Effect of Ginsenoside Rh-2 via Activation of Caspase-3 and Bcl-2-Insensitive Pathway in Ovarian Cancer Cells

Jin Hee KIM[1], Jae-Sun CHOI[2]

[1]School of Life Sciences and Biotechnology, Korea University, Seoul, Korea, [2]Department of Biomedical Laboratory Science, Far East University, Eumseong, Korea

Summary

Ginsenoside has been reported to have therapeutic effects for some types of cancer, but its effect on ovarian cancer cells has not been evaluated. In this study, we monitored the effects of ginsenoside-Rh2 (Rh2) on the inhibition of cell proliferation and the apoptotic process in the ovarian cancer cell line SKOV3 using an MTT assay and TUNEL assay. We found that Rh2 inhibited cell proliferation and significantly induced apoptosis. We confirmed the apoptotic effects of Rh2 using western blot analysis of apoptosis-related proteins. Specifically, the levels of cleaved poly ADP ribose polymerase (PARP) and cleaved caspase-3 significantly increased in SKOV3 cells treated with Rh2. Therefore, Rh2 clearly suppressed the growth of SKOV3 cells *in vitro*, which was associated with induction of the apoptosis pathway. Moreover, the migration assay showed that Rh2 inhibited the invasive ability of SKOV3 cells. Taken together, our results suggest that Rh2 has anticancer effects in SKOV3 cells through inhibition of cell proliferation and induction of apoptosis. Considering the therapeutic potential of Rh2, more studies should be carried out to facilitate the future application of this natural product as a potential anti-cancer agent.

Key words

Ginsenoside-Rh2 • Ovarian cancer cells • Apoptosis • Epithelial-mesenchymal transition

Corresponding author

J.-S. Choi, Department Biomedical Laboratory Science, Far East University, 76-32 Daehakgil, Gamgok-myeon, Eumseong-gun, Chungbuk-Do, 369-700, Korea. Fax: +82-43-880-3876. E-mail: jaenny123@ gmail.com

Introduction

Ovarian cancer is the most lethal gynecologic malignancy and exists predominantly in the form of epithelial ovarian cancer (Cho and Shih 2009, Swisher *et al*. 2012). Natural products are a valuable resource for the development of anticancer drugs and recent studies have revealed the anti-cancer properties of several types of natural products traditionally used as medicines (da Rocha and Lopes 2001, Mann 2002). One of the main mechanisms by which these products exert their therapeutic effects is *via* induction of apoptosis in cancer cells.

Apoptosis is a type of programmed cell death involving the activation of caspases through either an intrinsic mitochondria-dependent or an extrinsic mitochondria-independent cellular pathway (Salvesen and Dixit 1997, Fulda and Debatin 2006). Decreased cell volume is a typical morphological phenotype of apoptotic cells (Jacobson *et al*. 1997, Nagat 1997, Taatjes *et al*. 2008). Apoptosis involves a cascade of molecular changes, including morphologically changes, chromatin condensation, and DNA fragmentation (Chung *et al*. 2007, Sanjiv *et al*. 2012). Abnormal regulation of apoptosis leads to many human disorders, including cancers. Therefore, targeting and exploiting the mechanism of apoptosis is an important strategy for the treatment of cancer (Kelloff *et al*. 2000, Sun *et al*. 2007). Caspase activation is often regulated by various cellular factors, including members of the Bcl-2 family. The caspase cascade is a key pathway in apoptotic signal transduction, and its components can be divided into two subfamilies: upstream initiator caspases and downstream

effector caspases, which directly induce the final events in apoptosis (Lavrik *et al.* 2005, Lamkanfi *et al.* 2007, Lu *et al.* 2012). A central component of the programmed cell death process *via* the caspase cascade is apoptosis proteinase-activating factor-1 (Apaf-1) complex formation, and heterodimerization of Bcl-2 family proteins (Green and Reed 1998, Liu *et al.* 1996), which control the activation of caspases (Vaux and Korsmeyer 1999). Bcl-2-related proteins fall into two groups that either repress or promote apoptosis. During apoptosis, translocation of cytochrome c to the cytosol promotes Apaf-1 complex formation, which initially results in activation of caspase-9 followed by the activation of other caspases. Eventually, the caspases cleave the cellular proteins resulting in programmed cell death. Poly ADP ribose polymerase (PARP) is a substrate for caspases and is cleaved into fragments during apoptosis fragments.

Ginseng (*Panax ginseng*) has a variety of pharmacological and therapeutic applications, and has thus become one of the most popular traditional Chinese medicines. Ginsenosides are considered as the main bioactive components of herbal medicines derived from ginseng, which are triterpene saponins. In particular, the ginsenosides have been reported to have anti-cancer, anti-inflammatory, antioxidative, and vasorelaxing properties (Jang *et al.* 2014, Luo *et al.* 2008, Wang *et al.* 2007, Wang *et al.* 2008). Nevertheless, the exact molecular mechanism of the anti-cancer effect of the ginsenosides Rh2 remains unclear.

In this study, we investigated the effects of Rh2 on inhibition of cell proliferation and the apoptotic process in ovarian cancer *in vitro*.

Materials and Methods

Cell line and reagent

The cells used in this study were human ovarian adenocarcinoma cells SKOV3 (American Type Culture Collection, Rockville, MD, USA). The cells were cultured in a mixture of RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (FBS) (HyClone, Thermo Scientific) and 1 % penicillin-streptomycin (Invitrogen) in a humidified chamber with 5 % CO_2 at 37 °C. 1×10^6 cells were seeded per well into a 12-well plate (1 ml/well) with glass coverslips.

Ginsenoside Rh2 (purity~98.7 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of Rh2 (final concentration 0.1 M) was prepared in dimethyl sulfoxide (DMSO), stored at -20 °C, and diluted with fresh complete medium immediately before use. An equal volume of DMSO (final concentration <0.1 %) was added to the controls.

Cell proliferation assay

A cell survival analysis was performed according to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cell Titer 96 Aqueous Cell Proliferation Assay kit; Promega, Madison, WI, USA) assay method.

Briefly, cells were plated at 1×10^5 cells per well of the 96-well plate with Rh2 and 10 μl of 4 mg/ml MTT solution was added to each well. The cells were subsequently incubated for 4 h in the dark. The absorbance was measured in a microplate reader at 490 nm, and the results were expressed as a percentage of the control.

TUNEL assay

The terminal deoxynucleotidyl transferase mediated digoxigenin-dUTP-biotin nick-end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche, Penzberg, Germany) was used to confirm apoptosis by demonstrating apoptotic bodies in SKOV3. Briefly, cells were fixed with 4 % formaldehyde and incubated at room temperature for 40 min. This was followed by several rinses in PBS and permeabilization in 0.2 % Triton X-100 solution on ice for 5 min. Then, 50 μl of TUNEL reaction mixture was added on coverslips before being incubated for 60 min at 37 °C in a dark humidified chamber. Finally, the coverslips were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for 20 min at room temperature and examined with a model LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Western blot analysis

Cells were resuspended in ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) with the Protease Inhibitor Cocktail (complete mini tablet, Roche). Protein concentrations were determined with a BCA assay kit (Pierce, IL, USA) according to the manufacturer's instructions. Protein was separated *via* 10 % SDS-PAGE, transferred to PVDF membrane, and blocked with 5 % non-fat milk. Membranes were incubated with anti-cleaved caspase-3 (Cell signaling systems), anti-cleaved PARP (Cell signaling systems),

anti-Bcl-2 (Cell signaling systems) and beta-actin (Sigma) antibodies. Next, the membranes were incubated with HRP-conjugated anti-secondary IgG (Invitrogen) antibody and visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce, IL, USA).

Wound assays to assess cell motility

A sterile tip was used to wound a cell layer, and the cells were treated with Rh2. The photographs were taken by using a microscope at each time point. The monolayers were scratched with a 200 µl pipette tip and washed with media to remove the detached cells. The wounded areas were then imaged after incubation for an indicated period.

Statistical analyses

Data are shown as average and standard deviation. When comparing means between two groups, Student's t-test was applied. The level of statistical significance was based on the *p* values (p<0.05).

Results

Effect of ginsenoside-Rh2 on cell proliferation and apoptosis of SKOV3 cells

To examine the effect of Rh2 on the cell viability of ovarian cancer cells, we treated SKOV3 cells with 30 µM and 60 µM Rh2 for 0, 24 and 48 h and determined the proportion of surviving cells with an MTT assay. Forty-eight hours of exposure to Rh2 induced a toxic, dose-dependent effect on SKOV3 cells, with a maximal effect of ~50 % inhibition noted at a concentration of 60 µM. Thus, 60 µM was used as the concentration for Rh2 in all further experiments (Fig. 1; p<0.05).

Ginsenoside-Rh2 induces apoptosis in SKOV3 cells

The TUNEL assay is a sensitive assay for visualizing DNA damage in single cells resulting from apoptotic signaling cascades (Chen *et al*. 2013). To assess whether Rh2 exerts its anti-cancer effects *via* apoptosis, apoptotic cells were detected using TUNEL assays. We assessed the apoptotic rate of cells treated with 60 µM Rh2 for 24 h and 48 h. After incubation, cultured cells were fixed and stained for TUNEL along with DAPI. Confocal microscopy was used to compare the numbers of apoptotic cells in the groups cultured with and without Rh2. The number of apoptotic cells significantly increased in the SKOV3 cells treated with Rh2 in a time-

dependent manner, as compared to the control group (Fig. 2).

Caspase-3 activation is the key event in apoptotic cell death. To further evaluate the apoptotic mechanism of Rh2, we determined the levels of cleaved caspase-3, cleaved PARP, and Bcl-2, an important anti-apoptotic protein, by western blot analysis. The levels of cleaved caspase-3 and cleaved PARP were significantly higher in SKOV3 cells treated with 60 µM Rh2 for 48 h compared to control cells. Furthermore, the Bcl-2 level was significantly decreased in SKOV3 cells treated with 60 µM in a time-dependent manner (Fig. 3).

Ginsenoside-Rh2 affects SKOV3 cell mobility

To further verify that Rh2 blocked epithelial-mesenchymal transition (EMT), cell mobility was evaluated with migration assays. As shown in Figures 4A and 4B, Rh2 treatment significantly inhibited cell mobility and migration. Taken together, our findings strongly indicate that Rh2 is capable of reversing EMT-induced changes in cell mobility, underscoring its potential function as an inhibitor of EMT-mediated cancer metastasis.
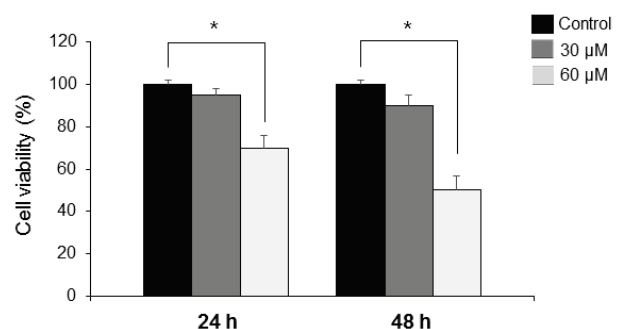

Fig. 1. Effects of Rh2 on viability and apoptosis in SKOV3 cells. Cells were seeded in 96-well plates and treated with various concentrations of Rh2, and cell viability was determined with the MTT assay. The values are calculated relative to the control group (0 µM Rh2). The results are shown as mean ± SD and are representative of three independent experiments. *p<0.05 versus the control group.

Discussion

In this study, we demonstrated the time-dependent development of apoptosis changes in SKOV3 ovarian cancer cells following exposure to ginsenoside-Rh2. Rh2 was found to induce significant apoptotic death of ovarian cancer cells using a TUNEL assay. Furthermore, we showed that the Rh2-induced apoptosis involves activation of caspase-3 and PARP, and the

treatment with specific inhibitors of Bcl-2 significantly induced the Rh2-induced cell apoptotic effects. Thus, our results demonstrated that the Rh2-induced apoptosis mechanism occurs through a mitochondria-dependent response. Recent studies have demonstrated the anticancer effect of ginsenoside for many types of cancer. We further found that Rh2 is significantly more potent at inducing cell death in ovarian cancer cells compared to Rg3 (data not shown). These results indicate that Rh2 may be a potent anticancer agent.
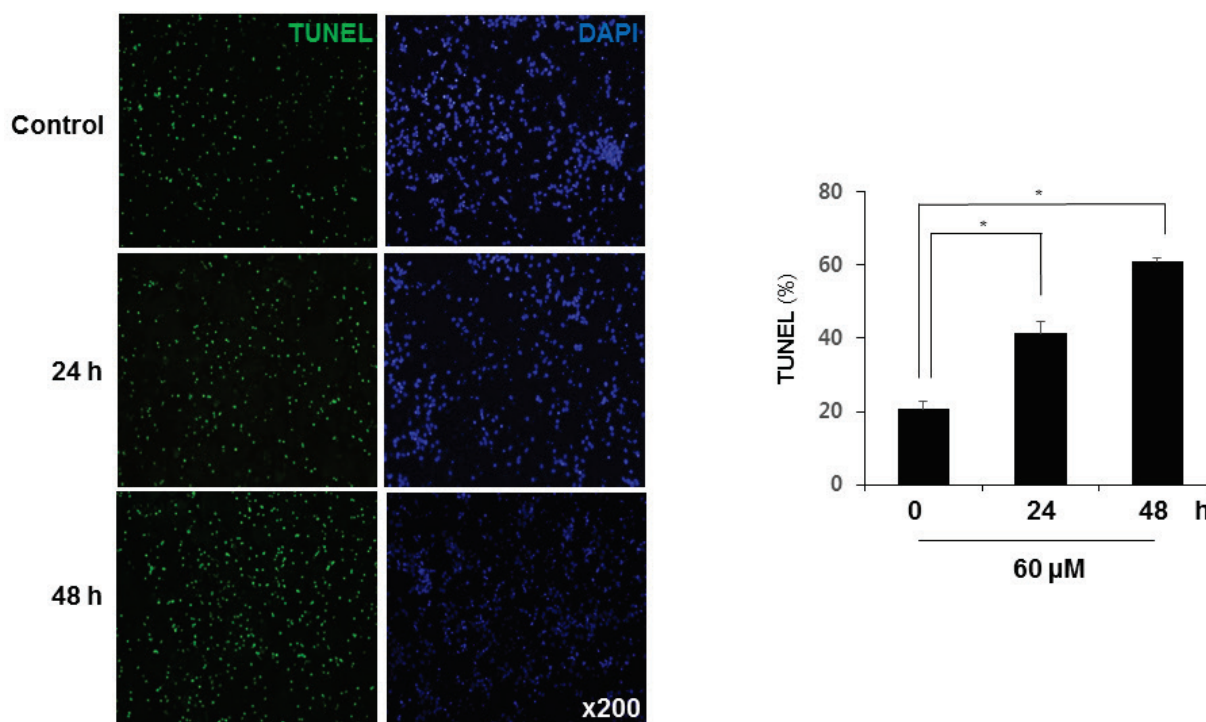

Fig. 2. Rh2 induces apoptosis in SKOV3 cells. Cells were treated with Rh2 and subsequently stained with TUNEL and DAPI. After treatment, the number of apoptotic cells was determined with the TUNEL assay. The TUNEL assay is representative of three independent experiments with similar results.
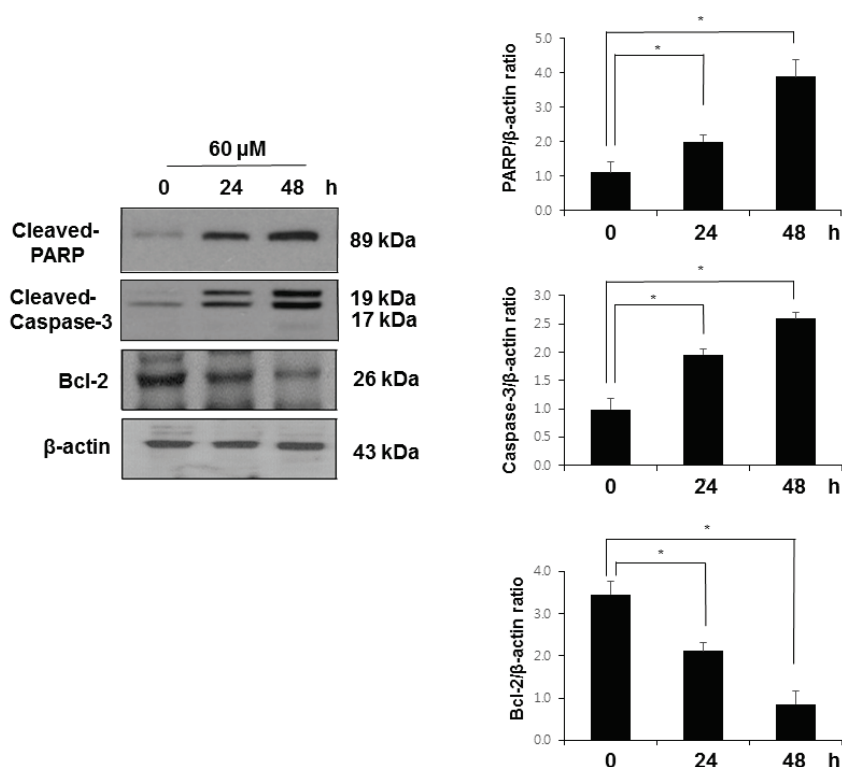

Fig. 3. The level of cleaved caspse-3 was significantly higher in SKOV3 cells. SKOV3 cells were analyzed for cleaved-caspase-3, and Bcl-2 expression by western blotting after treatment with Rh2. The western blots are representative of three independent experiments with similar results.
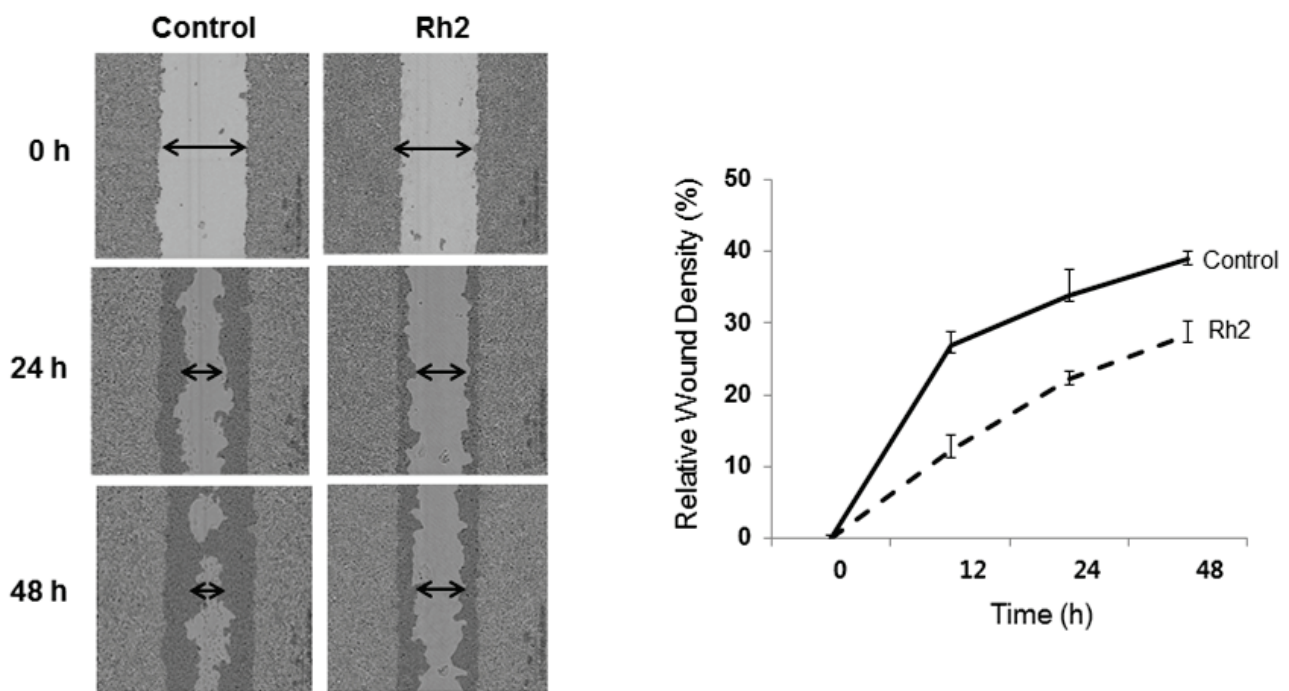
Fig. 4. Rh2 inhibited the mobility of SKOV3 cells. Cell mobility detected by a migration assay. Cells were incubated in media without Rh2, followed by scratching the confluent cell layer and exposing the cells to Rh2. The closure of the scratch was monitored and photographs were shown at 24 h and 48 h. All of the experiments were carried out in triplicate.

Ginsenosides can be classified into Rg1, Rg3, Rh1, Rh2, and other components. Anticancer activities tend to increase with decrease of the number of sugar moieties in a ginsenoside molecule. Although the underlying mechanisms are still poorly defined, Rh2 has been shown to inhibit the growth of a number of types of human cancer cells (Popovich and Kitts 2004, Wu *et al*. 2011). Rg3 and Rh2 are the main components in ginsenosides, which have shown potent anti-proliferative effects on cancer cells. Rh2, with one sugar residue, has shown 5- to 15-fold relatively stronger anti-apoptotic effects than Rg3, with two sugar residues (Nag *et al*. 2012, Yu *et al*. 2007, Wang *et al*. 2007). Rh2 may inhibit carcinoma cell growth through mediating coordinated autophagy, β-catenin signaling and inhibiting MMP13 (Guan *et al*. 2015, Kumar *et al*. 2016, Yang *et al*. 2016).

Moreover, the EMT is an important process in cancer metastasis, in which epithelial cells lose their properties resulting in migration to other sites. Therefore, we investigated the effects of Rh2 on EMT in ovarian cancer cells. The migration assays showed that Rh2 inhibited the cell mobility of SKOV3 cells. The results from the present study suggest that the anticancer actions of Rh2 are associated with its effects on the EMT. The EMT is regulated by transcription factors, including Snail, Twist, and ZEB. Induced EMT can promote the metastasis of cancers *via* several signaling pathways such as TGF-β, MAPK, and NF-κB signaling (Liu *et al*. 2015, Hou *et al*. 2014). Furthermore, given that EMT promotes motility, it can promote cancer progression (Ahmed *et al*. 2010). We demonstrated that the Rh2-induced suppression of EMT may contribute to the observed increases in cell-cell interactions and decreased capacity for motility in ovarian cancer cells. Thus, reversing the EMT may be an alternative strategy for cancer therapy.

In conclusion, ginsenoside-Rh2 was shown to suppress the growth of SKOV3 ovarian cancer cells *in vitro*, which is associated with inductions of apoptotic cell death. Therefore, Rh2 appears to be a useful natural material for enhancing the apoptosis of cancer cells. Furthermore, Rh2 was found to influence cell mobility in SKOV3 cells. Considering these properties, Rh2 appears to be a promising natural anti-cancer agent, which warrants further research for potential clinical application.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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