Cancer Chemother Pharmacol (2005) 55: 531–540
DOI 10.1007/s00280-004-0919-6

ORIGINAL ARTICLE

Chi-Chih Cheng · Shu-Mei Yang · Chi-Ying Huang
Jung-Chou Chen · Wei-Mao Chang · Shih-Lan Hsu

Molecular mechanisms of ginsenoside Rh2-mediated G_1 growth arrest and apoptosis in human lung adenocarcinoma A549 cells

Received: 20 March 2004 / Accepted: 26 May 2004 / Published online: 1 March 2005

Abstract Ginsenoside Rh2 (Rh2), a purified ginseng saponin, has been shown to have antiproliferative effects in certain cancer cell types. However, the molecular mechanisms of Rh2 on cell growth and death have not been fully clarified. In this study, the antiproliferative effect of Rh2 in human lung adenocarcinoma A549 cells was investigated. Treatment of A549 cells with 30 μg/ml Rh2 resulted in G_1 phase arrest, followed by progression to apoptosis. This Rh2-mediated G_1 arrest was accompanied by downregulation of the protein levels and kinase activities of cyclin-D1, cyclin-E and Cdk6, and the upregulation of pRb2/p130. In addition, Rh2-induced apoptosis was confirmed by TUNEL assay and DNA fragmentation analysis. Administration of Rh2 caused an increase in the expression levels of TRAIL-RI (DR4) death receptor but did not alter the levels of other death receptors or Bcl-2 family molecules. Furthermore, the Rh2-induced apoptosis was significantly inhibited by DR4:Fc fusion protein, which inhibits TRAIL-DR4-mediated apoptosis. In addition, caspase-2, caspase-3 and caspase-8 were highly activated upon Rh2 treatment. Inhibitors of caspase-2, caspase-3 and caspase-8 markedly prevented the cell death induced by Rh2. Inhibitor of caspase-8 significantly inhibited the activation of caspase-2, caspase-3 and caspase-8. These observations indicate that multiple G_1-related cell cycle regulatory proteins are regulated by Rh2 and contribute to Rh2-induced G_1 growth arrest. The increase in the expression level of DR4 death receptor may play a critical role in the initiation of Rh2-triggered apoptosis, and the activation of the caspase-8/caspase-3 cascade acts as the executioner of the Rh2-induced death process.

Keywords Apoptosis · Caspase · Cyclin · Ginsenoside Rh2 · pRb2/p130 · TRAIL-RI

Abbreviations Adv: Adenovirus · Cdk: Cycline-dependent kinase · Rb: Retinoblastoma · Rh2: Ginsenoside Rh2 · TRAIL: TNF-related apoptosis-inducing ligand · TUNEL: Terminal transferase-mediated dUTP-fluorescein nick end-labelling · z-VDVAD-fmk: z-Val-Asp-Val-Ala-Asp-fluoromethyl ketone · z-DEVD-fmk: z-Asp-Glu-Val-Asp-fluoromethyl ketone · z-IETD-fmk: z-Ile-Glu-Thr-Asp-fluoromethyl ketone · z-LEHD-fmk: z-Leu-Glu-His-Asp-fluoromethyl ketone

Introduction

Lung cancer is widespread in the world, and has been reported to be the leading cause of cancer death in females and the second highest cause of cancer death in males in Taiwan [1]. However, many patients are found to have advanced cancer at the time of diagnosis, and are treated with chemotherapy, which often has severe side effects and other limitations. This prompted us to search for novel effective therapeutic compounds for lung cancer treatment. Recently, several Chinese medicines have been used to treat cancers, but the potent active compound(s) and their mode of action at the cellular and molecular levels are largely undefined. Ginsenoside Rh2 is one such potent compound; it is

C.-C. Cheng · S.-L. Hsu (✉)
Department of Education and Research,
Taichung Veterans General Hospital, Taichung, 407, Taiwan
E-mail: h2326@vghtc.gov.tw
Tel.: +886-4-23592525
Fax: +886-4-23592705

S.-M. Yang · J.-C. Chen · S.-L. Hsu
Institute of Chinese Medical Science,
China Medical University, Taichung, Taiwan

C.-Y. Huang
Division of Molecular and Genomic Medicine,
National Health Research Institutes, Taipei, Taiwan

W.-M. Chang
Savelife Bioscience Company, Taichung, Taiwan

isolated from the root of *Panax ginseng* and has been shown to have anticancer effects [2, 3] and to be capable of inhibiting the growth and inducing apoptosis in mammalian tumor cells [4, 5]. It has been reported that Rh2 blocks cell proliferation and causes G_1 phase arrest in several tumor cells [6–9].

The cell cycle in eukaryotes is regulated through a precise balance of positive and negative regulatory components that exert their effects during the G_1 phase. The most critical positive-acting components are G_1 cyclins (cyclin-D and cyclin-E) and cyclin-dependent kinases (Cdks) [10, 11]. In addition, some Cdk inhibitors (CdkI) have been identified, such as INK and CIP/KIP family molecules. Several studies have shown the effect of Rh2 on cell cycle-regulatory molecules, such as cyclins, Cdks and CdkIs. A previous study has indicated that the expression of cyclin-E protein is decreased by Rh2 [8]. Ota et al. have demonstrated that Rh2-induced G_1 phase arrest is associated with a reduction in Cdk2 kinase activity in B16 murine melanoma cells [9]. Another study has shown that Rh2 induces apoptosis by reducing protein expression of cyclin-D and $p21^{CIP1}$, downregulating cyclin/Cdk complex kinase activity and decreasing phosphorylation of pRb [4].

In spite of modulation of cell cycle regulatory molecules, apoptosis-mediated molecules are also important in the determination of the Rh2-mediated antitumor effect. Apoptosis is a fundamental form of programmed cell death characterized by a series of morphological features, such as chromatin condensation, cell shrinkage, nuclear fragmentation and formation of apoptotic bodies, and is changed by biochemical features, such as expression of Bcl-2 family proteins, increasing death receptors and activation of caspase cascades [12]. Proteins of the Bcl-2 family may act as a rheostat of apoptosis by their ligands, lead to recruitment of adaptor proteins and activation of the downstream caspases [13]. Not only death receptors, but also molecules associated with mitochondria could induce apoptosis by associating with and activating the caspase cascades [14, 15].

Accumulating evidence indicates that members of the Bcl-2 family [16, 17] and caspase cascades can be regarded as regulators of the apoptotic response [16, 18–20]. Several previous studies have demonstrated that Rh2 can induce apoptotic cell death in MCF-7 human breast cancer cells [4], human neuroblastoma SK-N-BE(2) cells [21], SK-Hep-1 human hepatoma cells [19], human leukemia THP-1 cells [5] and A375-S2 human malignant melanoma cells [20]. Thus far, the cellular and molecular events involved in Rh2-induced apoptotic process have not been fully characterized.

In this study, human lung adenocarcinoma A549 cells were used to explore the effector mechanism of the antiproliferative effects induced by Rh2. We found that Rh2-mediated G_1 phase arrest was accompanied by a decrease in cyclin-D1, cyclin-E, and Cdk6, and an increase in pRb2/p130 protein. Moreover, we showed that Rh2-induced apoptosis occurs via a TRAIL-RI (DR4) death receptor and caspase-dependent pathway in human adenocarcinoma A549 cells.

Materials and methods

Reagents

Rh2 was provided by Dr. W.-M. Chang (Savelife Bioscience Company, Taichung, Taiwan). The purity of the Rh2 was more than 95%; its chemical structure is shown in Fig. 1. The drug was dissolved in a DMSO–ethanol solution (1:5 DMSO/100% ethanol). Anti-cyclin-D1, anti-cyclin-D3, anti-Cdk2, anti-Cdk4, anti-Fas and, anti-pRb1 and anti-pRb2 anti-Bcl-X_L antibodies were purchased from Transduction Laboratories (Lexington, Ky.). Anti-cyclin-A, anti-cyclin-E, anti-FasL, anti-DR4, anti-TNFR I, anti-TNFR II and anti-Bak antibodies were purchased from BD-Pharmingen (San Diego, Calif.). Anti-Cdk6, anti-$p15^{INK4B}$, anti-$p16^{INK4A}$, anti-$p27^{KIP1}$, anti-Bcl-2, anti-Bad, anti-Bak, anti-Bid and anti-Mcl-1 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, Calif.). Anti-$p21^{CIP1/WAF1}$ and anti-p53 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-TRAIL-RII (DR5) antibody was obtained from Chemicon International (Temecula, Calif.). Propidium iodide (PI) was purchased from Sigma Chemical Company (St. Louis, Mo.). The TUNEL assay kit was obtained from Boehringer Mannheim (Mannheim, Germany). Caspase activity assay kits were purchased from R&D Systems (Minneapolis, Minn.). Caspase-2 inhibitor (z-VDVAD-fmk), caspase-3 inhibitor (z-DEVD-fmk), caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEHD-fmk), broad-spectrum caspase inhibitor (z-VAD-fmk), and DR4:Fc (an inhibitor of DR4 death signal) were obtained from Kamiya Biomedical Company (Seattle, Wash.).

Cell culture and cytotoxicity assay

Cells of the human lung cancer cell lines A549 and CH27, and the human fibroblast-like lung cell line WI-38, were cultured in RPMI 1640 medium, and of the lung cancer cell line H460 were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 m *M* glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), at 37°C in a humidified

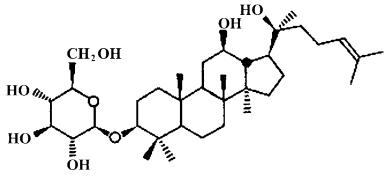

Fig. 1 Chemical structure of Rh2

atmosphere containing 5% CO_2. The medium was changed every 2 days. Rat hepatic stellate cells and rat heart endothelial cells were isolated as described previously [22, 23]. For cell viability assay, cells were seeded in 12-well plates at a density of 3×10^4 cells per well. After 24 h incubation, cells were treated with various concentrations of Rh2 for the indicated times. Some cells were pretreated with the caspase inhibitors z-VDVAD-fmk, z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk or z-VAD-fmk for 2 h prior to treatment with 30 μg/ml Rh2. The control cultures were treated with the vehicle (0.1% DMSO/ethanol mixture). Other cells were infected with recombinant Bcl-2-adenoviral and control adenoviral vectors for 16 h prior to exposure to Rh2. After treatment, cell viability was assessed by the trypan blue dye exclusion method and the cells were counted using a hemocytometer.

Cdk kinase activity assay

Cell lysates (300 μg total protein) were immunoprecipitated with anti-Cdk2 or anti-Cdk6 antibodies in the presence of 20 μl protein A Sepharose beads, then rotated at 4°C overnight and washed twice with kinase buffer. Histone H1 and Rb kinase activities were determined as described previously [24].

Apoptotic cell detection

A549 cells were treated without or with 30 μg/ml Rh2 for the indicated times. Control cultures were treated with the vehicle. After treatment, cells were washed with cold PBS and fixed in 2% paraformaldehyde at room temperature for 30 min. The cells were then permeabilized with 0.1% Triton X-100/PBS solution at room temperature for another 30 min. Morphological changes were observed under a fluorescence microscope. For the determination of apoptotic cells, the cells were washed and fixed. The TUNEL assay was then performed according to the manufacturer's instructions (Boehringer Mannheim). Labelled cells were examined using a fluorescence microscope. TUNEL-positive cells were counted as apoptotic cells.

DNA fragmentation assay

A549 cells were treated without or with 30 μg/ml Rh2 for 72 h then lysed in extraction buffer (50 m *M* Tris, pH 7.5, 10 m *M* EDTA and 0.3% Triton X-100) for 30 min on ice. Cell lysates were treated with RNase A (100 μg/ml) for 30 min at 55°C, and then with proteinase K (400 μg/ml) for 1 h at 55°C. The supernatant was extracted with phenol/chloroform. The DNA was precipitated and electrophoresed on a 2% agarose gel.

Flow cytometry

Cells (1×10^6 cells) were centrifuged at 1200 rpm for 10 min, and the cell pellets were fixed with 70% ethanol for 4 h. The cells were washed twice with PBS and resuspended in 1 ml of a solution containing 3.4 m *M* sodium citrate, 20 μg/ml propidium iodide, and 100 μg/ml RNase A, and stored in the dark at 37°C for 30 min. Cells were analyzed using a FACS-Calibur flow cytometer (Becton Dickinson, Mountain View, Calif.)

Protein preparation and Western blot analysis

A549 cells were treated without or with 30 μg/ml Rh2 for indicated time points. After treatment, both adherent and floating cells were harvested, total proteins were extracted as described elsewhere [24]. Protein concentration was determined using the Bradford method. For Western blot analysis, equal amounts of protein were loaded and separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were equilibrated in transfer buffer (50 m *M* Tris, pH 9.0–9.4, 40 m *M* glycine, 0.375% SDS, 20% methanol) and electrophoretically transferred to PVDF membrane (Millipore, Billerica, Mass.). The membrane was blocked with 5% nonfat dried milk in TBST buffer (20 m *M* Tris-HCl, pH 7.4, 150 m *M* NaCl and 0.1% Tween 20) and incubated overnight at 4°C with specific primary antibodies. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, and proteins were determined using an enhanced chemiluminescence detection kit (Amersham Life Science, Arlington Heights, Ill.)

Caspase activity assay

A549 cells were treated without or with 30 μg/ml Rh2 for the indicated times. Cells were lysed in lysis buffer (1% Triton X-100, 0.32 *M* sucrose, 5 m *M* EDTA, 10 m *M* Tris-HCl, pH 8, 2 m *M* dithiothreitol, 1 m *M* PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) for 20 min at 4°C and centrifuged at 10,000 *g* for 30 min. Caspase activity was determined according to the manufacturer's protocol (R&D Systems).

Statistical analysis

Figures were obtained from at least four independent experiments with similar patterns. All data are presented as the means ± SD of nine replicates from three separate experiments. The significance of differences were evaluated using Student's *t*-test, with the levels of significance at $P<0.05$, $P<0.01$ and $P<0.001$.

Results

Antiproliferative effect of Rh2

Treatment of A549, CH27 and H460 human lung cancer cells with Rh2 resulted in a dose-dependent and time-dependent antiproliferative effect (Fig. 2a). At 30 μg/ml Rh2, cell growth inhibition was observed within 24 h, followed by progression to cell death (Fig. 2a). Under the phase contrast microscope Rh2-treated cells exhibited a rounded and granulated morphology, and eventually detached from culture plates after 72 h of treatment (Fig. 2b). Moreover, WI-38 cells, rat heart endothelial cells and rat hepatic stellate cells were more resistant to the Rh2-mediated cytotoxicity than lung cancer cells (Fig. 2c).

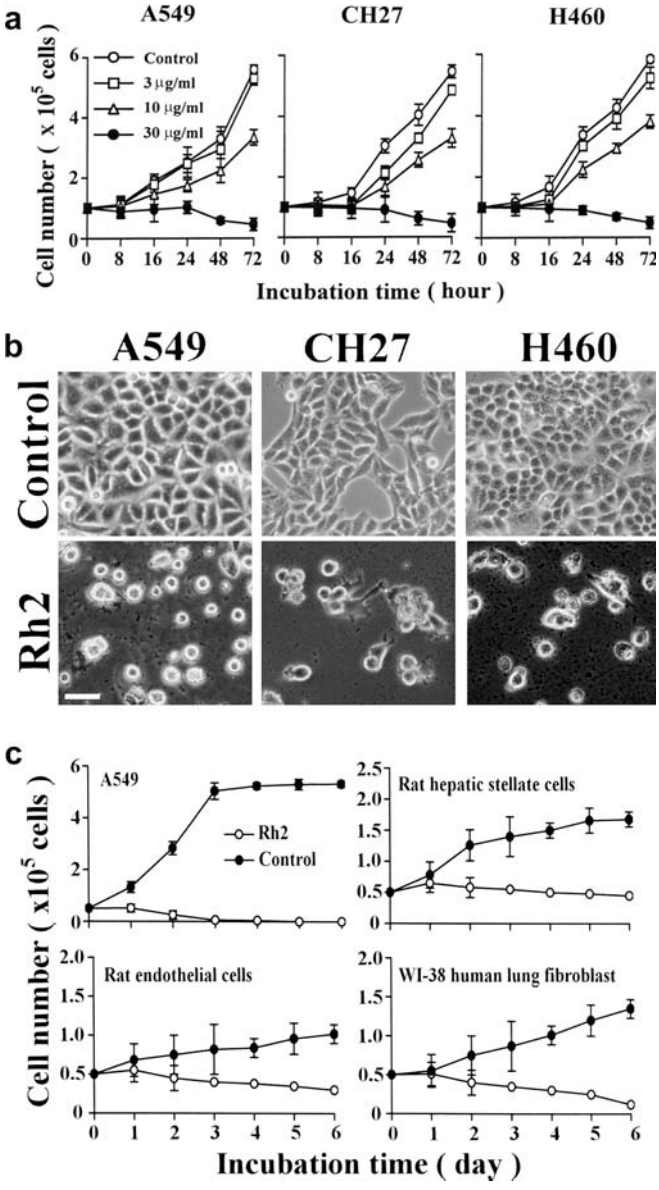

Fig. 2 Antiproliferative effect of Rh2. **a** Dose-dependent and time-dependent response: human lung cancer cells A549, CH27 and H460 were treated with a series concentrations (0, 3, 10 and 30 μg/ml) of Rh2 for 0, 8, 16, 24, 48 and 72 h. Cell viability was estimated using direct counting of cells by the trypan blue dye exclusion method. **b** Morphological changes: A549, CH27 and H460 cells were treated with or without 30 μg/ml Rh2. Phase contrast micrographs are shown for 48 h of treatment (×100, *scale bar* 20 μm). **c** Effect of Rh2 on normal cells. A549, WI-38 human lung fibroblast, rat heart endothelial cells and rat hepatic stellate cells were treated with or without 30 μg/ml Rh2 for 1, 2, 3, 4, 5 and 6 days. Cell viability was determined as previously described

Effect of Rh2 on cell cycle distribution

To examine the effect of Rh2 on cell cycle progression, cells were untreated or treated with Rh2 for 16, 24 or 48 h and the cell cycle distribution was analyzed by flow cytometry. As shown in Fig. 3, treatment with Rh2 resulted in accumulation of cells in G_1 phase, the G_1 blockage peaking at 16 h, with approximately $75.6 \pm 2.1\%$ of cells in G_1 at that time compared to $58.4 \pm 1.5\%$ in control cultures.

Regulation of cell cycle-related proteins by Rh2

To determine the molecular mechanism of Rh2-mediated G_1 phase arrest, we examined the status of G_1-regulatory proteins in A549 cells after Rh2 treatment. Cells were untreated or treated with 30 μg/ml Rh2 and protein extracts analyzed by immunoblot assay. As shown in Fig. 4a, after 16 h of Rh2 treatment, A549 cells showed a significant decrease in the levels of cyclin-D1, cyclin-E, and Cdk6 proteins in comparison with controls. The levels of $p21^{CIP1/WAF1}$ and p53 proteins were slightly downregulated (Fig. 4b). However, the increase of pRb2/p130 protein was detected by Rh2 treatment (Fig. 4b). The expression levels of cyclin-D3, cyclin-A, Cdk4, Cdk2, p15, p16, p27 and pRb1 were not altered upon Rh2 treatment. In addition, Rh2 treatment also caused a downregulation of cyclin-D1 and Cdk6, and an upregulation of pRb2/p130, in a concentration-dependent manner (Fig. 4c).

To define whether the Rh2-induced decreases in Cdk2, Cdk4, and Cdk6 protein levels were associated with changes in the kinase activities of various cyclin-Cdk complexes, in vitro kinase assay were performed. Histone H1 or a recombinant pRb fragment was used as substrate in immunoprecipitation experiments performed with antibodies against Cdk2, Cdk6, cyclin-D1 and cyclin-E. As depicted in Fig. 4d, treatment of A549 cells with 30 μg/ml Rh2 for 16 h and 24 h resulted in a marked decrease in kinase activities, indicating the Rh2-mediated decrease in Cdk6, cyclin-D1 and cyclin-E protein levels was consistent with a reduction in their kinase activities. These results indicate that decreases in the levels and activities of cyclin-D1, cyclin-E, and Cdk6, and an increase in pRb2/p130 may be involved in Rh2-mediated G_1 growth arrest.

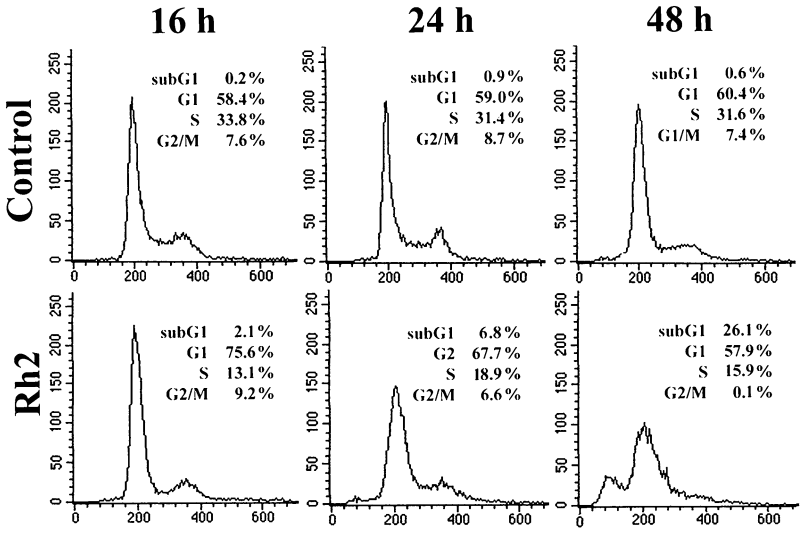

Fig. 3 Induction of G_1 phase arrest by Rh2. Cells were treated with or without 30 μg/ml Rh2 for 16, 24 and 48 h. After treatment, cells were fixed and stained with propidium iodide, and the cell cycle distribution was determined by flow cytometry

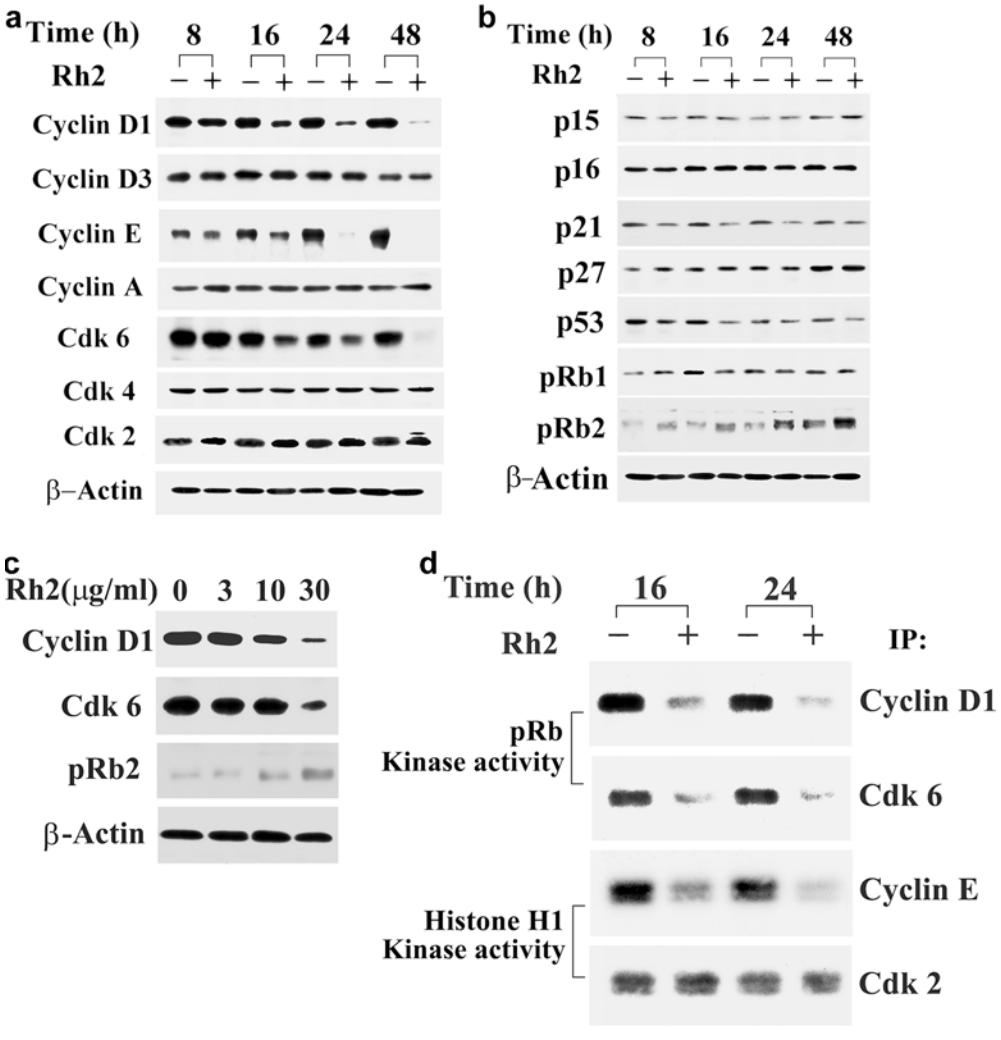

Fig. 4 Regulation of cell cycle-related molecules by Rh2. **a** Expression of Cdk and cyclin proteins. Western blot analysis of lysates from untreated and Rh2-treated A549 cells tested with antibodies to cyclin-D1, cyclin-D3, cyclin-E, cyclin-A, Cdk4, Cdk6 and Cdk2. **b** Expression of CdkI, p53 and pRb proteins. Western blot analysis of lysates from untreated and Rh2-treated A549 cells tested with antibodies to $p15^{INK4B}$, $p16^{INK4A}$, $p21^{CIP1/WAFì}$, $p27^{KIP1}$, p53, pRb1 and pRb2/p130. Blots were reprobed for *β*-actin to normalize each lane for protein content. **c** Concentration-dependent effect. A549 cells were treated with various concentrations (0, 3, 10 and 30 μg/ml) of Rh2 for 16 h. Western blot analysis was performed as described above. **d** In vitro kinase activity assay. Cyclin-D1, cyclin-E, Cdk6 and Cdk2 immunoprecipitates (*IP*) from control and Rh2-treated cell extracts were assayed for kinase activity using Rb fragment and histone H1 as substrate, respectively

Induction of apoptosis by Rh2 in A549 cells

As shown in Fig. 2b, treatment with 30 μg/ml Rh2 for 48–72 h resulted in the morphological features of apoptosis, rounded morphology and eventually detachment from the substratum. Flow cytometry analysis showed the presence of a marked sub-G_1 population (apoptotic cells) after 48 h of Rh2 treatment (Fig. 3). To obtain further support for the induction of apoptotic cell death by Rh2 in A549 cells, the in situ TUNEL assay and DNA fragmentation assay were performed. TUNEL-positive cells (Fig. 5a) and internucleosomal DNA fragments (Fig. 5b) were detected after 72 h of Rh2 treatment. However, in control cultures neither green fluorescent apoptotic cells nor ladder DNA fragments were observed throughout the 72-h incubation time. These results suggest that Rh2 indeed triggers cell death via an apoptotic process.

Effect of Rh2 on Bcl-2 family proteins

The Bcl-2 family proteins are important regulators in both the inhibition and the promotion of apoptosis [25]. To elucidate whether Bcl-2 family molecules were involved in Rh2-induced apoptosis, we examined the expression of several members of this family by Western blot analysis. The levels of apoptotic Bcl-2-related proteins, such as Bax, Bak, Bid, and Bad, and the antiapoptotic proteins Bcl-2, Bcl-X_L and Mcl-1, were unaffected by treatment with Rh2 (data not shown). To examine whether overexpression of Bcl-2 is associated with an antiapoptotic or a proapoptotic effect on Rh2-treated cells, we infected A549 cells with Bcl-2-adenoviral and control adenoviral vectors at a multiplicity of infection (moi) of 50. The expression of Bcl-2 protein was analyzed by Western blot analysis and the resulting cytotoxicity was determined by cell counting. The expression level of Bcl-2 protein was not significantly deferent between adenovirus infected and untreated control cultures (data not shown). However, infection with adeno-Bcl-2 vector resulted in a threefold increase in Bcl-2 expression. Neither control adenoviral nor Bcl-2-adenoviral infection could protect against Rh2-triggered apoptosis (data not shown). These results demonstrate that Bcl-2 family molecules might not be involved in Rh2-induced apoptosis.

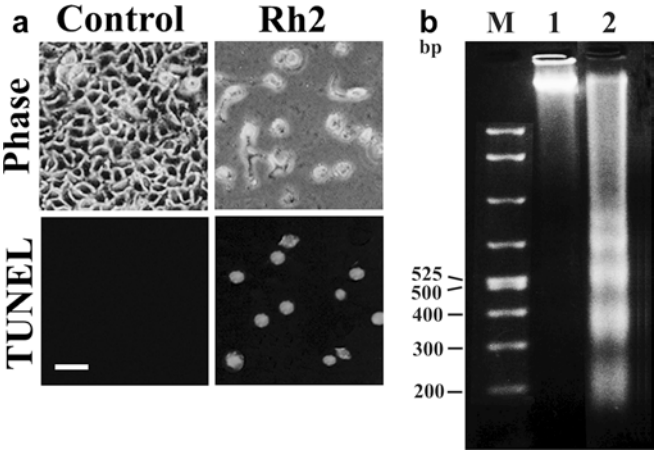

Fig. 5 Induction of apoptotic death by Rh2. **a** In situ TUNEL assay. A549 cells were treated with 30 μg/ml Rh2 for 48 h. The TUNEL assay was performed according to the manufacturer's protocol. TUNEL-positive cells were counted as apoptotic cells. **b** DNA fragmentation analysis. Treatment of A549 cells without or with 30 μg/ml Rh2 for 48 h. DNA was isolated and separated on 2% agarose gel (*lane M* DNA size marker, *lane 1* control culture, *lane 2* Rh2 treatment)

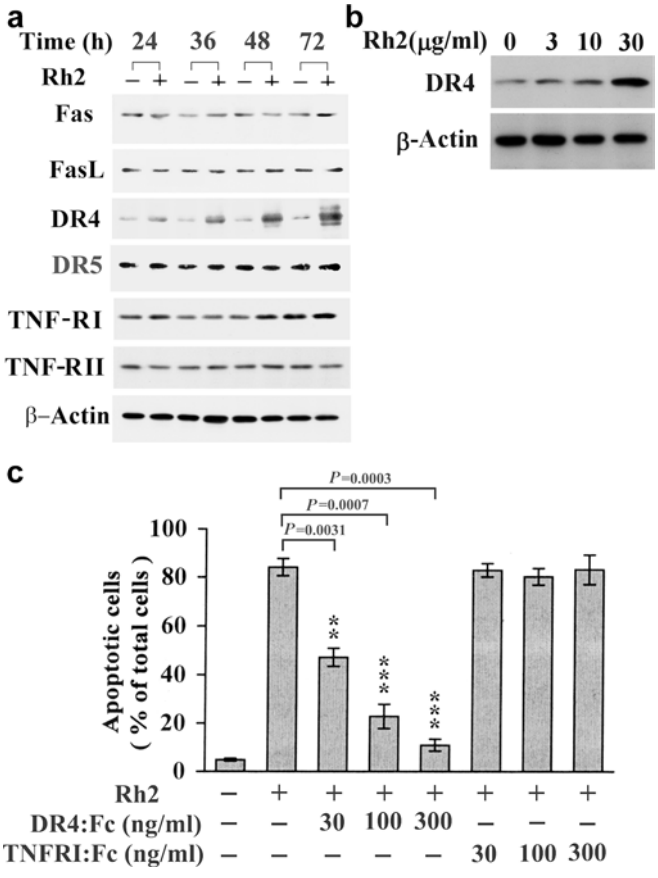

Fig. 6 DR4 signalling is involved in the Rh2-triggered death process. **a** Expression of death receptors. A549 cells were treated without or with 30 μg/ml Rh2 for 24, 36, 48 and 72 h. After treatment, Western blot analysis was performed using anti-Fas, anti-FasL, anti-DR4, anti-DR5, anti-TNF-RI and anti-TNF-RII antibodies. To confirm equal loading, *β*-actin was used as an internal loading control. **b** Concentration-dependent effect. Cells were treated with various concentrations (0, 3, 10 and 30 μg/ml) of Rh2 for 48 h. Western blot analysis was performed as above described. **c** The effect of DR4:Fc (an inhibitor of DR4 death signalling) on Rh2-induced apoptosis. Cotreatment of A549 cells with Rh2 and DR4:Fc or TNFR1:Fc for 72 h; apoptotic cells were estimated by the TUNEL assay

Rh2-induced apoptotic cell death via a DR4 death receptor pathway

To determine whether the expression levels of death receptors were regulated by Rh2, Western blot analysis was performed using the anti-Fas, anti-FasL, anti-DR4, anti-DR5, anti-TNFRI and anti-TNFRII antibodies. As indicated in Fig. 6a, exposure of A549 cells to 30 μg/ml Rh2 caused a significant increase in DR4 protein expression. However, the levels of Fas, FasL, DR5, TNF-RI and TNF-RII proteins were unaffected by Rh2 treatment. Treatment of A549 cells with various concentrations of Rh2 for 48 h resulted in a marked increase in the level of DR4 protein only at 30 μg/ml Rh2 (Fig. 6b). To determine if upregulation of DR4 plays a role in Rh2-induced apoptosis, a fusion protein having the DR4 extracellular domain fused to the immunoglobulin Fc region (DR4:Fc, used as a DR4 death signalling inhibitor) was added to the culture. As shown in Fig. 6c, treatment with DR4:Fc caused a dose-dependent prevention of Rh2-triggered cell death, administration of 300 ng/ml DR4:Fc almost completely blocked Rh2-induced cell death. As a negative control, TNFR1:Fc, an inhibitor of TNFR1-death signalling, failed to block Rh2-triggered apoptosis (Fig. 6c). These results suggest that activation of DR4 signalling is required for Rh2-mediated apoptosis.

Involvement of caspase activation in Rh2-induced apoptosis

It is well documented that the caspase family plays an important role in triggering the apoptotic process [26]. To determine whether the caspase cascade is activated during Rh2-induced apoptotic process, the specific caspase activity was measured using fluorogenic peptide substrates. As depicted in Fig. 7a, Rh2 caused a significant increase in caspase-2, caspase-3 and caspase-8 specific activities. In contrast, the activities of caspase-6 and caspase-9 in A549 cells were not altered by Rh2 treatment. To clarify whether activation of caspase was needed for induction of apoptotic death triggered by Rh2, A549 cells were cotreated with caspase inhibitor and Rh2. As shown in Fig. 7b, the broad-spectrum caspase inhibitor z-VAD-fmk almost totally abolished Rh2-triggered apoptotic death. Administration of the caspase-2 inhibitor z-VDVAD-fmk, the caspase-3 inhibitor z-DEVD-fmk, and the caspase-8 inhibitor z-IETD-fmk drastically inhibited Rh2-induced cell death. At 100 μM, the inhibitor of caspase-2, caspase-3 and caspase-8 restored cell viability by 30, 48 and 65%, respectively (Fig. 7b). These observations indicate that caspase-2, caspase-3 and caspase-8 are integrally involved in Rh2-triggered apoptosis. Accumulating evidence indicates that the major action pathway of DR4 proceeds through the activation of caspase-8. To further examine the role of caspase-8 in Rh2-induced apoptosis, the activity of caspase-2, caspase-3, and caspase-8 in the cell lysates after Rh2 treatment in the presence of the caspase-8 inhibitor was examined. As depicted in Fig. 7c, caspase-8 inhibitor significantly inhibited the activation of all three caspases in Rh2-treated A549 cells. These observations suggest that caspase-8 could act as an upstream initiator to activate caspase-2 and caspase-3 in Rh2-triggered apoptotic pathway.

Discussion

Rh2, a triterpene saponin, is a bioactive component from the root of *P. ginseng*, which has been shown to have antiproliferative activity against human hepatoma cells [19], HeLa cells [27] and mouse B16 melanoma cells [28]. Previous studies have demonstrated that Rh2 has numerous effects on tumor cells including cell cycle arrest [6, 9, 29, 30], promoting cell differentiation [7, 30], and increasing apoptosis [5, 16, 17, 19–21, 31, 32]. In this study, we observed prominent G_1 arrest of A549 cells after exposure to 30 μg/ml of Rh2. This result is consistent with that of other investigators who have shown Rh2-induced G_1 arrest in several types of human cancer cell lines [6, 8, 9, 30].

Cell cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of the cell cycle-regulatory proteins (including cyclins, Cdks, CdkIs, p53 and pRb) provides an important mechanism for inhibition of growth [33]. In mammalian, distinct cyclins are associated with and activate different Cdks throughout the cell cycle. During progression through the G_1 phase of the cell cycle two major types of cyclins (cyclin-D and cyclin-E) and three types of Cdks (Cdk4, Cdk6 and Cdk2) are required. D cyclins (D1, D2 and D3) bind Cdk4/Cdk6 to regulate cell cycle progression through mid-G_1. Cyclin-E bind Cdk2 in late G_1 and its activity is rate-limiting for progression from G_1 to S phase [34]. In the present study, we showed that Rh2 strongly downregulated the expression of Cdk6, cyclin-D1 and cyclin-E proteins, reduced the levels of these G_1-related Cdks and cyclins in A549 cells, and may facilitate cell cycle blocking in mid-G_1 and the G_1/S border.

The retinoblastoma (Rb) gene products pRb, p107 and pRb2/p130 are negative regulators of the transition between the phases G_1 and S of the cell cycle [35]. Like the other members of the family, pRb2/p130 strongly inhibits the cell cycle in several human cancer cell lines [36]. Here, we demonstrated that Rh2-induced G_1 growth arrest was accompanied by upregulation of pRb2/p130, suggesting that pRb2/p130 may also be involved in Rh2-mediated G_1 blockade in A549 cells. It is well documented that pRb1 and pRb2 tumor suppressor genes are differentially expressed during embryogenesis [37]. Functional studies have indicated that the expression of the pRb2/p130 protein is tightly cell cycle regulated. pRb2/p130 is highly expressed in quiescent and differentiated cells, and its levels drop rapidly when quiescent cells are stimulated to enter the cell cycle [38,

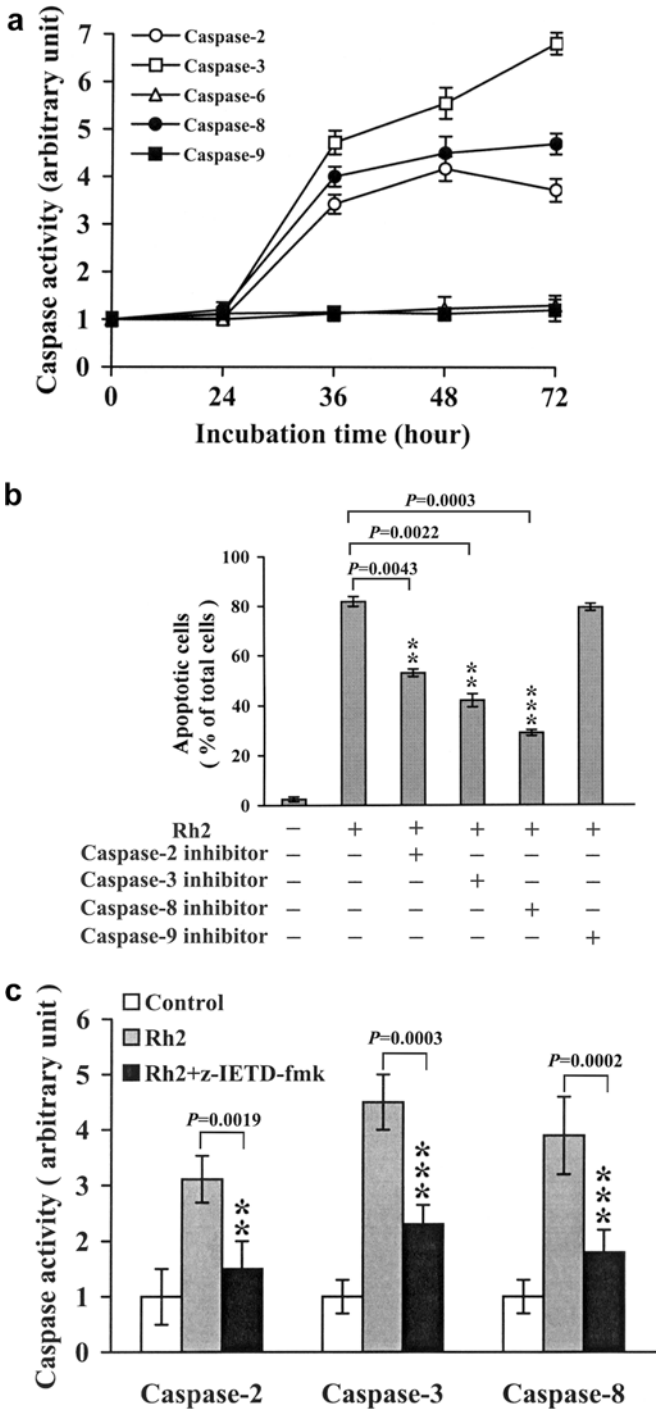

Fig. 7 Requirement for caspases in Rh2-induced apoptosis. **a** Extracts from untreated or Rh2-treated A549 cells were assayed for caspase-2, caspase-3, caspase-6, caspase-8 and caspase-9 activity using fluorogenic peptide substrates. **b** Blocking Rh2-induced apoptosis by caspase inhibitor. A549 cells were treated with 100 μM of each cell-permeable inhibitor of caspase-2 (z-VDVAD-fmk), caspase-3 (z-DEVD-fmk), caspase-8 (z-IETD-fmk), and caspase-9 (z-LEHD-fmk) 2 h prior to Rh2 treatment (30 μg/ml). Apoptotic cells were determined 72 h after treatment. **c** Blockade of activation of caspase-2, caspase-3, and caspase-8 by a caspase-8 inhibitor. Cells were treated with 30 μg/ml Rh2 and/or 100 μM caspase-8 inhibitor (Z-IETD-fmk) for 48 h. The activities of caspase-2, caspase-3, and caspase-8 in cell lysates were measured using their specific fluorogenic substrates

39]. In contrast, moderate levels of pRb can be found in most cell types as well as in both quiescent and cycling cells [38, 39]. Levels of pRb increase somewhat when quiescent cells begin to proliferate, only slightly decreasing when cells exit the cell cycle [38, 39]. At present, it is unclear which molecular pathway is involved in Rh2-induced upregulation of pRb2/p130. The precise mechanism of the Rh2-mediated increase in pRb2/p130 protein expression is the subject of ongoing research in our laboratory.

Another study has indicated that Rh2 blocks the cell cycle at the G_1/S boundary by inducing the protein expression of $p27^{KIP1}$ in human hepatoma SK-HEP-1 cells [8]. Ota et al. have demonstrated that Rh2-induced G_1 phase arrest is accompanied by suppression of Cdk2 kinase activity in cultured murine cells [9]. It has been reported that Rh2 mediates G_1 phase blockage in human breast cancer MCF7 cells by inducing protein expression of p21CIP1/WAF1 and reducing the protein levels of cyclin-D which results in a reduction of cyclin/Cdk complex kinase activity, decreasing phosphorylation of pRb [4]. The possible reasons for certain discrepancies between our observations and those of others may be due to differences in the cell types used and the assay conditions.

Accumulating evidence shows that Rh2 not only reduces cell proliferation but also induces cell death in tumor cells [5, 16, 17, 19–21, 31, 32]. In our experiments, Rh2 induced cell death at a concentration of 30 μg/ml after 48–72 h. It is interesting to note that human lung fibroblasts (WI-38 cell line), primary cultured rat heart endothelial cells and primary cultured rat hepatic stellate cells were more resistant to Rh2-mediated cytotoxicity at the same concentration (30 μg/ml) (Fig. 1c). In addition, human lung cancer cells treated with 30 μg/ml Rh2 exhibited features of apoptosis. In situ DNA fragmentation was detected by the TUNEL assay. A549 cells had a basal level of DNA breaks in the nucleus ($< 0.1\%$) but Rh2 treatment dramatically increased the number of TUNEL-positive cells. The fragments of 180–200 bp DNA ladders were also observed following Rh2 treatment by agarose gel electrophoretic assay. Similar to those of previous studies [5, 16, 17, 19–21, 31, 32], our data suggest that treatment with Rh2 induced apoptotic cell death in lung cancer A549 cells.

Many genes participate in the regulation of apoptosis. Activation of the caspase cascade is a central effector mechanism promoting apoptosis in response to death-inducing signals from cell surface receptors, from mitochondria or from endoplasmic reticulum stress [14]. Activation of caspases during Rh2-induced apoptosis has been detected in human hepatoma SK-HEP-1 cells [16, 32] and in rat C6 gliomal cells [31]. Fei et al. have reported that activation of caspase-8 and caspase-3 is involved in Rh2-induced apoptosis in human melanoma A375-S2 cells [20]. In this study, Rh2 induced the elevation of caspase-2, caspase-3 and caspase-8 proteolytic activities in human lung adenocarcinoma A549 cells. Treatment with the inhibitor of caspase-2, caspase-3 and

caspase-8 markedly attenuated Rh2-triggered apoptosis. Inhibitor of caspase-8 significantly prevented the activation of caspase-2, caspase-3, and caspase-8, indicating that caspase-8 acts as an upstream initiator of caspase-2 and caspase-3.

Moreover, our data demonstrate that Rh2 did not alter the expression of Fas/FasL, TNF-RI, TNF-RII or DR5 proteins, but markedly increased the level of DR4 protein. Treatment with DR4:Fc, which can inhibit TRAIL-DR4 death signalling, significantly prevented Rh2-triggered apoptosis. Our observations indicate that the upregulation of DR4 death receptor as well as activation of the caspase-8 cascade by Rh2 results in apoptosis in human lung adenocarcinoma A549 cells. DR4 is a type I transmembrane protein belonging to the cell surface death receptor family, and is activated by the common ligand TRAIL. DR4 contains a conserved cytoplasmic death domain responsible for recruiting adaptor proteins such as FADD to the receptor complex, and subsequently activates caspase-8 and downstream caspases, leading to the cleavage of death substrates and finally cell apoptosis [40]. Overexpression of DR4 leads to either ligand-dependent [41] or ligand-independent [42] apoptosis. It has been reported that some DNA-damaging agents are able to induce DR4 expression [43]. Other studies have shown that DR4 expression is regulated by p53 [44], NFkB [45], and AP-1 [46]. At present, the molecular mechanism involved in the upregulation of DR4 by Rh2 remains unclear and needs further exploration.

It is well documented that Bcl-2 family members play a regulatory role in apoptosis induced by several chemotherapeutic drugs. Previous studies have demonstrated that Bcl-2 family molecules are also essential for TRAIL-DR4/5 death receptor-induced apoptosis signalling [47–50]. However, at the concentrations used in this study, Rh2 did not alter the expression levels of Bcl-2, Bcl-X_L, Bax or Bak proteins. Moreover, overexpression of Bcl-2 protein in A549 cells by Adv-Bcl-2 vector infection did not block Rh2-induced apoptosis, suggesting that these Bcl-2 family members are not involved in the Rh2-mediated death process. Consistent with our observations, Rh2 has been shown to induce apoptosis independently of Bcl-2 family molecules in several cancer cell lines, such as human hepatoma SK-HEP-1 cells [16], rat gliomal C6 cells [31] and C6Bu-1 cells [17].

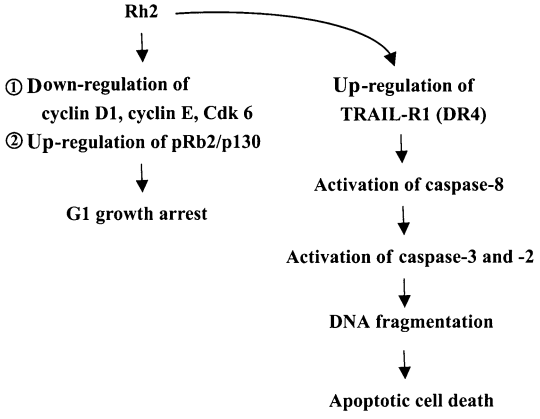

Fig. 8 Schematic diagram of the molecular events of Rh2-mediated antiproliferation in human lung adenocarcinoma A549 cells. Rh2 induces G_1 growth arrest by downregulation of cyclin-D, cyclin-E and Cdk-6, and upregulation of pRb2/p130. In addition, Rh2 triggers apoptosis via a caspase-8 activation pathway dependent on TRAIL-RI (DR4)

In conclusion, our present observations provide a clear picture of the molecular ordering of Rh2-induced antiproliferative events in lung adenocarcinoma A549 cells (Fig. 8). Rh2 arrests the cell cycle at the G_1 phase by downregulation of the protein levels and functions of cyclin-D1, cyclin-E and Cdk6, and upregulation of pRb2/p130 protein. Furthermore, Rh2 upregulated the DR4 death receptor protein and then activated caspase-8 and caspase-3 cascade, consequently leading to apoptosis. Our findings provide the first evidence that Rh2 induces apoptosis of A549 cells through a DR4-dependent but Bcl-2-independent activation of a caspase-8/caspase-3 pathway. Future studies focusing on cell signalling and the biological significance of Rh2-induced apoptosis would greatly extend our understanding of the mechanisms of chemotherapeutic potency of Rh2 in human cancers.

Acknowledgements This work is supported by grants from the Taichung Veterans General Hospital TCVGH-927312D and the National Science Council NSC 91-2311-B075A-001, Taiwan, ROC.

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