Emodin was isolated from Rheum palmatum L. and exhibits an anticancer effect on human cancer cell lines, however, the molecular mechanisms of emodin-mediated apoptosis in human tongue cancer cells have not been fully investigated. In this study, treatment of human tongue cancer SCC-4 cells with various concentrations of emodin led to G2/M arrest through promoted p21 and Chk2 expression but inhibited cyclin B1 and cdc2; it also induced apoptosis through the pronounced release of cytochrome c from mitochondria and activations of caspase-9 and caspase-3. These events were accompanied by the generation of reactive oxygen species (ROS), disruption of mitochondrial membrane potential (ΔΨm) and a decrease in the ratio of mitochondrial Bcl-2 and Bax content; emodin also promoted the levels of GADD153 and GRP78. The free radical scavenger N-acetylcysteine and caspase inhibitors markedly blocked emodin-induced apoptosis. Taken together, these findings suggest that emodin mediated oxidative injury (DNA damage) based on ROS production and ER stress based on the levels of GADD153 and GRP78 that acts as an early and upstream change in the cell death cascade to caspase- and mitochondria-dependent signaling pathways, triggers mitochondrial dysfunction from Bcl-2 and Bax modulation, mitochondrial cytochrome c release and caspase activation, consequently leading to apoptosis in SCC-4 cells.

It is well known that cell death can be divided into necrosis and apoptosis (1). Apoptosis is a highly regulated, organized and programmed cell death process controlling the development and homeostasis of multicellular organisms (2). Apoptosis can be divided into two pathways: the extrinsic (death receptor) and intrinsic (mitochondria) pathway (3, 4). The extrinsic pathway involves Fas and tumor necrosis factor receptor 1 and an initiator caspase such as caspase-8 which drives its activation through self cleavage and then activates downstream caspases such as caspase-9 and caspase-3 (5). The intrinsic pathway involves the death signals to mitochondria, which lead to the release of several mitochondrial intermembrane space proteins such as cytochrome c, which then associate with Apaf-1 and procaspase-9 to form the apoptosome (6). The mitochondrial pathway is controlled and regulated by Bcl-2 family proteins such as the antiapoptotic subfamily comprising Bcl-2 and Bcl-xl and the proapoptotic subfamily comprising Bax and Bak (7, 8).

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a naturally occurring anthraquinone, present in the roots and barks of numerous plants, is an active ingredient of various Chinese herbs including Rheum officinale and Polygonum cuspidatum.
It was reported that emodin acted as a purgative and has antibacterial, immunosuppressive, vasorelaxant, cardiotonic, anti-hepatoma protective properties (11-13). However, there is no available information to address how emodin affects human tongue cancer cells in vitro.

In the present study, it was investigated whether the observed toxic effects of emodin could also be extended to human tongue squamous cancer cells.

**Materials and Methods**

**Cell culture and chemicals.** Human tongue squamous cancer SCC-4 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were cultured at 37°C under a humidified 5% CO₂ and 95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) in 75 cm² tissue culture flasks with 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) and...
Assessment of cell morphology and viability. SCC-4 cells at a density of 2×10^5 cells/well were placed in 12-well plates and incubated at 37°C overnight. Different concentrations of emodin (0, 10, 20, 30, 40 and 50 μM) for 72 h before the cells were harvested by centrifuging and the percentage of cells in the sub-G1 (apoptosis), G0/G1-, S- and G2/M-phases were determined by flow cytometry, as described previously (14).

Flow cytometry analysis for cell cycle. SCC-4 cells at a density of 2×10^5 cells/well were placed in 12-well plates and were incubated with different concentrations of emodin (0, 10, 20, 30, 40 and 50 μM) for 48 h before isolation of the cells for 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, as described and 50 μM) for 48 h before isolation of the cells for 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, as described and 50 μM) for 48 h before isolation of the cells for 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, as described and 50 μM) for 48 h before isolation of the cells for 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, as described previously (16). Approximately 5×10^6 SCC-4 cells/ml were treated with 30 μM emodin for 48 h before isolating the cells to extract DNA to be used in DNA gel electrophoresis, as described previously (14).

Detections of reactive oxygen species (ROS), Ca^{2+} production and mitochondrial membrane potential. A total of 5×10^5 SCC-4 cells/ml were treated with 30 μM emodin for 0, 12, 24, 48 and 72 h before isolating the cells to determine specific proteins associated with the cell cycle (p21, cyclin B1, Cdc2, Chk2) and apoptosis (cytochrome c, apoptosis-inducing factor (AIF), growth arrest and DNA damage-inducible gene 153 (GADD153), glucose-regulated protein 78 kDa (GRP78), Bax, Bcl-2, caspase-9, caspase-3). Isolated cells with or without emodin treatment were lysed and the protein levels quantified. All samples were separated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis, as described previously (14).

Statistical analysis. All data are presented as mean±S.D. of three experiments. Statistical differences were evaluated using Student’s t-test and considered significant at p<0.001.

Results

Effects on cell morphology and viable SCC-4 cells. SCC-4 cells were used for investigating the potential cytotoxic effects of emodin on human tongue squamous cells in vitro. The normal cells appeared in a well spread pentagonal shape when observed and photographed with a phase-contrast microscope (Figure 1A). After being treated with different concentrations of emodin for 48 h, a significant proportion of cells were of a round shape and the cell density was reduced in a dose-dependent manner (Figure 1A). After calculation, emodin was found to induce cell death in a dose-dependent manner (Figure 1B). SCC-4 cells incubated with 30 μM emodin for 48 h resulted in a significant decrease in cell viability by approximately 92 and 48%, respectively.
Effects on cell cycle arrest and apoptosis in SCC-4 cells.

The inhibition of cell growth by emodin was further investigated with flow cytometric analysis of cellular DNA content. As shown in Figure 2, the percentage of cells in the G2/M-phase was 20% in control cells, whereas it significantly increased to 22-34% when cells were treated with 20-50 μM of emodin for 48 h and apoptosis was determined by DAPI staining by fluorescence microscopy (×200) as described in Materials and Methods.

Figure 3. Emodin-induced apoptosis and DNA damage in SCC-4 cells as examined by DAPI staining. SCC-4 cells were incubated with different concentrations of emodin for 48 h and apoptosis was determined by DAPI staining by fluorescence microscopy (×200) as described in Materials and Methods.

Figure 4. Emodin induced production of reactive oxygen species (ROS) (A) and Ca2+ (B) and decreased the levels of mitochondrial membrane potential (ΔΨm) (C) in SCC-4 cells. SCC-4 cells were treated with 30 μM emodin for various time periods, before being collected, stained by 2,7-dichlorodihydrofluorescein diacetate for ROS levels, by Indo 1/AM for Ca2+ levels and DiOC6 for ΔΨm levels determined as described in Materials and Methods. Data represent mean±S.D. of three experiments. *p<0.001 Compared to control (0 μM).

Effects on cell cycle arrest and apoptosis in SCC-4 cells. The inhibition of cell growth by emodin was further investigated with flow cytometric analysis of cellular DNA content. As shown in Figure 2, the percentage of cells in the G2/M-phase was 20% in control cells, whereas it significantly increased to 22-34% when cells were treated with 20-50 μM of emodin for 48 h. Apoptosis was determined by DAPI staining by fluorescence microscopy (×200) as described in Materials and Methods.
Emodin induced apoptosis and DNA damage in SCC-4 cells as shown by DAPI staining. The induction of DNA damage by emodin was further investigated with DAPI staining. As shown in Figure 3, the proportion of cells being stained by DAPI increased when the emodin dose was increased; the
number of the cells in total was less than that of control based on unstained DAPI cells.

Emodin induced production of reactive oxygen species (ROS) and Ca\(^{2+}\) while reducing the mitochondrial membrane potential (ΔΨ\(_m\)) in SCC-4 cells. After SCC-4 cells were exposed to 30 μM emodin for various time periods, ROS production was analyzed and quantified by flow cytometry. The data demonstrated that emodin induced ROS production early and time-dependently (Figure 4A) up to 6 h of treatment, with ROS levels remaining significantly higher than the controls.

Emodin-promoted Ca\(^{2+}\) production was time dependent (Figure 4B) up to 48 h treatment, with Ca\(^{2+}\) levels generally being significantly higher than in controls.

Emodin increased the loss of mitochondrial membrane potential in a time-dependent manner (Figure 4C).

Emodin induced the activity of caspase-9 and -3 in SCC-4 cells. In order to examine whether emodin induced apoptosis in SCC-4 cells through a caspase-dependent pathway, SCC-4 cells were exposed to 30 μM emodin for various time periods and the activities of caspase-9 and caspase-3 were determined by flow cytometric analysis. The results demonstrated that emodin promoted the activities of caspase-9 and caspase-3 and these effects were time dependent (Figure 5A and 5B). To explore whether the activation of caspases was required for induction of apoptosis by emodin, SCC-4 cells were co-treated with N-acetylcysteine and pan-caspase, caspase-9 and caspase-3 inhibitors, and emodin. As shown in Figure 5C, incubation with the N-acetylcysteine and pan-caspase, caspase-9 and caspase-3 inhibitors significantly blocked emodin-triggered apoptosis in SCC-4 cells. These results indicated that emodin induced apoptosis in SCC-4 cells through a mitochondria- and caspase-3-dependent pathway.

Emodin affected the G\(_2\)/M phase and levels of apoptosis-associated proteins in SCC-4 cells. In order to determine the molecular mechanism of emodin-induced G\(_2\)/M arrest and apoptosis in SCC-4 cells, cells were exposed to 30 μM emodin for various time periods, harvested and proteins levels were determined by Western blotting. The results are shown in Figure 6A, emodin promoted p21 and Chk2 levels but inhibited the levels of cyclin B1 and Cdc2 which led to G\(_2\)/M arrest. As shown in Figure 6B, emodin promoted the levels of cytochrome c, AIF, Bax, caspase-9 and caspase-3, but reduced the level of Bcl-2, which led to apoptosis. Results also showed that emodin promoted GADD153 and GRP78 levels, indicating that emodin-induced apoptosis also involved ER stress.

Discussion

The best strategy for chemotherapeutic agents is largely dependent on their ability to trigger cell death in tumor cells; therefore, novel inducers of apoptosis may offer a new therapeutic approach for cancer therapy. Emodin has an anticancer effect on human cancer in vitro (17) but it had no cytotoxic effect on MCF-7 (18), Hepa-1 (19) and HepG2 cell lines (20). Here we reported that emodin was active
against human tongue cancer SCC-4 cells, based on apoptotic cell death. Emodin induced apoptotic features, including nuclear condensation, DNA fragmentation and caspase activation, all hallmarks of apoptosis. These observations have been reported in emodin-treated human cervical cancer cells (21), promyeloleukemic HL-60 cells (22), hepatocarcinoma cell lines (20) and lung squamous carcinoma cells (23).

Our results demonstrated emodin induced ROS and Ca\textsuperscript{2+} production, but induced dysfunction of mitochondria in SCC-4 cells. The generation of ROS may contribute to mitochondrial damage and lead to cell death by their acting as apoptotic signaling molecules (24). Other investigators have indicated that emodin can act as an ROS generator to increase the susceptibility of tumor cells to cytotoxic therapeutic agents (17). Our results also showed that the effect of emodin on mitochondrial membrane potential (ΔΨ\textsubscript{m}) and apoptosis in SCC-4 cells is apparently dependent on ROS generation because the emodin-mediated ΔΨ\textsubscript{m} disruption and apoptosis was abolished by the antioxidant N-acetylcysteine (Figure 5C). Disruption of ΔΨ\textsubscript{m} is recognized to be an indicator of mitochondrial damage and is considered as an early stage of apoptosis, preceding the efflux of cytochrome c, AIF and cIAPs from the mitochondrial which is then followed by caspase-9 and caspase-3 cascade activation (25). Our results demonstrated that the marked activation of caspase-9 and -3 by emodin depended on mitochondrial cytochrome c release to the cytosol and that it follows the breakdown of ΔΨ\textsubscript{m}, suggesting that emodin-induced apoptosis takes place through the mitochondria-mediated pathway. This is in agreement with other reports which indicated that emodin-induced apoptosis occurs through a mitochondria-dependent activation of caspase-3 and -9 in human cervical cancer cells (24) and lung squamous carcinoma CH27 cells (26). Here, the treatment of SCC-4 cells with the pan-caspase, caspase-9 and caspase-3 inhibitors drastically blocked apoptosis induced by emodin. These observations suggest that the intrinsic mitochondria-mediated caspase activation signaling pathway plays a crucial role in emodin-induced apoptosis in SCC-4 cells.

The dysfunctions of the mitochondrial membrane were associated with changes of the ratio of Bax/Bcl-2. ROS might modulate the cellular distribution and content of Bax and Bcl-2 (27). Our results also showed that emodin promoted the levels of Bax but decreased the levels of Bcl-2, which led to dysfunction of the mitochondrial membrane. The increase of pro-apoptotic protein Bax was followed by the translocation of this protein to the mitochondria. After Bax is translocated to the mitochondria, it can cause the release of cytochrome c and induce cell death. The antiapoptotic protein Bcl-2 probably acts to prevent apoptosis by scavenging oxygen-derived free radicals inside the cells (27, 28).

Another report demonstrated that emodin-triggered apoptosis occurs through a mechanism involving inactivation of Akt (also name protein kinase B, PKB) (29). Inhibition of this kinase by LY294002 or wortmannin potentiates apoptotic cell death (29). However, whether the translocation of Bax was regulated by Akt in emodin-treated HL-60 cells remains to be defined. Huang et al. (30) previously presented data demonstrating that inactivation of extracellular signal-regulated kinase (ERK) but not p38 is an important determinant of apoptotic cell death induced by emodin in cultured human breast cancer MDA-MB-231 cells and human skin squamous carcinoma HSC5 cells.

In conclusion, the possible apoptotic pathways induced by emodin can be summarized by the sequence presented in Figure 7. In this model, emodin-mediated ROS generation takes place causing DNA damage, changes to the ratio of the Bax/Bcl-2, dysfunction of mitochondrial membrane, cytochrome c release, activation of caspase-9 and -3, then finally causing apoptosis in SCC-4 cells.

Acknowledgements

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References

Figure 7. The proposed model of emodin-mediated cell cycle arrest and apoptosis in human tongue squamous cancer SCC-4 cells. Schematic diagram showing emodin-induced signaling concentrating on activation of G2/M arrest and of apoptotic machinery in SCC-4 cells.


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