 and caspase-9 are the most apical caspases in receptor-mediated and chemical-induced apoptosis, respectively. To investigate the pathway of aloe-emodin-induced CH27 cell death, the activation of caspase-3, -8, and -9 was assessed. The proforms of caspase-3 (32 kDa) and caspase-9 (46 kDa) were significantly decreased after aloe-emodin (40 μM) treatment until 24 h and caspase-8 (55 kDa) levels were decreased after 2 h, as assessed by Western blotting analysis. The time point of activation of caspase-3 is consistent with that of caspase-9. This indicates that caspase-9 may be an upstream activator of caspase-3 during aloe-emodin-induced apoptosis in CH27 cells. This study also demonstrated that the activation of caspase-8 is involved in aloe-emodin-induced CH27 cell death and occurs after the activation of caspase-9. Based on the above results, the activation of caspase-3, caspase-8, and caspase-9 is an important determinant of apoptotic death induced by aloe-emodin. Proteolysis of caspase substrates provides a marker of apoptosis in general and of caspase activity in particular. Caspase substrates, such as δ protein kinase C and poly(ADP-ribose)polymerase, were proteolyzed after aloe-emodin-treatment. These results suggest that aloe-emodin induces CH27 cell death by the Bax death pathway and the Fas pathway.

In summary, the present study demonstrated that aloe-emodin induced apoptotic cell death in CH27 cells. The expression of Bcl-2 family proteins is involved in aloe-emodin-induced apoptosis of CH27 cells. The release of cytochrome *c* from the mitochondria into the cytosol by aloe-emodin was regulated in part by Bak and Bax regulation of mitochondrial function. Caspase activation in this model occurs, at least in part, via the mitochondrial death pathway, as evidenced by the translocation of cytochrome *c* from the mitochondria to the cytosol and the activation of both caspase-9 and caspase-3. This study also demonstrated that the activation of caspase-8 is involved in aloe-emodin-induced CH27 cell death and occurs after the activation of caspase-9.

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