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Aloe-emodin　對肺癌細胞凋亡機轉之探討

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（一）計畫中文摘要

Aloe-emodin 是大黃植物之根及根莖中所含之活性成分。文獻中記載其有抗腫瘤的效果。在 2000 年 Pecere 等報告 aloe-emodin 有專一的抗神經外胚層腫瘤活性，本實驗也證實 aloe-emodin 引起肺癌細胞株(H460)的細胞進行典型的細胞凋亡，而此細胞凋亡與 caspase-3 的活化，caspase-3 上游之 cytochrome *c* 的釋放，及 protein kinase C 之表現有密切的關係。如今本實驗將更深入探討 aloe-emodin 引起 H460 細胞凋亡之更深入的機轉，其中包含各種的第二訊號途徑蛋白激脢 A、蛋白激脢 C、Mitogen-activated protein kinase 家族及 caspases。我們已探討 aloe-emodin 所引起肺癌細胞 H460 之細胞凋亡中，在第二訊號的所有成員中那些成員是重要的及闡明第二訊號途徑中每個途徑互相間的關係。

關鍵詞: Aloe-emodin; 肺癌細胞株(H460); 第二訊號途徑; 細胞凋亡; 蛋白激脢 A; 蛋白激脢 C; Mitogen-activated protein kinase 家族; caspases

（二）計畫英文摘要

Aloe-emodin, an active component contained in the root and rhizome of Rheum palmatum L. (Polygonaceae), was found to have antitumor effects. Pecere et al. (2000) have reported that aloe-emodin has a specific antineuroectodermal tumor activity. Our previous study also demonstrated that aloe-emodin induced cell death, which was indicative of a typical apoptosis on lung carcinoma cell lines H460. Our findings indicate that activation of caspase-3 is critical for aloe-emodin-induced apoptosis of H460 cells, that cytochrome c is one of the main upstream activators of this pathway, and that the expression of protein kinase C has involved in aloe-emodin-induced apoptosis. The present study would investigate deeply mechanisms of aloe-emodin-induced apoptosis of H460 cells, including various second messenger pathways such as protein kinase A, protein kinase C family, Mitogen-activated protein kinase family, and caspases. We has investigated that which members of the second messengers are important and the relationship between the second messenger pathways might more clearly on the aloe-emodin-induced apoptosis of lung cancer cells H460.

Keywords: Aloe-emodin; Lung cancer cell lines H460; Second messenger

pathways; Apoptosis; Protein kinase A; Protein kinase C; Mitogen-activated protein kinase family; Caspases

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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* that has been reported to exhibit antitumor effects through an unknown mechanism. The study investigated the mechanisms of aloe-emodin-induced cell death in the human lung nonsmall cell carcinoma cell line H460. Aloe-emodin (40 μM)-induced apoptosis of H460 cells involves modulation of cAMP-dependent protein kinase, protein kinase C, Bcl-2, caspase-3, and p38 protein expression. The relationship of various signals involved in cell death, such as cAMP-dependent protein kinase, protein kinase C, Bcl-2, caspase-3, and p38, has been investigated in the regulation of apoptotic cell death of aloe-emodin. We demonstrated that the expression of p38 is an important determinant of apoptotic death induced by aloe-emodin.

INTRODUCTION

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, clarifying 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.,[1] has been demonstrated to possess anti-tumor activity.[2-4] However, the mechanisms of the aloe-emodin-produced anticancer effects remain unknown. The present study served to investigate the mechanisms of aloe-emodin-induced apoptosis on human nonsmall cell lung carcinoma cell H460.

Apoptosis is a major form of cell death, which involves many factors such as expression and translocation of Bcl-2 family proteins, activation of caspases, expression of protein kinase C (PKC) family, cAMP-dependent protein kinase (PKA), and mitogen-activated protein kinase (MAP kinase) members signal transduction pathways. The Bcl-2 family proteins, such as Bcl-2, Bcl-X_L, Bak, and Bax, are the best-characterized regulators of apoptosis.[4,5] Caspases, a family of cysteine proteases, play a critical role during apoptosis. There are at least two major mechanisms (one

involving caspase-8 and the other involving caspase-9)[6,7] which directly or indirectly activate "effector" caspases such as caspase-3.[8,9] Many investigators have reported that activation of cAMP signaling is involved in apoptosis.[10,11] The role of cAMP in various cell responses is mediated by the cAMP-dependent protein kinase. However, the dissociation of catalytic subunit from inactive PKA is the marker of the activity of PKA. The present study determined the change of PKAc (PKA catalytic subunit) in aloe-emodin-induced apoptosis. PKC represents 11 isozymes that have been implicated in the regulation of apoptosis.[12,13] However, the contribution of individual PKC isozymes to this process is not well understood. Many reports suggested that the degradation of δPKC was directly concerned in apoptosis in all of the PKC isozymes.[13,14] The relationship between activation of caspase and δPKC was investigated in many reports. These reports suggest that δPKC should lie upstream or downstream of caspase-3.[13,14] The present study examined the changes of δPKC and caspase-3 and the specificity of the PKC-caspase-3 relationship on aloe-emodin-induced apoptosis.

The mitogen-activated protein kinase (MAP kinase) members can be grouped into three major classes - the extracellular signal-regulated protein kinases (ERKs), the c-jun N-terminal kinases (JNKs), and the p38s.[15,16] The ERK pathway is predominantly activated by mitogens through a Ras-dependent mechanism, and it is required for cell proliferation and differentiation.[17] However, JNK and p38, involving apoptotic cell death, are regulated by pro-inflammatory cytokines and various environmental stresses such as UV-light, DNA-damaging agents, and oxidative stresses.[18-21] This study examined whether MAP kinase members are involved in aloe-emodin-induced apoptosis. It also examined the relationship between the expression of the MAP kinase members and the expression of PKA or PKC.

Since the mechanism of aloe-emodin's antitumor effect was unclear, the major purpose of this study was to investigate the mechanisms of aloe-emodin-induced cancer cell death. In this study, we evaluated aloe-emodin's effect on factors that play important role in apoptosis, such as PKAc in the cAMP-dependent protein kinases, δPKC in PKC family, Bcl-2 in Bcl-2 members, caspase-3 in caspase family, and p38, JNK, and ERK in MAP kinase pathway. Furthermore, we examined the relationship of various signaling pathways in aloe-emodin-induced apoptosis in the human lung nonsmall carcinoma cell line H460.

MATERIALS AND METHODS

Cell culture

The human lung nonsmall cell line H460 was kindly provided by S.L. Hsu, Ph.D. (Taichung Veterans General Hospital, Taichung, Taiwan). The cells were grown in

monolayer culture in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD) containing 5 % fetal bovine serum (Hyclone, Logan, Ut), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Rockville, MD), and 2 mM glutamine (Merck, Darmstadt, Germany) at 37℃ in a humidified atmosphere comprised of 95 % air and 5 % CO_2. When H460 cells were treated with aloe-emodin (Sigma Chemical Co., St. Louis, MO), the 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.

Cell viability assay

Cells were seeded at a density of 1×10^5 cells/well onto a 12-well plate (Falcon, Franklin Lakes, NJ) 24 h before treatment. Drugs were added to medium, at various indicated times and concentrations. The control cultures were treated with 0.1 % DMSO (dimethylsulfoxide; Merck). After incubation, cells were washed with PBS (phosphate-buffered saline). The number of viable cells was determined by staining the cell population with Trypan blue (Sigma Chemical Co.). 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.

Measurement of intracellular cyclic AMP

Cyclic AMP was measured by enzymeimmunoassay (Amersham Pharmacia Biotech) as previously described.[22] H460 cells were seeded at a density of 1×10^5 cells onto 12-well plates 24 h before drug treatment. After incubation for the indicated time, the medium was removed completely and the cells were washed twice with PBS containing 0.5 mM isobutylmethylxanthine to inhibit phosphodiesterase and to prevent subsequent breakdown of the cyclic AMP during cell solubilization, sample collection, and processing. The amount of cyclic AMP was measured by enzymeimmunoassay system.

Caspase-3 activity assay

Caspase-3 was measured by fluorometric caspase assay kit (BioVision, Mountain View, CA). H460 cells were seeded at a density of 3×10^6 cells onto 10 cm dish 24 h before drug treatment. 40 μM aloe-emodin was added to medium at various times. The control cultures were treated with 0.1 % DMSO. Cells were lysed in lysis buffer [1 % Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8, 2 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin] for 20 min at 4℃ followed by centrifugation (100000 × g) for 30 min. Caspase activities were assayed in 50 μl reaction mixtures with fluorogenic report substrate peptides DEVD-AFC of

caspase-3. The substrate peptide (200 μM) was incubated at 37°C with cytosolic extract (20 μg of total protein) in reaction buffer [100 mM HEPES, 10 % sucrose, 10 mM DTT, and 0.1 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma Chemical Co.)]. Fluorescence was measured after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a fluorescence plate reader (Fluoroskan Ascent; Labsystems).

Protein preparation

Protein was extracted as previously described.[4] 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 DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml antipain (Sigma Chemical Co.)] 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.[23]

Western blot analysis

Samples were separated by various appropriate concentrations (9, 10, 11, 12, and 13 %) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Life Science Products, Hercules, CA). The SDS-separated proteins were equilibrated in transfer buffer [50 mM Tris, pH 9.0-9.4, 40 mM glycine (Bio-Rad Life Science Products), 0.375 % SDS (Bio-Rad Life Science Products), 20 % methanol (Merck)] and electrotransferred to Immobilon-P Transfer Membranes (Millipore Corporation, Bedford, MA). The blot was blocked with a solution containing 5 % nonfat dry milk in Tris-buffered saline [10 mM Tris, 150 mM NaCl (Sigma Chemical Co.)] with 0.05 % Tween 20 (TBST; Merck) for 1 h, washed, and incubated with antibodies to β-actin (1:5000; Sigma Chemical Co., the detection of β-actin was used as an internal control in all of the data of Western blotting analysis), ATF-2 (1:1000; Cell Signaling Technology, Beverly, MA), Bcl-2 (1:500; BioVision, Mountain View, CA), caspase-3 (1:1000; PharMingen), δPKC (1:500; Transduction Laboratory), ERK1/ERK2 (1:1000; Santa Cruz), JNK1 (1:1000; Santa Cruz), p38 (1:1000; Santa Cruz), and PKAc (1:1000; Transduction Laboratory). Secondary antibody (Amersham, Buckinghamshire) consisted of a 1:20,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (for ATF-2, Bcl-2, ERK1/ERK2, JNK1, and p38) or HRP-conjugated goat anti-mouse IgG (for

caspase-3, PKAc, and δPKC). The enhanced chemiluminescent (NEN Life Science Products, Boston, MA) detection system was used for immunoblot protein detection.

RESULTS

Aloe-emodin induces H460 cell death

This study determined the effect of aloe-emodin on cell viability by Trypan blue dye exclusion. Viable cells were counted at 6, 12, 24, 36, 48, 60, and 72 h after the addition of control medium or various concentrations (1, 3, 10, 30, and 50 μM) of aloe-emodin. In this study, 48 h of continuous exposure to various concentrations of aloe-emodin resulted in time- and dose-dependent decreases in cell number relative to control cultures (data not shown). After the incubation of H460 cells with 30 and 50 μM aloe-emodin for 48 h, almost all the cells were dead. IC50 value, the aloe-emodin concentration lethal to 50% of the H460 cells, was about 40 μM. Therefore, 40 μM aloe-emodin was chosen for further experiments.

Effects of forskolin, isobutylmethylxanthine, chelerythrine, and SB202190 on aloe-emodin-induced apoptosis in H460 cells

To further investigate whether the induction of cell death by aloe-emodin could be linked the influence of forskolin (adenylate cyclase activator), isobutylmethylxanthine (a phosphodiesterase inhibitor), chelerythrine (a PKC inhibitor), and SB202190 (a p38 MAP kinase inhibitor), cell viability was observed. This study determined the effects of forskolin (1, 10, 30, 50, and 100 μM, pretreatment 1 h), isobutylmethylxanthine (1, 10, 30, 50, and 100 μM, pretreatment 1 h), chelerythrine (0.01, 0.1, and 1 μM, pretreatment 1 h), and SB202190 (0.01, 0.1, 0.5, and 1 μM, pretreatment 2 h) on aloe-emodin (40 μM, 40 h)-induced cell death by Trypan blue dye exclusion. Treatment with chelerythrine (more than 1 μM) or SB202190 (more than 1 μM) alone induced H460 cell death after 24 h treatment (data not shown). Preteatment with various indicated concentrations of forskolin, isobutylmethylxanthine, or chelerythrine for 1 h had no effect on the aloe-emodin (40 μM, 40 h)-induced cell death (data not shown). However, cells were pretreated with indicated concentrations (0.01, 0.1, 0.5, and 1 μM) of SB202190 for 2 h and then 40 μM aloe-emodin for 40 h. As shown in Table I, SB202190 caused the best inhibition of aloe-emodin-induced cell death at 0.1 μM. Based on the above results, 0.1 μM SB202190, 30 μM forskolin, and 1 μM chelerythrine were chosen for Western blotting experiments.

Effects of aloe-emodin or forskolin on intracellular cAMP concentration in H460 cells

To determine the possible role of cAMP as an intracellular mediator of aloe-emodin-induced cell death, intracellular cAMP was measured in H460 cells. Cells were treated with 0.5 % DMSO, 40 μM aloe-emodin, or forskolin (30 and 50 μM) for 1, 2, 4, 8, 16, and 24 h. Forskolin induced an increase in the intracellular cAMP concentration during 24 h, whereas aloe-emodin induced a decrease after 2 h of treatment with aloe-emodin (Table II). In this study, the levels of cAMP concentration inducement by 30 and 50 μM forskolin were almost equal. Therefore, 30 μM forskolin was chosen for Western blotting experiments.

Effects of aloe-emodin on PKAc and of forskolin, chelerythrine, and SB202190 on the aloe-emodin-induced expression of PKAc in H460 cells

Since aloe-emodin induced a decrease in the intracellular cAMP concentration, this study served to investigate whether aloe-emodin-induced the change of the only known target of cAMP, PKA. The change of PKA was detected with PKAc antibody by Western blotting analysis (Fig. 1). Treatment with aloe-emodin (40 μM) resulted in a decrease of PKAc at 1, 2, 4, and 8 h (Fig. 1). We also investigated the effects of forskolin (30 μM, pretreatment 1 h), chelerythrine (1 μM, pretreatment 1 h), and SB202190 (0.1 μM, pretreatment 2 h) on the aloe-emodin-induced decrease of PKAc by Western blotting analysis. Pretreatment with forskolin abolished the aloe-emodin-induced decrease of PKAc (Fig. 1). However, pretreatment with chelerythrine and SB202190 then aloe-emodin had no effect on the aloe-emodin-induced decrease in PKAc (Fig. 2 and 3). The degree of PKAc expression was quantified by AlphaEase image software in Table III.

Effects of aloe-emodin on δPKC and of forskolin, chelerythrine, and SB202190 on the aloe-emodin-induced expression of δPKC in H460 cells

In our previous study, we demonstrated that the expression of δPKC was decreased in aloe-emodin treated H460 cells during 24 h. The present study investigated that the effects of forskolin (30 μM, pretreatment 1 h), chelerythrine (1 μM, pretreatment 1 h), and SB202190 (0.1 μM, pretreatment 2 h) on the aloe-emodin-induced degradation of δPKC by Western blotting analysis. Pretreatment with forskolin prevented δPKC from degradation during aloe-emodin treatment at 8 h (Fig. 1). However, pretreatment with chelerythrine and SB202190 then aloe-emodin had no effect on the aloe-emodin-induced decrease in the expression of δPKC (Fig. 2 and 3). As shown in Table III, the expression of δPKC was quantified by AlphaEase image software.

Effects of aloe-emodin on the expression of Bcl-2 protein and of forskolin, chelerythrine, and SB202190 on the aloe-emodin-induced expression of Bcl-2 in H460 cells

To elucidate whether the expressions of these cell Bcl-2 protein is involved in aloe-emodin-induced apoptosis, this study examined the regulation of Bcl-2 protein levels during aloe-emodin-mediated apoptosis by Western blotting techniques. Exposure of H460 cells to 40 μM aloe-emodin resulted in decreased in Bcl-2 protein levels after 2 h of treatment (Fig. 1). This study also investigated the effects of forskolin (30 μM, pretreatment 1 h), chelerythrine (1 μM, pretreatment 1 h), and SB202190 (0.1 μM, pretreatment 2 h) on the aloe-emodin-induced down-regulation of Bcl-2 by Western blotting analysis. The pretreatment with forskolin and chelerythrine was prevented from the aloe-emodin-induced down-regulation of Bcl-2 (Fig. 1 and 2). However, pretreatment with SB202190 then aloe-emodin had no effect on the aloe-emodin-induced decrease in Bcl-2 (Fig. 3). The degree of Bcl-2 expression was quantified by AlphaEase image software in Table III. These data show that the expression of Bcl-2 protein was consistent with the onset of apoptosis in this cell line and Bcl-2 lies downstream of either PKA or PKC.

Effects of aloe-emodin on caspase-3 and of forskolin, chelerythrine, and SB202190 on the aloe-emodin-induced expression of caspase-3 in H460 cells

Since the cleavage and activation of caspase-3 is required during apoptosis, the activation of caspase-3 was detected on aloe-emodin-induced H460 cell death. The levels of the proform of caspase-3 (32 kDa) was significantly decreased at all times after aloe-emodin (40 μM) treatment by Western blotting analysis (Fig. 1). Treatment with aloe-emodin resulted in a processing of caspase-3 accompanied by the formation of 17 kDa fragments (Fig. 1). To gain further insights into the activity of caspase-3 on aloe-emodin-induced apoptosis, caspase-3 protease activity was measured with DEVD-AFC using a fluorometric assay. As shown in Table IV, 40 μM aloe-emodin induces a biphasic change in caspase-3 activity of H460 cells. The initial phase exhibited an increase after the addition of aloe-emodin for 40 min, which decreased after 2 h. In this study, we also investigated the effects of forskolin (30 μM, pretreatment 1 h), chelerythrine (1 μM, pretreatment 1 h), and SB202190 (0.1 μM, pretreatment 2 h) on the aloe-emodin-induced activation of caspase-3 by Western blotting analysis. Because aloe-emodin induced the activation of caspase-3 within 8 h of treatment of aloe-emodin, Western blotting analysis for forskolin, chelerythrine, and SB202190 on the aloe-emodin-induced activation of caspase-3 was determined at 1, 2, 4, and 8 h. Pretreatment with forskolin and chelerythrine prevented cleavage of caspase-3 (Fig. 1 and 2). However, pretreatment with SB202190 followed

by aloe-emodin had no effect on the aloe-emodin-induced decrease in the activation of caspase-3 (Fig. 3). As shown in Table III, the Western blot results of caspase-3 was quantified by AlphaEase image software.

Effects of aloe-emodin on MAP kinase and of forskolin, chelerythrine, and SB202190 on the aloe-emodin-induced expression of p38 in H460 cells

To elucidate whether the expression of MAP kinase is involved in aloe-emodin-induced apoptosis, this study examined the expression of ERK1/ERK2, JNK, and p38 MAP kinase by Western blotting techniques during aloe-emodin-mediated apoptosis. Exposure of H460 cells to 40 μM aloe-emodin resulted in decreases in ERK1/ERK2 and p38 protein levels after 2 h of treatment (Fig. 4 and 5). However, the levels of JNK1 did not change during 24 h of aloe-emodin treatment (Fig. 6). This study also determined the expression of phosphorylation of ERK1/ERK2, JNK, and p38 on aloe-emodin-induced apoptosis by Western blotting techniques. The results showed that the aloe-emodin-induced expression of phosphorylation of ERK1/ERK2, JNK, and p38 MAP kinase were similar in expression to those in their protein (Fig. 4, 5, and 6). Furthermore, the present study investigated the effects of forskolin (30 μM, pretreatment 1 h), chelerythrine (1 μM, pretreatment 1 h), and SB202190 (0.1 μM, pretreatment 2 h) on the aloe-emodin-induced change of p38. Pretreatment with forskolin and chelerythrine prevented p38 degradation at 1 and 2 h (Fig. 1 and 2). However, forskolin or chelerythrine did not have as much effect on the aloe-emodin-induced decrease in p38 as SB202190 did. Pretreatment with SB202190 followed by aloe-emodin had a significant effect on the aloe-emodin-induced decrease in p38 expression during 24 h (Fig. 5). ERK, JNK1, and p38 protein levels were quantified by AlphaEase image software in Table V.

Effects of aloe-emodin on ATF-2 and of SB202190 on the aloe-emodin-induced expression of ATF-2 in H460 cells

Our study also determined the expression of the target of JNK and p38, ATF-2, by Western blotting techniques during aloe-emodin-mediated apoptosis. Exposure of H460 cells to 40 μM aloe-emodin resulted in decreases in the protein levels of ATF-2 after 2 h of treatment (Fig. 7). Our study also determined the expression of phosphorylation of ATF-2 on aloe-emodin-induced apoptosis. The results showed that the expressions of phosphorylation of ATF-2 were similar in expressions of ATF-2 protein on aloe-emodin-induced apoptosis during 24 h (Fig. 7). Furthermore, the present study investigated that the effects of SB202190 (0.1 μM, pretreatment 2 h) on the aloe-emodin-induced change of ATF-2. Pretreatment with SB202190 followed

by aloe-emodin had a significant effect on the aloe-emodin-induced decrease in ATF-2 expression during 24 h (Fig. 7). The Western blot results of ATF-2 and phosphorylation of ATF-2 were quantified by image software in Table V.

Aloe-emodin-induced H460 cell death is irreversible

In order to determine if the inducement of aloe-emodin cell death is reversible or irreversible, this study treated H460 cells 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 washed off 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 cells without treatment of aloe-emodin as 100 %. When 10 μM aloe-emodin was washed out after 1 h treatment, the cells started to grow and the growth rate was similar to the untreated cells. However, the fraction of viable cells decreased compared with untreated cells after cells were treated with 10 μM aloe-emodin for 2 h (Fig. 8). After cells were treated with 40 μM of aloe-emodin for indicated time intervals, the viable cells significantly decreased compared with untreated cells in the subsequent time course (Fig. 8). These results indicate that aloe-emodin-induced H460 cell death is irreversible.

DISCUSSION

Our previous study demonstrated that aloe-emodin induced cytotoxicity, which was indicative of a typical apoptosis on lung carcinoma cell lines H460. Our findings indicate that activation of caspase-3 is critical for aloe-emodin-induced apoptosis of H460 cells and that the expression of protein kinase C has involved in aloe-emodin-induced apoptosis.[4] The purpose of the study was to investigate the mechanisms of aloe-emodin-induced apoptosis of H460 cells. Cyclic adenosine monophosphate (cAMP) has been previously shown to modulate a variety of cellular responses in many cell types, including cell death. The involvement of cAMP in the regulation of apoptosis is implicated. Some studies suggested that cAMP has a significantly protective effect on agents-induced apoptosis.[10] Activation the cAMP signaling pathway leading to an increase in apoptosis has also been reported.[11] The present study demonstrated a direct role of cAMP, decreasing cAMP level, in the regulation of aloe-emodin-induced apoptotic pathway. Forskolin did not block aloe-emodin-induced cell death, but forskolin affected the protein levels of signaling pathway by aloe-emodin-induced apoptosis, such as the expression of PKAc, δPKC, Bcl-2, caspase-3, and p38. This seems to indicate that increasing cAMP concentration does not reverse aloe-emodin-induced apoptosis, once cell death was triggered by

aloe-emodin. The regulation of cAMP in cellular responses was associated with the activation of cAMP-dependent protein kinase (PKA). PKA is composed of two distinct subunits: catalytic and regulatory. Following binding of cAMP, the regulatory subunits dissociate from the catalytic subunits, rendering the enzyme active. The up- or down-regulation of PKA is known to be associated with the regulation of drug-induced apoptosis.[24,25] However, the mechanisms by which PKA regulates apoptosis is unclear. In our study, PKAc is down-regulated in aloe-emodin-induced apoptosis.

Protein kinase C is an attractive target for modulation of apoptosis as there is mounting evidence implicating PKC as a multifaceted regulator of cellular sensitivity to chemotherapeutic agents. We found that the PKC inhibitor, chelerythrine, had no effect on aloe-emodin-induced down-regulation of PKAc. Since forskolin blocked the aloe-emodin-induced decrease in δPKC, our study is consisted with other observations that PKC may function downstream of PKA in the apoptotic signaling pathway.[26] The Bcl-2 family proteins are the best-characterized regulators of apoptosis.[5,27] Some members of this family, such as Bcl-2 and Bcl-X_L, suppress apoptosis, whereas others, such as Bak and Bax, promote apoptosis.[5] The present study has demonstrated that aloe-emodin resulted in the decreased in Bcl-2 protein levels in which Bcl-2 overexpression performs anti-apoptotic function.[25,28] Administration of forskolin or chelerythrine prevented the aloe-emodin-induced decrease in Bcl-2, suggesting that the expression of Bcl-2 protein should be regulated by PKA and PKC.

Caspases, a family of cysteine proteases, play a critical role in the apoptosis.[29,30] Caspases have been proposed that “initiator” caspases, such as caspase-8 and caspase-9, either directly or indirectly activate caspase-3.[8,9] Therefore, the activation of caspase-3 is required during apoptosis. Mitogen-activated protein kinase (MAP kinase) cascades play a central role in the cellular response to various extracellular stimuli. Activation of MAP kinase members has been implicated in the regulation of apoptotic cell death. In this study, not only the expression of p38, which is regulated by various environmental stresses, but also ERK, which is associated with cell proliferation and differentiation, is regulated by aloe-emodin. This result is consistent with previous observations in which p38 and ERK appear to be important signal transduction pathways leading to apoptosis in a human T-cell line.[31] Because early reports suggest that PKA or PKC lie upstream of either caspase-3 or MAP kinase to protect drug-induced apoptotic cell death,[14,32] we investigated the possibility that PKA or PKC was responsible for caspase-3 or p38 degradation by aloe-emodin. In our previous study, we examined the specificity of the PKC-caspase-3 relationship on aloe-emodin-induced apoptosis. We suggested that the activation of caspase-3 and

PKC proceed through two distinct mechanisms in the aloe-emodin-induced apoptosis, PKC activation involves different signaling pathways or occurs at a site upstream of caspase-3.[4] The present study found that both forskolin and chelerythrine abolished the aloe-emodin-induced degradation of caspase-3 and p38. This study suggested that the activation of caspase-3 is regulated by PKC. Our study also demonstrated that pretreatment with the p38 inhibitor, SB202190, did not block the aloe-emodin-induced change in PKAc, δPKC, and caspase-3. It suggested that p38 lies downstream of PKA, δPKC, and caspase-3. Since pretreatment with forskolin or chelerythrine had no effect on aloe-emodin-induced cell death, we determined whether the induction of cell death by aloe-emodin is reversible or irreversible. Data showed that cell death once triggered by aloe-emodin was irreversible. However, administration of SB202190 could diminish aloe-emodin-induced cell death. It seems to indicate that proteins that lie upstream of p38 are less influential than those that lie downstream of p38 on aloe-emodin-induced apoptosis. P38 is an important determinant of apoptotic death induced by aloe-emodin. ATF-2 has been shown to be a target of the JNK and p38 MAP kinase signaling pathways. Various forms of cellular stress including genotoxic agents, inflammatory cytokines, and UV irradiation stimulate the transcriptional activity of ATF-2. The recent study has suggested that the activation of ATF-2 should follow the activation of ERK and p38.[33] Our study also demonstrated that ATF-2 (activating transcription factor-2) was regulated after aloe-emodin treatment.

In summary, the present study demonstrates aloe-emodin induced apoptotic cell death in H460 cells. PKA, PKC, Bcl-2, caspase-3, and p38 expression was involved in aloe-emodin-induced apoptosis of H460 cells. The order of signaling in the aloe-emodin-induced cell death pathway may be PKA, PKC, Bcl-2, caspase-3, and then p38. P38 clearly is an important determinant of apoptotic death induced by aloe-emodin.

ACKNOWLEDGEMENTS

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TABLE I - EFFECT OF SB202190 ON ALOE-EMODIN-INDUCED APOPTOSIS IN H460

Reagents	Viable cells (% of control)
Control	100
SB202190 (1 μM)	97 ± 9
Aloe-emodin	27 ± 6†
SB202190 (1 μM) + Aloe-emodin	26 ± 4
SB202190 (0.5 μM) + Aloe-emodin	40 ± 5*
SB202190 (0.1 μM) + Aloe-emodin	54 ± 5**
SB202190 (0.01 μM) + Aloe-emodin	38 ± 4*

Cells were cultured 24 h before drug treatment in 12-well plates. Cells were treated with 0.1 % DMSO, SB202190, aloe-emodin (40 μM), or pretreated with indicated concentrations of SB202190 for 2 h and then 40 μM aloe-emodin for 40 h at 37℃, and cells were washed and counted by Trypan blue exclusion with hemocytometer. All determinations are expressed as the mean % control ± S.D. of duplicate from three

independent experiments. †(P < 0.01): Statistically different from control. * (P < 0.05), ** (P < 0.01): Statistically different from aloe-emodin alone.

TABLE II - EFFECT OF ALOE-EMODIN OR FORSKOLIN ON INTRACELLULAR CAMP LEVELS OF H460

Cyclic AMP (% of control)

Time (h)	1	2	4	8	16	24
Control	100	100	100	100	100	100
Forskolin (30 μ M)	123 ± 2**	127 ± 5**	137 ± 4**	214 ± 10**	116 ± 7	117 ± 5
Forskolin (50 μ M)	121 ± 7**	128 ± 4**	140 ± 6**	164 ± 8**	124 ± 9*	112 ± 5
Aloe-emodin	101 ± 5	80 ± 2**	75 ± 6**	70 ± 8**	68 ± 4**	53 ± 7**

Cells were incubated 0.5 % DMSO, 30 μ M forskolin, 50 μ M forskolin, or 40 μ M aloe-emodin in the presence of 1 % serum for various time periods. The amount of cyclic AMP was measured by enzymeimmunoassay system. All determinations are expressed as the mean percentage of control ± S.D. of triplicate from three independent experiments. Asterisks indicate values significantly different from control values (* P < 0.05, ** P < 0.01).

TABLE III – DENSITOMETRIC ANALYSIS OF THE WESTERN BLOT RESULTS OF PKAc, δPKC, BCL-2, CASPASE-3, AND P38

Intensity (% control)

Forskolin (F) + Aloe-emodin (A)

	PKAc			δPKC			Bcl-2			Cas-3			p38		
	C	A	F+A	C	A	F+A	C	A	F+A	C	A	F+A	C	A	F+A
1 h	100	51±5[a]	53±4[a]	100	50±3[a]	68±5[b]	100	60±5[a]	84±6[b]	100	82±5[a]	76±3[a]	100	63±3[a]	74±8[b]
2 h	100	48±3[a]	70±5[b]	100	52±4[a]	107±9[b]	100	50±4[a]	101±8[b]	100	73±6[a]	116±7[b]	100	57±5[a]	79±3[b]
4 h	100	48±4[a]	73±4[b]	100	57±3[a]	78±7[b]	100	78±3[a]	102±4[b]	100	47±7[a]	110±5[b]	100	46±2[a]	44±5[a]
8 h	100	52±5[a]	69±7[b]	100	64±3[a]	78±4[b]	100	67±5[a]	98±6[b]	100	57±4[a]	108±6[b]	100	52±2[a]	47±4[a]

Chelerythrine (Ch) + Aleo-emodin (A)

	PKAc			δPKC			Bcl-2			Cas-3			p38		
	C	A	Ch+A	C	A	Ch+A	C	A	Ch+A	C	A	Ch+A	C	A	Ch+A
1 h	100	69±6[a]	62±3[a]	100	60±4[a]	60±3[a]	100	80±5[a]	120±9[b]	100	72±7[a]	82±2[b]	100	50±3[a]	65±5[b]

2 h	100	70±2 [a]	56±8 [a]	100	75±3 [a]	71±5 [a]	100	71±2 [a]	108±7 [b]	100	70±9 [a]	92±8 [b]	100	48±4 [a]	83±3 [b]
4 h	100	44±7 [a]	36±2 [a]	100	76±7 [a]	78±5 [a]	100	58±4 [a]	108±3 [b]	100	33±4 [a]	45±3 [b]	100	54±3 [a]	46±2 [a]
8 h	100	70±8 [a]	70±4 [a]	100	61±4 [a]	64±1 [a]	100	84±4 [a]	111±6 [b]	100	38±5 [a]	44±8 [a]	100	53±5 [a]	41±7 [a]

SB 202190 (S) + Aloe-emodin (A)

	PKAc				δPKC				Bcl-2				Cas-3			
	C	S	A	S + A	C	S	A	S + A	C	S	A	S + A	C	S	A	S + A
1 h	100	104±3	39±3 [a]	41±5 [a]	100	97±4	76±3 [a]	62±6 [a]	100	86±3	57±5 [a]	62±2 [a]	100	90±4	54±6 [a]	50±1 [a]
2 h	100	98±2	50±4 [a]	48±1 [a]	100	96±2	54±6 [a]	41±4 [a]	100	88±5	52±4 [a]	41±3 [a]	100	95±5	54±3 [a]	49±7 [a]
4 h	100	98±5	54±3 [a]	54±6 [a]	100	98±9	48±4 [a]	57±5 [a]	100	84±1	39±7 [a]	40±4 [a]	100	97±6	38±8 [a]	35±4 [a]
8 h	100	92±2	63±7 [a]	62±3 [a]	100	102±4	62±7 [a]	66±9 [a]	100	92±5	57±6 [a]	53±7 [a]	100	87±8	30±4 [a]	28±6 [a]

The degree of protein expression was quantified by AlphaEase image software. a: Statistically different from control values. b: Statistically different from aloe-emodin alone.

TABLE IV - EFFECT OF ALOE-EMODIN ON CASPASE-3 ACTIVATION IN H460

Caspase-3 activity (% of control)

Time	20 min	40 min	1 h	2 h	4 h	8 h
Control	100	100	100	100	100	100
Aloe-emodin	98 ± 5	134 ± 7**	178 ± 12**	198 ± 8**	111 ± 7*	92 ±6

Cells were incubated 0.1 % DMSO or 40 μM aloe-emodin in the presence of 1 % serum for various time periods. The caspase-3 activity was determined as described in Materials and Methods. All determinations are expressed as the mean percentage of control ± S.D. of triplicate from three independent experiments. Asterisks indicate values significantly different from control values (* $P < 0.05$, ** $P < 0.01$).

TABLE V – DENSITOMETRIC ANALYSIS OF THE WESTERN BLOT RESULTS OF ERK, P38, JNK1, AND ATF-2

Intensity (% control)

SB 202190 (S) + Aloe-emodin (A)

	ERK				ERK-phos				p38				p38-phos			
	C	S	A	S + A	C	S	A	S + A	C	S	A	S + A	C	S	A	S + A
1 h	100	90±4	85±8	81±8	100	67±4	59±8 [a]	39±3 [a]	100	85±9	62±8 [a]	62±4 [a]	100	103±4	89±9 [a]	78±5 [a]

2 h	100	95±5	88±7	85±10	100	118±10	82±6 [a]	54±4 [a]	100	99±5	57±9 [a]	88±3 [b]	100	94±5	81±6 [a]	96±7 [b]
4 h	100	83±9	57±5 [a]	55±7 [a]	100	124±8	47±3 [a]	87±5 [b]	100	109±4	48±4 [a]	98±6 [b]	100	95±5	36±5 [a]	84±9 [b]
8 h	100	105±8	70±9 [a]	82±5 [a]	100	132±9	89±5 [a]	110±9 [b]	100	98±5	58±4 [a]	126±7 [b]	100	98±10	36±8 [a]	95±3 [b]
16 h	100	80±8	48±3 [a]	53±5 [a]	100	104±8	75±5 [a]	87±4 [b]	100	99±10	52±6 [a]	70±7 [b]	100	100±11	55±4 [a]	60±3 [a]
24 h	100	66±4	51±3 [a]	61±3 [a]	100	127±9	80±5	90±6	100	101±8	75±6 [a]	97±9 [b]	100	101±5	76±10 [a]	87±6 [a]
	JNK1				JNK1-phos				ATF-2				ATF-2-phos			
	C	S	A	S + A	C	S	A	S + A	C	S	A	S + A	C	S	A	S + A
1 h	100	100±2	98±8	101±7	100	100±5	90±4	75±8	100	99±5	78±6[a]	98±7[b]	100	105±5	96±7	95±4
2 h	100	118±10	100±6	110±8	100	98±3	108±7	110±6	100	102±7	62±6 [a]	83±8 [b]	100	98±4	55±4[a]	73±5[b]
4 h	100	100±8	89±5	88±5	100	92±4	93±6	93±7	100	92±6	54±4 [a]	70±4 [b]	100	101±5	74±6 [a]	120±9 [b]
8 h	100	121±5	112±13	102±7	100	115±7	110±9	98±4	100	99±3	48±4 [a]	83±9 [b]	100	100±6	63±5 [a]	88±5 [b]
16 h	100	107±9	100±5	92±4	100	106±8	98±4	95±9	100	95±4	40±8 [a]	75±6 [b]	100	105±5	56±3 [a]	106±8 [b]
24 h	100	107±6	100±7	95±6	100	102±6	95±6	90±8	100	97±6	68±7 [a]	81±5 [b]	100	102±2	87±4 [a]	120±8 [b]

The degree of protein expression was quantified by AlphaEase image software. a: Statistically different from control values. b: Statistically different from aloe-emodin alone.

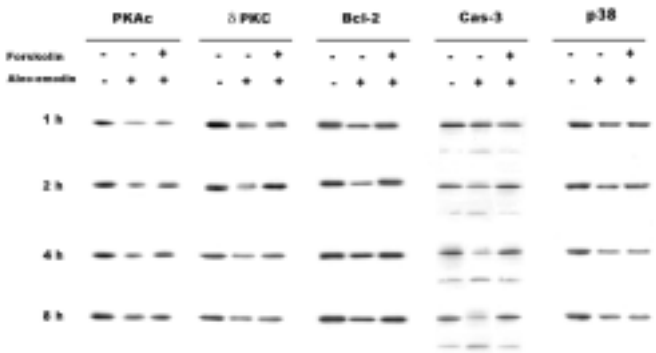

FIGURE 1 - Effects of aloe-emodin on the expression of PKAc, δPKC, Bcl-2, caspase-3, and p38 and of forskolin on the aloe-emodin-induced expression of PKAc, δPKC, Bcl-2, caspase-3, and p38 in H460 cells. The effect of aloe-emodin (40 μM) on PKAc, δPKC, Bcl-2, caspase-3 (Cas-3), and p38 proteins was detected by Western blot analysis. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1 % serum for 1, 2, 4, and 8 h. In forskolin treatment, cells were pretreated with forskolin (30 μM, 1 h), and then 40 μM aloe-emodin for the indicated times. Cell lysates were analyzed by 9 % (δPKC), 11 % (PKAc), 12 % (caspase-3 and p38), and 13 % (Bcl-2) SDS-PAGE, and then probed with primary antibody as described in Materials and Methods. Results are representative of three independent experiments.

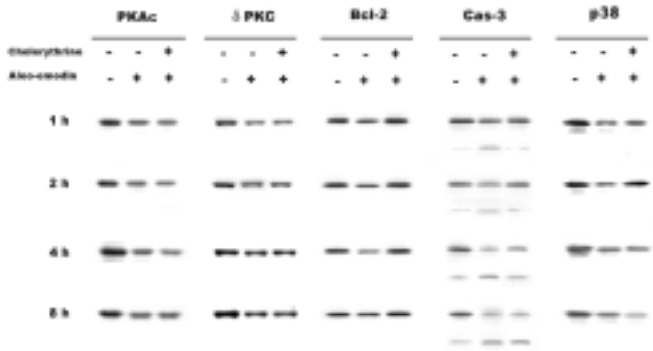

FIGURE 2 - Effects of chelerythrine on the aloe-emodin-induced expression of PKAc, δPKC, Bcl-2, caspase-3 (Cas-3), and p38 in H460 cells. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1 % serum for 1, 2, 4, and 8 h. In chelerythrine treatment, cells were pretreated with chelerythrine (1 μM, 2 h), and then 40 μM aloe-emodin for the indicated times. Cell lysates were analyzed by 9 % (δPKC), 11 % (PKAc), 12 % (caspase-3 and p38), and 13 % (Bcl-2) SDS-PAGE, and then probed with primary antibody as described in Materials and Methods. Results are representative of three independent experiments.

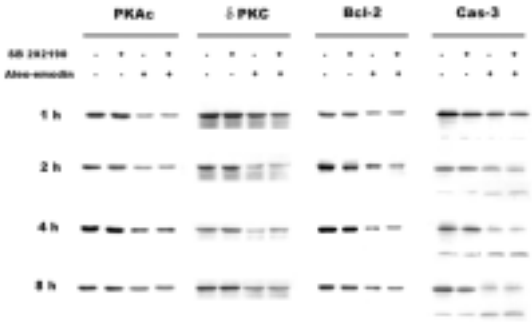

FIGURE 3 - Effects of SB202190 on the aloe-emodin-induced expression of PKAc, δPKC, Bcl-2, and caspase-3 (Cas-3) in H460 cells. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1 % serum for 1, 2, 4, and 8 h. In SB202190 treatment, cells were pretreated with SB202190 (0.1 μM, 2 h), and then 40 μM aloe-emodin for 1, 2, 4, and 8 h. Cell lysates were analyzed by 9 % (δPKC), 11 % (PKAc), 12 % (caspase-3), and 13 % (Bcl-2) SDS-PAGE, and then probed with primary antibody as described in Materials and Methods. Results are representative of three independent experiments.

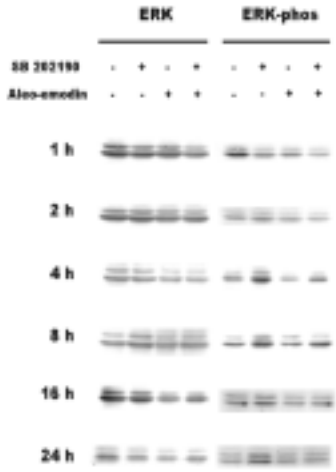

FIGURE 4 - Effects of aloe-emodin on ERK1/ERK2 and the phosphorylation of ERK1/ERK2 and of SB202190 on the aloe-emodin-induced expression of ERK1/ERK2 in H460 cells. The effects of SB202190 on the aloe-emodin-induced expression of ERK1/ERK2 and phosphorylation of ERK1/ERK2 were detected by Western blot analysis in H460 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. In SB202190 treatment, cells were pretreated with SB202190 (0.1 μM, 2 h), and then 40 μM aloe-emodin for the indicated times. Cell lysates were analyzed by 12 % SDS-PAGE, and then probed with primary antibodies as described in Materials and Methods. Results are representative of three independent experiments.

FIGURE 5 - Effects of aloe-emodin on p38 and the phosphorylation of p38 and of SB202190 on the aloe-emodin-induced expression of p38 in H460 cells. The effects of SB202190 on the aloe-emodin-induced expression of p38 and phosphorylation of

p38 were detected by Western blot analysis in H460 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. In SB202190 treatment, cells were pretreated with SB202190 (0.1 μM, 2 h), and then 40 μM aloe-emodin for the indicated times. Cell lysates were analyzed by 12 % SDS-PAGE, and then probed with primary antibodies as described in Materials and Methods. Results are representative of three independent experiments.

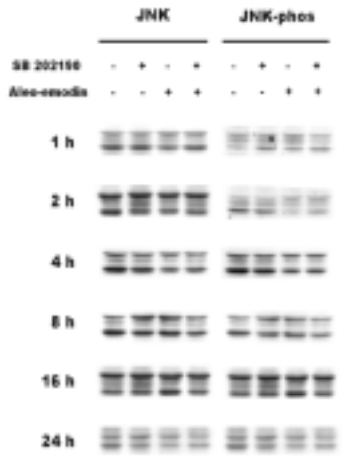

FIGURE 6 - Effects of aloe-emodin on JNK and the phosphorylation of JNK and of SB202190 on the aloe-emodin-induced expression of JNK in H460 cells. The effects of SB202190 on the aloe-emodin-induced expression of JNK and phosphorylation of JNK were detected by Western blot analysis in H460 cells. Cells were incubated with or without 40 μM aloe-emodin in the presence of 1 % serum for 2, 4, 8, 16, and 24 h. In SB202190 treatment, cells were pretreated with SB202190 (0.1 μM, 2 h), and then 40 μM aloe-emodin for the indicated times. Cell lysates were analyzed by 11 % SDS-PAGE, and then probed with primary antibodies as described in Materials and Methods. Results are representative of three independent experiments.

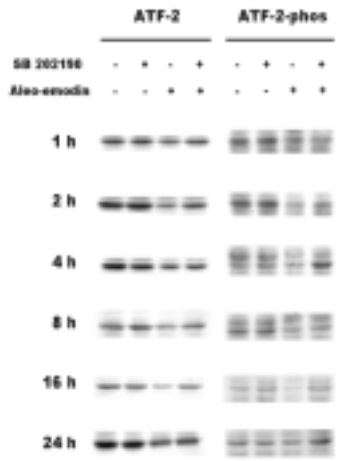

FIGURE 7 - Effects of aloe-emodin on ATF-2 and the phosphorylation of ATF-2 and of SB202190 on the aloe-emodin-induced expression of ATF-2 in H460 cells. The effects of aloe-emodin (40 μM) on ATF-2 and SB202190 on the aloe-emodin-induced expression of ATF-2 were detected by Western blot analysis in H460 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. In SB202190 treatment, cells were pretreated with SB202190 (0.1 μM, 2 h), and then 40 μM aloe-emodin for the indicated times. Cell lysates were analyzed by 10 % SDS-PAGE, and then probed with primary antibodies as described in Materials and Methods. Results are representative of three independent experiments.

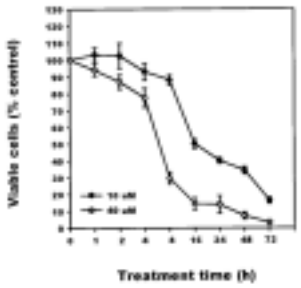

FIGURE 8 - Induction of cell death by aloe-emodin is irreversible. Cells were treated with vehicle alone or with 10 μM or 40 μM aloe-emodin in the presence of 1% serum for 1, 2, 4, 8, 16, 24, 48, and 72 h, and then washed. After that, the cells were incubated with fresh medium without aloe-emodin for 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.