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Induction of Apoptosis by Ginsenoside Rk1 in SK-MEL-2-Human Melanoma

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Ginsenosides are active compounds isolated from *Panax ginseng* Meyer. Among these ginsenosides, less polar ginsenosides such as ginsenoside Rg3 and ginsenoside Rh2 have been demonstrated to have tumor inhibitory effects because of their cytotoxicity. In this study, we evaluated the apoptotic effects of ginsenoside Rk1 in SK-MEL-2 human melanoma. Ginsenoside Rk1 isolated from red ginseng is one of the novel ginsenosides that shows strong cytotoxicity compared to ginsenoside Rg3 in dose- and time-dependent manners. The results of DNA fragmentation, 4',6-diamidino-2-phenylindole staining, and flow cytometric analysis are corroborated that ginsenoside Rk1 induced apoptosis in SK-MEL-2 cells. Western blot analysis revealed up-regulation of Fas, FasL, and Bax protein expression and down-regulation of procaspase-8, procaspase-3, mutant p53 and Bcl-2 protein expression. These findings suggest that ginsenoside Rk1 might be a promising compound to induce apoptosis through both extrinsic and intrinsic pathways in SK-MEL-2 cells.

Key words: Apoptosis, Ginsenoside Rk1, Mutant p53, SK-MEL-2 human melanoma

INTRODUCTION

Panax ginseng has been used for thousands of years because of its numerous pharmacological functions as an immune stimulant, memory enhancer and anticancer agent. Recent research has indicated that most of those pharmacological effects are attributed to ginsenosides, which are the most prominent constituents of ginseng. Among more than 60 kinds of ginsenosides, some characteristic ginsenosides have been focused on apoptotic effects in various cancer cells because of less polarity and strong cytotoxicity. There are several reports demonstrating the potential uses of these compounds as anticancer agents, such as the inhibition of tumor angiogenesis and metastasis by ginsenoside Rb2 (Sato et al., 1994) and the induction of apoptosis by ginsenoside Rg3 in various cancer cell lines such as breast (Wang et al., 2008), prostate (Kim et al., 2010), lung (Liu et al., 2009), and skin melanoma (Chen et al., 2008).

Skin cancer is the general term for malignant tumor cell growth on the outer layers of the skin. As one subtype of skin cancer, skin melanoma is becoming more common. For example, in the United States, the percentage of patients with melanoma has more than doubled in the past 30 years. Therefore, the early detection and treatment of malignant melanoma are extremely important because it is often lethal. The expression of proteins such as Fas, FasL, and the Bcl-2 family as well as p53 in skin cancer could contribute in part to the outcome of chemotherapy and treatment (Erb et al., 2005; Benjamin and Ananthaswamy, 2007).

Ginsenoside Rk1 was discovered in heat-processed ginseng called Sun Ginseng (Park et al., 2002) and isolated by high-speed counter-current chromatography (Ha et al., 2007). Its pharmacological activity has been studied on antitumor activity in human hepatocellular carcinoma cells (Kim et al., 2008) and arachidonic acid-induced platelet aggregation (Lee et al., 2009). In this study, we confirmed the apoptotic effects of ginsenoside Rk1 in SK-MEL-2 human

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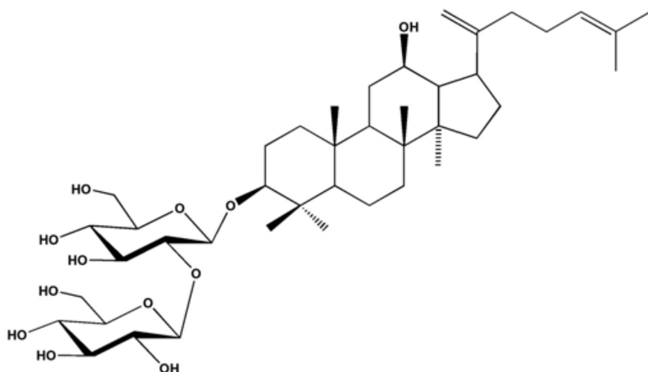

Fig. 1. Chemical structure of the ginsenoside Rk1.

melanoma. Although ginsenoside Rk1 (Fig. 1) has a similar structure to ginsenoside Rg3, its cytotoxic effect on SK-MEL-2 cells is much stronger than that of ginsenoside Rg3 within 24 h of treatment. Furthermore, the results of morphological observation, fluorescence-activated cell sorting and Western blotting provide clear evidence for the apoptotic mechanism of ginsenoside Rk1.

MATERIALS AND METHODS

Cell line and chemicals

SK-MEL-2 human melanoma cell line was obtained from the American Type Culture Collection. Ginsenoside Rk1 and Rg3 were isolated using a high-speed counter-current chromatography described in a previous study (Ha et al., 2007). RPMI 1640 medium, Dulbecco's phosphate buffer saline (D-PBS), 4',6-diamidino-2-phenylindole (DAPI) and dimethyl sulfoxide (DMSO) were acquired from Sigma Chemical Co. A Cell-Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories. Nitrocellulose membranes (NC membranes) were obtained from Pall Corporation. An enhanced chemiluminescence (ECL) plus detection kit was purchased from AbFrontier. The Fas/FasL Antagonist, Kp7-6, was purchased from Calbiochem. Other chemicals and solvents were obtained from Aldrich Chemical Co. Primary antibodies to Fas, Fas-L, procaspase-3, procaspase-8, procaspase-9, cytochrome C, Bax, Bcl-2, mutant p-53 and β-actin and secondary antibody were acquired from Santa Cruz Biotechnology.

Cell viability assay

SK-MEL-2 cells were seeded in 96-well plates at a density of 2×10^4 cells/well and allowed to adhere for 24 h at 37°C under 5% CO_2. After incubation, cells were treated with the investigated compounds for 24 h, and then 10 μL of CCK-8 solution was added to each well, followed by incubation for 2 h at 37°C. The resulting color was analyzed at 450 nm using Emax microplate reader (Molecular Devices).

DNA fragmentation

SK-MEL-2 cells were seeded in 6-well plates and were treated with ginsenoside Rk1 (0, 25, 50, 75, and 100 μM) for 24 h. Whole cells were washed with D-PBS and resuspended with Triton X-100 lysis buffer (40 mL of 0.5 M EDTA; 5 mL of 1 M TrisCl buffer; pH 8.0; 5 mL of 100% Triton X-100; 50 mL of H_2O). The DNA was extracted using a 25:24:1 (v/v/v) equal volume of neutral phenol-chloroform-isoamyl alcohol and precipitated in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. All DNA was analyzed electrophoretically on 2% agarose gel containing ethidium bromide. The gel was visualized under ultraviolet transillumination.

Cell morphological observation

SK-MEL-2 cells (1×10^5 cells/well) were seeded in 6-well plates. After incubation for 24 h, cells were treated with ginsenoside Rg3 (100 μM) and ginsenoside Rk1 (0, 25, 50, 75, and 100 μM) for 24 h. The cells were washed with D-PBS three times each for 10 min. The cells were then viewed under a CKX41 microscope (Olympus). For observation of typical apoptotic features such as nuclear condensation and nuclear fragmentation, the cells were treated with each compound, fixed with 4% paraformaldehyde solution for 15 min and stained with 4',6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 15 min in the dark. After washing with D-PBS, cells were viewed with a CKX41 microscope (Olympus).

Fluorescence-activated cell sorting

An Annexin V-FITC/PI apoptosis detection kit (BD Bioscience) was used to observe the apoptotic effect of ginsenoside Rk1. SK-MEL-2 cells (4×10^5 cells/well) were seeded in 6-well plates and incubated for 24 h. After treatment with ginsenoside Rg3 (100 μM) and ginsenoside Rk1 (0, 25, 50, 75, and 100 μM) for 24 h, adherent and floating cells were collected and washed with D-PBS. The cells were then centrifuged. The pellet was resuspended in 1 × binding buffer, stained with Annexin V-FITC and PI, respectively, and incubated for 15 min at room temperature in the dark. After incubation, the cells were added to 400 μL of 1 × binding buffer and analyzed by flow cytometry (FACS) Vantage SE (Becton Dickinson) using a CellQuest software.

FAS/FASL antagonist analysis

SK-MEL-2 cells were seeded in 96-well plates at a

density of 2×10^4 cells/well and allowed to adhere for 24 h. After incubation, the cells were treated with 1 mM FAS/FASL antagonist Kp 7-6 in each well. After incubation for 1 h, media containing the antagonist were removed and the cells were treated with ginsenoside Rk1 (0, 25, 50, 75, and 100 μM) for 24 h. Next, 10 μL of CCK-8 solution was added to each well, followed by incubation for 2 h at 37°C. The resulting color was assayed at 450 nm using an Emax microplate reader (Molecular Devices).

Western blot analysis

SK-MEL-2 cells (4×10^5 cells/well) were treated with various concentrations of ginsenoside Rk1 for 24 h. After incubation, total cytoplasmic extracts (except nuclear proteins) were prepared with lysis buffer (20 mM HEPES, 350 mM NaCl, 20% Glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT (dithiothreitol), 0.1 mM PMSF (phenylmethylsulfonyl fluoride) and 0.1 mM PI (protease inhibitors cocktails)). SDS-PAGE was performed, and the proteins were transferred to NC membranes. After being blocked in 5% skim milk, the membranes were incubated with primary and secondary antibodies sequentially. Finally, the blots were developed for visualization using the ECL Plus detection kit.

Statistical analysis

The results were expressed as means ± S.E.M. Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) and the Student's *t*-test. Statistical significance was assessed as $p < 0.05$ [$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$].

RESULTS

Ginsenoside Rk1 shows strong cytotoxicity in SK-MEL-2 human melanoma

When SK-MEL-2 cells were incubated with ginsenoside Rk1 (5, 10, 25, 50, 75, and 100 μM) for 24 and 48 h, the values of IC_{50} were 82.33 and 69.60 μM, respectively. For the 24 h treatment, ginsenoside Rk1 had a stronger cytotoxic effect than did ginsenoside Rg3 in SK-MEL-2 human melanoma (Fig. 2A).

Ginsenoside Rk1 induces DNA fragmentation and morphological changes by apoptosis

Condensation and fragmentation of nuclei has been considered as a hallmark of apoptosis. To determine whether the cytotoxicity of ginsenoside Rk1 was associated with apoptosis, SK-MEL-2 cells were treated with ginsenoside Rk1 (0, 25, 50, 75, and 100 μM) for 24 h and DNA was isolated and visualized on a agarose gel. DNA ladder was confirmed in cells treated with 75 and 100 μM ginsenoside Rk1 (Fig. 2B). To further observe the morphological changes in apoptosis, SK-MEL-2 cells were treated with ginsenoside Rk1 (25, 50, 75, and 100 μM) and ginsenoside Rg3 100 μM for 24 h. Using an optical microscope, the shapes of the cells treated with ginsenoside Rk1 appeared to become rounder and smaller compared to the control (Fig. 3A). As DAPI staining was then performed to verify apoptosis, the characteristics of apoptotic cell bodies were clearly observed upon treatment with 25 μM ginsenoside Rk1 by fluorescence microscopy. The cells incubated with ginsenoside Rg3 were also weakly

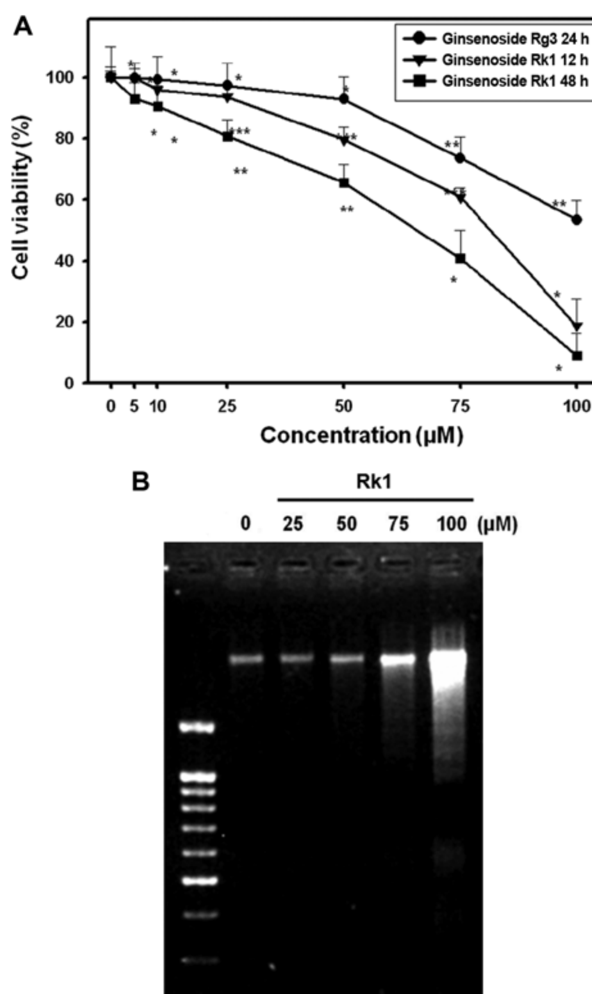

Fig. 2. (**A**) The cytotoxic effect of ginsenoside Rk1 in SK-MEL-2 human melanoma cells. The cells (2×10^4 cells/well) were seeded in 96-well plates and treated with ginsenoside Rg3 for 24 h and ginsenoside Rk1 for 24 and 48 h under the same concentrations (0, 5, 10, 25, 50, 75, and 100 μM). (**B**) The effect of ginsenoside Rk1 on DNA fragmentation in SK-MEL-2 cells. The cells were incubated for 24 h in the presence of ginsenoside RK1 (0, 25, 50, 75, and 100 μM). DNA was extracted and separated using 2% agarose gel electrophoresis.

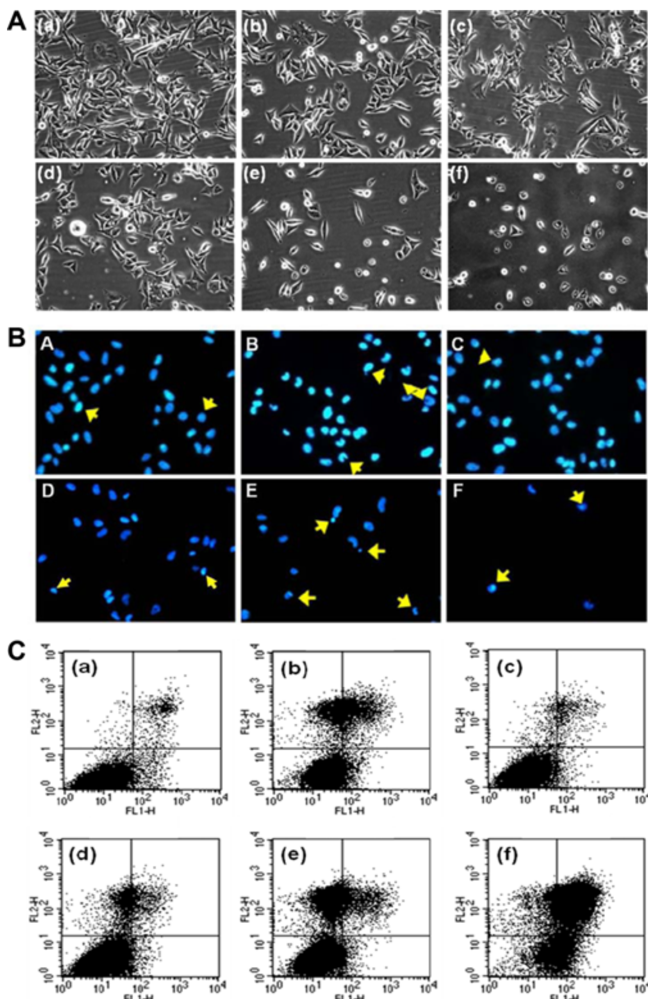

Fig. 3. (**A**) Morphologic changes of ginsenoside Rk1 on SK-MEL-2 cells. Cells treated with ginsenoside Rg3 and Rk1 were viewed under an optical microscope at the magnification of 40×10. a: cells were treated with vehicle. b: cells were treated with ginsenoside Rg3 (100 μM). c-f: cells were treated with ginsenoside Rk1 (c: 25 μM; d: 50 μM; e: 75 μM; f: 100 μM) for 24 h. (**B**) The apoptotic bodies by ginsenosides were assessed by a fluorescence microscope. a: cells were treated with vehicle. b: cells were treated with ginsenoside Rg3 (100 μM). c-f: cells were treated with ginsenoside Rk1 (c: 25 μM; d: 50 μM; e: 75 μM; f: 100 μM) for 24 h. (**C**) Measurement of apoptotic rates by Annexin V/PI analysis. Apoptotic cells were evaluated by FACS analysis. a: cells were treated with vehicle. b: cells were treated with ginsenoside Rg3 (100 μM). c-f: cells were treated with ginsenoside Rk1 (c: 25 μM; d: 50 μM; e: 75 μM; f: 100 μM) for 24 h.

shown to have fragmented chromatin and apoptotic bodies (Fig. 3B).

Ginsenoside Rk1 leads to apoptosis in a concentration-dependent manner

To investigate whether the phenomena such as morphological changes result from apoptosis by ginsenoside Rk1, cells were treated with ginsenoside Rk1, stained with Annexin V/PI and analyzed by FACS. As the concentration of ginsenoside Rk1 was increased, the number of apoptotic cells also increased. The corresponding quantities of total cell apoptosis were 15.72% at 100 μM ginsenoside Rg3 and 4.96%, 6.25%, 8.42%, and 78.97% at 25, 50, 75, and 100 μM ginsenoside Rk1, respectively (Fig. 3C).

Treatment with the Fas/FasL antagonist partially inhibits apoptosis by ginsenoside Rk1

There has been a lot of controversy regarding the involvement of FAS and/or FASL in human malignant melanomas (Erb et al., 2005). To investigate how ginsenoside Rk1 induces apoptosis via an extrinsic pathway, cells were treated with a Fas/FasL antagonist before sample treatment. The cells were treated with 1 mM Fas/FasL antagonist Kp 7-6 and incubated for 1 h. Cells were then treated with ginsenoside Rk1 (1, 5, 10, 50, and 100 μM) for 24 h. Only Kp 7-6 treatment did not induce cell proliferation or cell death compared to the cells with media control (data not shown). The treatment of cells with Kp 7-6 reduced the cytotoxic effect of 100 μM ginsenoside Rk1 by about 32% compared to the control (no treatment of Kp 7-6). The result showed that although Fas/FasL antagonist could disturb the apoptosis induced by ginsenoside Rk1 in SK-MEL-2 cells, it was not completely suppressed. In other words, it was demonstrated that the apopto- tic effects of ginsenoside Rk1 might be influenced by other pathways (Fig. 4A).

Ginsenoside Rk1 triggers apoptosis through both extrinsic and intrinsic pathways

To verify the mechanism of ginsenoside Rk1 in SK-MEL-2 cells, Western blot analysis for the expected proteins was conducted. Consistent with Fig. 4A, the expression levels of Fas and FasL, the signaling mediators that function at the beginning of the pathways responsible for cellular death, were increased by ginsenoside Rk1 treatment. Procaspase-8, procaspase-9, and downstream initiator caspases, were decreased (Fig. 4B). In order to investigate involvement of the mitochondria-related intrinsic pathway, the blots were also probed for the amounts of Bcl-2 and Bax. Down-regulation of anti-apoptotic Bcl-2 and up-regulation of pro-apoptotic Bax were observed. Also, cytochrome C obtained from total fraction was increased. The function of p53 in cancer depends on multiple factors such as the cell line and the cancerous stage (Benchimol, 2001; Latonen and Laiho, 2005). Mutations in p53 have been commonly detected in skin carcinomas (Stretch et al., 1991). Ginsenoside Rk1 caused the expression

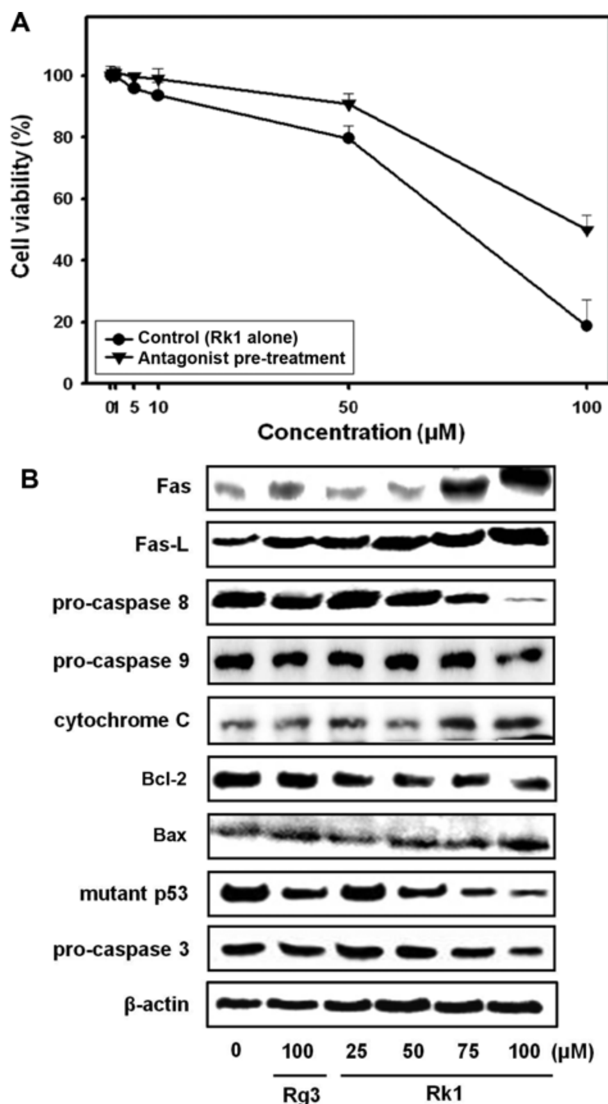

Fig. 4. (**A**) The inhibitory effects of FAS/FASL antagonist against apoptosis. Cells (2×10^4 cells/well) were pre-treated with 1 mM FAS/FASL antagonist Kp 7-6 each well. After 1 h incubation, media containing the antagonist were removed and the cells were treated with ginsenoside Rk1 (0, 25, 50, 75, and 100 μM) for 24 h. And then 10 μL of CCK-8 solution was added to each well, followed by incubation for 2 h at 37°C. (**B**) Effects of ginsenoside Rk1 on protein expression related to apoptosis. After treatment with ginsenoside Rg3 (100 μM) and ginsenoside Rk1 (0, 25, 50, 75, and 100 μM) for 24 h, the cells were lysed and performed to western blotting. The equal amount of loading was confirmed by the quantification of β-actin.

of p53 to decrease significantly. Effector procaspase-3 also decreased in a concentration-dependent manner. Consequently, it seems that ginsenoside Rk1 affects both extrinsic and intrinsic pathways to induce apoptosis in SK-MEL-2 human melanoma.

DISCUSSION

We have demonstrated that ginsenoside Rk1 induces apoptosis in SK-MEL-2 human melanoma. All results were compared with the effects of ginsenoside Rg3 because ginsenoside Rg3 has been proven to have potent antitumor activity in various cancer cell lines. Ginsenoside Rk1 inhibited SK-MEL-2 cell proliferation in a dose- and time-dependent manner. It was established through cell viability assay and cell morphology observation using optical microscope. The induction of apoptosis was confirmed through DAPI staining and FACS analysis. For the mechanism study, Western blot analysis was performed for Fas, FasL, procaspase-8, procaspase-3, Bcl-2, Bax and mutant p-53. Fas, an important cell surface receptor protein, induces apoptosis by binding to FasL. Ginsenoside Rk1 up-regulated the expression of Fas and FasL protein expression. Pretreatment with a Fas/FasL antagonist inhibited the cytotoxic effect of ginsenoside Rk1 on SK-MEL-2 cells. Moreover, ginsenoside Rk1 down-regulates procaspase-8 and procaspase-3 protein expression. These results show that ginsenoside Rk1 induces apoptosis in SK-MEL-2 cells through the extrinsic apoptosis pathway. In addition, ginsenoside Rk1 down-regulates Bcl-2 and up-regulated Bax protein. The death-suppressing activity of Bcl-2, a characteristic marker as the intrinsic apoptosis pathway, is regulated by Bax, which promotes cell death. The ratio of these two proteins is considered to be important for determining whether a cell undergoes apoptosis after an appropriate stimulus. Meanwhile, p53 was the first tumor suppressor gene linked to apoptosis. p53 muta- tions occur in the majority of human tumors and are often associated with advanced tumor stage and poor patient prognosis (Wallace-Brodeur and Lowe, 1999; Vousden and Lane, 2007). Lots of skin melanomas have mutant forms of p53 and SK-MEL-2 cells also keep the high levels of the mutant p53 (O'Connor et al., 1997). When ginsenoside Rk1 was treated to the cells, the expression of mutant p53 decreased signifi- cantly. Therefore, we tried to suggest that 'gain of function (GOF)' of mutant p53 might be one of the apoptotic effects by ginsenoside Rk1.

In conclusion, ginsenoside Rk1 has an apoptotic effect on SK-MEL-2 cells via both extrinsic and intrinsic pathways. Although further studies are needed to confirm out results, ginsenoside Rk1 could be a potential candidate compound for treatment of skin cancers.

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