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Apoptotic effects of ginsenoside Rh_2 on human malignant melanoma A375-S2 cells[1]

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KEY WORDS ginseng; saponins; apoptosis; caspases; melanoma

ABSTRACT

AIM: To study the mechanism of ginsenoside-Rh_2 (G-Rh_2)-induced growth inhibition of A375-S2 cells. **METHODS**: A375-S2 cell viability and the effect of caspase inhibitors on G-Rh_2-induced apoptosis were measured by crystal violet assay. Changes in cellular morphology were observed by phase-contrast microscopy. Apoptosis-specific nucleosomal DNA fragmentation was assayed by agarose gel electrophoresis. Cell cycle distribution was measured by flow cytometry. **RESULTS**: G-Rh_2 inhibited the A375-S2 cell growth in concentration- and time-dependent manners. Caspase family inhibitor, z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), caspase-3 inhibitor, z-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk), and caspase-8 inhibitor, z-Ile-Glu-Asp-fluoromethylketone (z-IETD-fmk), partially inhibited G-Rh_2-induced apoptosis. But caspase-1 inhibitor, Ac-Tyr-Val-Ala-Asp-chloromethyl-ketone (Ac-YVAD-cmk), did not antagonize G-Rh_2 induced-cell death. **CONCLUSION**: G-Rh_2 suppresses the growth of A375-S2 cells *in vitro* by inducing apoptosis. G-Rh_2-induced apoptosis is partially dependent on caspase-8 and caspase-3 pathway in A375-S2 cells. Other apoptotic pathways might be also related to the induction of apoptosis by G-Rh_2.

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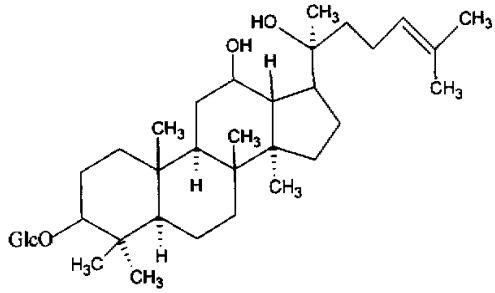
INTRODUCTION

The root of *Panax ginseng* CA Mey has traditionally been used as an herbal medicine in the East Asia countries for the treatment of various diseases, such as liver dysfunction, hypertension, cerebrovascular diseases, atherosclerosis, and postmenopausal disorder. Ginsenoside Rh_2 isolated from *Panax ginseng* belongs to protopanaxadiol dammarene glycosides. G-Rh_2 has been reported to have suppressive effect on growth of various cancer cells[1-6]. In addition, G-Rh_2 has been observed to block the cell cycle of SK-HEP-1 human hepatoma cells at the G_1/S boundary by selective induction of $p27^{kipl}$ expression[7]. It has been reported that G-Rh_2 induces a G_1 arrest in cell cycle progression, but does not induce apoptosis in MCF-7 human breast cancer cells[8]. However, the mechanisms of G-Rh_2 in malignant cells are still unclear.



Chemical structure of G-Rh_2 (20 S)

Human melanoma cell proliferation depends on growth factors and is inhibited by several cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), or

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transforming growth factor-β (TGF-β)[9]. A375 human melanoma cells are directly killed by IL-1β[10,11]. But IL-1-treated A375 cells did not exhibit the DNA fragmentation and other hallmarks of apoptosis[11].

Previous reports have demonstrated that caspase family proteases play essential roles in the process of apoptosis[12,13]. All the known caspases, a family of cysteine protease, specifically cleave protein substrates after their Asp residues. Caspases ase synthesized as zymogens that require cleavage adjacent to aspartates to liberate one large (α) and one small (β) subunit, which associate into a $\alpha_2\beta_2$ tetramer to form the active enzyme. The requirement for cleavage next to aspartates enables caspases to activite other caspases, thereby, setting the stage for an amplifying cascade. Caspase may be divided into two classes based on the lengths of their N-terminal prodomains. Since procaspase-1 and caspase-8 have long prodomains and localize at or near the cell membrane, it appears to be involved in targeting or regulating activation of apoptotic signal transmission. These interactions recruit procaspase-8 to the "death-inducing signaling complex" that forms in conjunction with the cytoplasmic domain of the death receptor, CD95 and tumor necrosis factor (TNF) receptors[14]. Whereas caspase-3 has short prodomain and localizes near the nuclei. It has been suggested that at the downstream end of the caspase cascade, caspase-3 operates to cleave the substrates[15].

In the present study, we demonstrated the anti-proliferative activity of G-Rh_2 against A375-S2 human malignant melanoma cells and investigated its inhibitory mechanism of action.

MATERIALS AND METHODS

Chemical reagents Ginsenoside-Rh_2 (20 S) was obtained from Prof MA Xing-Yuan (Department of Organic Chemistry, Faculty of Medical Sciences, Jilin University). The structure of G-Rh_2 was assigned by comparing the chemical and spectral data (^{1}H NMR, IR) with those reported in the literature[16]. The purity of G-Rh_2 was measured by HPLC and determined to be about 96 % (the chemical structure of G-Rh_2 is shown). G-Rh_2 was dissolved in dimethyl sulfoxide (Me_2SO) to make a stock solution. Me_2SO concentration was kept below 0.001 % in all the cell culture and did not exert any detectable change in cell growth or apoptosis.

Cell culture The A375-S2 cell line was purchased from American Type Culture Collection (ATCC, # CRL 1872, USA). Cells were cultured in RPMI-1640 medium (GIBCO, USA) supplemented with 5 % newborn bovine serum (NBS) (Dalian Biological Reagent Factory, Dalian, China) and 0.03 % *L*-glutamine (GIBCO) and maintained at 37 ℃ in 4 % CO_2.

***In vitro* growth inhibition test** A375-S2 cells (2×10^3/well) were seeded into 96-well culture plates (NUNCTM, Denmark). After 6 h incubation with 2 % NBS, various concentrations of G-Rh_2 and caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk (Medical & Biological Lab, Japan), and Ac-YVAD-cmk (Bachem, Japan) were added to the plates. Following incubation, cell growth was measured at 1, 3, and 6 h by crystal violet assay[17]. Optical density at 595 nm (A_{595}) was measured with an enzyme-linked immunosorbent assay plate reader (Bio-Rad, USA). Calculate the percentage of nonviable cells as follows:

Nonviable cells (%) = [A_{595}(control) − A_{595}(G-Rh_2)]/A_{595}(control) × 100 %

Observation of morphological changes A375-S2 cells in RPMI-1640 containing 2 % NBS were seeded into 6-well culture plates and cultured overnight. G-Rh_2 (10 μmol/L) was added to the cell culture and the cellular morphology was observed using phase contrast microscopy at 3, 6, and 12 h (Olympus, Japan).

DNA extraction and detection of DNA fragments[18] A375-S2 cells (1×10^6 cells) were collected by centrifugation at 1000 × g for 5 min, and washed once with Ca^{2+}- and Mg^{2+}-free phosphate buffered-saline (PBS). The cell pellet was suspended in 100 μL cell lysis buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 10 mmol/L pH 8.0, Triton X-100 0.5 %) and kept at 4℃ for 10 min. The lysate was centrifuged at 25 000 × *g* for 20 min. The supernatant was incubated with RNase A 40 μg/L (Sigma, USA) at 37 ℃ for 60 min, then incubated with proteinase K 40 μg/L (Merck, USA) at 37 ℃ for 60 min. The supernatant was again mixed with NaCl 0.5 mol/L and 50 % 2-propanol and incubated overnight at − 20 ℃, then centrifuged at 25 000 × *g* for 15 min. After drying, DNA was dissolved in TE buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 1 mmol/L pH 8.0), separated by 2 % agarose gel electrophoresis at 100 V for 40 min and stained with 0.1 mg/L ethidium bromide.

Assay of caspase activity[19] The A375-S2 cells (5×10^5) were incubated with or without G-Rh_2 as described above. All the cells were collected, washed as

described above and homogenized in 0.5 mL of extraction buffer containing *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid]-KOH (HEPES-KOH) 10 mmol/L pH 7.4, ethylenediamine tetraacetic acid 2 mmol/L, CHAPS 0.1 % (Sigma), dithiothreitol (DTT) 5 mmol/L (Wako, Japan), phenylmethylsulfonyl fluride (PMSF) 100 μmol/L (Sigma), leupeptin 10 mg/L (Sigma), pepstain A 1 mg/L (Sigma), and antipain 10 mg/L (Sigma). The cell extracts (100 μg of protein) were diluted with 1 mL of reaction buffer (HEPES-KOH 50 mmol/L pH 7.4, sucrose 10 %, CHAPS 0.1 %, DTT 10 mmol/L, ovalbumin 0.1 g/L), and incubated for 30 min at 30 ℃ with fluorescent substrates 10 μmol/L in the presence or absence of caspase-1 inhibitor Ac-YVAD-cmk 100 μmol/L or caspase-3 inhibitor z-DEVD-fmk 2 μmol/L. MOCAc-Tyr-Val-Ala-Asp-Ala-Pro-Lys (Dnp)-NH_2 and MOCAc-Asp-Glu-Val-Asp-Ala-Pro-Lys (Dnp)-NH_2 (Peptied Institute, Japan) were used as substrates for caspase-1 and caspase-3, respectively. Cells (5×10^5) were incubated with or without G-Rh_2 (0, 5, 10, 15, and 20 μmol/L) for 6 h. Cells (5×10^5) were also incubated with or without G-Rh_2 (20 μmol/L) in the presence of z-DEVD-fmk 2 μmol/L and Ac-YVAD-cmk 100 μmol/L for 3 h, then caspase-3 and caspase-1 activities were measured. The fluorescence of the cleaved substrates was determined with a spectro-fluorometer (Hitachi Type 850, Japan) set at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Specific caspase-1- and caspase-3-like activities were determined by subtracting the values obtained in the presence of inhibitors. One unit of enzyme activity corresponds to the activity that cleaves 1 pmol of the respective substrate at 30 ℃ in 1 min per mg protein.

Flow cytometric analysis of cell cycle Flow cytometric analysis was performed as described in previous experiments[7]. In brief, A375-S2 cells, both adherent and floating, were pelleted and washed with PBS. Cells were fixed in 75 % ethanol at 4 ℃ overnight. After washing twice with PBS, the cells were stained with 1.0 mL of propidium iodine (PI) solution containing PI 50 mg/L (Sigma, USA), RNase A 1 g/L (Sigma, USA), and 0.1 % Triton X-100 in sodium citrate 3.8 mmol/L and incubated on ice for 30 min in the dark.

RESULTS

Growth inhibition of A375-S2 cells G-Rh_2 5 to 80 μmol/L inhibited A375-S2 cell growth in a time- and concentration-dependent manner (Fig 1, 2). As shown in Fig 1, G-Rh_2 had potent inhibitory effect on A375-S2 cell growth. By 6 h after G-Rh_2 40 μmol/L treatment, cell death rate reached almost 100 %.

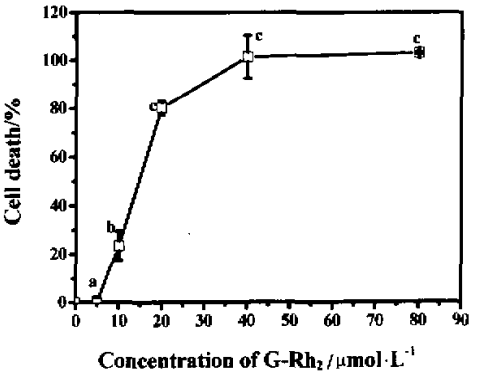

Fig 1. Inhibitory effect of G-Rh_2 on A375-S2 cell growth. The cells (2×10^4) were incubated with G-Rh_2 for 6 h. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control (G-Rh_2 0 μmol/L group).

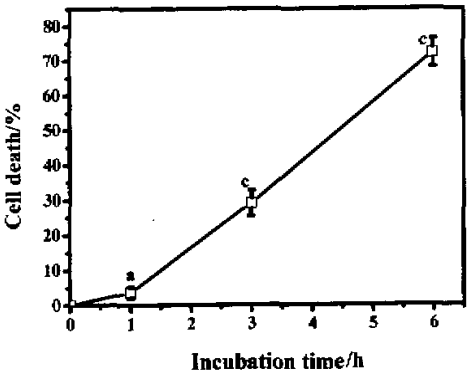

Fig 2. Time-dependent growth inhibition of G-Rh_2 (15 μmol/L)-treated A375-S2 cells (2×10^4). $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control (G-Rh_2 0 μmol/L group).

G-Rh_2-induced morphological changes and DNA fragmentation of A375-S2 cells When tumor cells were cultured for 3, 6, or 12 h with G-Rh_2 (10 μmol/L), marked morphological changes were observed as compared with the untreated control (Fig 3). G-Rh_2-treated A375-S2 cells underwent retraction of cellular processes and became round in shape at 3 h (Fig 3d). By 6 h, the majority of A375-S2 became round with

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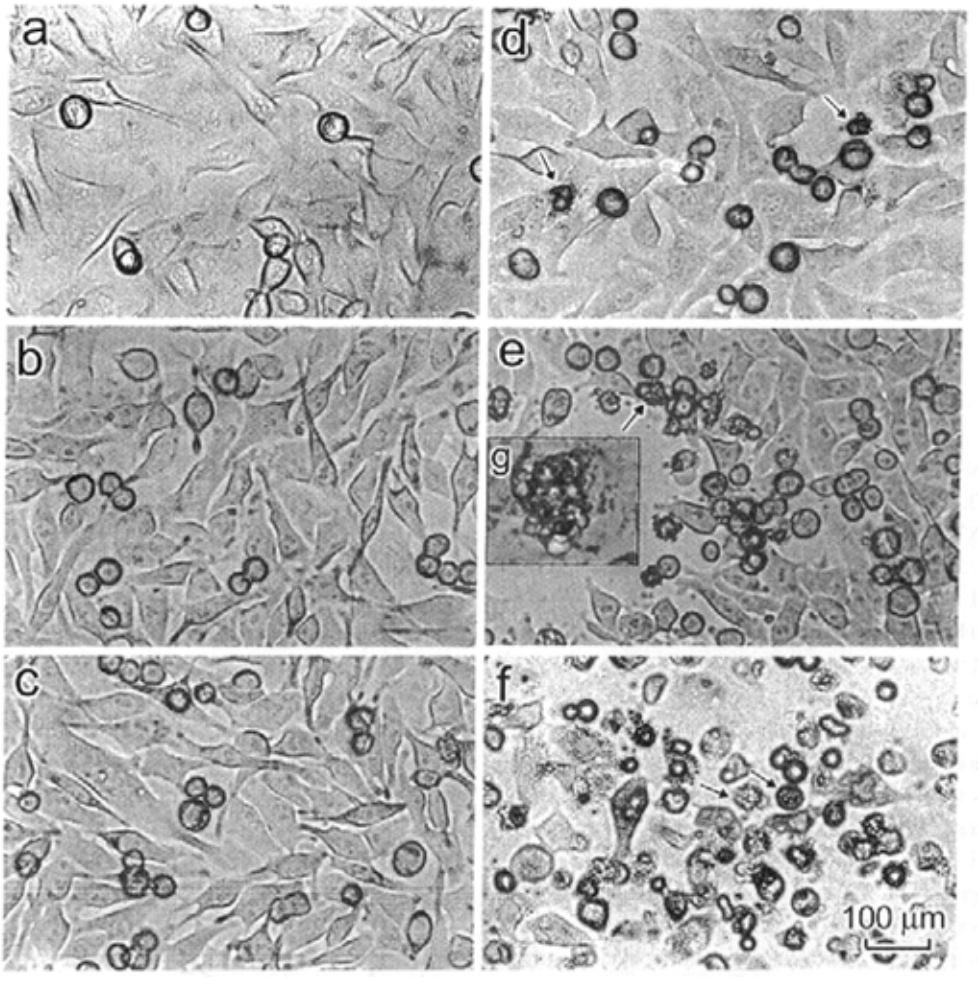

Fig 3. G-Rh_2-induced morphological changes of A375-S2 cells. The cells were incubated in a 6 well culture plate. Changes of cellular morphology were examined at 3 h (a, d), 6 h (b, e), 12 h (c, f) in the absence (a, b, c) or the presence (d, e, f) of G-Rh_2 10 μmol/L with × 100 magnification. Black arrows in (d) indicate multiblebbing cells and black arrows in (e) and (f) indicate apoptotic bodies and chromatin condensation, respectively. Inserted (g) in (e) shows apoptotic bodies with × 400 magnification.

shrunken nuclei (Fig 3e). Some of these cell showed membrane blebbing and nuclei were fragmented into apoptotic bodies (Fig 3g). Eventually, dead cells were floating at the later stages (Fig 3f). However, the untreated cells did not show these apoptotic characteristics (Fig 3a, b and c). After 6 h treatment of G-Rh_2(0, 5, 10, 15, or 20 μmol/L) treatment, A375-S2 cells began to generate DNA fragmentation that is another hallmark of apoptosis (Fig 4).

Effect of caspase inhibitors on G-Rh_2-induced apoptosis In Fig 5, when cells were pre-treated with z-VAD-fmk, z-IETD-fmk, or z-DEVD-fmk, G-Rh_2-induced cell death was effectively inhibited in G-Rh_2-treated cells (Fig 5C, E, F). But Ac-YVAD-cmk did not block G-Rh_2-induced cell death (Fig 5D). As shown in Fig 6D, z-DEVD-fmk effectively decreased G-Rh_2-induced DNA fragmentation. Addition of Ac-YVAD-cmk did not inhibit DNA fragmentation (Fig 6E). z-IETD-fmk weakly reduced G-Rh_2-induced DNA fragmentation (Fig 6F). G-Rh_2(20 μmol/L) increased caspase-3 activity to about 2.5 times of the control value at 6 h incubation, but did not increase caspase-1 activity.

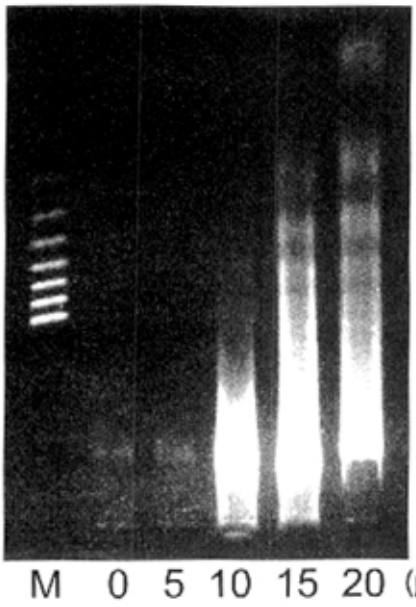

Fig 4. G-Rh_2-induced DNA fragmentation of A375-S2 cells. A375-S2 cells (1×10^6) were cultured in a 6-well plate in the absence or presence of G-Rh_2 (0, 5, 10, 15, and 20 μmol/L). After 6 h culture, cells were collected for the detection of DNA fragmentation. *Hae*-Ⅲ-digested ΦX-174 DNA fragments were used as molecular marker (M).

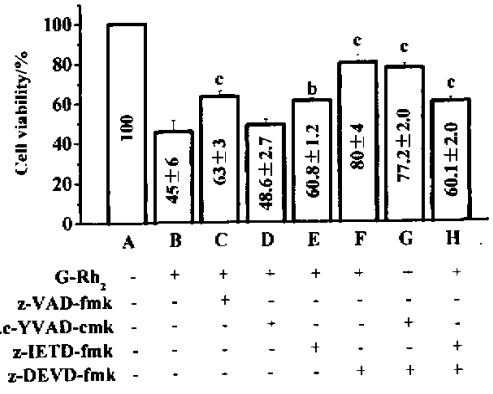

Fig 5. Effect of caspase inhibitors on G-Rh_2-induced apoptosis. A375-S2 cell were cultured in the absence or the presence of the caspase inhibitors, z-VAD-fmk (2 μmol/L), Ac-YVAD-cmk (100 μmol/L), z-IETD-fmk (2 μmol/L), or z-DEVD-fmk (2 μmol/L), 30 min prior to the addition of G-Rh_2 20 μmol/L, then incubated for 6 h. $n = 3$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* B group.

Moreover, G-Rh_2 (5 and 10 μmol/L) did not increase caspase-3 activity markedly, which was consistent with our finding that it did not induce DNA fragmentation (Tab 1).

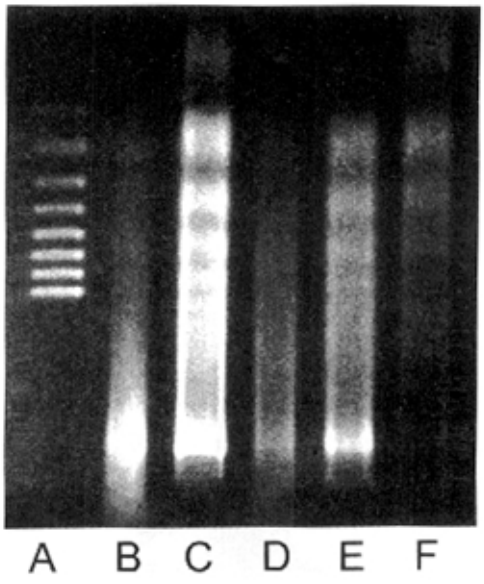

Fig 6. Effects of caspase inhibitors on G-Rh_2-induced DNA fragmentation of A375-S2 cells. A375-S2 cell were cultured in the absence or the presence of caspase inhibitors, 30 min prior to the addition of G-Rh_2 20 μmol/L, then incubated for 6 h. Cells were collected for the detection of DNA fragmention. *Hae*-Ⅲ-digested ΦX-174 DNA fragments were used as molecular marker (A). G-Rh_2 0 μmol/L (B); G-Rh_2 20 μmol/L (C); z-DEVD-fmk (2 μmol/L) and G-Rh_2 20 μmol/L (D); Ac-YVAD-cmk (100 μmol/L) and G-Rh_2 20 μmol/L (E); z-IETD-fmk (2 μmol/L) and G-Rh_2 20 μmol/L (F).

Tab 1. Activities of caspase-1 and caspase-3 in G-Rh_2-treated A375-S2 cells. $n = 3$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* G-Rh_2 0 μmol/L group.

G-Rh_2/μmol·L^{-1}	Caspase-1 activity unit	Caspase-3 activity unit
0	6.5±1.4	7.5±2.2
5	6.4±1.2	7.5±0.9
10	7.7±1.0	8.6±0.7
20	8.1±1.0	19±3^c
20 + Ac-YVAD-cmk	6.4±1.5	
20 + z-DEVD-fmk		8.6±0.7

Effect of G-Rh_2 on cell cycle arrest In Fig 7, in flow cytometric analyses 43.67 % of cells underwent apoptosis at 6 h in G-Rh_2 60 μmol/L-treated group. In

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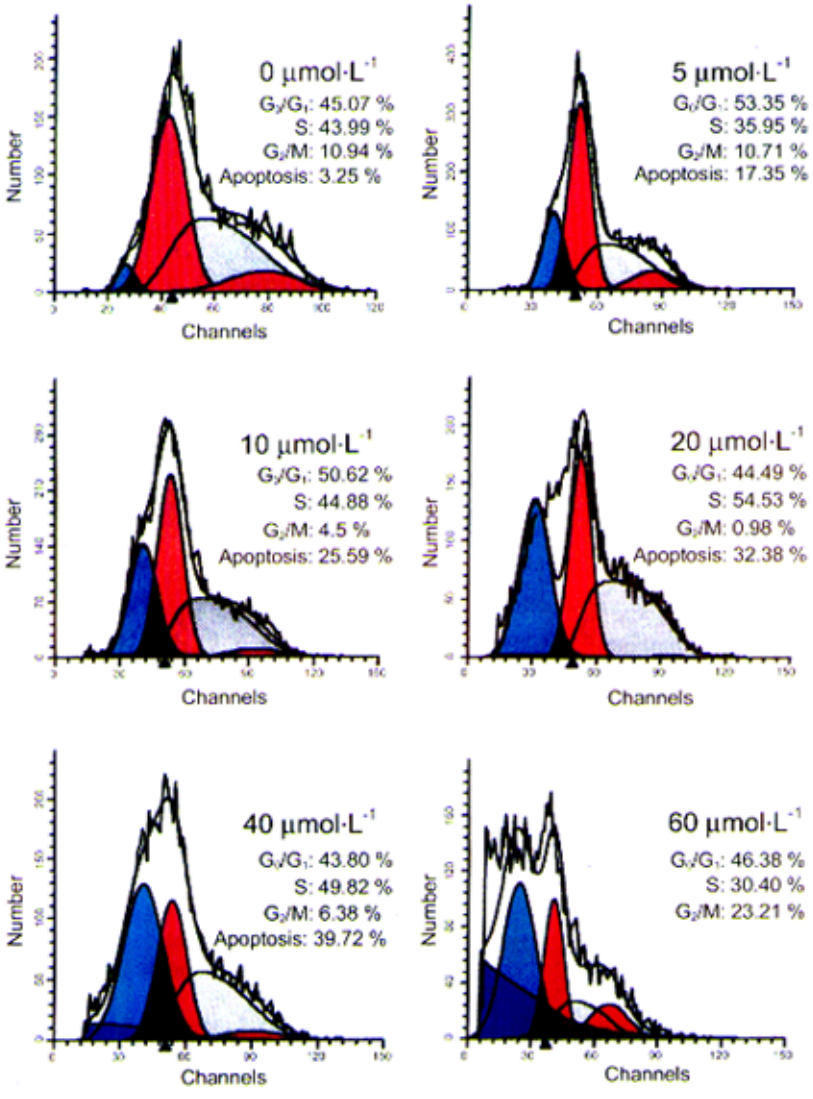

Fig 7. Flow cytometric analysis of the cell cycle distribution of the A375-S2 cells with or without G-Rh_2 for 6 h.

the control, only a minor cell population (3.25 %) underwent apoptosis. The cell number in G_0/G_1 phase and S phase has no change in all groups treated with different concentrations of G-Rh_2. Cell number in G_2/M phase decreased in G-Rh_2 0 to 20 μmol/L-treatment group, but increased again after G-Rh_2 40 μmol/L to 60 μmol/L treatment. The results demonstrated that G-Rh_2 treatment did not induce marked accumulation of cell cycle at a specific stage, suggesting that G-Rh_2-induced cell apoptosis might not be related to the cell cycle arrest of A375-S2 (Fig 7).

DISCUSSION

The purpose of the present study was to elucidate the mechanism of action by which G-Rh_2 induces the proliferation arrest of A375-S2 cells. We have shown that G-Rh_2 exerts a potent inhibitory effect on the cell growth in a concentration-dependent manner. G-Rh_2-induced cell death of A375-S2 was characterized by a

variety of apoptotic features as determined by morphological or biochemical criteria.

The results in this study demonstrated that z-VAD-fmk, z-DEVD-fmk, and z-IETD-fmk effectively inhibited the G-Rh_2-induced cell death, whereas Ac-YVAD-cmk did not inhibit cell death of G-Rh_2-treated A375-S2. Capase-3 inhibitor (z-DEVD-fmk) effectively prevented G-Rh_2-induced DNA fragmentation, and caspase-8 inhibitor (z-IETD-fmk) slightly reduced cell death, but caspase-1 inhibitor failed to do it. G-Rh_2 increased caspase-3 activity during G-Rh_2-induced apoptosis. These results showed that the process of G-Rh_2-induced apoptosis required the activation of caspase-3 protease. Because caspase-8 localized at or near the cell membrane, its function was considered to be regulating apoptotic signal transmission[17]. The result also showed that caspase-8 inhibitor (z-IETD-fmk) inhibited the G-Rh_2-induced cell death and did not inhibit G-Rh_2-induced DNA fragmentation. These results might be attributed to the falt that caspase-8 localizes at or near the cell membrane and it activates upper stream of apoptotic signal transmission, therefore, it might not induce DNA fragmentation directly. Caspase-3 localizes near the nuclei at the downstream end of the caspase cascade and induces increased DNase activity, resulting in DNA fragmentation[17]. Previous reports have demonstrated that caspase-8 can cleave radiolabeled precursors for caspase-3, -4, -7, and -9 *in vitro*. To examine whether action of caspase-8 correlated with caspase-3, simultaneous administration of z-DEVD-fmk, z-IETD-fmk, and G-Rh_2 was tried. The result showed that cotreatment of z-DEVD-fmk and z-IETD-fmk did not induce cell viability higher than z-IETD-fmk alone. Caspase-8 activates other apoptosis-related proteases that function between caspase-8 and caspase-3 stages. It is also probable that other pathways except for caspase-8 pathway activate caspase-3 in the downstream of the cascade. Since z-IETD-fmk and z-DEVD-fmk did not completely inhibit G-Rh_2-induced cell death, other apoptotic pathways might be required for the induction of apoptosis by G-Rh_2. In addition, it was reported that mitochondrial oncogene expression regulated cytochrome C release from mitochondria and activates caspase-9 and caspase-3 pathway[20]. Therefore, there might exist probable relationship between G-Rh_2 and mitochondrial function.

It has been reported that G-Rh_2 induced G_1 arrest in cell cycle progression of human breast cancer MCF-7 cells[8]. We expected that G-Rh_2-induced apoptosis also might be related to cell cycle arrest at some stages. G-Rh_2 0 – 20 μmol/L-treated A375-S2 cells showed marked decrease in cell number at G_2/M phase, however, G-Rh_2 40 – 60 μmol/L-treatment led to a significant increase in cell number at the same stage. Therefore, it is possible that the higher doses of G-Rh_2 might stimulate the expression of proteins regulating cell cycle at G_2/M boundary. Cytometric analysis showed that G-Rh_2-induced A375-S2 apoptosis was not directly associated with the specific cell cycle arrest.

G-Rh_2 induced A375-S2 cell apoptosis *in vitro*. G-Rh_2-induced apoptosis is partially dependent on caspase-8 and caspase-3 pathway in A375-S2 cells. Studies on anti-cancer activity of ginsenoside Rh_2 and the elucidation of molecular mechanisms of apoptosis might be useful tool for developing new therapeutic strategies.

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人参皂苷 Rh_2 对人黑色素瘤 A375-S2 细胞的促凋亡作用[1]

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关键词 人参; 皂苷类; 细胞凋亡; caspases; 黑色素瘤

目的: 研究人参皂苷 G-Rh_2 诱导人黑色素肿瘤细胞 A375-S2 凋亡的分子生物学机制. **方法**: 用结晶紫染色的方法测定细胞的死亡率. 用倒置显微镜观察细胞形态学的变化. 用琼脂糖凝胶电泳检测核酸片断. 用流式细胞仪检测细胞凋亡和细胞周期. **结果**: G-Rh_2 抑制 A375-S2 细胞增殖并在 20 μmol/L 可以诱导 A375-S2 细胞产生凋亡. Caspase 抑制剂 z-VAD-fmk (caspase 家族), z-DEVD-fmk (caspase-3) 或 z-IETD-fmk (caspase-8)能部分抑制细胞凋亡. 但是 Ac-YVAD-cmk (caspase-1)不能抑制 A375-S2 细胞凋亡. **结论**: G-Rh_2 在体外抑制 A375-S2 细胞的增殖, 通过细胞形态学和核酸片断分析, G-Rh_2 能够诱导 A375-S2 细胞产生凋亡. 这种作用是通过细胞内 caspase 一类半胱氨酸蛋白酶进行信号传导的. G-Rh_2对 A375-S2 细胞的周期没有影响.

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