wthsieh

寄件者: onbehalfof+anne.kopp+taylorandfrancis.com@manuscriptcentral.com 代理 anne.kopp@taylorandfrancis.com
寄件日期: 2010年10月11日星期一 上午 2:57
收件者: jgchung@mail.cmu.edu.tw
主旨: Nutrition and Cancer: An International Journal - Decision on Manuscript ID N&C-10-09-0629.R4

10-Oct-2010

Dear Prof. Chung:

The review of the final revision of your manuscript entitled "Latex of Euphorbia antiquorum induces apoptosis in human cervical cancer cells via c-Jun N-terminal kinase activation and reactive oxygen species production" is now complete. I am pleased to accept your manuscript for publication in Nutrition and Cancer: An International Journal. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter. We will let you know when your manuscript is assigned to an issue.

Thank you for your fine contribution to Nutrition and Cancer: An International Journal. I hope you will consider the journal as an outlet for your work in the future.

Please read the following text to familiarize yourself with the procedures that will be implemented to publish your article:

Keeping in Touch

The Editor of the journal is your primary source of information and advice. However, if you have a question the Editor is unable to answer, feel free to contact the Taylor and Francis Production Editor for the journal or the Taylor and Francis Electronic Journal Publishing Manager. (The Editor can put you in touch with both people.)

The journal is on a tight production schedule, so . . .

1. If you move or are away for an extended period during the time your manuscript is in production, let the Editor and Production Editor know where you can be reached (postal and e-mail addresses; telephone and fax numbers). You can imagine the delays that occur when the Production Editor e-mails typeset proofs to an address or phones a contributor with an important question, only to learn that the contributor is on sabbatical and did not leave forwarding information.
2. Contact the Production Editor if you are unable to return page proofs corrections within 2 days.

Understanding the Publication Process

1. You have now submitted your accepted manuscript to the journal. If you have not already done so, please e-mail (a) signed publication agreement, (b) disclosure of interest form, and (c) any permissions to adapt or reprint tables, figures, and quotations to Anne Kopp at anne.kopp@taylorandfrancis.com. Please go to http://mc.manuscriptcentral.com/nc and click on the Instructions and Forms link to access the forms.

2. The Editor compiles an issue of manuscripts from several contributors and provides the Taylor and Francis Production Editor with the issue.

3. The Production Editor reviews the files. If your manuscript is not prepared correctly (see the Instructions for Authors link at http://www.leajournals.com/nc/instructions_for_authors_nc.htm), the Production Editor notifies the Editor so you can revise it. (Production on the issue is suspended until all manuscripts are ready.)
4. When all manuscripts are ready, the Production Editor oversees copyediting and typesetting of the issue and sends via e-mail to each contributor the PDF files of (a) the page proofs of the respective article, (b) a list of queries concerning the article, and (c) a reprint request form.
5. You proofread the typeset article; record any changes; and return to the Production Editor your responses to the queries, any other corrections, and the reprint request form (with payment if ordering reprints).
6. The Production Editor finalizes the issue and sends it to the printer.

We are pleased that you have considered Nutrition and Cancer for the publication of your work. If you have any questions, please contact the Managing Editor, Anne Kopp, at Anne.Kopp@taylorandfrancis.com.

On behalf of the Editors of Nutrition and Cancer: An International Journal, we look forward to your continued contributions to the Journal.

Sincerely,

Ms. Anne Kopp
Nutrition and Cancer: An International Journal anne.kopp@taylorandfrancis.com

Reviewer(s)' Comments to Author:

Euphorbia antiquorum-induced Apoptosis through Fas/FasL Expression, Reactive Oxygen Species Production, and MAPK pathways in human cervical adenocarcinoma HeLa cells

Journal:	*Nutrition and Cancer: An International Journal*
Manuscript ID:	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	
Complete List of Authors:	Hsieh, Wen-Tsong; China Medical University Lin, Hui-Yi; China Medical University Chen, Jou-Hsuan; China Medical University Chung, Jing-Gung; China Medical University, Biological Science and Technology
Keywords:	Apoptosis, Cervical cancer, Herbs

Euphorbia antiquorum-induced Apoptosis through Fas/FasL Expression, Reactive Oxygen Species Production, and MAPK pathways in human cervical adenocarcinoma HeLa cells

Wen-Tsong Hsieh[a], Hui-Yi Lin[b], Jou-Hsuan Chen[a], and Jing-Gung Chung[c,d,*]

[a] Department of Pharmacology, [b] School of pharmacy, [c] Department of Biological Science and Technology, [d] Department of Microbiology, China Medical University, Taichung, Taiwan

*Corresponding author at: Department of Biological Science and Technology, China Medical University, No 91, Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C. Tel: +886-4-2205 3366-2161; Fax: +886-4-2205 3764. E-mail address: jgchung@mail.cmu.edu.tw

Keywords: *Euphorbia antiquorum*; Apoptosis; Mitochondrial; HeLa cells

Running title: *Euphorbia antiquorum*-induced cell cycle arrest and apoptosis in HeLa human cervical cancer cells

Abstract

Euphorbia antiquorum (EA) has been found to induce apoptosis in several types of human cancer cells. We investigated the EA-induced apoptotic signaling cascade pathway in human cervical carcinoma cells. EA induced-apoptosis is associated with a dose- and time-dependent in HeLa and Ca Ski cells. EA reduced mitochondrial membrane potential ($\Delta\Psi m$) and induced ROS generation in HeLa cells. Allopurinol (an effective scavenger of ROS) and cyclosporine A (Inhibitor of the mitochondrial permeability transition), reversed the EA-induced ROS generation. EA not only activated the intrinsic apoptotic pathway to releasing of cytochrome c through increased Bid and Bax levels, decreased anti-apoptosis protein Bcl-2 family levels, but activated the extrinsic signaling and death FasL/Fas receptor. EA-induced the mitochondrial stress pathway beginning with the release of cytochrome c from mitochondria, which activates caspase-9 and caspase-3. In addition, EA-induced apoptosis could, via decreased extracellular signal- regulated kinase (ERK) 1/2-p, increase p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) apoptotic pathway. Furthermore, the induction of cleaved caspase-8, -9 and -3 were alleviated by SP600125 (inhibitor for JNK). Based on these results, EA-induced apoptotic cell death may be conducted through ROS production and activation of the FasL/Fas, MAPK pathway in human cervical adenocarcinoma HeLa cells.

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide. The clinical therapy of cervical cancer includes surgery, radiotherapy, and chemotherapy (1). However, patients often develop tolerance, metastatic and recurrent on cisplatin-based chemotherapy (2,3). Increasing awareness and chemoprevention suggest using natural substances to prevent initial and promotional events associated with cancer development and this strategy has been considered to be the most direct way to counteract malignancy development (4,5).There are many natural plants or herbal formulas which could provide some clues for the identification of new anticancer compounds and a variety of plant secondary metabolites have been used as agents for chemoprevention of cancer (6,7). Many studies have focused on selectively killing tumor cells through the induction of apoptosis (6). Our preliminary study shows that the extract of *Euphorbia antiquorum* (EA) induced apoptosis and reduced cell growth specific in human cervical HeLa cells.

Euphorbia antiquorum L. called 'Crown of Thorns', is a traditional Chinese herbal medicine and useful in diuretic, rheumatism, dropsy, gout, neuropathy, carbuncle and cancer (8-11). It is also cultivated for fences in the tropic and subtropics regions of the world (12). The latex has been reported to contain euphol, euphol 3-O-cinnamate, antiquol A, antiquol B, 24-methylene-cycloartanol, and cycloeucalenol as the triterpene constituents (13). However, there is no available information to address the effects of EA on human cervical cancer HeLa cells.

Euphorbia antiquorum L. called 'Crown of Thorns', is a traditional Chinese herbal medicine and useful in diuretic, rheumatism, dropsy, gout, neuropathy, carbuncle and cancer (8-11). It is also cultivated for fences in the tropic and subtropics regions of the

world (12). The latex has been reported to contain euphol, euphol 3-*O*-cinnamate, antiquol A, antiquol B, 24-methylene- cycloartanol, and cycloeucalenol as the triterpene constituents (13). However, there is no available information to address the effects of EA on human cervical cancer HeLa cells.

Therefore, in this study, we investigated the induction of apoptosis and the role of the mitochondrial (intrinsic) or the receptor mediated (extrinsic) apoptosis pathway in human cervical cancer HeLa cell.

MATERIALS AND METHODS

Chemical and Reagents

Allopurinol, cyclosporine A, propidium iodide, ribonuclease A, Trypan blue, Tris-HCl, SP600125 (inhibitor for JNK), and Triton X-100 were from Sigma-Aldrich Chemical Company (St Louis, MO). DMEM medium, Fetal bovine serum (FBS), penicillin/streptomycin (P/S), L-glutamine and the primary antibody for phospho-p38 were from invitrogen™ (Grand island, N.Y.). Acrylamide/Bis 40% solution (ACRYL/BISTM 29: 1), Ammonium persulfate (APS), Glycine, SDS-EAGE running buffer (TG-SDS buffer), N,N,N',N'-Tetramethyl-ethylenediamine (TEMED), Tris (hydroxyl- methly)- aminomethane, and SDS (Sodium dodecyl sulfate), were from Amresco Inc. (Cochran Road Solon, OH). H2DCFDA (ROS detection reagent), SB203580 (p38 inhibitor), and primary antibodies for anti-p38 and anti-Bid were from Calbiochem, Novabiochem, & Novagen (Merck KGaA, Darmstadt, Germany). The primary antibody for anti-Erk2 and Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The primary

antibodies for anti-Bax, anti-p38 phosphorylation, anti-Erk phosphorylation anti-JNK phosphorylation and ECL detection system were from Amersham (GE Healthcare). The primary antibodies for anti-Fas, anti-caspase-9, anti-caspase-3, and anti-Bcl-2 were from Upstate (Millipore). The primary antibody for anti-JNK1/2 was from Cell Signaling Technology, Inc. (Danvers, MA). Protein assay-Dye reagent concentrate was from Bio-Rad Laboratories Inc. (Hercules, CA).

Preparation of EA plant extracts

The fresh *Euphorbia antiquorum* was collected from Nan-Tau County in Taiwan in March, 2006. The fresh juice was extracted by juicer from stem of *Euphorbia antiquorum.* The extract of *Euphorbia antiquorum* (EA) was centrifuged at 4000 rpm for 15 min. EA was filtered with 0.22 micron FGLP Teflon and frozen to dry (2.5 % yield) and stored at −20°C for used. About 5.0 kg of Euphorbia antiquorum 20.0 L of cold (25+2°C) methanol was immersed twice above the herb for 72 h each time, and the methanolic supernatant extract were filtered and put onto as the mixture extract. Then the same procedure was conducted for warm methanol (70°C ±2°C) extraction for 4 h, respectively. All the mixtures of mathenolic extracts were evaporated under 45℃ and vacuumed to dryness. The yield of Euphorbia antiquorum methanolic crude extract (EAM) was 605 g. The produce yield of extract was near about 12.1%. The extract was separated into several portions and stored under -20°C to be prepared for use.

Cell lines and culture

All cell lines were obtained from the Bioresource Collection and Research Center

(Hsinchu, Taiwan). Human cervical adenocarcinoma HeLa cells were grown in sterile DMEM medium. Epidermoid cervical carcinoma CaSki cells, human breast adenocarcinoma MDA-MB 231 cells, human promyelocytic leukemia HL-60 cells were grown in sterile RPMI 1640 medium. The entire medium contained 0.2% sodium bicarbonate, antibiotics (1:100 penicillin: streptomycin) and 10% heat-inactivated fetal bovine serum. All the cells were incubated under a fully humidified atmosphere of 95% room air and 5% CO_2 at 37°C.

Cell viability of HeLa cells treated with or without EA were determined by using flow cytometric analysis

The HeLa cells were plated in 12-well plates at a density of 2×10^5 cells per well and grown for 24 h. Different concentrations of EA were then added to cells for final concentration 0.5, 1.0, 2.0 and 3.0 μg/ml, while only adding medium for the control regimen and grown at 37°C, 5% CO_2 and 95% air for a different period of time. To determine cell viability, the trypan blue exclusion protocol was used. Briefly, approximately 10 μl of cell suspensions in PBS were mixed with 40 μl of trypan blue, and the numbers of stained (dead cells) and unstained cells (live cells) were counted using the flow cytometric assay as described previously (14).

Apoptosis from HeLa cells treated with different concentrations of EA were examined by using flow cytometric analysis

About 5×10^5 cells per well of HeLa cells in 12-well plate with various concentrations (0, 0.5, 1.0, 2.0 and 3.0 μg/ml, respectively) of EA were incubated in an

incubator for different time periods. Cells were harvested by centrifugation and were fixed gently (drop by drop) by putting 70% ethanol (in PBS) in 4°C overnight and then re-suspended in PBS containing 40 µg/mL propidium iodide and 0.1 mg/mL RNase and 0.1% Triton X-100 in dark room. After 30 min at 37°C, the cells were analyzed with a flow cytometric analysis equipped with an argon ion laser at 488 nm wavelength and monitored through a 630/22-nm band pass filter using a Becton–Dickinson FACS-Calibur flow cytometry (15).

Analysis of DNA Damage Using the Comet Assay

The protocol used for single cell gel (comet) assay for each treatment and control followed the guidelines recommended by Tice et al., 2000 (6). Briefly, HeLa cells were incubated individually with 0, 0.5, 1.0, 2.0 and 3.0 µg/ml of EA for different time periods, 10 µl of cells (1×10^5 cells) were added to 120 µl of 0.5% low-melting point agarose at 37ºC, layered onto a pre-coated slide with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and the slides were immersed in a lysing solution consisting of 2.5 M sodium chloride, 100 mM ethylenediamine- tetraacetic (EDTA), 10 mM Tris-HCl buffer at pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% dymethylsulfoxide (DMSO) for about 1 h. Prior to electrophoresis, the slides were left in alkaline buffer containing 0.3 mM NaOH and 1 mM EDTA (pH>13) for 20 min and electrophoresed for another 20 min at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5), fixed in absolute ethanol and stored at room temperature until blind analysis in a fluorescence microscope (Olympus; Optical Co. Ltd, Tokyo, Japan) at X400

magnification. To minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination (17).

Detection of mitochondrial membrane potential (ΔΨm) in HeLa cells after treated with EA by flow cytometric analysis

Approximately 2.5×105 cells per well of HeLa cells in 12-well plate with various concentrations (0, 0.5, 1.0, 2.0 and 3.0 μg/ml, respectively) of EA were incubated for 24 h. The cells were washed twice, re-suspended in 500 μl of DiOC6 (4 mol/l) and incubated at 37°C for 30 min. The level of $\Delta\Psi m$ of the HeLa cells was determined by flow cytometric analysis, using the DiOC6 (4 mol/L).The fluorescence intensity was determined at an excitation wavelength: 488 nm, emission wavelength: 500 nm with flow cytometric analysis. To determine ΔΨm, the flow cytometric assay was used as described previously (15).

Detection of reactive oxygen species (ROS) in HeLa cells after treated with EA by flow cytometric analysis

Approximately 2.5×105 cells per well of HeLa cells in 12-well plate with various concentrations (0, 0.5, 1.0, 2.0 and 3.0 μg/ml, respectively) of EA were incubated for 24 h. Following the exposure to EA, the cells were trypsinized and washed with ice-cold PBS. We then added 1 ml PBS containing 50 μg/ml 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma), and incubated the cells for 30 min at 37℃. The level of ROS of the HeLa cells was examined by flow cytometric analysis. To determine ROS, the flow cytometric assay was used as described previously (15).

Western blotting for examining the effect of EA on proteins level of HeLa cells

HeLa cells were plated at a density of 1×106 cells/well onto 6-well plates 24 h and then treated with 0, 0.5, 1.0, 2.0 and 3.0 μg/ml EA 24 h. Cells were harvested by scraping and washed twice with PBS. Cells were counted and 1×106 cells were resuspended in 1 ml of 50 mM resuspension buffer, followed by the addition of 200 μl of extraction agent for 30 min on ice with occasional vortexing. Lysates were prepared by centrifugation at 14000xg for 30 min at 4°C and stored at -80°C until assayed. The total amount of protein was determined in samples using the Bradford method. Equal amounts of total protein of each sample were loaded onto SDS-polyacrylamide gels and the proteins electrophoretically transferred onto a PVDF membrane (Millipore, Bedford, MA). Immunoblots were analyzed using specific primary antibodies to p53, Fas, Bax, caspase-3, c-Jun. p-p38, and p-JNK followed by exposure to horseradish peroxidase-conjugated secondary antibody for 1 h. Proteins were visualized using an enhanced chemiluminescence detection kit (ECL Kits; Amersham Life Science, UK)(18).

Inhibitors inhibit viability

HeLa cells were seeded at a density of 5×105 cells/well onto 6-well plates 24 h before cells were pre-treated with 10 μM of SP600125 (JNK inhibitor) and 10 μM of SB203580 (p38 inhibitor) for 1 h followed by treatment with EA and medium as a control. Cells were then harvested at 24 and 48 h to determine the percentage of viable cells as previously described (15).

Statistical analysis

Data were expressed as means ± S.D. ANOVA and Student's t test were used to analyze the difference between the means of test samples and controls with $P<0.05$ were considered statistically significant.

RESULTS

EA decreased the percentage of viable and induced apoptosis in HeLa cells

After HeLa cells were treated with increasing concentrations of EA for various time periods, viable cells were examined by flow cytometric methods. The cell viability was determined by formazan dye uptake and expressed as percent of untreated control cells. The results demonstrated that EA decreased the percentage of viable cells dose-dependent and time-dependent (Figure 1A). The fresh juice extract from Euphorbia antiquorum (EA) was more potent than methanolic of Euphorbia antiquorum extract decreasing cells viability (Data not shown).

HeLa cells were treated with increasing concentrations of EA for 24 h. The apoptosis (sub-G1 group) was examined and analyzed by flow cytometric methods. As compared to control, the results demonstrated that sub-G1 group significantly increased after cells were cultured with EA (0.5 μg/ml: 4.1%, 1.0 μg/ml: 8.1%, 2.0 μg/ml: 14.1%, respectively) (Figure 1B). Results indicate that EA can induce apoptosis in HeLa cells in a dose-dependent manner.

EA induced DNA damage and apoptosis in HeLa cells

The comet assay was used to measure DNA damage in human cervical carcinoma

HeLa cells in vitro. Although other studies have shown that EA induced DNA damage in some cell lines, here, in order to examine whether or not EA induced DNA damage in HeLa cells, Comet assay was used and the results indicated that EA induced DNA damage based on the DNA damage tail production which is shown in Figure 2A. Increasing the dose of EA led to an increase of DNA damage and those effects were dose-dependent. As shown in Figure 3A the photographs from DNA gel electrophoresis assays was observed in EA- treated HeLa cells compared with intact control cells and this effect was dose-dependent (Figure 2B).

EA suppressed the levels of mitochondria membrane potential ($\Delta\Psi m$), in HeLa cells

After HeLa cells were treated with 2.0 μg/ml of EA for 1, 24, and 48 h the levels of $\Delta\Psi_m$ were analyzed and quantified by flow cytometric methods. As shown in Figure 3A, A significant decrease of $\Delta\Psi_m$ was detected during one hour in cells treated with EA 2.0 μg/ml. The representative profiles given in Figure 3A and Figure 3B indicated that increased time led to increase the levels of $\Delta\Psi_m$ after cells were treated with EA 2.0 μg/ml for 48 h.

EA-induced generation of reactive oxygen species (ROS) in HeLa cells

Generation of ROS by oxidative damage plays an important role in apoptosis (19,20). We and others have shown that chemopreventive agents induce apoptosis in cancer cells through generation of ROS (21-23). To investigate the mechanism by which EA induced apoptosis in HeLa cells, we examined whether EA might be able to induce generation of ROS in HeLa cells during apoptosis. Using flow cytometric methods, the production of

ROS was monitored by measuring the fluorescent intensity of DCF. The results demonstrated that 2.0 μg/ml of EA promoted the production of ROS (Figure 4A) and exhibited maximum effects at 4 to 6 hour (Figure 4B), which was approximately 67 % higher than that of the control cells. ROS generation was markedly induced in EA-treated HeLa cells (Figure 4C). The results showed that the EA-induced ROS generation could be significantly prevented by treatment with the allopurinol (an effective scavenger of ROS) (24) (Figure 4D) and Cyclosporine A (CsA: Inhibitor of the mitochondrial permeability transition) (15) (Figure 4E). The data demonstrated that EA induced ROS production quite early and time-dependently (Figure 4a) and pretreatment with allopurinol and Cyclosporine A decreased the ROS after treatment with EA.

Berberine affects the levels of caspase- 3, -8 and -9 activity of HeLa cells

Caspases mediate both apoptosis through aspartate-specific cleavage of a wide number of cellular substrates (26).The cleaved caspase 3 and cleaved caspase 8 are the characteristic hallmarks of apoptosis (27). Based on our results, which show that EA causes strong apoptotic death (Figure 2), in order to examine whether or not EA induced apoptosis in HeLa cells through caspase-dependent or -independent pathways, cells were treated with 2.0 μM EA for various time periods before harvesting for Western blotting examination. In order to examine whether or not EA induced apoptosis in HeLa cells through caspase-activation, cells were treated with increasing concentrations of EA, marked increase in cytochrome c release and induction of cleavage of caspase 8, cleavage of caspase 9, and cleavage of caspase 3 production (Figure 5A). The results are shown in Figure 5A, which indicates that EA promoted the activation of caspase-8, -9 and -3 in

HeLa cells and these effects were dose-dependent.

EA-activated the mitochondria relative apoptosis pathway in HeLa cells

Cells treated with various concentration of EA for 24 h showed significantly dose-dependent down-regulation in Bcl-2 expression and up-regulation in Bid, Bax and cytochrome c expression. The Bax/Bcl-2 ratio was significantly increased at dose dependent manner (Figure 5B). EA induced Bax, and Bid cleavage of proapoptotic proteins and inhibited antiapoptotic proteins Bcl-2 expression as measured by Western blot analysis (Figure 5B). These data suggest that EA regulates both pro- and antiapoptotic members of Bcl-2 family, and may link death receptor pathway through cleavage and activation of Bid. These data suggest that EA regulates both proapoptotic and antiapoptotic members of EA treatment in HeLa cells and these effects were dose-dependent.

EA-activated the Fas/FasL and JNK/SAPK apoptosis pathway in HeLa cells.

ROS, which are known to be an important component of cytotoxic action, were involved in apoptotic cell death (28) and JNK activation (29).Because caspase-8, an initiator caspase linked with Fas, was involved in EA-mediated apoptosis, we examined surface Fas and FasL expression after EA stimulation by western blotting analysis. The kinase activity was also increased with the various doses of EA treatment (Figure 5A). Thus, EA-induced apoptosis includes JNK activation. We found that FasL was scarcely expressed on unstimulated HeLa cells although EA stimulation clearly induced surface expression of FasL. It is feasible that induced FasL after EA stimulation can trigger

apoptosis of adjacent cells in a Fas/FasL mechanism. Cross-linking of EA quickly evoked cleaved caspase-3, -8 and -9 generation on HeLa cells (Figure 5A).

To confirm the involvement of the MAPK signaling pathways Erk 1/2, JNK and p38, we used the JNK inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one), and p38 MAPK inhibitors, SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5 -(4-pyridyl)-1H-imidazole) (31), respectively. As depicted in Figure 5C, abolishment of cleaved caspase-3, -8 and -9 generation by SP600125 was able to block both up-regulation of JNK and induction of FasL expression after EA stimulation. We characterized the JNK assay by using HeLa cells (Figure 5B). The kinase activity increased at 24 h.

The immunoblotting results showed that EA increased the protein levels both of extrinsic apoptosis and MAP kinase signaling pathways associated proteins such as JNK-p, p38, p38-p and c-jun (Figure 5C). The inhibition of apoptosis by SP600125, but not by SB203580 demonstrated the involvement of JNK pathways in the modulation of EA-induced apoptosis in cervical cells, but showed no effect of the p38 pathway (Figure 5D).

DISCUSSION

In the present study, we have demonstrated that EA inhibits growth and induces apoptosis in cervical melanoma HeLa cancer cells through activation of multiple apoptosis signaling pathways. Apoptosis can be divided into caspase-dependent and -independent pathways (31). A significant decrease of $\Delta\Psi m$ was detected during one hour in cells treated with EA. Cytochrome c release and the mitochondrial membrane potential

decrease as the result of the opening of permeability transition pores have been proposed as early irreversible events during apoptosis (32,33). Mitochondrial dysfunctions including the loss of MMP (ΔΨm), permeability transition, and release of cytochrome c from the mitochondrion into the cytosol are associated with apoptosis (34).

According to the result data, EA promoted the production of ROS. High levels of ROS can lead to necrotic cell death, whereas low levels of ROS have been shown to induce apoptotic cell death (35). Generation of the ROS may be the important event in the mitochondria for the release of cytochrome c, which has been found to activate caspases (36). ROS can cause MMP ($\Delta\Psi m$) loss by activating mitochondrial permeability transition, and induce apoptosis by releasing apoptogenic protein such as cytochrome c to cytosol (37). Mitochondria are major producers of free radical species (ROS) and major targets for oxidative damage (38,39). Our data have shown that EA causes decreased mitochondrial membrane potential and release of cytochrome c from mitochondria to cytosol. The results indicate that EA induces apoptosis mainly through mitochondrial-dependent pathway which was evident by drop in mitochondrial membrane potential, generation of reactive oxygen species (ROS), and release of mitochondrial proteins (cytochrome c) in cervical melanoma HeLa cells.

EA exhibited marked induction of the levels of cleavage of caspase 8, cleavage of caspase 9, and cleavage of caspase 3 (Figure 5A). Activation of caspase-dependent signaling pathways was via cleavage of the procaspase-9 and -8 and results in the downstream activation of caspase-3, -6, -7, finally leading to apoptosis (40). In addition, EA treatment resulted in marked increase in cytochrome c release (Figure 5B). There are at least one mitochondrial protein (Cytochrome c, Endo G and AIF) released following

mitochondrial depolarization is an activator of nuclear apoptosis, presumably through caspase activation (41).Cytochrome c release and induction of cleavage of caspase have been reported to play vital roles in intrinsic mitochondrial apoptosis (42). To activate and transfer cytochrome c from mitochondria to the cytosolic compartment, the generation of the mitochondrial membrane potential might be involved at the beginning of the cell death process (43).

Bcl-2 family prevents the redistribution of cytochrome c which plays a key role in regulating apoptosis mainly at the level of mitochondria, and thus controlling the release of mitochondrial proteins, and caspase activation (44,45). Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells (46). Indeed, the ratio between these two subsets determines, in part, the susceptibility of cells to a death signal (47). Bc1-2 and Bcl-xL protect mitochondria against the loss of mitochondrial membrane potential during both apoptosis and at least some forms of necrotic cell death (48). Our results indicate that EA induces expression of proapoptotic proteins (Bax, and Bid) and inhibits expression of antiapoptotic proteins (Bcl-2).

The multidomain proapoptotic proteins Bax and Bak are constitutively expressed and only induce permeabilization of outer mitochondrial membrane following apoptotic stimuli, suggesting that they are inactive in non-apoptotic cells (49). Furthermore, EA enhances translocation of Bax to mitochondria where it may interact with other Bcl-2 family members to cause MOMP and release of mitochondrial proteins leading to caspase activation and apoptosis.

ROS generation is associated with increased expression of the mitogen-activated protein kinase (MAPK) and caspase pathways have been implicated in apoptosis (50).

ROS dependent sustained JNK activation can promote cell death, although there is still some controversy on the molecular targets of ROS-activated JNK, a number of hypotheses (51-53) have been proposed to explain this JNK-mediated event. All of these observations indicate that sustained JNK activation is essential to induce apoptosis in some types of cell death (54). The EA-induced persistent JNK activation may be produced by the continued stimulation of upstream activators of the JNK pathway due to cellular damage by ROS. The sustained JNK activation is ROS dependent and may serve as a death signal in EA-induced apoptosis. EA induces a persistent activation of the JNK enzyme, which may play a critical role in the apoptotic suicide program. EA stimulates JNK activity after a 24-h exposure of cells, and the kinase activity persists throughout apoptosis. Cyclosporine A prevented the EA-induced JNK activation and apoptosis, confirming the mediating role of ROS during EA-induced apoptosis.

Although there are variable effects on p38 MAP kinase and ERK activities in different cell systems, the JNK activation is persistent and shows a direct correlation with apoptosis (55). EA increases JNK activity after 24 h having a significant effect on p38 MAP kinase and ERK activity in HeLa cells. Finally, EA enhances the apoptosis-inducing potential by up-regulating death receptors (Fas/FasL) and cleaving Bid.

In conclusion, EA-induced apoptosis was accompanied by sustained phosphorylation of JNK, c-Jun, and p38 MAPK. EA also reduced mitochondrial membrane potential ($\Delta\Psi m$) and induced ROS generation in HeLa cells. Both proapoptosis factors Bid and Bax levels were increased and antiapoptosis factor Bcl-2 was decreased. The activation of the caspase 9, caspase 8, caspase 3 and the release of cytochrome c; whereas, the expression of proapoptosis factor were involved in EA-induced apoptosis in HeLa cells. These

results lead us to speculate that EA-induced apoptotic cascade and cell death may go through activation of the FasL/Fas, JNK pathway and induction of ROS production in human cervical adenocarcinoma HeLa cells.

ACKNOWLEDGEMENTS

This work was supported by the grant from the China Medical University (CMU96-125).

REFERENCES

1. Magné N, Chargari C, Vicenzi L, Gillion N, Messai T, et al.: New trends in the evaluation and treatment of cervix cancer: the role of FDG-PET. *Cancer Treat Rev* **34**, 671-681, 2008.
2. Torres MA, Jhingran A, Thames HD Jr, Levenback CF, Bodurka DC, et al.: Comparison of treatment tolerance and outcomes in patients with cervical cancer treated with concurrent chemoradiotherapy in a prospective randomized trial or with standard treatment. *Int J Radiat Oncol Biol Phys* **70**,118-125, 2008.
3. Tao X, Hu W, Ramirez PT, Kavanagh JJ: Chemotherapy for recurrent and metastatic cervical cancer. *Gynecol Oncol* **110,** (3 Suppl 2):S67-71, 2008.
4. Sasieni P: Chemoprevention of cervical cancer. *Best Pract Res Clin Obstet Gynaecol* **20***,* 295-305, 2006.
5. Charles AL, Chang CK, Wu ML, Huang TC: Studies on the expression of liver detoxifying enzymes in rats fed seaweed (Monostroma nitidum). *Food Chem Toxicol* **45**, 2390-2396, 2007.
6. Ferreira CG, Epping M, Kruyt FA, Giaccone G: Apoptosis: target of cancer therapy.

Clin Cancer Res **8**, 2024-34, 2002.

7. Peng B, Chang Q, Wang L, Hu Q, Wang Y, et al.: Suppression of human ovarian SKOV-3 cancer cell growth by Duchesnea phenolic fraction is associated with cell cycle arrest and apoptosis. *Gynecol Oncol* **108**, 173-181, 2008.
8. Huang CK: The Pharmacology of Chinese Herbs 2nd edit, CRC Press, 494, 1998.
9. Akihisa T, Kithsiri Wijeratne EM, Tokuda H, Enjo F, Toriumi M, et al.: Eupha-7,9(11),24-trien-3beta-ol ("antiquol C") and other triterpenes from Euphorbia antiquorum latex and their inhibitory effects on Epstein-Barr virus activation. *J Nat Prod* **65**,158-162, 2002.
10. Riddle JM: Ancient and Medieval Chemotherapy for Cancer. *Isis* **76**, 319-330, 1985.
11. United States Patent Application 20030165579
12. Batanouny KH, Stichler W, Ziegler H: Photosynthetic pathways and ecological distribution of Euphorbia species in Egypt. *Oecologia* **87**, 565-569, 1991.
13. Gewali, MB, Hattori M, Tezuka Y, Kikuchi T, Namba T: Constituents of the Latex of Euphorbia antiquorum L. *Phytochemistry* **29**, 1625-1628, 1990.
14. Lu HF, Sue CC, Yu CS, Chen SC, Chen GW, et al.: Diallyl disulfide (DADS) induced apoptosis undergo caspase-3 activity in human bladder cancer T24 cells. *Food Chem Toxicol* **42**, 1543-1552, 2004.
15. Li TM, Chen GW, Su CC, Lin JG, Yeh CC, et al.: Ellagic acid induced p53/p21 expression, G1 arrest and apoptosis in human bladder cancer T24 cells. *Anticancer Res* **25**, 971-979, 2005.
16. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, et al.: Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing.

Environ Mol Mutagen **35**, 206-221, 2000.

17. Angelis KJ, Dusinská M, Collins AR: Single cell gel electrophoresis: detection of DNA damage at different levels of sensitivity. *Electrophoresis* **20**, 2133-2138, 1999.
18. Chung JG, Yang JS, Huang LJ, Lee FY, Teng CM, et al.: Proteomic approach to studying the cytotoxicity of YC-1 on U937 leukemia cells and antileukemia activity in orthotopic model of leukemia mice. *Proteomics* **7**, 3305-3317, 2007.
19. Kroemer G, Reed JC: Mitochondrial control of cell death. *Nat Med* **69**, 513-519, 2000.
20. Jacobson MD: Reactive oxygen species and programmed cell death. *Trends Biochem Sci* **21**, 83-86, 1996.
21. Shankar S, Srivastava RK: Involvement of Bcl-2 family members, phosphatidyl-inositol 3′-kinase/AKT and mitochondrial p53 in curcumin (diferulolylmethane)-induced apoptosis in Prostate Cancer. *Inter J Oncol* **30**, 905-918, 2007.
22. Fujisawa S, Kadoma Y: Anti- and pro-oxidant effects of oxidized quercetin, curcumin or curcumin-related compounds with thiols or ascorbate as measured by the induction period method. *In Vivo* **20**, 39-44, 2006.
23. Azam S, Hadi N, Khan NU, Hadi SM: Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol In Vitro* **18**, 555–561, 2004.
24. Müller-Peddinghaus R, Wurl M: The amplified chemiluminescence test to characterize antirheumatic drugs as oxygen radical scavengers. *Biochem Pharmacol* **36**, 1125-1132, 1987.

25. Broekemeier KM, Pfeiffer DR: Inhibition of the mitochondrial permeability transition by cyclosporin A during long time frame experiments: relationship between pore opening and the activity of mitochondrial phospholipases. *Biochemistry* **34**, 16440-16449, 1995.

26. Green DR, Reed JC: Mitochondria and apoptosis. *Science* **281**, 1309-1312, 1998.

27. Decker P, Isenberg D, Muller S: Inhibition of caspase-3-mediated poly(ADP-ribose) polymerase (PARP) apoptotic cleavage by human PARP autoantibodies and effect on cells undergoing apoptosis. Inhibition of caspase-3-mediated poly(ADP-ribose). *J Biol Chem* **275**, 9043-9046, 2000.

28. Kadowaki H, Nishitoh H, Urano F, Sadamitsu C, Matsuzawa A, et al.: Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death Differ* **12**, 19-24, 2005.

29. Ventura JJ, Cogswell P, Flavell RA, Baldwin AS Jr, Davis RJ: JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. *Genes Dev* **18**, 2905-2915, 2004.

30. Ward SG, Parry RV, Matthews J, O'Neill L: A p38 MAP kinase inhibitor SB203580 inhibits CD28-dependent T cell proliferation and IL-2 production. *Biochem Soc Trans* **25**, 304S, 1997.

31. Mühlenbeck F, Haas E, Schwenzer R, Schubert G, Grell M, et al.: TRAIL/Apo2L activates c-Jun NH2-terminal kinase (JNK) via caspase-dependent and caspase-independent pathways. *J Biol Chem* **273**, 33091-33098, 1998.

32. Spierings D, McStay G, Saleh M, Bender C, Chipuk J, et al.: Connected to death: the (unexpurgated) mitochondrial pathway of apoptosis. *Science* **310**, 66-67, 2005.

33. Thomas WD, Zhang XD, Franco AV, Nguyen T, Hersey P: TNF-related apoptosis-inducing ligand-induced apoptosis of melanoma is associated with changes in mitochondrial membrane potential and perinuclear clustering of mitochondria. *J Immunol* **165**, 5612-5620, 2000.

34. Hengartner MO: The biochemistry of apoptosis. *Nature* **407**,770-776, 2000.

35. Yeo JE, Kim JH, Kang SK: Selenium attenuates ROS-mediated apoptotic cell death of injured spinal cord through prevention of mitochondria dysfunction; in vitro and in vivo study. *Cell Physiol Biochem* **21**, 225-238, 2008.

36. Li M, Wang AJ, Xu JX: Redox state of cytochrome c regulates cellular ROS and caspase cascade in permeablized cell model. *Protein Pept Lett* **15**, 200-205, 2008.

37. Woo SH, Park IC, Park MJ, An S, Lee HC, et al.: Arsenic trioxide sensitizes CD95/Fas-induced apoptosis through ROS-mediated upregulation of CD95/Fas by NF-kappaB activation. *Int J Cancer* **112**, 596-606, 2004.

38. Duchen MR: Mitochondria in health and disease: perspectives on a new mitochondrial biology. *Mol Aspects Med* **25**, 365-451, 2004.

39. Monsalve M, Borniquel S, Valle I, Lamas S: Mitochondrial dysfunction in human pathologies. *Front Biosci* **12**, 1131-1153, 2007.

40. Broekemeier KM, Pfeiffer DR: Inhibition of the mitochondrial permeability transition by cyclosporin A during long time frame experiments: relationship between pore opening and the activity of mitochondrial phospholipases. *Biochemistry* **34**, 16440-16449, 1995.

41. Widłak P: Apoptosis: nuclease, caspases, cytochrome c. *Postepy Biochem* **44**, 252-254, 1998.

42. Nie Z, Phenix BN, Lum JJ, Alam A, Lynch DH, et al.: HIV-1 protease processes procaspase 8 to cause mitochondrial release of cytochrome c, caspase cleavage and nuclear fragmentation. *Cell Death Differ* **9**, 1172-1184, 2002.

43. La Piana G, Fransvea E,;zulli D, Lofrumento NE: Mitochondrial membrane potential supported by exogenous cytochrome c oxidation mimics the early stages of apoptosis. *Biochem Biophys Res Commun* **246**, 556-561, 1998.

44. Cory S, Adams JM: The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**, 647-656, 2002.

45. Kim CN, Wang H, Huang Y, Ibrado AM, Liu L, et al.: Overexpression of Bcl-X_L inhibits Ara-C-induced mitochondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. *Cancer Res* **57**, 3115-3120, 1997.

46. Murphy E, Imahashi K, Steenbergen C: Bcl-2 regulation of mitochondrial energetics. *Trends Cardiovasc Med* **15**, 283-290, 2005.

47. Oltvai ZN, Milliman CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619, 1993.

48. Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, et al.: Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* **13**, 21-29, 1996.

49. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, et al.: Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**, 183-192, 2002.

50. Ranawat P, Bansal MP: Apoptosis induced by modulation in selenium status involves p38 MAPK and ROS: implications in spermatogenesis. *Mol Cell Biochem*, 2009 Apr 12. (in press)

51. Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P: RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell Death Diff* **14**, 400-410, 2007.

52. Chang L, Kamata H, Solinas G, Luo JL, Maeda S, et al.: The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. *Cell* **124**, 601-613, 2006.

53. Deng Y, Ren X, Yang L, Lin Y, Wu X: A JNK-dependent pathway is required for TNFalpha- induced apoptosis. *Cell* **115**, 61-70, 2003.

54. Chen YR, Wang X, Templeton D, Davis RJ, Tan TH: The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem* **271**, 31929-31936, 1996.

55. Liu J, Lin A: Role of JNK activation in apoptosis: a double-edged sword. *Cell Res* **15**, 36-42, 2005.

FIGURE LEGENDS

Figure 1. EA decreased cell viability and induced apoptosis in human cervical HeLa cells. (A) EA decreased cell viability in HeLa cells. Cells were cultured with various concentrations of EA for 24, 48 and 72 h and were collected by centrifugation. Then the viable cells were determined by PI-incorporation and flow cytometric analysis as described in Methods and Materials. (B) EA induced apoptosis in HeLa cells. Cells were treated with various concentrations of EA for 24 h and the cells were harvested and analyzed for cell cycle and the sub-G1 group (the percent of cells in apoptosis) was analyzed by flow cytometric analysis. Data represents mean ± S.D. of three experiments. ***p <0.001.

Figure 2. EA induced DNA damage and apoptosis in human cervical HeLa cells. Cells were incubated with various concentrations (0, 0.5, 1.0, 2.0 or 3.0 μg/ml, respectively) of EA and 0.5% H2O2 for 24 h. DNA damage was determined using DNA gel electrophoresis. DNA strand breaks were represented by the mean tail moment and tail intensity for 50 comets per sample. Under compare with control, hydrogen peroxide, or EA induced strand breakage in DNA of HeLa cells in a dose-dependent manner. In contrast, DNA from the untreated cells remained intact and was devoid of comet like tails (control).

Figure 3. EA decreased the levels of mitochondrial membrane potential ($\Delta\Psi m$) and release of mitochondrial proteins to cytosol. (A) EA decreased the $\Delta\Psi m$. The $\Delta\Psi m$ were

measured after incubation in EA (2.0 μg/ml) for 1, 24 and 48 h in HeLa cells. The zero concentration was defined as control. (B) The statistics results indicate that EA decrease ΔΨm during one hour in HeLa cells. The percentage of cells that were stained by DiOC6 dye, and the stained cells were determined by flow cytometric analysis. (C) HeLa cells were treated with EA (20 μg/ml) for 24 h, and cytoplasmic fractions were prepared. Crude proteins were subjected to SDS-PAGE and immunoblotted with anti-cytochrome c (Cyto C), anti-Bcl-2, anti-Bax or anti-Bid antibody. Data presented means ± SD (n = 3).

Figure 4. EA induces production of reactive oxygen species (ROS) in HeLa cells. (A) The HeLa cells were treated with 2.0 μg/ml of EA and we detected the changes of ROS activity by staining with 2,7-dichlorodihydrofluorescein diacetate for various period by flow cytometric analysis. (B) EA promoted the production of ROS and the maximum effects between 4 to 6 hour. (C) EA promoted the production of ROS and the maximum effects between 4 to 6 hour. (D) Allopurinol (5.0 μg/ml) EA promoted the production of ROS and the maximum effects between 4 to 6 hour. (E) HeLa cells were pretreated with cyclosporine A (1.0 μg/ml). for 2 h and exposure to EA for 4 h then to detect the changes of ROS. (F) EA-induced ROS generation could be prevented by treatment with the allopurinol and Cyclosporine A was found to prevent ROS production from EA treated. Data shown are mean ± S.E (n = 3). of two separate experiments performed in duplicate. *, $P < 0.05$.

Figure 5. EA-induced apoptosis involvement of caspases through activating the Fas/FasL and JNK/SAPK pathway in HeLa cells (A) Interactive effects of EA on cleavage of

caspase-8, caspase-3, and caspase-9. HeLa cells were treated with EA (0–3.0 μg/ml) for 24 h. At the end of incubation period, cells were harvested. Crude proteins were run on SDS-PAGE, and immunoblotted with anti-caspase-8, anti-caspase-3, anti-caspase-9, and anti-PARP antibodies. β-actin was used as a loading control. (B) EA activated MAP kinase relative protein JNK, JNK-p, c-jun (-p63 and -p73), and extrinsic pathway protein (ERK2, ERK1/2-p, p38, p38-p, Fas and FasL). (C) The influenced levels of apoptotic caspases were pre-treated with SP600125 (inhibitor of JNK) and/or SB203580 (inhibitor of p38) then treated with EA for 24 h. β-Actin is included as a protein-loading normalization control. This experiment was repeated three times with a similar outcome. The data shown here are mean ± SEM from a representative experiment as compared to the vehicle group.

Figure 6. Model for EA-induced apoptosis in human cervical melanoma HeLa cells. We found that EA stimulation activated the FasL, EA-induced ROS generation, phosphorylation of JNK, c-jun, p38-P and down-regulated ERK1/2. EA-induced ROS generation could be prevented by treatment with the allopurinol (an effective scavenger of ROS) and Cyclosporine A (CsA: Inhibitor of the mitochondrial permeability transition) and protected the cells from apoptosis. Bid and Bax levels are increased and Bcl-2 level is reduced. The protein levels of cytochrome c and cleavage of pro-caspase-8, -9, and -3 levels are increased in the EA-induce apoptotic cells. The JNK inhibitor SP600125 abrogated EA-induced caspase upregulation, and protected the cells from apoptosis. Two main pathways lead to caspase-dependent apoptosis (see figure). The extrinsic pathway involves stimulation of members of the tumour necrosis factor receptor (TNFR)

superfamily, such CD95/Fas, TNFR or TRAILR (death receptors). The intrinsic pathway is characterized by mitochondrial outer membrane permeabilization (MOMP) and the release of mitochondrial cytochrome c, which results in assembly of a caspase-activating complex between caspase-9 and APAF1 (the apoptosome). Death-receptor stimulation typically results in the recruitment and activation of caspase-8 by the Fas-associated via death domain (FADD)/TNFR1-associated death domain protein (TRADD) to form a death-inducing signalling complex (DISC) that can further propagate death signals in three ways: via proteolysis of the BCL2 homology-3 (BH3)-only protein BID, which provokes translocation of truncated BID to mitochondria and consequent MOMP; by direct proteolytic activation of downstream effector caspases; or via activation of the kinase RIP93.

Figure 1

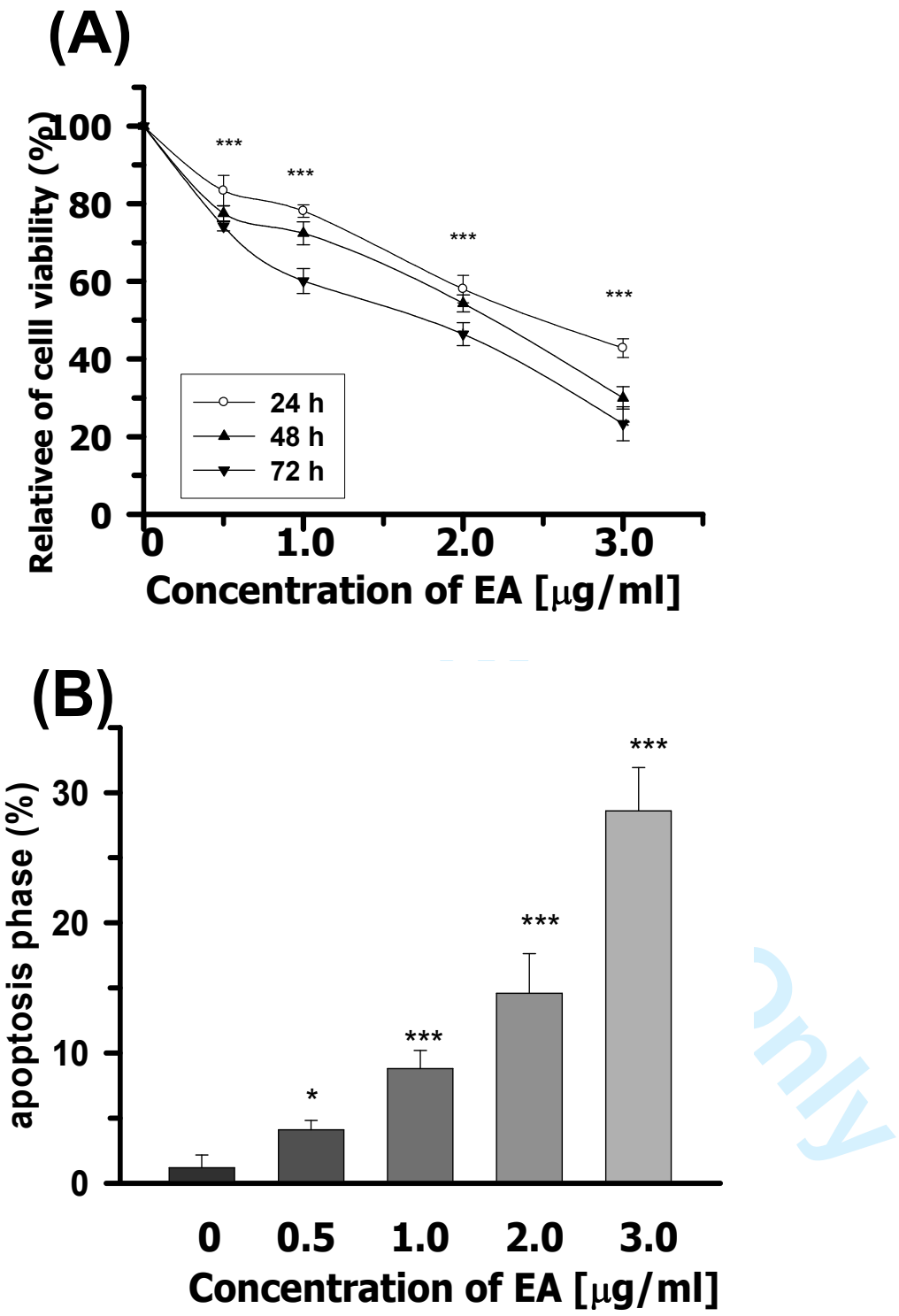

(A)
Relativee of cellI viability (%)
100
80
60
40
20
0
***
***
***
***
24 h
48 h
72 h
0
1.0
2.0
3.0
Concentration of EA [μg/ml]
(B)
apoptosis phase (%)
30
20
10
0
*
***
***
***
0
0.5
1.0
2.0
3.0
Concentration of EA [μg/ml]

Figure 2

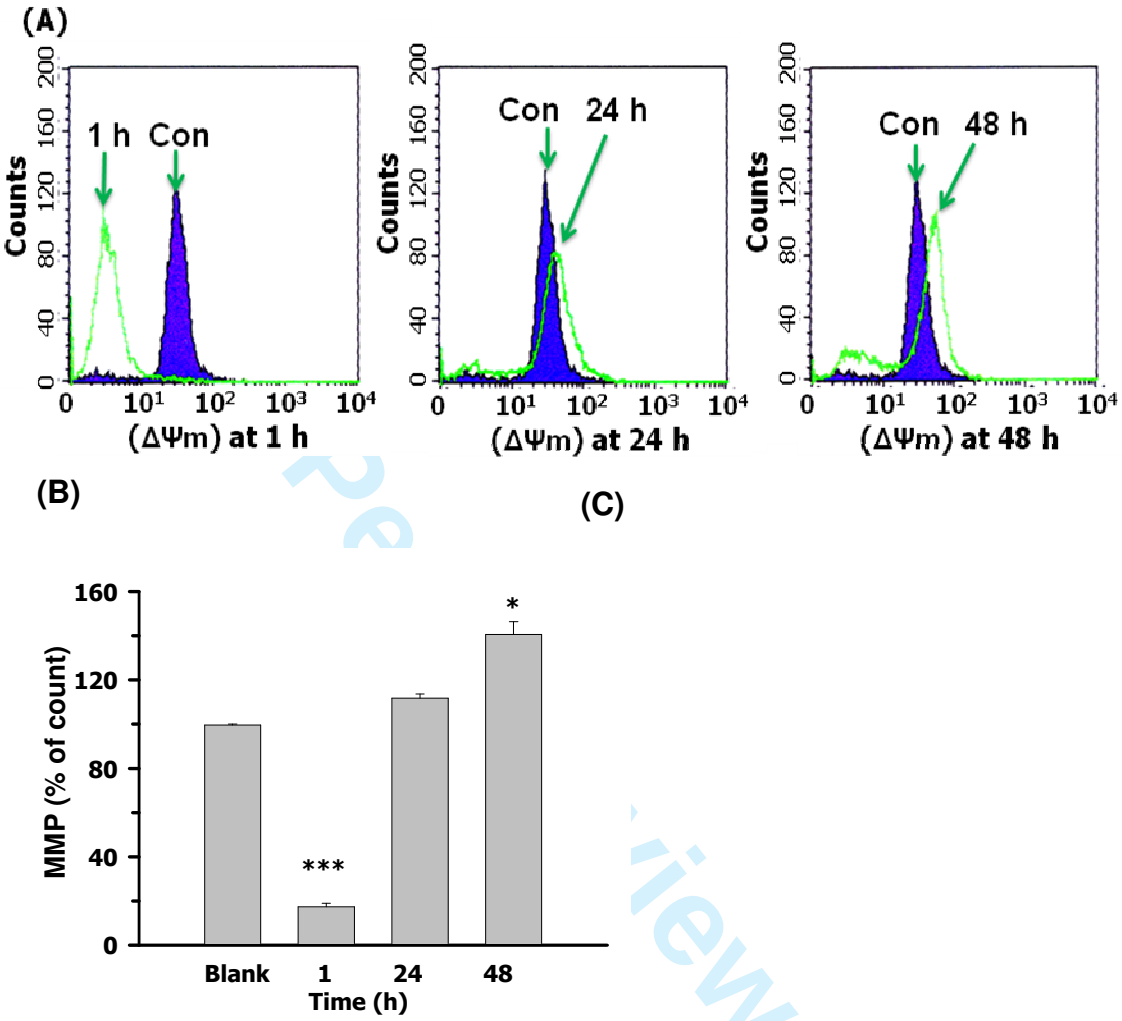


Figure 3

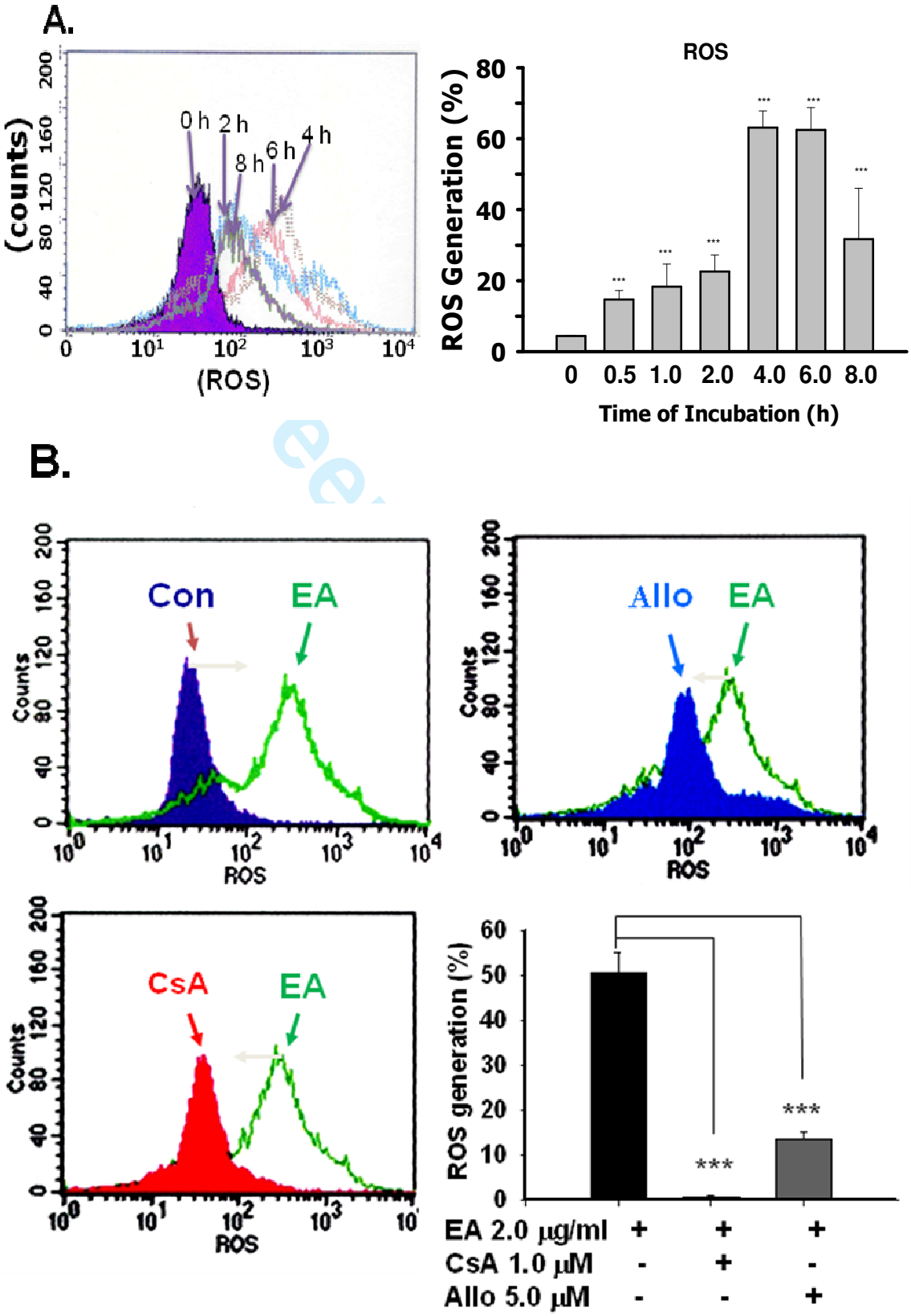
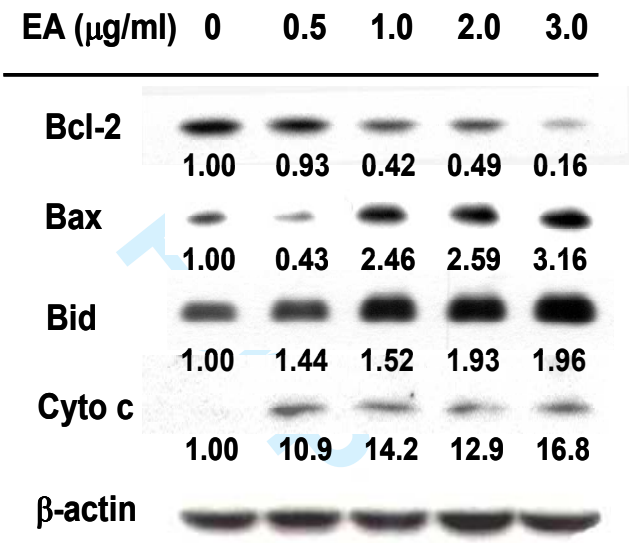
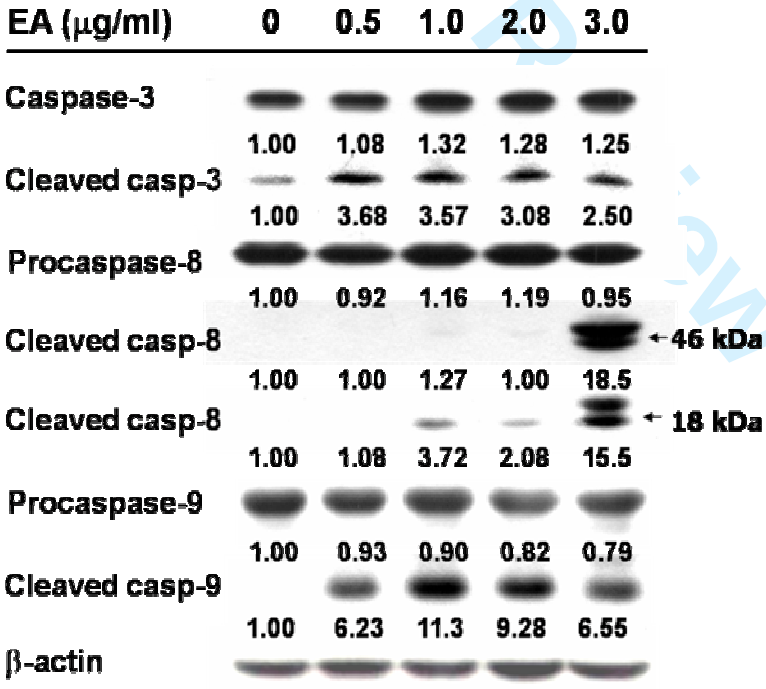

A.
(counts)
0 h
2 h
4 h
6 h
8 h
(ROS)
ROS
ROS Generation (%)
Time of Incubation (h)
0
0.5
1.0
2.0
4.0
6.0
8.0
B.
Con
EA
Allo
EA
CsA
EA
Counts
ROS
ROS generation (%)
EA 2.0 μg/ml + + +
CsA 1.0 μM - + -
Allo 5.0 μM - - +

Figure 4

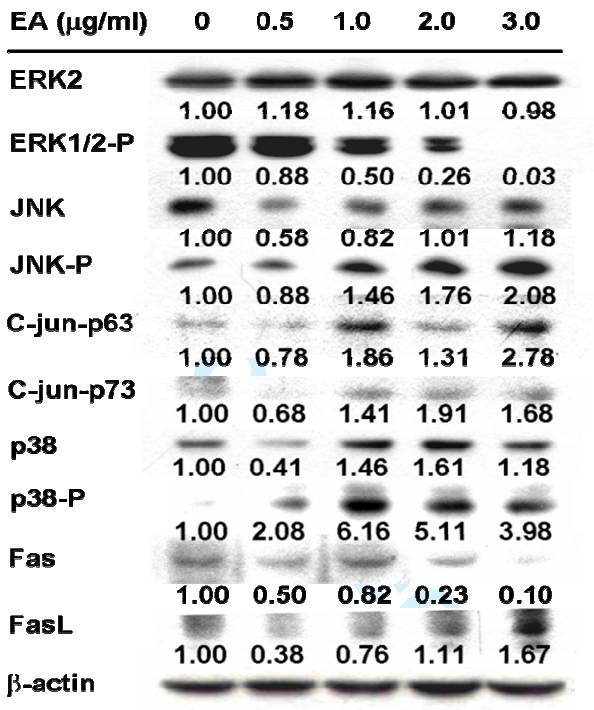
(A)



(B)



(C)


EA (μg/ml) 0 0.5 1.0 2.0 3.0
ERK2
1.00 1.18 1.16 1.01 0.98
ERK1/2-P
1.00 0.88 0.50 0.26 0.03
JNK
1.00 0.58 0.82 1.01 1.18
JNK-P
1.00 0.88 1.46 1.76 2.08
C-jun-p63
1.00 0.78 1.86 1.31 2.78
C-jun-p73
1.00 0.68 1.41 1.91 1.68
p38
1.00 0.41 1.46 1.61 1.18
p38-P
1.00 2.08 6.16 5.11 3.98
Fas
1.00 0.50 0.82 0.23 0.10
FasL
1.00 0.38 0.76 1.11 1.67
β-actin

(D)

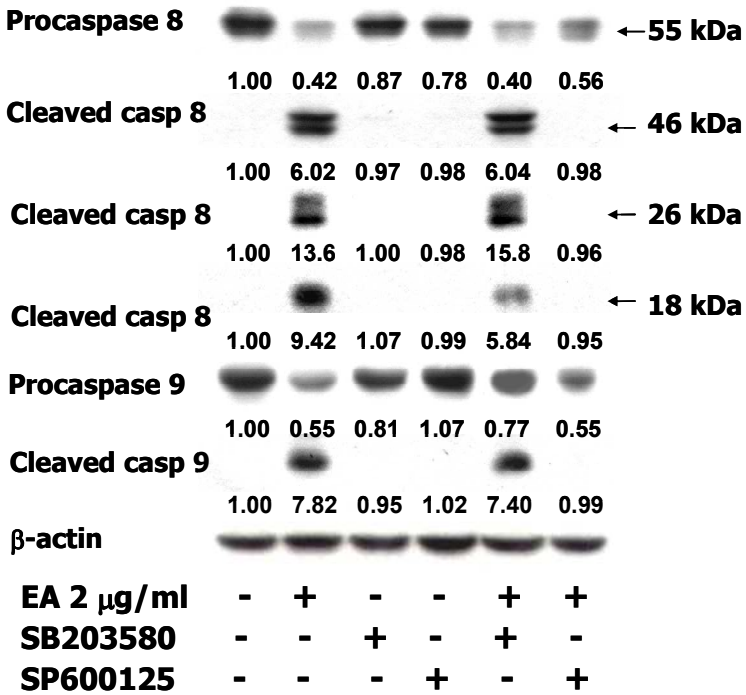

Procaspase 8
1.00 0.42 0.87 0.78 0.40 0.56
55 kDa
Cleaved casp 8
1.00 6.02 0.97 0.98 6.04 0.98
46 kDa
Cleaved casp 8
1.00 13.6 1.00 0.98 15.8 0.96
26 kDa
Cleaved casp 8
1.00 9.42 1.07 0.99 5.84 0.95
18 kDa
Procaspase 9
1.00 0.55 0.81 1.07 0.77 0.55
Cleaved casp 9
1.00 7.82 0.95 1.02 7.40 0.99
β-actin
EA 2 μg/ml - + - - + +
SB203580 - - + - + -
SP600125 - - - + - +

Figure 5