Abstract. Although rhein has been shown to induce apoptosis in several cancer cell lines, the mechanism of action of rhein-induced cell cycle arrest and apoptosis at the molecular level is not well known. In this study, the mechanism of rhein action on A-549 human lung cancer cells was investigated. Rhein induced G0/G1 arrest through inhibition of cyclin D3, Cdk4 and Cdk6. The efficacious induction of apoptosis was observed at 50 μM for 12 h and up to 72 h as examined by a flow cytometric method. Flow cytometric analysis demonstrated that rhein increased the levels of GADD153 and GRP78, both hallmarks of endoplasmic reticulum stress, promoted ROS and Ca2+ production, induced the loss of mitochondrial membrane potential (ΔΨm), promoted cytochrome c release from mitochondria, promoted caspase-3 activation and led to apoptosis. Rhein also increased the levels of p53, p21 and Bax but reduced the level of Bcl-2. The Ca2+ chelator BAPTA was added to the cells before rhein treatment, thus blocking the Ca2+ production and inhibiting rhein-induced apoptosis in A-549 cells. Our data demonstrate that rhein induces apoptosis in A-549 cells via a Ca2+-dependent mitochondrial pathway.

Antitumor research was based on the ability of agents to induce cellular lesions through cytotoxic mechanisms for many past years, however, evidence suggested that some antitumor agents can induce apoptosis, which leads to the rapid elimination of tumor cells (1-3). Apoptosis is a well-regulated and organized death process and is a general mechanism for removal of unwanted cells in the development and homeostasis of multicellular organisms (4). The characters of apoptosis include membrane blebbing, nuclear and cytoplasmic shrinkage, DNA fragmentation, translocation of phosphatidylserine of the plasma membrane from the inner to the outer leaflet, and activation of a family of caspases (5, 6). It is well documented that caspase activation is considered to be a key hallmark of apoptosis. Apoptosis is also accompanied by a loss of mitochondrial membrane potential (ΔΨm), before induction of cytochrome c release and activation of caspase-3 (7-9).

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), an anthraquinone found especially in the root of rhubarb (Rheum palmatum L. or R. tanguticum Maxim), was used to treat chronic liver disease in China. Many studies in vivo showed that rhein inhibits the growth of tumor cells in rat liver (10), human glioma (11) and Ehrlich ascites tumor (12). Rhein inhibits superoxide anion production, chemotaxis and phagocytic activity of neutrophils, and macrophage migration and phagocytosis, and these effects are dose dependent (13). Rhein reduced the concentration of Ca2+ and inhibition of protein kinase C that led to blocking of interleukin-12 mRNA transcription (14). Rhein generated nitric oxide in vitro and led to induced apoptosis in human...
Materials and Methods

Chemicals and reagents. Rhein, RNase, trypsin blue, propidium iodide (PI), Tris-HCl and Triton® X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BAPTA, potassium phosphates and dimethyl sulfoxide (DMSO) were obtained from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was bought from OncoImmunin, Inc (Gaithersburg, MD, USA).

Human lung carcinoma cell line (A-549). A-549 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75 cm² tissue culture flasks and grown at 37°C under a humidified 5% CO₂ and 95% air for different periods of time. Cells were harvested and washed twice, re-suspended in 12-well plates at concentrations of 0, 25, 50 and 100 μM of rhein were incubated for 24 hours. Cells were harvested by centrifugation and the medium was removed. A 50 μl of 10 μM PHIPhlux substrate solution (a unique class of substrates for caspase-3) was then added to the cell pellet (1×10⁵ cells per sample) without vortexing the cells. Cells were then incubated at 37°C for 60 minutes. Cells were the washed once by adding 1 ml of ice-cold PBS and re-suspended in a fresh 1 ml. Cells were analyzed with flow cytometry (Becton-Dickinson) with an argon ion laser at 488 nm. Finally, the caspase-3 activity was determined and analyzed (21).

Inhibition of rhein-induced apoptosis by the caspase-3 inhibitor z-DEVD-fmk in A-549 cells. In order to examine whether or not caspase-3 activation was involved in apoptosis triggered by rhein, A-549 cells were pretreated with the caspase-3 inhibitor z-DEVD-fmk (20 μM) 3 h prior to treatment with 50 μM rhein. Apoptosis and caspase-3 activity were then determined by flow cytometry as described above (21).

Detection of reactive oxygen species (ROS) in A-549 cells after treatment with rhein by flow cytometry. The level of ROS of the A-549 cells was examined by flow cytometry (Becton Dickinson FACS Calibur), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma). Approximately 2×10⁵ cells/well of A-549 cells in 12-well plates were incubated for 0, 15, 30, 60, 120, 240 and 480 minutes with 50 μM rhein to detect the changes in ROS. The cells were harvested and washed twice, re-suspended in 500 μl of DCFH-DA (10 μM) and incubated at 37°C for 30 min before being analyzed by flow cytometry (20).

Detection of mitochondrial membrane potential (ΔΨm) in A-549 cells after treatment with rhein by flow cytometry. The level of cell ΔΨm in A-549 was determined by flow cytometry (Becton Dickinson FACS Calibur) using DiOC₆ (4 μmol/l). Approximately 2×10⁵ cells/well of A-549 cells in 12-well plates with 0, 25, 50 and 100 μM of rhein were incubated for 24 hours to detect the changes in ΔΨm. The cells were harvested and washed twice, re-suspended in 500 μl of DiOC₆ (4 μmol/l) and incubated at 37°C for 30 min before being analyzed by flow cytometry (17).

Detection of Ca²⁺ concentrations in A-549 cells after treatment with rhein by flow cytometry. The level of Ca²⁺ of the A-549 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using Indo 1/AM (Calbiochem; La Jolla, CA, USA). Approximately 2×10⁵ cells/well of A-549 cells in 12-well plates with 0, 25, 50 and 100 μM of rhein were incubated for 24 hours to detect the changes in Ca²⁺ concentration. The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/ml) and incubated at 37°C for 30 min before being analyzed by flow cytometry (22).

Detection of Ca²⁺ concentrations and ΔΨm levels in A-549 cells after pre-treatment with BAPTA. The level of Ca²⁺ and ΔΨm of the A-549 cells were determined by flow cytometry (Becton Dickinson FACS Calibur) using Indo 1/AM (Calbiochem; La Jolla, CA). Approximately 2×10⁵ cells/well of A-549 cells in 12-well plate were pre-treated with BAPTA, a Ca²⁺ chelator (10 μM), before adding 50 μM of rhein for incubation for 24 hours to
detect the changes in Ca^{2+} concentration and the levels of ΔΨm. The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/ml) and incubated at 37˚C for 40 min before being analyzed by flow cytometry (22).

Western blotting for examining the effect of rhein on Cdk4 and 6, cyclin E and Cdk2, p53, p21, Bax, Bcl-2 and cytochrome c of A-549 cells. The total proteins were collected from A-549 cells treated with or without various concentrations of rhein for 48 hours before p53 p21, Bax, Bcl-2, Cdk1, Cdk2, cyclin B1, D3 and cytochrome c (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described elsewhere (18-20).

Confocal laser microscopy. A-549 cells (5x10^4 cells/well) plated on 4-well chamber slides were treated with or without 30 μM rhein for 24 hours then the cells were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton®-X 100 in PBS for 1 h with blocking of non-specific binding sites using 2% boven serum albumin. Fixed cells were then incubated with antihuman GADD153 and GRP78 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (1:100 dilution) overnight and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution) (Santa Cruz Biotechnology Inc.), followed by DNA staining with PI. Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope (23)
Statistical analysis. Student’s t-test was used to analyze the differences between the rhin treated and control groups.

Results

Effects of cell viability by rhin on A-549 cells. The results from PI staining experiments indicated increasing the dose of rhin and/or increasing the time of incubation with 50 μm rhin led to a significant decrease in the percentage of viable cells. These effects of rhin were dose- and time-dependent (Figure 1A and B).

Induction of cell cycle arrest and apoptosis by rhin on A-549 cells. We investigated the occurrence of cell cycle arrest and apoptosis of A-549 cells by treatment with rhin.

Figure 3. Flow cytometric analysis of the effects of rhin on caspase-3 activity and apoptosis in A-549 cells. The A-549 cells were incubated with 50 μM rhin and/or with or without z-DEVD-fmk treatment for caspase-3 activity (A) and apoptosis determination (B) as described in Materials and Methods. Data represent mean±S.D. of three experiments. *p<0.05. Significantly different from the control at *p<0.05.

Figure 4. Effects of calcium antagonist BAPTA (Ca^{2+} chelator) on the rhin effects on the levels of ΔΨ_{m}, Ca^{2+} and apoptosis in A-549 cells. The A-549 cells were pre-treated with BAPTA for 3 hours then treated with rhin and cells were harvested for ΔΨ_{m} (A), Ca^{2+} (B) and apoptosis (C) determinations as described in Materials and Methods. Data represent mean±S.D. of three experiments. Significantly different from the control at *p<0.05.
As shown in Figure 2A and B, rhein induced G0/G1 arrest in a concentration- and induction of apoptosis in a time-dependent manner. The percentage of cells in the G0/G1 phase and apoptosis increased significantly in A-549 cells by treatment with rhein. The percentage of apoptosis reached 58% with 50 μM rhein treatment for 48 hours. Inhibition of rhein-induced caspase-3 activity and apoptosis by the caspase-3 inhibitor z-DEVD-fmk on A-549 cells. This was conducted in order to examine whether or not caspase-3 activation is involved in the apoptosis of cells triggered by rhein. The results indicate that rhein increased caspase-3 activity and apoptosis (Figure 3A and B). The A-549 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor (z-DEVD-fmk) 3 hours prior to the treatment with rhein. After treatment with rhein and z-DEVD-fmk, inhibition of rhein-mediated caspase-3 activation in A-549 cells was accompanied by the marked attenuation of rhein-induced apoptotic cell death (Figure 3B).

Detection of reactive oxygen species (ROS) in A-549 cells after treatment with different concentrations of rhein by flow cytometry. The percentage of ROS was higher in the rhein-treated groups than in the control. The ROS production was maximal at 15 minutes after treatment with rhein before decreasing for up to 6 hours (Table I).

Effects of rhein on the levels of Ca2+ and mitochondrial membrane potential (ΔΨm) of A-549 cells. The percentage of Ca2+ production was significantly lower in the rhein-treated groups than in the control. Increasing the time of rhein treatment led to an increase in the Ca2+ production in A-549 cells (Table II). The ΔΨm was significantly lower in the rhein-treated groups than in the control. Increasing the dose of rhein led to greater reduction of the ΔΨm in A-549 cells (Table II). The effects of rhein on the levels of Ca2+ and ΔΨm were dose dependent.

Effects of BAPTA on rhein-induced production of ΔΨm, Ca2+ and apoptosis of A-549 cells. The ΔΨm, Ca2+ and apoptosis production levels were significantly different between rhein- and/or pretreated with BAPTA treated and control groups. Pre-treatment with BAPTA mitigated the ΔΨm induced by rhein (Figure 4A) and reduced Ca2+ levels (Figure 4B) and apoptosis (Figure 4C) in A-549 cells.

Western blotting for examining the effect of rhein on Cdk1, Cdk4, Cdk6, Cdk2, cyclins D3 and E, p53, p21, Fas, FasL, bax, Bcl-2 and cytochrome c of A-549 cells. The results are shown in Figure 5A. Western blot indicated that the levels of Cdk4, Cdk6, Cyclin D3 and cyclin E were reduced, which may have led to G2/M arrest. Rhein increased the expressions of p53, p21, Bax and cytochrome c but reduced the expression of Bcl-2, which may have led to apoptosis in these cells.

Confocal laser microscopy for examining the levels of GADD153 and GRP78. As illustrated in Figure 6A, rhein-treated cells reacted with GADD153 antibodies and PI staining results showed that rhein treatment for 24 h increased the levels of GADD153, which was translocated to nuclei. The results from Figure 6B also showed that rhein treatment for 24 h increased the levels of GRP78, which was translocated to nuclei.

Discussion

Many anthraquinones have been reported to possess tumor cell inhibitory effects through a variety of mechanisms, including induction of apoptosis (24, 25), intercalation and binding with cellular DNA (26), redox-cycling radical...
Figure 5. Representative Western blots showing changes in the levels of cyclin D3 and E, and Cdk4 and 6 (A), p53 and p21 (B), Fas and FasL (C), GADD153 and GRP78 (D), cytochrome c, Apaf-1, Bcl-2, Bax and Bid (E) and caspases 8, 9 and 3 (F) in A-549 cells after treatment with rhein. The A-549 cells (5×10⁶/ml) were treated with 50 μM rhein for 6, 12, and 24 h, then cytosolic fraction and total protein were prepared as described in Materials and Methods followed by evaluation of the levels of associated proteins by Western blotting as described in Materials and Methods.
formation (27, 28) and inhibition of topoisomerase (29). A comparison of the results obtained from viability assays suggest that the antitumor activity of rhein was through the induction of apoptosis in A-549 cells. Rhein is an anthraquinone existing in rhubarb which inhibits the growth of tumor cells, which may be associated with apoptotic cell death. In this study, we demonstrated that rhein reduced the percentage of viable cells and induced apoptosis in A-549 cells in dose- and time-dependent manners. Comparing the inhibition of viable cells (A-549) (IC₅₀ is 45 μM) to other
cells from other investigators, IC50 values of rhein for KB, hepatoma BEL-7402 and mammary carcinoma MCF-7 cells were 11.5 μg/ml, 14.0 μg/ml and 18.4 μg/ml, respectively (30) and 25 μM for Ca Ski cells (17). Apparently, IC50 values of rhein are dependent on cell lines.

Dietary antioxidants may play a role in potentiating the effects of certain antitumor agents including anthraquinone derivatives (31). Therefore, we also examined the ROS in A-549 cells after treatment with rhein and results showed that rhein quickly induced ROS production, but 8 hours later levels returned to normal levels. We also added vitamin C with rhein to the cells and saw a significant increase of cell viability (data not shown). This is in agreement with other reports that co-treatment of breast cancer cells with both anthraquinones and vitamins C and E generally resulted in a significant increase in cell viability. Similar effects have been found when cancer cells were treated with paclitaxel and carboplatin and an antioxidant vitamin mixture containing vitamins C and E and β-carotene (32). These data indicate that anthraquinones might play various redox reaction roles among different cancer cell lines.

Incubation of A-549 cells with rhein led to G0/G1 arrest and apoptosis (sub-G1 group), which appeared to be mediated by a mitochondria-dependent and caspase-3 activation pathway in a dose- and time-dependent fashion. Other investigators showed that rhein induced apoptosis in human colonic adenocarcinoma cell monolayer (15) and human promyelocytic leukemia cells (HL-60) (16) and our previous studies also had similar results in Ca Ski cells (17).

The root of rhubarb has been used at an average daily dosage of 2-20 g of powered root for stomach cancer, leukemia and liver cancer in the Chinese population (15, 33). Anthraquinone comprise 3-12% of rhubarb root, 60-80% of which include rhein, emodin, and aloe-emodin (equivalent of approximate 400 μM in blood concentration). Although many experiments demonstrated that emodin and aloe-emodin induced cell cycle arrest and apoptosis in human and animal cancer cell lines, there are no studies that show rhein affecting the A-549 human lung cancer cell line. In the present study, 50 μM rhein induced apoptosis at 12, 24, 48 and 72 hours in A-549 cells in a dose- and time-dependent manner. This concentration can be obtained from the daily therapeutic dosage of rhubarb root (25-100 μM). The induction of apoptosis and cell cycle arrest by rhein in animal model in vivo for lung cancer needs to be investigated.
Our data showed that rhein induced G0/G1 phase arrest in A-549 cells and this is in agreement with other investigators who demonstrated that rhein blocked cell cycle progression in the G1-phase of human hepatoblastoma G2 (Hep G2) cell line through the significantly increased expressions of p53 and p21/WAF1 protein (34). However, the further check point enzymes that may or may not be affected by rhein are not known. Our previous studies showed that rhein induced G0/G1 arrest in human cervical cell through a caspase-dependent and mitochondria-dependent pathway (17). Currently our data showed that rhein induced the expression of p53 and p21 and inhibited the expressions of Cdk2, 4 and 6 and cyclin E that led to G0/G1 arrest in A-549 cells.

It is well known that caspases play critical roles in the initiation of apoptosis. Two major apoptosis pathways, caspase-dependent and -independent, are well established. Caspases can be grouped into apoptotic initiators, such as caspase-8, and apoptotic effectors, such as caspase-3, based on their substrate specificities and target proteins (35). We showed rhein induced activation of caspase-3, promoted Bax expression and reduced Bcl-2 expression in A-549 cells. We also showed that adding the caspase-3 inhibitor (z-DEVD-fmk), thus inhibiting rhein-induced caspase-3 activity, led to a decrease of apoptosis in these cells.

The mitochondrion plays an important role in the regulation of apoptosis via mitochondria-dependent and -independent pathways (36, 37). The characters of mitochondrial dysfunctions are the loss of ΔΨm, permeability transition and release of cytochrome c from the mitochondrion into the cytosol, and these are associated with apoptosis (38). In these studies, rhein induced the rapid loss of ΔΨm, the release of cytochrome c and promoted capase-3 activity in A-549 cells. Western blot also showed that rhein increased Bax protein which is a pro-apoptotic protein associated with apoptosis. Our data showed that rhein also reduced Bcl-2 protein which is an anti-apoptotic protein. Caspase-8 can be activated either by cytokines or chemicals that in turn induce Bid cleavage. Cleaved Bid causes cytochrome c efflux from mitochondria leading to caspase-3 activation and PARP cleavage, which in turn results in apoptosis (39, 40). Our data also showed that rhein induced Ca2+ production and pre-treatment with a Ca2+ chelator (BAPTA) led to a decrease in the levels of Ca2+ and ΔΨm in A-549 cells. After pre-treating A-549 cells with BAPTA for 3 hours and adding rhein to the cells, the levels of ΔΨm and caspase-3 activation decreased, and the percentage of apoptosis also decreased. Apparently, Ca2+ plays an important role in rhein-induced apoptosis in A-549 cells.

A number of chemopreventive agents had been shown to induce apoptosis in part with the generation of ROS and the disruption of redox homeostasis (38, 41). Our data showed that rhein induced ROS production up to 15 minutes treatment, before decreasing so that by six hours, there was only ~20% left. By 8 hours’ treatment, the ROS level was close to that of the control group. The ROS production may lead to ER stress and DNA damage causing p53 levels to increase in A-549 cells. We used confocal microscopy to show rhein increased the levels of GADD153 and GRP78, a hallmark of ER stress. When the antioxidant catalase was added to the rhein-treated cells, the results showed lower ROS production and also lower rhein-induced apoptosis (data not shown).

In conclusion, this is the first report that rhein induced G0/G1-phase arrest and induced apoptosis in A549 human lung cancer cells. The proposed flow chart of rhein-induced G0/G1 arrest and apoptosis is shown in Figure 7. Rhein appears to induce G0/G1 arrest by the inhibition of Cdk2, 4 and 6 and cyclin E, but promotes the expressions of p53 and p21. It also induces apoptosis through increasing the level of Bax and decreasing the level of Bcl-2, before decreasing the levels of ΔΨm. Caspase-3 activation then occurs and ROS and Ca2+ levels are also involved in this process in these cells. These findings may offer some information regarding the probable therapeutic function of rhein in human lung cancer.

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