Aloe-emodin Induces Cell Death through S-Phase Arrest and Caspase-dependent Pathways in Human Tongue Squamous Cancer SCC-4 Cells

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Abstract. Aloe-emodin, one of the anthraquinones, has been shown to have anticancer activity in different kinds of human cancer cell lines. Therefore, the purpose of this study was to investigate the anti-cancer effect of aloe-emodin on human tongue squamous carcinoma SCC-4 cells. The results indicated that aloe-emodin induced cell death through S-phase arrest and apoptosis in a dose- and time-dependent manner. Treatment with 30 μM of aloe-emodin led to S-phase arrest through promoted p53, p21 and p27, but inhibited cyclin A, E, thymidylate synthase and Cdc25A levels. Aloe-emodin promoted the release of apoptosis-inducing factor (AIF), endonuclease G (Endo G), pro-caspase-9 and cytochrome c from the mitochondria via a loss of the mitochondrial membrane potential (ΔΨm) which was associated with a increase in the ratio of B-cell lymphoma 2-associated X protein (Bax)/B cell lymphoma/leukemia-2 (Bcl-2) and activation of caspase-9 and -3. The free radical scavenger N-acetylcysteine (NAC) and caspase inhibitors markedly blocked aloe-emodin-induced apoptosis. Aloe-emodin thus induced apoptosis in the SCC-4 cells through the Fas/death-receptor, mitochondria and caspase cascade. Aloe-emodin could be a novel chemotherapeutic drug candidate for the treatment of human tongue squamous cancer in the future.

Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone) is a natural active compound found in the leaves of Aloe vera (1). Aloe-emodin has antiviral, antimicrobial and hepatoprotective activities (2) and anticancer activity in neuroectodermal tumors (3), lung squamous cell carcinoma (4), hepatoma cells (5) and in a glia cell line (6) and a human glioma cell line (7). It has been reported that aloe-emodin suppressed N-methyl-D-aspartate (NMDA)-induced apoptosis of retinal ganglion cells through regulation of extracellular signal-regulated kinase (ERK) phosphorylation (8) and aloe-emodin-induced apoptosis in rat hepatic stellate cells transformed by simian virus 40 (t-HSC/CI-6) involved a mitochondria-mediated pathway (9). Recently, it has been reported that aloe-emodin suppressed N-methyl-D-aspartate (NMDA)-induced apoptosis of retinal ganglion cells through regulation of extracellular signal-regulated kinase (ERK) phosphorylation (8) and aloe-emodin-induced apoptosis in rat hepatic stellate cells transformed by simian virus 40 (t-HSC/CI-6) involved a mitochondria-mediated pathway (9). Recently, it has been reported that aloe-emodin-induced apoptotic cell death was mediated via oxidative stress and sustained jun N-terminal kinase (JNK) activation (10) and aloe-emodin-induced apoptosis in human gastric carcinoma cells by a reduced phosphorylation of BH3 interacting domain death agonist (Bid), a downstream substrate of casein kinase II and a pro-apoptotic molecule (11). However, the effect of aloe-emodin on human tongue cancer cells has not been studied.

Cell death can be categorized as necrosis or apoptosis with apoptosis being the best focus for anticancer agents.

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Apoptosis is an actively regulated process of cell death and occurs via an extrinsic or intrinsic pathway and the intrinsic pathway involves the mitochondria (12). Members of the caspase family that are produced and activated during apoptosis hasten the cell death process involving the caspase-dependent apoptotic pathway (13). After cell death stimulation, the mitochondrial outer membrane is permeabilized under the regulation of Bax and Bcl-2 allowing the release of cytochrome c from the mitochondria to the cytoplasm where it initiates apoptosis (13). Another protein named apoptosis-inducing factor (AIF), which is released from the mitochondria into the cytosol and nucleus then also induces apoptosis (14).

Here, the human tongue squamous SCC-4 cell line was used to evaluate the anticancer effect of aloe-emodin.

Materials and Methods

Drugs and reagents. Aloe-emodin, dimethyl sulfoxide (DMSO), N-acetylcysteine (NAC) and ribonuclease A (RNase A) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). The aloe-emodin was dissolved in 1% DMSO to a concentration of 10.0 mM and stored at –20˚C until used. Caspase-3, -8 and -9 activity assay kits were purchased from OncolImmun, Inc. (Gaithersburg, MD, USA). The caspase-3 inhibitor (z-DEVD-fmk), caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were obtained from R&D Systems, Inc (Minneapolis, MN, USA).

Cell culture. The human tongue squamous carcinoma cell line (SCC-4), used in all the experiments, was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured at 37˚C under a humidified 5% CO2 and L-glutamine as described elsewhere (15).

Assessment of cell morphology and viability. SCC-4 cells at a density of 2x10^5 cells/well were plated onto 12-well plates and incubated at 37˚C for 24 h. Different concentrations of aloe-emodin (0, 10, 20, 30, 40 or 50 μM) were added and the cells were incubated for 48 h. DMSO (vehicle) was used as the control. For morphological assessment, the cells were examined under a phase-contrast microscope and were photographed (16). For cell viability, a flow cytometric protocol was used, as previously described (16).

Flow cytometry analysis of the cell cycle. SCC-4 cells at a density of 2x10^5 cells/well were plated onto 12-well plates and incubated with different concentrations of aloe-emodin (0, 10, 20, 30, 40 or 50 μM) for 48 h. The cells were then harvested by centrifugation and the percentage of cells in the sub-G1 (apoptosis), G0/G1-, S- and G2/M-phases were determined by flow cytometry, as previously described (17).

DAPI staining. DAPI staining was performed as previously described (18). DAPI-positive nuclei were visualized and photographed using an Olympus fluorescence microscope (Olympus, Tokyo, Japan) (17).

Detection of reactive oxygen species (ROS), Ca^{2+} levels and mitochondrial membrane potential (ΔΨm). Cells were plated onto 12-well plates and treated with 30 μM of aloe-emodin for 0, 6, 12, 24, 48 or 72 h. The cells were then harvested, washed twice, and re-suspended in the ROS indicator 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA), calcium probe 1H-indole-6-carboxylic acid, 2-[4-[(acetyloxy)methoxy]-2-oxoethyl]amino]-3-2-[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxyl]-ethoxy)phennyl]-, (acetyloxy)methyl ester (Indo 1/AM) or the ΔΨm indicator 3,3'-dihexyloxycarbocyanine iodide (DiOC6) and incubated at 37˚C for 30 min to flow cytometry was used to detect changes in ROS, Ca^{2+} levels and ΔΨm as previously described (15, 17). The SCC-4 cells were also treated with 30 μM aloe-emodin in the presence or absence of the ROS inhibitor NAC (1 mM) as previously described (15).

Western blotting (total protein preparation and immunoblotting). Cells were treated with 30 μM aloe-emodin for 0, 12, 24, 48 or 72 h. The levels of the following proteins were determined: cell cycle proteins (p53, p21 and p27, Cyclin A, E, thymidylate synthase, Cdk2 and Cdc25A) and apoptosis (Fas, FasL, caspase-8, caspase-9, caspase-3, Bid, cytochrome c, AIF, Poly (ADP-ribose) polymerase (PARP), Bax, Bcl-2, Activating transcription factor 6α (ATF-6α) and glucose-regulated protein 78 (GRP78)). The total proteins were extracted with a protein extraction reagent (Pierce Biotechnology, Inc. Rockford, IL, USA), according to the instructions of the manufacturer. All the samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (17, 18).

Immunostaining assay. SCC-4 cells were cultured onto 12-well plates for 24 h, 30 μM of aloe-emodin was added, or only DMSO (solvent) for the control regimen, and the cells were grown at 37˚C in a humidified 5% CO2 for 24 h. The cells were fixed and stained with primary antibodies to Endo G (Monoclonal Antibody Assay; Alexis, San Diego, CA, USA). The cells were washed twice with PBS. The supernatant was removed and 50 μl 1% FITC-conjugated goat anti-mouse IgG antibody (secondary antibody) was added for 30 min in the dark, following washing with PBS. The cells were examined under a confocal laser scanning fluorescence microscopy (17).

Statistical analysis. The unpaired one-way ANOVA was used to identify means that were significantly different between the control and aloe-emodin treatments, p<0.05 was regarded as significant.

Results

Effects of aloe-emodin on morphology and cell viability. The control cells had a well spread pentagonal shape under a phase-contrast microscopy (Figure 1A). Exposure of the SCC-4 cells to aloe-emodin resulted in a dose-dependent...
effect and decrease in cell viability compared to the control cells (Figure 1B). Aloe-emodin-induced 50% cell death at a concentration of 30 μM as shown in Figure 1B and this concentration was used in subsequent experiments.

**Effects of aloe-emodin on cell cycle arrest and apoptosis.** To investigate whether the aloe-emodin-mediated cell death was due to cell cycle arrest and an apoptotic mechanism, the nuclear morphological changes that occurred during aloe-emodin treatment were examined. The treatment with 30 μM aloe-emodin for 48 h resulted in S-phase arrest and sub-G1 phase production as determined by flow cytometric analysis (Figure 2A and B) and changes in the nuclear morphology as evidenced by DAPI staining (Figure 3).

**Effects of aloe-emodin on ROS and Ca2+ levels and the mitochondria membrane potential (ΔΨm).** Aloe-emodin induced ROS production quite early and time-dependently (Figure 4A) up to 12 h of treatment; the ROS levels remained high when compared to the control. Aloe-emodin increased the Ca2+ levels, which was time-dependent (Figure 4B) up to 48 h of treatment. Then mitochondrial membrane potential was reduced by aloe-emodin in a time-dependent manner (Figure 4C). As shown in Figure 4D, incubation with the free radical scavenger NAC significantly blocked aloe-emodin-triggered apoptosis in the SCC-4 cells.

**Effects of aloe-emodin on caspase-3, -8 and -9 activity.** The results shown in Figure 5A, B and C demonstrated that aloe-emodin increased the activity of caspase-3, caspase-8 and caspase-9 and these effects were time-dependent. To determine if caspase activation was required for the induction of apoptosis by aloe-emodin, the SCC-4 cells were pre-treated with inhibitors of caspase-3, caspase-8 or caspase-9 and aloe-emodin. As shown in Figure 5D, the caspase inhibitors significantly blocked the aloe-emodin-triggered apoptosis.

**Effects of aloe-emodin on cell cycle and apoptosis associated protein levels.** Aloe-emodin increased p53, p21 and p27, but inhibited cyclin A, E, thymidylate synthase, Cdk2 and Cdc25A (Figure 6A). Aloe-emodin treatment also increased the levels of Fas, FasL, caspase-3, -8 and -9, Bid, cytochrome c and AIF, but reduced the levels of PARP and Bcl-2 which was associated with an increase in apoptosis (Figure 6B and C). Figure 6D also shows that aloe-emodin treatment promoted ATF-6α and GRP78 levels which may be indicative that aloe-emodin induced apoptosis involved ER stress and the mitochondria.

**Effects of aloe-emodin on Endo G release from mitochondria.** As shown in Figure 7, the aloe-emodin-treated SCC-4 cells reacted with the Endo G antibodies and PI staining showed that aloe-emodin treatment for 48 h increased the levels of Endo G; which was released from mitochondria and translocated/moved to the nuclei.

**Discussion**

In the present study, aloe-emodin had anticancer effects on the SCC-4 human tongue squamous carcinoma cells and the cytotoxic mechanism involved the induction of apoptosis. The aloe-emodin-induced apoptosis occurred by the release
Figure 2. Aloe-emodin effects on the cell cycle and sub-G1 (apoptosis) population. A) Representative cytometric profiles and B) percentage of each phase. Data represents mean±S.D. of three experiments. *p<0.05.
Figure 3. Aloe-emodin-induced apoptosis and DNA damage in SCC-4 cells examined by DAPI staining, and photographed by fluorescence microscopy (×200) as described.

Figure 4. Effects of aloe-emodin on the production of reactive oxygen species (ROS) (A), Ca^{2+} (B), the mitochondria membrane potential (ΔΨ_m) (C) and ROS inhibitor (N-acetylcysteine; NAC) on aloe-emodin-induced apoptosis (D). Control cells set at 100%. Data represent mean±S.D. of three experiments. *p<0.05, **p<0.01, ***p<0.001.
of AIF and mitochondrial dysfunction and the release of cytochrome c, activation of caspase-9 and -3 and also reduced the ratio of Bax/Bcl-2 (i.e. increase of the Bax and decrease of Bcl-2 levels).

It was previously reported that apoptosis induced by aloe-emodin was associated with changes in the expression of Bcl-2 family members, apoptosis regulators, and that aloe-emodin caused cytochrome c release from mitochondria in a human lung squamous carcinoma cell line CH27 (19). Those findings were similar to the present results which also showed that aloe-emodin promoted the levels of p53 and p21 in the human tongue squamous carcinoma SCC-4 cells. This was in agreement with other reports showing that aloe-emodin induced apoptosis in the human hepatocellular carcinoma cell lines, HepG2 and Hep3B, accompanied by the induction of p53 and p21 expression (5). Additionally, p53-mutant cell lines have been shown to be less sensitive to aloe-emodin than p53 wild-type cell lines (12).

The present results demonstrated that aloe-emodin-induced apoptosis in the SCC-4 cells involved the Fas receptor, mitochondria and caspase cascade. Aloe-emodin also caused the release of AIF and cytochrome c from the mitochondria, followed by the activation of caspase-3. The present results also showed that aloe-emodin promoted the activation of caspase-8, connecting with the...
Fas (CD95) receptor by promoted Fas and FasL levels. It was reported that aloe-emodin can act through Fas to induce apoptosis in cancer cells (20). It is well known that cytochrome c mediates the allosteric activation of apoptotic protease activating factor-1 (Apaf-1) which is required for the proteolytic maturation of caspase-9 and caspase-3 (13, 21). Aloe-emodin also affects casein kinase II activity (11), and in the present study, aloe-emodin was also found to affect casein kinase levels (data not shown). Further studies are needed for more detailed understanding of the relationship between casein kinase II activity and caspase-3 activity.

Remarkably, aloe-emodin did not cause any detectable acute or chronic toxic effects in various normal cell lines and in animal model systems (3). A selective uptake of aloe-emodin by neuroectodermal tumor cells, but not by other tumor cells tested, was observed (3). Nevertheless, a detailed investigation of the effects of aloe-emodin on normal healthy cells in a human body still needs to be performed. In addition, in order to use aloe-emodin as a chemotherapeutic agent to ameliorate specific types of human carcinoma, it might be a challenge to engineer methods that allow the specific and efficient delivery of aloe-emodin to a given tumor in a human body.

In summary, aloe-emodin, isolated from Aloe vera leaves, was demonstrated for the first time to exhibit an anticancer effect against human tongue squamous carcinoma cells in vitro. The proposed molecular mechanism and pathway of aloe-emodin-induced apoptosis in SCC-4 cells is outlined in Figure 8. Aloe-emodin induces ROS and Ca$^{2+}$ production,
ER stress, dysfunction of mitochondria, cytochrome c release, caspase-9 and -3 activation and it also induces AIF release, finally leading to apoptosis. Aloe-emodin may be a chemotherapeutic drug candidate for the treatment of tongue squamous cancer in the future.

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