Safrole Induces Apoptosis in Human Oral Cancer HSC-3 Cells

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ABSTRACT

Phytochemicals have been used as potential chemopreventive or chemotherapeutic agents. However, there are data suggesting a mutagenic effect of some phytochemicals. We hypothesized that safrole would have anticancer effects on human oral squamous cell carcinoma HSC-3 cells. Safrole decreased the percentage of viable HSC-3 cells *via* induction of apoptosis by an increased level of cytosolic Ca^{2+} and a reduction in the mitochondrial membrane potential ($\Delta\Psi_m$). Changes in the membrane potential were associated with changes in the Bax, release of cytochrome *c* from mitochondria, and activation of downstream caspases-9 and -3, resulting in apoptotic cell death. *In vivo* studies also showed that safrole reduced the size and volume of an HSC-3 solid tumor on a xenograft athymic nu/nu mouse model. Western blotting and flow cytometric analysis studies confirmed that safrole-mediated apoptotic cell death of HSC-3 cells is regulated by cytosolic Ca^{2+} and by mitochondria- and Fas-dependent pathways.

KEY WORDS: safrole, oral cancer HSC-3 cells, apoptosis, caspase cascades, xenograft model.

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INTRODUCTION

Tobacco and alcohol consumption are major contributors to oral cancer (Franco *et al*., 1989; Schlecht *et al*., 1999). In addition, diets low in carotenoids and vitamin A, poor oral hygiene, and indoor air pollution are also reported to be associated with oral cancer (Franceschi *et al*., 1991; Pintos *et al*., 1998; Velly *et al*., 1998). In Taiwan, betel quid chewing is a significant factor associated with oral cancer. In Taiwan, 9.6 individuals *per* 100,000 died of oral cancer in 2008, and it is the fourth most frequent cause of cancer death among males in Taiwan (Department of Health) (Ho *et al*., 2009a). Conventional treatments for patients with oral cancer are surgery, radiotherapy, and chemotherapy (Ichimiya *et al*., 2005), but the cure rates are not satisfactory.

Betel quid consists of areca nut, slaked lime, and *Piper betle* inflorescence or leaf instead of tobacco (Chen *et al*., 1999). It has been reported that chewing betel quid with tobacco or combining it with cigarette smoking is recognized as a major risk factor for the development of oral squamous cell carcinoma (OSCC) (Ko *et al*., 1995). Chewing betel quid containing *Piper betle* inflorescence can generate a high concentration of safrole (420 μM) in the saliva (Wang and Hwang, 1993). The International Agency for Research on Cancer (IARC) categorized safrole as a group 2B carcinogen (safrole is a documented rodent carcinogen). There are, however, no adequate studies elucidating the relationship between exposure to safrole and human cancers (No Authors Listed, 1976). Carcinogenicity of safrole has typically been found to be caused by safrole-DNA adduct formation (Daimon *et al*., 1997, 1998; Chen *et al*., 1999; Liu *et al*., 2004).

In human oral cancer cells, safrole induced a $[Ca^{2+}]i$ rise by causing the release of stored Ca^{2+} from the endoplasmic reticulum in a phospholipase C- and protein kinase C-independent fashion and by inducing Ca^{2+} influx *via* nifedipine-sensitive Ca^{2+} entry (Huang *et al*., 2005). It has also been reported that safrole caused marked $[Ca^{2+}]i$ elevation and decreased cell viability in human osteosarcoma cells (Lin *et al*., 2006). Therefore, we investigated the facts surrounding safrole promoting cytosolic Ca^{2+} increase, and whether safrole induces the cell death of HSC-3 cells. Safrole can also bind to DNA, resulting in safrole-DNA adduct formation (Daimon *et al*., 1997, 1998; Lee *et al*., 2005), but there is no information on whether safrole can reduce cell viability and induce apoptosis in human oral cancer cells. HSC-3 cells, a human oral squamous cell carcinoma cell line, exhibit high metastatic and migratory potential (Kawahara *et al*., 1999; Shigeta *et al*., 2008). The aim of this study was to examine the hypothesis that safrole is able to trigger

apoptosis and to have anticancer responses in HSC-3 cells *in vitro* and *in vivo*. Therefore, we examined the apoptotic cells in HSC-3 cells after exposure to safrole, and the results indicated that safrole induced apoptosis through a mitochondria-dependent cell death pathway.

MATERIALS & METHODS

Cell Culture

The human oral squamous cell carcinoma HSC-3 cell line was provided by Professor Pei-Jung Lu (National Cheng Kung University). Cells were plated in 75 cm^2 tissue culture flasks at 37°C under a humidified 5% CO_2 and 95% air atmosphere in DMEM/F-12 (1:1) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, as described previously (Lin *et al*., 2007).

In vitro Studies

Morphological Changes and Cell Viability

Cells (2 x 10^5 *per* well) were placed in 12-well plates, and then safrole (Sigma Chemical Co., St. Louis, MO, USA) was added to each well at final concentrations of 10, 25, 50, 75, and 100 μM. DMSO, 0.2% in media, served as a vehicle control. Cells were incubated for 24 and 48 hrs. Cell viability was determined by propidium iodide (PI, Sigma Chemical Co.) exclusion and flow cytometric procedures as previously described (Lin *et al*., 2007). For morphological changes, cells in each well were examined for 24 and 48 hrs and photographed by phase-contrast microscopy (Chen *et al*., 2009; Tsou *et al*., 2009).

4′,6′-Diamidino-2-Phenylindole Hydrochloride (DAPI) Staining and Comet Assay

Cells (2 x 10^5 *per* well) in 12-well plates were treated with 0 and 75 μM of safrole for 24 hrs. Cells were then individually stained by DAPI (Molecular Probes/Invitrogen Corp., Eugene, OR, USA) and photographed by fluorescence microscopy as described elsewhere (Yang *et al*., 2009). The levels of DNA damage were determined by the Comet assay as previously described (Lu *et al*., 2009). Results were expressed and quantified with TriTek CometScore™ software (Tritek Corp., Sumerduck, VA, USA).

Flow Cytometric Assays for Apoptotic Cells, Level of $\Delta\Psi_m$, and Cytosolic Ca^{2+} Elevations

Cells (2 x 10^5 *per* well) were placed in 12-well plates, and then safrole was added to each well at final concentrations of 0, 25, 50, 75, and 100 μM for 24 hrs. The cells were stained with PI and were analyzed by flow cytometry. Formation of cells in the sub-G1 phase was indicative of apoptotic cells (Chen *et al*., 2009). We exposed cells to safrole at 75 μM for 0, 1, 3, 6, 12, or 24 hrs to determine the level of $\Delta\Psi_m$ and the cytosolic Ca^{2+}. Cells were harvested and suspended in 500 μL of $DiOC_6(3)$ (4 μmol/L) and Fluo-3/AM (2.5 μg/mL) (Molecular Probes) for the levels of $\Delta\Psi_m$ and cytosolic Ca^{2+}, respectively, in a dark room for 30 min at 37°C, and then were analyzed by flow cytometry as previously described (Kuo *et al*., 2009; SY Lin *et al*., 2009).

Assessments of Caspases-3, -8, and -9 Activity and Determination of Viability of HSC-3 Cells after Inhibitors of Caspases-3 and -8 Pre-treatment

Cells (2 x 10^5 *per* well) in 12-well plates were pre-treated with or without specific caspase inhibitors (Z-IETD-FMK for caspase-8 or Z-DEVD-FMK for caspase-3) and then were incubated with safrole at 75 μM for 0, 6, 12, 18, and 24 hrs for determination of the caspases-3, -8, and -9 activity or viability of HSC-3 cells, as described elsewhere (Ji *et al*., 2009). The cells were in a 10 μM substrate solution (PhiPhiLux®-G_1D_2, CaspaLux®8-L_1D_2, CaspaLux®9-M_1D_2 kits, OncoImmunin, Inc., Gaithersburg, MD, USA) and incubated at 37°C for 60 min. The cells were washed again and then analyzed by flow cytometry (SY Lin *et al*., 2009).

Western Blotting for Protein Level Analyses

Cells (1 x 10^6 *per* dish) were treated with 75 μM safrole and incubated for 0, 6, 12, and 24 hrs. Abundance of selective proteins associated with apoptosis was determined by Western blotting analysis. The levels of apoptotic relative proteins were determined in cell lysates with antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). To ensure equal protein loading, each membrane was stripped and re-probed with anti-β-actin antibody (ML Lin *et al*., 2009).

Assay for mRNA Levels of Caspases-3, -8, and -9 and Endo G

Cells (1 x 10^6 *per* dish) were incubated with 75 μM safrole for 0 and 24 hrs, and mRNAs of caspases-3, -8, and -9 and Endo G were determined. Total RNA was extracted, RNA samples were reverse-transcribed, and then quantitative PCR was performed with the forward and reverse primers (Appendix Table 1) as described elsewhere (Ho *et al*., 2009a; Ji *et al*., 2009).

In vivo Studies: Mouse Xenograft Model

Twenty six-week-old male BALB/c athymic nude mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were housed in a regular 12-hour light/12-hour dark cycle. Water and food were autoclaved and provided *ad libitum*. HSC-3 cells (1 x 10^7/100 μL/mouse) were subcutaneously (*s.c.*) injected into the flanks of the mice. Animals bearing tumors were randomly assigned to a control or safrole treatment group (10 mice *per* group). Treatment was initiated when xenografts reached a volume of about 200 mm^3, and mice were then injected *i.p.* every 4 days (in the morning) with 30 μL of control vehicle (DMSO) or safrole (15 mg/kg body weight). When mice started to exhibit tumors, they were then monitored and counted, and tumor sizes were measured initially after 2 wks, with the final measurement taken 5 wks after tumor inoculation. At the end of the study (5 wks after cell inoculation), animals were sacrificed. For each animal, tumors were removed, measured, and weighed (Yang *et al*., 2008; Ho *et al*., 2009b). All animal studies were conducted according to institutional guidelines (Affidavit of Approval of Animal Use Protocol) approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan).

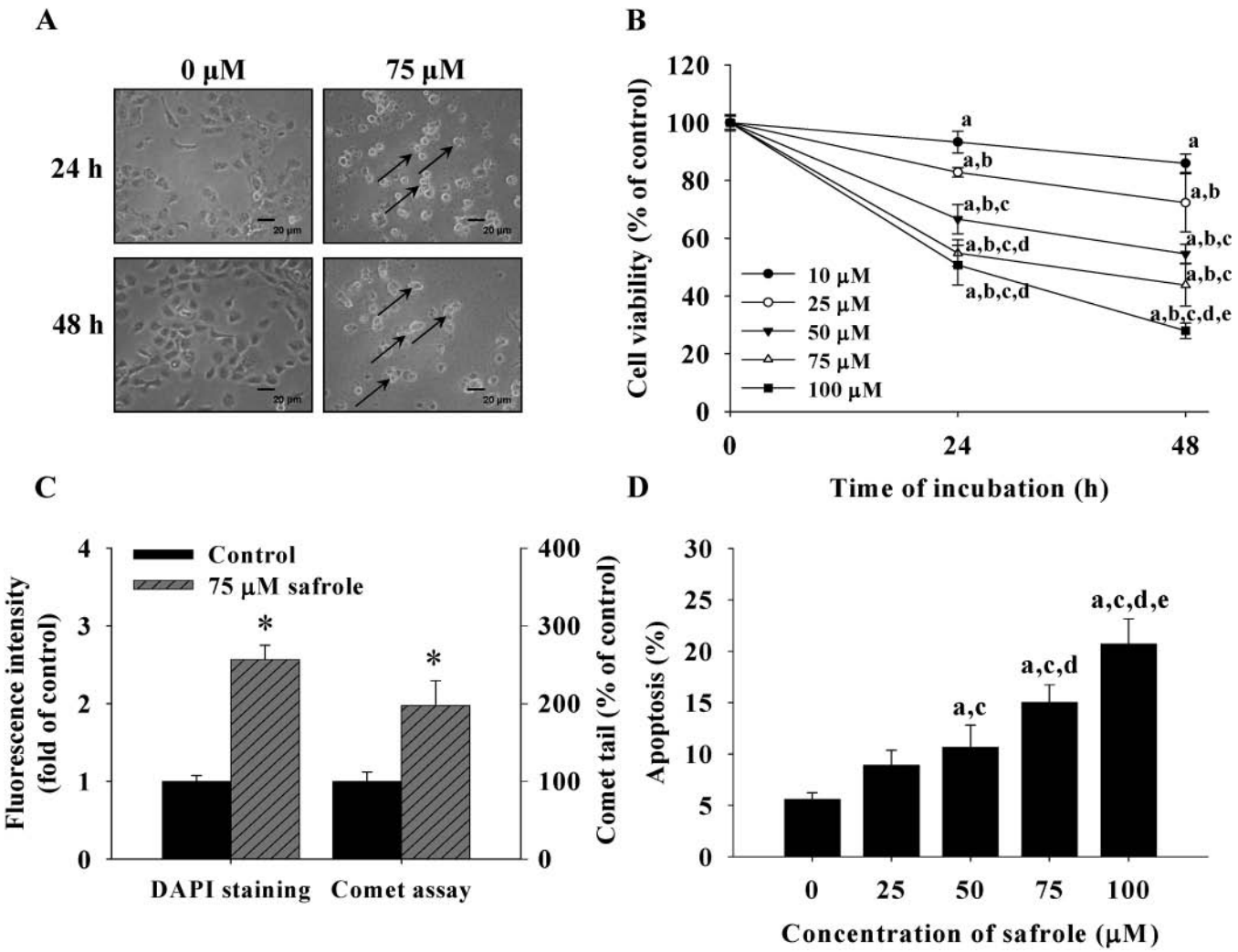

Figure 1. The effects of safrole on cell morphology, percentage of viable cells, apoptotic cells, and DNA damage in HSC-3 cells. Cells were cultured in DMEM/F-12 (1:1) + 10% FBS with various concentrations of safrole for 24 or 48 hrs. Cells were examined and photographed by phase-contrast microscopy for morphological changes. Scale bar, 20 μm **(A)**, percentage of viable cells **(B)**, quantification of fluorescence intensity and comet tail from DAPI staining and DNA damage, respectively **(C)**, and apoptosis **(D)** were determined as described in MATERIALS & METHODS. Each point is the mean ± SD of 3 experiments. a, $p < 0.05$, significantly different compared with DMSO-treated control; b, c, d, and e, $p < 0.05$, significantly different compared with 10, 25, 50, and 75 μM of safrole treatment, respectively, by one-way ANOVA followed by Bonferroni's multiple comparison test. *$p < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA.

Statistical Analyses

Differences between treatment groups were calculated by one-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. Data are presented as mean ± standard deviation (SD), and the results are representative of at least 2 or 3 independent experiments. A p value of < 0.05 was considered significant.

RESULTS

Safrole Induces Morphological Changes, Viability, Apoptosis, and DNA Damage

Safrole induced morphological changes (Fig. 1A) and decreased the percentage of viable cells in a dose- and time-dependent manner, with an IC_{50} value of about 75 μM after 48-hour treatment (Fig. 1B). Compared with DMSO-treated controls, each safrole treatment resulted in the formation of sub-G1 in HSC-3 cells. Analysis of the data showed that safrole induced apoptosis (sub-G1 phase) in a dose-dependent manner (Fig. 1D). DAPI staining assay confirmed chromatin condensation (an apoptotic characteristic) occurring in HSC-3 cells (Fig. 1C, Appendix Fig. 1A). Comet assay also demonstrated that safrole induced DNA damage in HSC-3 cells (Fig. 1C, Appendix Fig. 1B).

Safrole Alters $\Delta\Psi_m$ and Cytosolic Ca^{2+} Levels

The results showed that safrole significantly decreased the levels of $\Delta\Psi_m$ in HSC-3 cells in a time-dependent manner

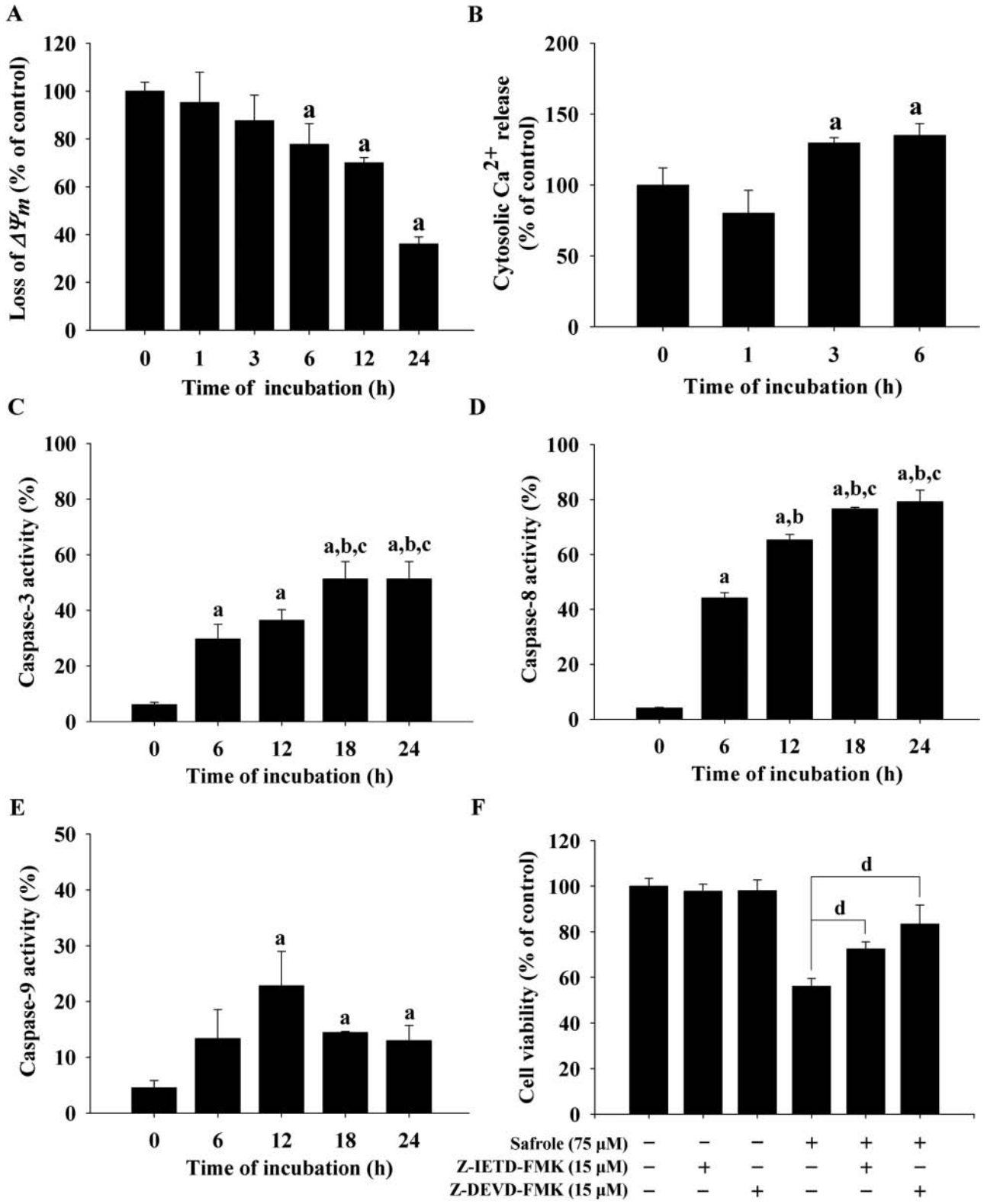

Figure 2. Safrole alters the levels of mitochondrial membrane potential ($\Delta\Psi_m$) and cytosolic Ca^{2+} and stimulates caspase cascade activity in HSC-3 cells. Cells were treated with 75 μM safrole for indicated time intervals, and the levels of $\Delta\Psi_m$ **(A)** and cytosolic Ca^{2+} release **(B)** were determined and calculated. Cells were pre-treated with inhibitors (Z-IETD-FMK for caspase-8 or Z-DEVD-FMK for caspase-3), and then were treated with 75 μM safrole for 0, 6, 12, 18, and 24 hrs. The cells were determined for caspases-3 **(C)**, -8 **(D)**, and -9 **(E)** activity and the percentage of viability **(F)**, as described in MATERIALS & METHODS. Columns, mean (n = 3); bars, SD. a, $p < 0.05$, significantly different compared with DMSO-treated control; b, c, and d, $p < 0.05$, significantly different compared with 6-, 12-, and 24-hour treatment of 75 μM safrole, respectively, by one-way ANOVA followed by Bonferroni's multiple comparison test.

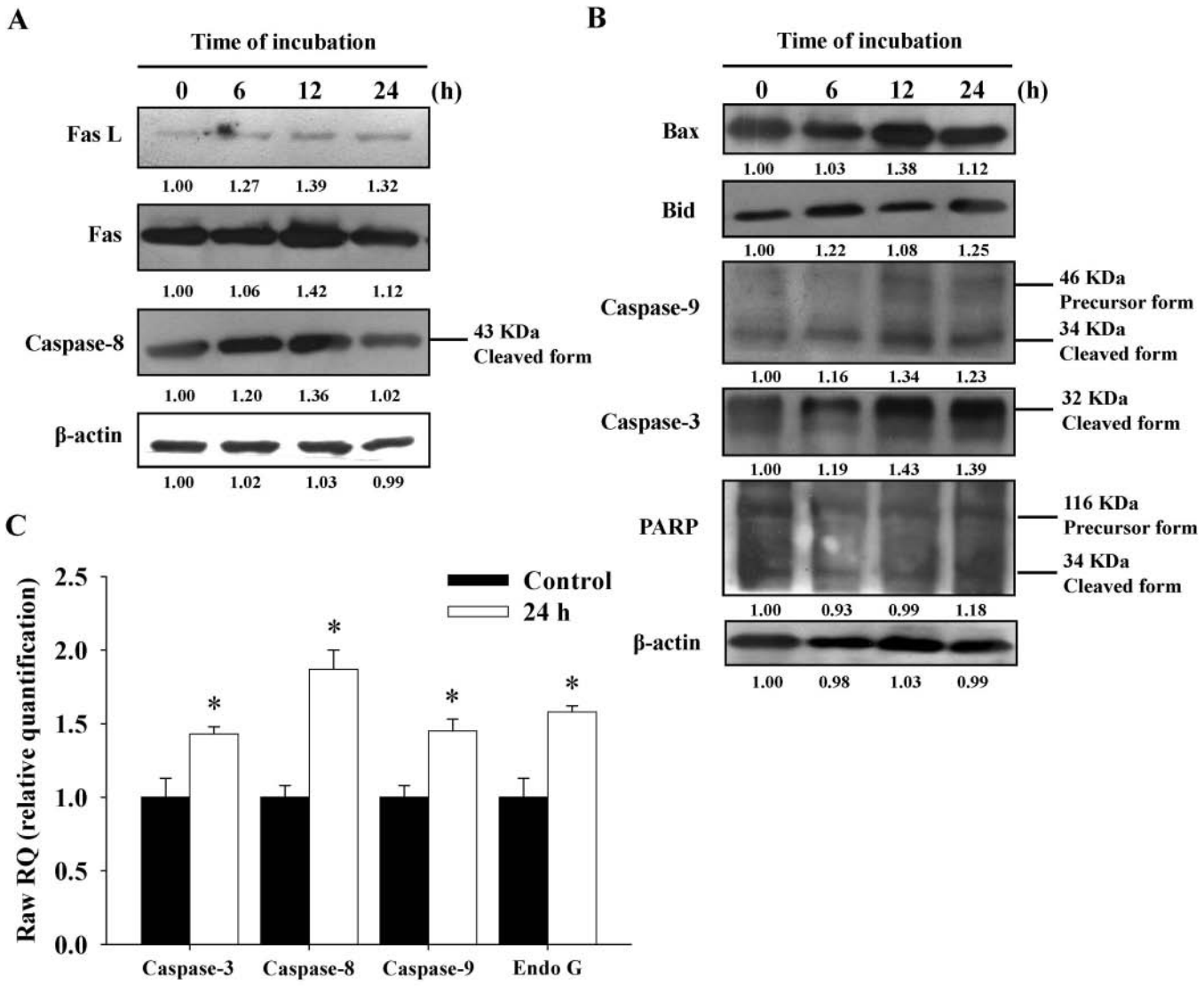

Figure 3. Representative Western blotting and real-time PCR analyses show changes in the levels of associated proteins and genes in the apoptotic HSC-3 cells after exposure to safrole. Cells were treated with 75 μM safrole for 0, 6, 12, or 24 hrs before the total proteins and mRNA were prepared and determined as described in MATERIALS & METHODS. The levels of apoptotic relative proteins (**A**, Fas L, Fas, and caspase-8; **B**, Bax, Bid, caspase-9, caspase-3, PARP) were estimated by Western blotting analysis. For real-time PCR assay, cells after exposure to 75 μM safrole for 24 hrs were isolated, and the gene expressions of mRNA *caspases-3, -8,* and *-9* and *Endo G* (**C**) were then determined and performed as described in MATERIALS & METHODS. $*p < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA.

(Fig. 2A). Safrole also significantly increased cytosolic Ca^{2+} levels (Fig. 2B).

Safrole Stimulates the Activity of Caspases-3, -8, and -9

Safrole significantly increased the activity of caspases-3, -8, and -9 (Figs. 2C, 2D, 2E). These effects were time-dependent with the exception of caspase-9, which, after an 18-hour incubation, showed a reduction in activity compared with the 12-hour treatment (Fig. 2E). HSC-3 cells were pre-treated with specific inhibitors (Z-IETD-FMK for caspase-8 or Z-DEVD-FMK for caspase-3), and then exposure to safrole led to promotion of the percentage of viable cells as compared with safrole-treatment-only cells (Fig. 2F).

Safrole Affects Abundance of Apoptotic-associated Proteins, Protein Translocation, and mRNA Expression

Results showed that safrole increased levels of pro-apoptotic proteins such as Bax and Bid, and the active forms of caspases-9 and -3 and PARP (Fig. 3B) were promoted. The levels of Fas ligand (Fas L) and Fas and caspase-8 proteins (Fig. 3A) were also up-regulated, but much earlier as compared with levels of the aforementioned proteins. Expression levels of mRNA of caspases-3, -8, and -9 and Endo G were increased (Fig. 3C). The results from confocal laser microscopy (Appendix Fig. 2) also indicated that safrole promoted the release of cytochrome *c* and Endo G from the mitochondria to the cytosol and nuclei, respectively.

Safrole Inhibits Tumor Size of HSC-3 Cells in a Mouse Xenograft Model

Safrole significantly decreased the tumor weight (Figs. 4A, 4B, 4C) and the percentage of inhibition of tumors (Appendix Table 2) as compared with control. Safrole induced a 40% inhibition of tumors compared with control treatment. Comparison of tumor volume between the control and safrole treatment groups showed that 15 mg/kg safrole significantly reduced the occurrence of tumors (Fig. 4D). Tumors in the treatment groups were significantly smaller than those in the control group. The final tumor sizes of mice treated with 15 mg/kg safrole were smaller than the tumor sizes of control mice after cell inoculation (treated for 20 days) with safrole.

DISCUSSION

Several studies have shown that safrole is a carcinogen and that it can induce carcinogenesis in animals (Daimon *et al*., 1997, 1998; Chen *et al*., 1999; Liu *et al*., 2004). However, there is no report to show that safrole induced apoptosis in any human cancer cell lines. Therefore, the purpose of the present study was to examine the effects of safrole in human oral cancer cells *in vitro* and its effects on solid tumors in a mouse xenograft model. We found that safrole can induce apoptosis and reduce viability in human oral squamous cell carcinoma HSC-3 cells, and it also can inhibit the size of oral cancer tumors in a mouse xenograft model *in vivo*. Safrole also promoted the activity of caspases-3, -8, and -9. It is well-known that the activation of these caspases is a major mechanism which promotes apoptosis in response to death-inducing signals from cell-surface receptors and mitochondrial stress (Eeva *et al*., 2009). HSC-3 cells were pre-treated with the inhibitors of caspases-8 and -3 (Z-IETD-FMK and Z-DEVD-FMK, respectively), and then were exposed to safrole. Results showed that the activities of caspases-8 and -3 were inhibited and the percentage of viable HSC-3 cells was increased. Apparently, safrole-induced apoptosis is involved in a caspase-dependent pathway. We also observed that safrole promoted cytosolic Ca^{2+} release and decreased the levels of $\Delta\Psi_m$. The importance of mitochondrial changes induced by safrole is that the mitochondria act as a nodal point for the execution of apoptosis and the permeability transition pore-opening, and collapse of the $\Delta\Psi_m$ leads to a rapid release of cytochrome *c* into the cytoplasm and activation of caspase-3 *via* caspase-9, culminating in cell death (Zou *et al*., 1999; Robertson and Orrenius, 2000). To examine whether safrole-inhibited apoptotic-associated protein levels also affected gene expression of mRNA, we isolated cells after exposure to safrole and then examined the levels of total RNA by real-time PCR. Results indicated that safrole also activated mRNA expression of caspases-3, -8, and -9 and Endo G in examined HSC-3 cells.

There is no information to show that safrole inhibits HSC-3 tumors in a mouse xenograft model. Therefore, for the *in vivo* effect of safrole on HSC-3 cells, a mouse xenograft model was used. Here, in the present study, we provide the first *in vivo* evidence to show that safrole decreased the size and weight of HSC-3 tumors in a mouse xenograft model. A novel finding of the present study was that safrole inhibited tumor growth in the model. Tumors in mice that received safrole alone were about 40% smaller than those of the control group, and tumors that received safrole treatment continued to grow slowly. Importantly, the growth-inhibitory effect of safrole on HSC-3 xenografts

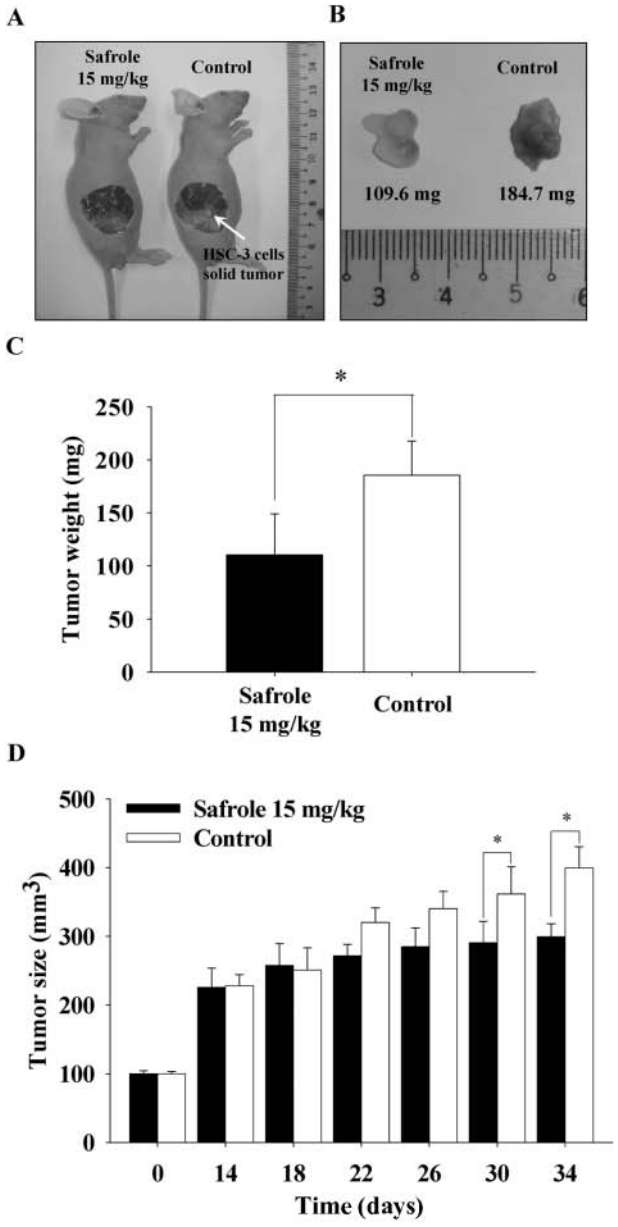

Figure 4. Representative tumor on the xenograft animal model and anti-tumor activity of safrole against HSC-3 oral squamous cell carcinoma tumors. Twenty nude mice were *s.c.* implanted with HSC-3 cells (1×10^7 *per* mice) for 14 days, and then randomly divided into 2 groups. Group 1 was treated with DMSO only. Group 2 was treated with 15 mg/kg safrole, and then, on the 34th day, all animals were killed. During the treatment, each animal was measured for tumor size and weight as described in MATERIALS & METHODS. **(A)** Representative animal with tumor; **(B)** representative solid tumor weight; **(C)** quantification of tumor weight. The tumor sizes observed in DMSO-treated control and safrole groups **(D)** were compared as analyzed by one-way ANOVA. A *p* value of < 0.05 was considered statistically significant.

in vivo was consistent with the results obtained *in vitro*. The *in vitro* and *in vivo* findings in the present study provide initial support for further investigation into the use of safrole in the treatment of oral cancer. In further study, we will use a chemical carcinogen to generate oral cancer in C57BL/6JNarl mice and then will treat it with safrole by oral administration for measurement of its efficiency *in vivo* (Chang *et al*., 2010).

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