Abstract. Rhein, an anthraquinone compound, can be found in the rhizome of rhubarb, a traditional Chinese medicine herb showing antitumor activity. In this study, it was observed that rhein induced S-phase arrest through the inhibition of p53, cyclin A and E and it induced apoptosis through the endoplasmic reticulum stress by the production of reactive oxygen species (ROS) and Ca²⁺ release, mitochondrial dysfunction, and caspase-8, -9 and -3 activation in human tongue cancer cell line (SCC-4). The most efficient induction of apoptosis was observed at 30 μM for 24 h. Mechanistic analysis demonstrated that rhein induced changes in the ratio of Bax/Bcl-2 based on the decrease of Bcl-2 levels, the loss of mitochondrial membrane potential, cytochrome c release from the mitochondria and the activation of caspase-9 and -3. The data demonstrated that rhein induces apoptosis in SCC-4 cells via caspase, ROS and mitochondrial death pathways.

Apoptosis is a highly organized and regulated death process (1) and its features include cellular morphological changes, membrane blebbing, chromatin condensation, oligonucleosomal DNA cleavage, apoptotic bodies, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, and activation of a family of cysteine proteases called caspase (2-4). Apoptosis can be divided into the extrinsic pathway involving membrane receptors and the intrinsic pathway involving mitochondria (5). They are both signal pathways leading to apoptosis depending on different apoptotic stimuli. A third pathway, termed endoplasmic reticulum stress, involves caspase-12 (6).

Caspase activation is generally considered to be a key hallmark of apoptosis. The human system contains 11 kinds of caspases, including caspase-2, -3, -6, -7, -8, -9 and -10, which are involved in chemical- and agent-induced apoptosis (7). Caspase-8 is an apoptosis initiator (5), activation of which initiates downstream events such as directly cleaving and activating pro-caspase-3, or cleaving of the cytoplasmic protein Bid generating a fragment that activates the mitochondrial pathway (6). Apoptosis-inducing factor (AIF) is released into the cytosol and nucleus of mitochondria inducing chromatin condensation and DNA fragmentation (8). After the dysfunction of mitochondria, cytochrome c is released and accumulated in the cytoplasm binding to the Apaf-1 protein causing Apaf-1 oligomerization followed by pro-caspase-9 binding to the Apaf-1 oligomers to form a high-molecular-mass complex named apoptosome (9, 10). The active caspase-9 in turn activates caspase-3 which can cleave poly(ADP-ribose)polymerase finally leading to apoptosis (11, 12). Reactive oxygen species (ROS) may play an important role during apoptosis induction (11) as it can directly activate the mitochondrial permeability transition and result in mitochondrial membrane potential (∆Ψₘ) loss (13).
Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), one of the anthraquinone compounds present in the root of rhubarb (R. palmatum L. or R. tanguticum Maxim) (14), has been extensively used for stomach diseases for a long time in Chinese populations. A previous study has shown that rhein can inhibit N-acetyltransferase activity (15) and decrease the percentage of viable cells and induced apoptosis in cervical cancer cells (16) and human nasopharyngeal carcinoma cells (17). Other reports also demonstrated that rhein suppresses the phorbol ester-induced tumor promotion in mouse epidermal cell line JB6 (21) and suppresses the growth of tumor cells in rat liver (18), human glioma (19) and Ehrlich ascites tumor (20) in vivo. Rhein was found to induce apoptosis through the generation of nitric oxide in human colonic adenocarcinoma monolayer cells in vitro (21). However, there are no studies addressing rhein effects in human tongue cancer cells. Therefore, the present study is the first to report rhein induced apoptosis in human tongue squamous cancer cells (SCC-4) via caspase- and mitochondria-dependent pathways.

Materials and Methods

Reagents. Rhein and other general reagents were obtained from Sigma (St. Louis, MO, USA).

Cell culture. Human tongue cancer cell line (SCC-4) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Moregate BioTech, Bulimba QLD, Australia), 1% penicillin-streptomycin (100 U/mL, penicillin and 100 μg/mL streptomycin) and 1% glutamine in 75 cm² tissue culture flasks and atmospheres as described elsewhere (22).

Morphological changes and viability examined by microscopy and flow cytometry. SCC-4 cells at a density of 2×10⁵ cells/well were placed in 12-well plates and incubated at 37°C for 24 h. Then 0, 10, 20, 30, 40 and 50 μM rhein were added and the cells were incubated for 6, 12, 24, 48 and 72 h. Cells were directly examined and photographed in the plates under a phase-contrast microscope. For total viable cells, cells from each sample were harvested and labeled with propidium iodide (PI). Live and dead cells were determined by flow cytometry (Becton Dickinson FACSCalibur) and data analysis was performed as previously described (17, 22).

Cell cycle and sub-G1 analysis. Rhein treated and incubated SCC-cells, as described above were treated with PI and then harvested. The percentage of cells in the sub-G1 (apoptosis), G2/G0- and G1/M-phases was determined by flow cytometry, as described previously (17, 22).

DNA damage examined by DAPI staining and DNA gel electrophoresis. Rhein treated and incubated SCC-cells, as described above were stained with DAPI performed as previously described (21, 22). The DAPI-positive nuclei were visualized and photographed using an Olympus fluorescence microscope (Olympus, Tokyo, Japan).

Cells from incubation with 30 μM rhein for 48 h were harvested and their DNA extracted as described previously (21). DNA gel electrophoresis was performed as described previously, and photographed under a fluorescence microscope (17, 23).

Reactive oxygen species (ROS), Ca²⁺ production levels and mitochondrial membrane potential detected by flow cytometric analysis. SCC-4 cells at 5×10⁵ cells/mL were placed in 12-well plates and treated with 30 μM of rhein for 0, 6, 12, 24, 48 and 72 h. Cells from each treatment were harvested and washed twice. Then re-suspended in 500 μL of 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA, Sigma) (dye contains fluorescence for staining of ROS), Indo I/AM (3 μg/mL) (dye contains fluorescence for staining of Ca²⁺) and DiOC₆ (4 μmol/L) (dye contains fluorescence for staining of ΔΨm), then incubated at 37°C for 30 min to detect changes in ROS, Ca²⁺ and ΔΨm levels by using flow cytometry as described previously (17, 22, 23).

Caspase-3,-8 and -9 activity determination by flow cytometric analysis. SCC-4 cells at 5×10⁵ cells/mL were placed in 12-well plates and treated with 30 μM of rhein for 0, 12, 24, 48 and 72 h. Cells were harvested and 50 μL of 10 μM solution of each substrate (21, 22) for caspase-3,-8 and -9 were added to the cell pellet (1×10⁵ cells per sample) and incubated at 37°C for 60 min in the dark. The cells were then washed once with 1 mL of ice-cold PBS and re-suspended in 1 mL fresh PBS. Cells were analyzed with a flow cytometer for caspase-3,-8 and -9 activity according to the manufacturer’s instructions (17, 22, 23).

Total protein preparation and Western blotting. SCC-4 cells at 5×10⁶ cells/mL were placed in 6-well plates and treated with 30 μM of rhein for 0, 12, 24, 48 and 72 h. In order to determine specific proteins associated with cell cycle (p53, p21, cyclin B1, cdk2, Chk2, Wee1, Cdc25c) and apoptosis (Bax, Bel-2, cytochrome c, caspase-9, -3, ATF-6α, GADD153, GRP78) the cells were isolated. Total proteins were extracted with an M-PER mammalian protein extraction reagent (Pierce Biotechnology, Inc.) according to the manufacturer’s instructions. All samples were separated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis, as described previously (17, 22, 23).

EndoG release from mitochondria determined by confocal laser microscopy. SCC-4 cells (5×10⁶) cells were plated on 4-well chamber slides and treated with 30 μM rhein for 24 h. Cells were then fixed in 4% formaldehyde in PBS for 15 min and permeabilized with 0.3% Triton-X 100 in PBS for 1 hour with blocking of non-specific binding sites using 2% BSA. The slides were then incubated overnight with anti-human EndoG antibody (1:100 dilution; Santa Cruz, USA) and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution: Santa Cruz), followed by DNA staining with PI. Photomicrographs were obtained as previously described (23).

Statistical analysis. The unpaired Student’s t-test was used to identify means that were significantly different from control and rhein treatment (p<0.05).
Results

Rhein effects on cell morphology, viability, cell cycle and sub-G1 group of SCC-4 cells. The effects of rhein on cell morphology and cell death were examined in SCC-4 cells. Treatment of SCC-4 cells with 10-50 μM rhein resulted in morphological changes (Figure 1A) and decreased the percentage of total viable cells (Figure 1B), approximately 25-65% inhibition was detected after 24 h treatment of SCC-4 cells. Both effects in SCC-4 cells are dose- and time-dependent. As shown in Figure 1C and D, the percentage of cells in S phase was 30% in control, whereas it increased to 38-65% when treated with 10-50 μM of rhein for 24 h. It was also observed that the relative proportion of cells in G0/G1 and G2/M phase decreased while the concentration of rhein increased. In addition, the presence of a remarkable apoptotic sub-G1 peak was detected at 40 μM of rhein.

Rhein-induced apoptosis and DNA damage in SCC-4 cells examined by DAPI staining DNA gel electrophoresis. Based on the appearance of a sub-G1 peak in cell cycle analysis, the rhein induced DNA damage and apoptosis was further characterized by DAPI staining and DNA gel electrophoresis. Rhein induced apoptosis in SCC-4 cells occurred in a dose-dependent manner (Figure 2A). Further, rhein induced a DNA ladder to occur in SCC-4 cells (Figure 2B).

Rhein induced production of ROS and Ca2+ and decreased the levels of mitochondrial membrane potential (ΔΨm) in SCC-4 cells. The production of ROS contributed to mitochondrial damage that may facilitate the further release of ROS into the cytoplasm. To address the possibility that rhein-induced apoptosis could be related to contributions from the mitochondrial pathway, SCC-4 cells were treated with 30 μM of rhein for various time periods and the change in ROS and...
Ca\(^{2+}\) production, and \(\Delta \Psi_m\) were examined. As shown in Table I rhein induced ROS production in early treatment (0.5-6 h) of SCC-4 cells. The relative levels of ROS were elevated as early as 0.5 h after rhein treatment and persistently increased with exposure time. However, rhein decreased the levels of \(\Delta \Psi_m\) and promoted the Ca\(^{2+}\) production for up to 48 h treatment. The levels of \(\Delta \Psi_m\) and ROS in untreated control cells were unchanged over all of the incubation time periods.

**Rhein induced the activities of caspase-3, -8 and -9 in SCC-4 cells.** Caspases are a family of cysteine proteases that play a central role during the executional phase of apoptosis. To explore whether rhein induced apoptosis by activation of caspases, fluorogenic peptide substrates for caspase-3, -8 and -9 were used to detect caspase activity. Caspase-3, -8 and -9 were activated in SCC-4 cells after 12 h rhein treatment (Figures 3A, B and C). A marked

![Flow cytometric analysis of intracellular ROS and Ca\(^{2+}\) levels and MMP in SCC-4 cells with Rhein treatment.](image.png)

Table I. Flow cytometric analysis of intracellular ROS and Ca\(^{2+}\) levels and MMP in SCC-4 cells with Rhein treatment.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ROS</th>
<th>Ca(^{2+})</th>
<th>(\Delta \Psi_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>100.00±0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>15.05±4.60*</td>
<td>25.55±4.10*</td>
<td>51.40±4.81*</td>
</tr>
<tr>
<td>1</td>
<td>25.34±3.21*</td>
<td>31.02±6.32*</td>
<td>48.60±8.83*</td>
</tr>
<tr>
<td>4</td>
<td>40.34±4.10*</td>
<td>40.41±2.69*</td>
<td>45.14±6.32*</td>
</tr>
<tr>
<td>8</td>
<td>41.32±3.21*</td>
<td>45.65±6.32*</td>
<td>38.62±4.11*</td>
</tr>
</tbody>
</table>

The SCC-4 cells (5x10\(^5\) cells/mL) were treated with different concentrations of Rhein. The zero concentration was defined as the control. The percentage of cells of stained by DCFH-DA, Indo-1/AM or DiOC6 dyes, respectively, for ROS, Ca\(^{2+}\) or MMP were determined by flow cytometry as described in the Materials and Methods section. Values are means±SD (n=3). Statistical calculations of the data were performed using an unpaired Student’s t-test. *Significantly different from the control at p<0.05.
Rhein affected the levels of associated proteins in cell cycle and apoptosis of SCC-4 cells. After SCC-4 cells were treated with 30 μM rhein for various time periods, cells were collected from each sample and then proteins were isolated for determination of associated proteins involved in cell cycle and apoptosis by Western blotting. As shown in Figure 4A, B, C, D and E, rhein promoted p21, p27 and Chk2 levels but inhibited the levels of cyclin B1, cyclin A, Cdc25A and thymidylate synthase, which led to S phase arrest. Rhein promoted FasL, active-caspase-3, -8 and -9, cytochrome c, and Bax levels but decreased Bcl-2 levels leading to apoptosis. Rhein promoted AIF levels (Figure 4C) but inhibited GADD153 and promoted ATF-6α and GRP78 levels (Figure 4E) indicating that ER stress and mitochondria were also involved in rhein-induced apoptosis.

Rhein affects EndoG release from mitochondria in SCC-4 cells. Rhein induced EndoG release from mitochondria to cytosol and nuclei (Figure 5A). The possible signaling pathways for rhein-induced apoptosis can be summarized as shown in Figure 5B indicating that rhein induced apoptosis also occurs through caspase-3-dependent and -independent pathways.

Discussion

In the presented study it was found that rhein induced S phase arrest and apoptosis in SCC-4 cells and induced morphological changes and decreased the percentage of viable SCC-4 cells. While alterations in cellular morphology are indicative of a cell death process, permeabilization of the cell membrane is required to confirm cell death. Annexin V was used for staining and to analyze apoptosis by flow cytometry (data not shown). The possible molecular mechanism of apoptosis which associated the signal pathways in SCC-4 cells after exposure to rhein was examined. While cells treated with rhein showed decreases in populations of cells in the G0/G1 and G2/M phases, there was a relative accumulation of cells in the S phase. This suggests blocking at the S phase in the cell cycle, confirming earlier reports. Since rhein definitely induced cell cycle arrest in SCC-4 cells, the levels of cell cycle-related proteins were determined in the cells exposed to the indicated doses of rhein. It was observed that rhein up-regulated cyclin-dependent kinase inhibitor (CDKI) p27 and down-regulated the proteins of CDK2. Treatment with rhein resulted in the down-regulation of cyclin A and E, which regulate the progression of the S phase. Collectively, rhein-induced S phase arrest in SCC-4 is accompanied by the alteration of various cell cycle-regulated proteins.

These results suggest that pharmacologics blocking progress down the apoptotic cascade could potentially serve as therapeutic agents. However, the mechanism of this S phase blockade remains unclear. The deregulation of cell cycle has been reported to correlate with the induction of apoptosis (24). Rhein induced apoptosis was also involved in caspase-3, -8 and -9 activity of caspase-3, -8 and -9 was observed after 24 h rhein treatment. These effects occurred in a time-dependent manner.

Figure 3. Rhein induced the activities of caspase-3, -8 and -9 in SCC-4 cells. Cells were treated with 30 μM rhein for various time periods and were collected for addition of 50 μL of 10 μM substrate solution for caspase-3 (A), -8 (B) and -9 (C). Activity was determined according to the manufacturer’s instructions as described in Materials and Methods. Data represents mean±S.D. of three experiments. ***p<0.001.
-9 based on the activation from flow cytometric analysis. The caspase family can be divided into major subgroups on the basis of their substrate specificity, sequence homology and biochemical function: caspase 1-like (caspase 1, 4 and 5), and caspase 3-like (caspase 2, 3 and 10) proteases (25). Caspase-3, in particular, plays a pivotal role in the terminal phase of apoptosis (26). To test whether caspase-3 activity was involved in rhein-induced apoptosis, a substrate for caspase-3-like proteases was used in this study. As shown in Figure 3, rhein increased caspase 3-like activity in a dose-dependent manner. Adding the peptide Ac-DEVD-CHO that possesses an inhibitory effect on caspase 3-like proteases abolished the increase in caspase-3 activity in the SCC-4 cells (data not shown). These data suggest that activation of caspase-3-like proteases was involved in rhein-induced apoptosis. Those observations were also seen for caspase-8 and -9.

It is generally recognized that after the loss of the outer mitochondrial membrane integrity and the release of cytochrome c from the mitochondria to the cytosol, the cells are committed to apoptosis (2-4). The results demonstrated that

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**Figure 4.** Rhein affected the levels of associated proteins in cell cycle and apoptosis of SCC-4 cell. Cells were treated with 30 μM rhein for various time periods and were then harvested for total protein isolation determined as described in Materials and Methods. The associated protein expressions (A: p53, p21, p27, cyclin E, thymidylate synthase, Cdk2, cyclin A and Cdc25A; B: Fas, FasL, pro-caspase-8, caspase-8, Bid, tBid; C: cytochrome c, pro-caspase-9, caspase-9, caspase-3, AIF and PARP; D: Bax and Bcl-2; E: ATF-6α, GADD153 and GRP78) were estimated by Western blotting.
rhein induced dysfunction of mitochondria (i.e. decreased levels of $\Delta \Psi_m$) in SCC-4 cells in a dose-dependent manner. The results from Western blotting showed that rhein inhibited the protein level of Bcl-2. The ratio of Bax/Bcl-2 changes which could lead to the changes of $\Delta \Psi_m$ then affected the release of cytochrome c. Bcl-2 has been shown to localize in the mitochondrial membrane and stabilize mitochondrial functions, thereby suppressing the release of pro-apoptotic effector molecules. Bax translocation to the mitochondria induces cytochrome c release from mitochondria (22, 27).

This observation indicates that cells treated with rhein undergo apoptosis involving mitochondrial depolarization prior to the loss of cell membrane integrity. The mitochondrial respiratory chain is an important site of ROS production in the cell. To elucidate the involvement of ROS in rhein-induced apoptosis, ROS levels were assessed by using H2DCFDA and DHE fluorescence (22, 23). Antioxidants such as NAC (N-acetylcystein) were used for pre-treatment of SCC-4 cells followed by rhein treatment leading to an increase of the percentage of viable cells compared to rhein treatment only. However, all of the caspase inhibitors, which significantly prevented cell death, did not completely block the percentage of apoptosis. Therefore, another pathway was also involved in rhein

Figure 5. Rhein affected EndoG release from mitochondria in SCC-4 cells. Cells were incubated with 30 μM rhein for 24 h and fixed and stained with primary antibodies to EndoG followed by FITC-labeled secondary antibodies (green fluorescence) detected by Confocal laser microscopy. Areas of co-localization of EndoG expressions in the merged panels are yellow. Scale bar, 10 μm (A). A model for summarizing rhein-induced apoptotic signaling pathway in human squamous cancer SCC-4 cells (B). Rhein induced the production of ROS and Ca$^{2+}$ and decreased $\Delta \Psi_m$ levels leading to cytochrome c release, promoted the activation of caspase-9 and -3 causing apoptosis in SCC-4 cells.
induced apoptosis such as EndoG release from mitochondria (Figure 5A).

In conclusion, rhein, an anthraquinone from rhubarb, induces dose-dependent apoptosis involving the mitochondria in SCC-4 cells. The possible signaling pathways include ROS and Ca\(^{2+}\) production, changes to the ratio of Bax/Bcl-2, dysfunction of mitochondria, cytochrome c release and activation of caspase-3 leading to apoptosis.

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References


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