Ginsenoside Rg3 induces apoptosis in the U87MG human glioblastoma cell line through the MEK signaling pathway and reactive oxygen species

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Abstract. Ginsenoside is known to have potential cancer-preventive activities. The major active components in red ginseng consist of a variety of ginsenosides including Rg3, Rg5 and Rk1, each of which has different pharmacological activities. Among these, Rg3 has been reported to exert anticancer activities through inhibition of angiogenesis and cell proliferation. However, the effects of Rg3 and its molecular mechanism on glioblastoma multiforme (GBM) remain unclear. Therefore, it is essential to develop a greater understanding of this novel compound. In the present study, we investigated the effects of Rg3 on a human glioblastoma cell line and its molecular signaling mechanism. The mechanisms of apoptosis by ginsenoside Rg3 were related with the MEK signaling pathway and reactive oxygen species. Our data suggest that ginsenoside Rg3 is a novel agent for the chemotherapy of GBM.

Introduction

In recent years, the discovery of new therapeutic reagents for cancer treatment has been studied in several countries. One of the most aggressive and malignant types of human cancer, glioblastoma multiforme (GBM), is a common brain tumor in humans (1,2). The prognosis of GBM after diagnosis remains dismal, even after aggressive treatment such as radiotherapy, chemotherapy and surgery (3-5). Thus, it is necessary to identify new treatment modalities for GBM to achieve more favorable results.

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Korean red ginseng (KRG; *Panax ginseng* C.A. Meyer), which is also generally known as Korean ginseng, is a native herbal remedy commonly used in Korea and China (6,7). Red ginseng has been recognized as a life prolonging herb in Asia for thousands of years (8-10). The major active components in red ginseng are the ginsenosides Rg3, Rg5 and Rk1, each of which has unique pharmacological activities (11,12).

Ginsenosides have been reported to exert cancer-preventive effects against various types of cancer, such as lung cancer (13), nasopharyngeal carcinoma (14) and prostate cancer (15). Various components of ginsenosides are expected to exert a similar preventive effect against various types of cancer, but their effects against GBM remain unknown. In the present study, we investigated the effects of individual ginsenosides, particularly those of Rg3, on the human glioblastoma cell line, U87MG, and their molecular signaling mechanism.

Materials and methods

Reagents. Rg3 was purchased from NPC BioTech (Korea). DAPI stain, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and modified Hanks' balanced salt solution (HBSS) were obtained from Sigma-Aldrich (USA). α-minimum essential medium (MEM) and fetal bovine serum (FBS) were acquired from Invitrogen (Canada). U0126, PD98059, SP600125, SB203580 and Z-VAD-fmk were obtained from Calbiochem (Denmark). An enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences (UK). Bcl-2, Bax and pro-caspase3 were acquired from Epitomics (USA). p-ERK, p-p38 and p-JNK were obtained from Cell Signaling Technology, Inc. (USA).

Cell culture conditions. U87MG cells (human glioblastoma cell line) were purchased from the Korean Cell Line Bank and cultured in α-MEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were plated in cell culture dishes and cultivated at 37˚C in a humidified 5% CO_2 incubator. These cells were then sub-cultured until confluence for 3-5 days using 0.05% trypsin. Cells were cultivated under serum starved conditions for 1-2 days before various reagents were added.

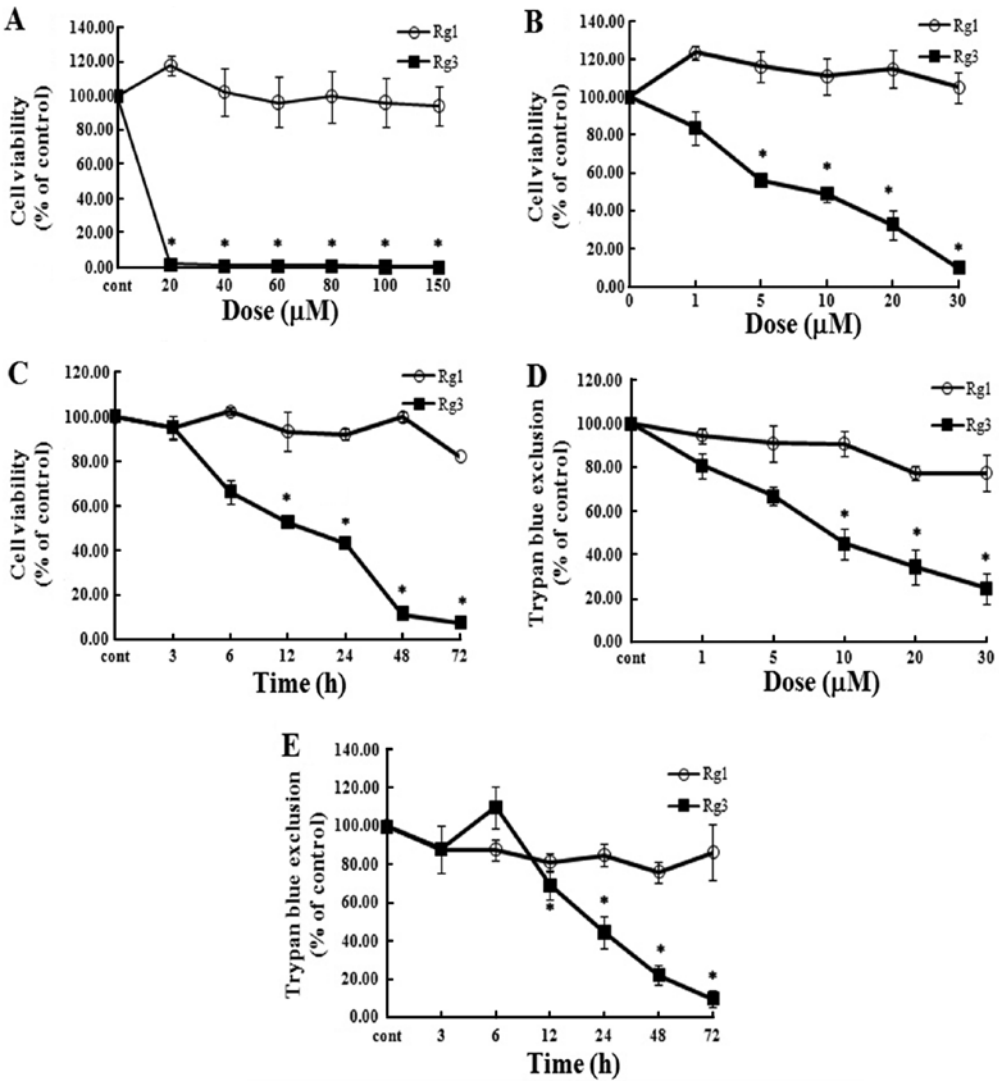

Figure 1. Viability of U87MG cells decreases in response to treatment with Rg3. Changes in viability in response to treatment with various concentrations of Rg3 or Rg1 for different time periods were measured using an MTT assay (A-C) and trypan blue exclusion assay (D and E). The cells were treated with 10 μM Rg1 and Rg3 (C and E). The data reported are the means ± SEM of four independent experiments. *P<0.05 when compared to the control without Rg3.

Measurement of cell growth

MTT assay. U87MG cells were seeded in 96-well plates at $5x10^2$ cells/well with various concentrations of Rg3 for the indicated time periods. Following cultivation under various conditions, 0.5 mg MTT/ml in α-MEM was added. The cells were then cultivated for 2 h, after which they were dissolved in DMSO and the absorbance of each well was measured at 570 nm using a 680 microplate ELISA reader (UK).

Measurement of cell death by a trypan blue dye exclusion assay. Rg3-treated cells were harvested using 0.05% trypsin solution and washed with HBSS buffer, after which they were suspended in 0.4% trypan blue solution. Cells that excluded the dye were considered viable. The cells were counted using a hemocytometer under light microscopy.

Flow cytometry. Cells were plated in 6-well plates at $5x10^4$ cells/well, after which they were treated with the indicated reagents for 24 h at 37˚C. The cells were then harvested using 0.05% trypsin solution and centrifuged at 10,000 x g for 15 min, after which the pellets were washed in HBSS buffer twice and fixing solution was added. The samples were then incubated overnight at 4˚C, stained with 50 μg propidium iodide/ml containing 100 μg RNase/ml for 20 min and analyzed using a FACSort Becton-Dickinson Flow Cytometer (USA).

Staining of the apoptotic cells. DNA fragmentation was evaluated by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay using an *in situ* Cell Death Detection kit (fluorescein) purchased from Roche Applied Science (USA). Briefly, cells were plated at a density of $5x10^4$ cells/cover slip (25 mm size), and then treated with Rg3. The cells were subsequently washed, after which freshly prepared 4% paraformaldehyde was added and they were incubated for 60 min at 37˚C. Next, the samples were permeabilized in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 5 min on ice, then subjected to the TUNEL reaction at 37˚C in a humidified atmosphere in the dark for 60 min. Finally, the fluorescent signal was detected using a Zeiss fluorescence microscope (Germany).

Immunocytochemistry. Cells were plated at $5x10^4$ cells/well cover slip (25 mm size), fixed in freshly prepared 4% para-

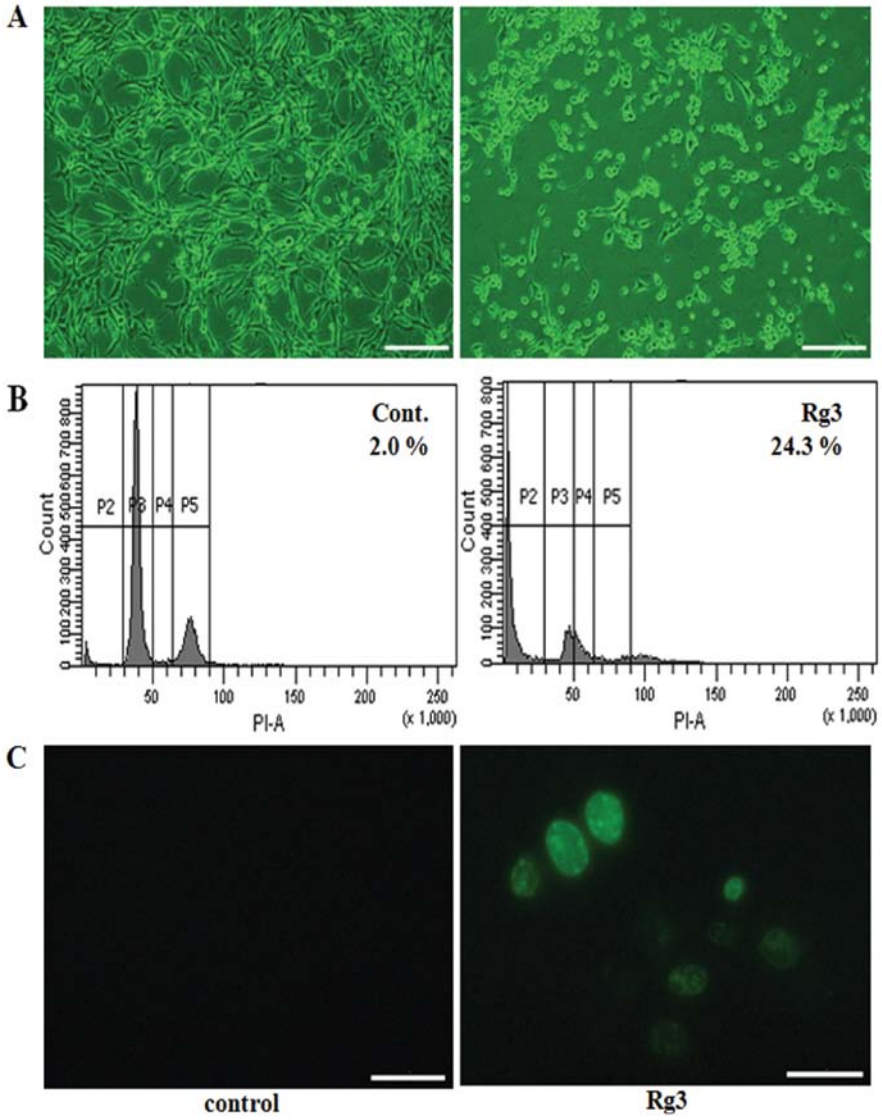

Figure 2. Rg3 induces apoptosis in U87MG cells. The cells showed clear morphological changes after 24 h of treatment with 10 μM Rg3 (A). The cells were treated with 10 μM Rg3 for 24 h and were examined by flow cytometric analysis (B) and TUNEL assay (C). The scale bar is 400 μm.

formaldehyde for 5 min and then washed. The cells were then blocked in 1% BSA blocking reagent for 30 min at room temperature, after which they were stained with primary antibodies such as Bax (1:500) and Bcl-2 (1:500) overnight at 4˚C and washed. Next, secondary antibody was added and the cells were incubated for 2 h, at which time they were subjected to DAPI staining. Fluorescent signals were detected using a Zeiss fluorescence microscope.

Western blot analysis. The cells were plated in 6-well plates at $5x10^4$ cells/cm^2 and then lysed on ice using lysis buffer (pH 7.4; 1 mM EGTA; 1 mM EDTA; 0.1 mM phenylmethylsulfonyl fluoride; 10 mM NaCl; 20 mM Tris-HCl; 1% Triton X-100). The lysates were then centrifuged at 10,000 x g for 20 min at 4˚C and loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, then transferred to nitrocellulose membranes. Membranes were immunoblotted with primary antibodies such as Bax (1:500), Bcl-2 (1:500) and β-tubulin (1:1,000). The membrane signals were visualized using an ECL kit.

Measurement of reactive oxygen species (ROS). The intracellular generation of ROS was detected using 2',7'-dichlorofluorescin diacetate (DCFH-DA). Briefly, cells were plated in 6-well plates at $5x10^4$ cells/cm^2, pre-treated with antioxidant enzymes and then treated with Rg3. Following treatment, the cells were washed using PBS and incubated with 30 μM DCFH-DA for 1 h at 37˚C, after which they were quickly washed, and the fluorescence signal intensity was monitored using a Zeiss fluorescence microscope.

Statistical analysis. All experiments were performed at least three times and statistical significance was determined using a Student's t-test (two-tailed). A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Rg3 exerts an inhibitory effect against U87MG cells. To test whether ginseng components exert inhibitory effects against U87MG cells, we conducted an MTT and a trypan blue assay.

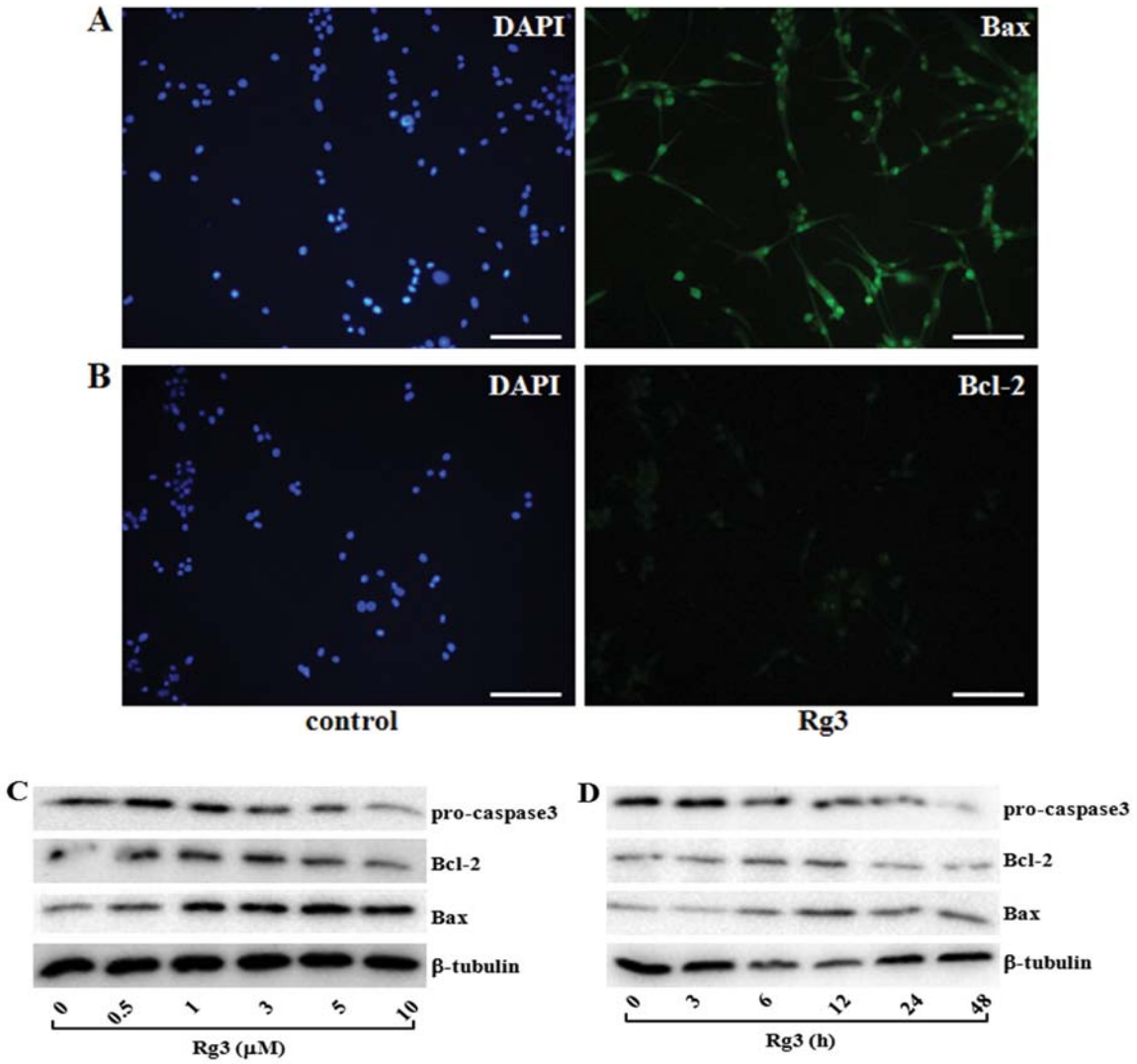

Figure 3. Rg3 induces apoptosis in U87MG cells. Cells were treated with 10 μM Rg3 for 24 h and were then examined by immunocytochemistry (A and B) and western blot analysis (C and D).

Under high-concentration treatment conditions, Rg1 and Rg3 showed markedly different patterns (Fig. 1A). We also treated U87MG cells with low concentrations of each of the two reagents. As shown in Fig. 1B-E, Rg3 clearly decreased the viability of cells in a dose- and time-dependent manner when compared to Rg1 treatment. These results provide evidence that Rg3 exerts an inhibitory effect against U87MG cells.

Rg3 induces apoptosis in U87MG cells. We used a variety of methods to clarify the findings of the observed Rg3 effects on cell viability and morphology. Cells that were treated with Rg3 exhibited cellular adhesion loss and morphological change undergoing rounding and shrinkage (Fig. 2A). In addition, flow cytometry was performed to reaffirm the inhibitory effects of Rg3 on the cells, and Rg3-treated cells showed apoptosis, with a peak from 2.9 to 54.6 (Fig. 2B). To identify the type of cell death, U87MG cells were treated with Rg3 and then stained by TUNEL assay (Fig. 2C). The numbers of positively stained cells (green) were visibly increased after treatment with Rg3 when compared to the untreated condition. These findings suggest that RG3 induced apoptotic changes in the cells. Moreover, the cells treated with Rg3 were stained by immunocytochemistry using a pro-apoptotic member, Bax, and an anti-apoptotic member, Bcl-2 (16) (Fig. 3A and B). The level of Bax expression was high, whereas Bcl-2 expression was very low. Similarly, western blot analysis indicated that the levels of pro-caspase3 and Bcl-2 expression were universally decreased in cells that were treated with different concentrations of Rg3 for various lengths of time, but the level of Bax expression increased in a dose- (Fig. 3C) and time- (Fig. 3D) dependent manner. These results indicate that Rg3 induced apoptosis in U87MG cells.

Effect of Z-VAD-fmk on U87MG cells during Rg3-induced apoptosis. To verify the involvement of the caspase cascade in Rg3-induced apoptosis, the cells were pre-exposed to the general caspase inhibitor, Z-VAD-fmk. Caspases, the interleukin-1 β-converting enzyme family proteases, are one of the major executors of the apoptotic process, and they convey the apoptotic signal via induction of death receptors (17,18). Caspases have been reported as a class of cysteine proteases including several representatives involved in apoptosis (19). However, the results of our present study indicate that the cell viability of the group that was pre-treated with the caspase

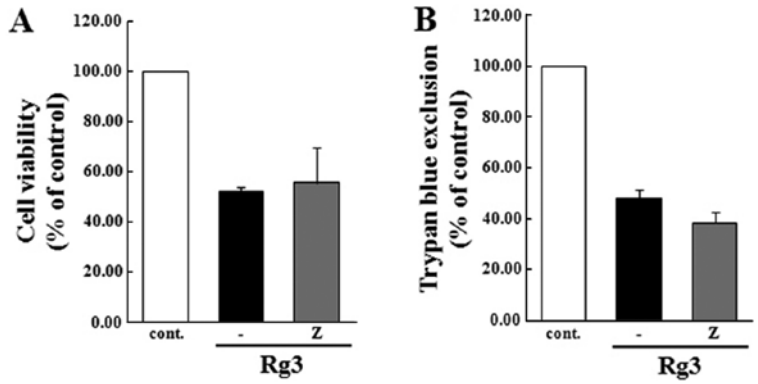

Figure 4. Rg3-induced apoptosis is not directly correlated with caspase cascade. Cells were pre-treated with 20 μM Z-VAD-fmk for 1 h, after which they were treated with 10 μM Rg3 for 24 h and analyzed by MTT assay (A) and trypan blue exclusion (B). The data are the means ± SEM of four independent experiments. *P<0.05 when compared to the control without Rg3. The scale bar indicates 400 μm.

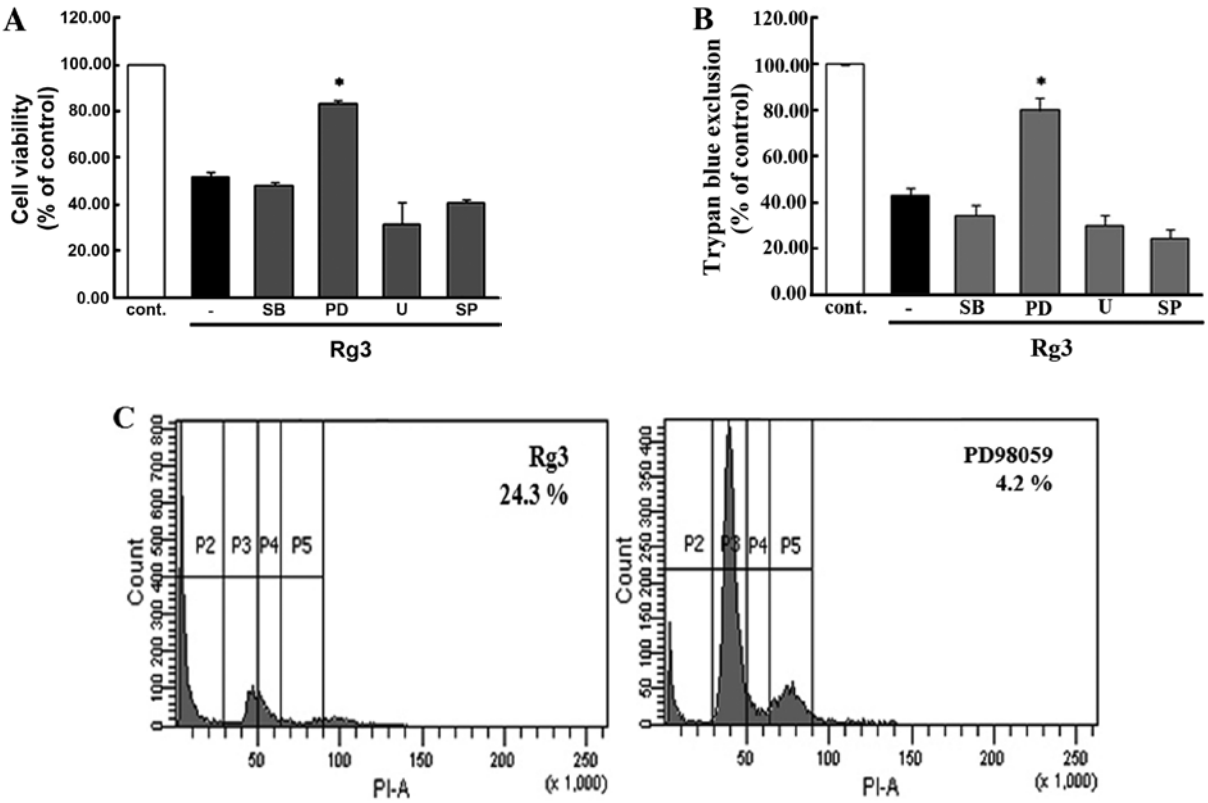

Figure 5. Rg3-induced apoptosis is related to MEK signaling. Cells were pre-treated with 25 μM SP600125 (SP), 20 μM SB203580 (SB), 20 μM PD98059 (PD) and 20 μM U0126 (U), and then treated with 10 μM Rg3 for 24 h. The U87MG cells were measured using an MTT assay (A) and trypan blue exclusion (B) and were examined by flow cytometric analysis (C). The data reported are the means ± SEM of four independent experiments. *P<0.05 when compared to the control without Rg3.

cascade inhibitor was sustained in the Rg3-treated group (Fig. 4), suggesting that the caspase cascade does not regulate Rg3-induced apoptosis in U87MG cells.

Effect of mitogen-activated protein kinases (MAPKs) on U87MG cells during Rg3-induced apoptosis. MAPK signaling cascades are composed of a large group of serine/threonine kinases. The MAPKs mediate signal transduction from the cell surface to the nucleus and are actively involved in converting a wide variety of extracellular stimuli commonly expressed in various cell types (20,21). Several studies have shown that the MAPK signaling pathway plays an important role in the regulation of cellular growth, survival, apoptosis and differentiation (22-24). Moreover, it has been established that MAPK consists of three parallel kinase modules, ERK, JNK and p-38-MAPK. Overall, MEK, a key kinase, is responsible for the upstream signals from Ras and Raf via activation of ERK (25). Based on these findings, we examined the effects of Rg3 on the viability of U87MG cells by conducting a variety of methods. Cells pre-treated with inhibitors of MAPK family members were measured by MTT assay (Fig. 5A) and trypan exclusion assay (Fig. 5B). To confirm these results, we

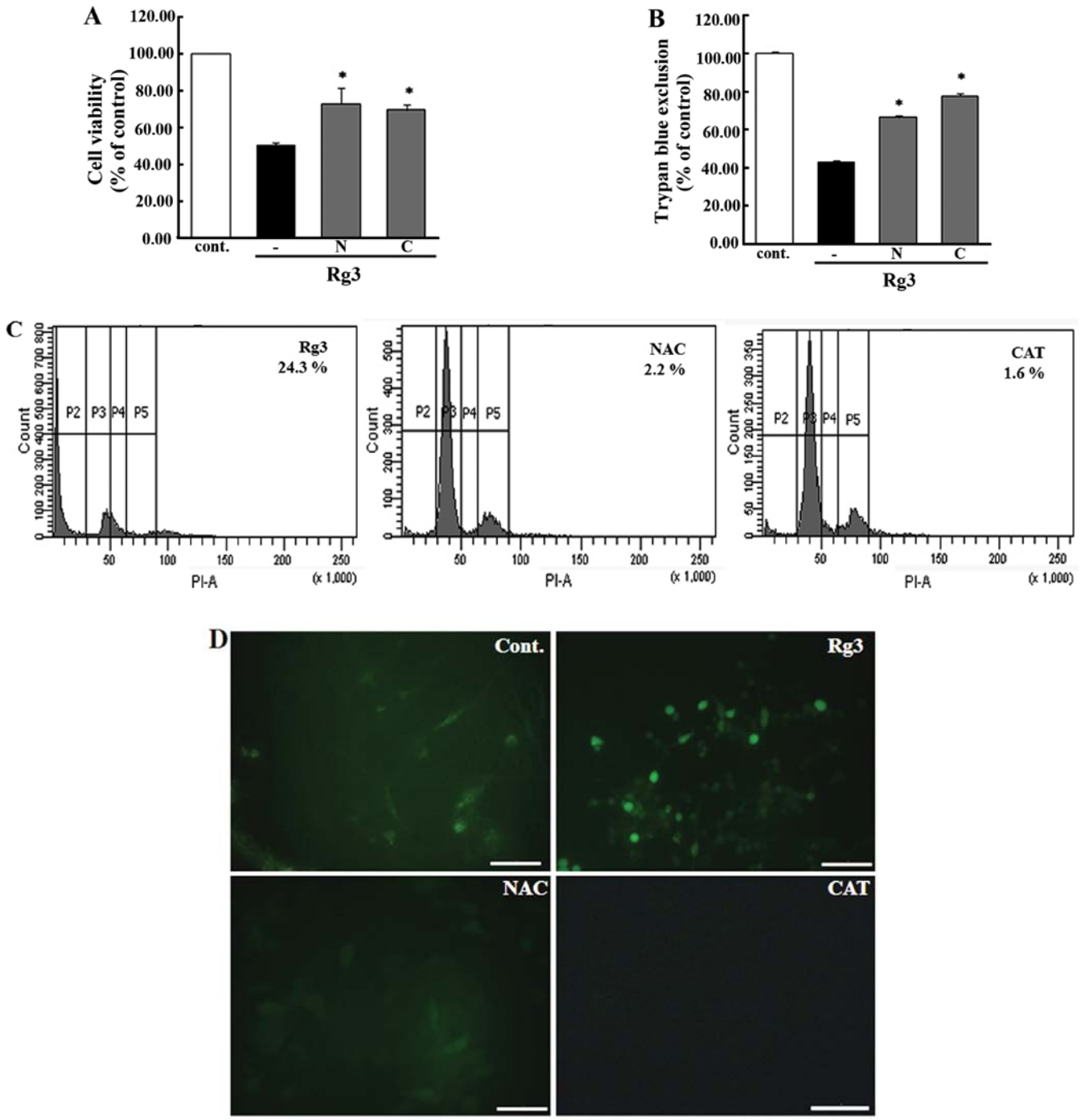

Figure 6. Rg3-induced apoptosis is specifically related to the antioxidant enzyme system. Cells were pre-treated with 10 μM NAC (N) and 800 U/ml CAT (C), after which they were treated with 10 μM Rg3 for 24 h. The U87MG cells were measured using an MTT assay (A) and trypan blue exclusion (B) examined by flow cytometry (C) and the DCF fluorescence intensity was assessed by fluorescence microscopy (D). The data reported are the means ± SEM of four independent experiments. *P<0.05 when compared to the control without Rg3.

conducted flow cytometry (Fig. 5C) to compare the inhibitor treatment group to the untreated control group. Our data clearly show that Rg3-induced apoptosis in U87MG cells through PD98059 (specific inhibitor of MEK1/2).

Effect of antioxidant enzyme system on U87MG cells during Rg3-induced apoptosis. Antioxidant enzymes are endogenous proteins that are well known for their involvement in protecting cells from ROS damage (26). ROS are extremely toxic to organisms (27,28) and oxidative stress can lead to damage to cellular structures and is related to a number of diseases, including cancer (29). The present study was conducted to verify whether ROS is associated with the regulation of Rg3-induced cellular apoptosis. To accomplish this, we used antioxidant enzyme inhibitor for the treatment of cells. As shown in Fig. 6A and B, in the group pre-treated with antioxidant enzymes such as N-acetylcysteine (NAC) and catalase (CAT), all cells were sustained as in the control group, except for those that received the Rg3 treatment, which showed decreased cell viability. To confirm these results, we

conducted flow cytometry (Fig. 6C) to compare the group that received the antioxidant pre-treatment to the untreated control group. Moreover, we measured the fluorescence activation of ROS using DCFH-DA staining (Fig. 6D) as DCFH-DA is generally used for detection of ROS formation (30). DCFH-DA expression was not detected in cells that were pre-treated with CAT and NAC, while the Rg3-treated group showed increased DCFH-DA expression when compared to the control. Taken together, these findings indicate that ROS is involved in Rg3-induced apoptosis, particularly through the work of antioxidant enzymes system.

Discussion

Studies of the chemo-preventive effect of anticancer reagents have recently begun to focus on naturally-occurring chemical compounds in plants and animals. KRG is well-recognized in traditional Korean medicine as having a pharmacological effect (6-8). Among various components of KRG, ginsenosides are the most widely known. These compounds have diverse, beneficial biochemical activities, including chemo-preventive effects against diseases. Rg3 has negative effects on cancer growth, such as inhibition of metastasis and angiogenesis (31), confirming its usefulness as a novel anticancer agent (32). Rg3 is also an effective chemical reagent of a saponin, a unique component of KRG that can be extracted from ginseng (12,33). It has been reported that Rg3 has potential cancer-preventive effects owing to its suppression of invasion, metastasis and growth of various forms of cancer and neovascularization (32,34). Moreover, Rg3 has been reported to induce the reduction of metastasis and the amount of tumor development in colon and ovarian cancer (35), as well as to inhibit angiogenesis in prostate (15) and lung cancer (36).

Effective and less-invasive alternatives for cancer treatment are actively being developed. To date, surgical techniques and chemotherapies such as reagents targeting specific molecules have been applied to improve the prognosis of cancer treatment.

Despite several novel trials for its treatment, GBM is one of the most aggressive and invasive malignant tumor forms of human cancer (1,2). As standard treatment methods for cancer such as chemotherapy, microsurgical techniques and radiotherapy have been shown to be ineffective at ameliorating GBM, the prognosis of GBM remains poor (5,37,38). Consequently, recent studies have focused on the development of new treatment modalities for cancer such as biological therapy and chemotherapy using novel substances that can be extracted from a variety of foods.

This study was conducted to investigate the effects of Rg3 on U87MG cells. Furthermore, we attempted to identify the molecular mechanisms of cell death induced by Rg3 using various inhibitory agents.

U87MG cells were used to explore the effects and regulatory mechanisms of Rg3 on the human glioblastoma cell line. First, we treated cells with Rg3 (panaxadiol group) and Rg1 (panaxatriol group). The results of these experiments revealed different effects on the cells, particularly under high concentration conditions (9). Specifically, the inhibition of U87MG cell growth was much greater when cells were treated with Rg3 than with Rg1, and these differences occurred in a dose-dependent manner (Fig. 1A). We also found that Rg1 had no inhibitory effect on cell proliferation or viability, while Rg3 did (Fig. 1B-E).

Some studies have reported that Rg1 promotes angiogenesis *in vivo* and *in vitro* by inducing vascular endothelial growth factor (VEGF), a mediator of angiogenesis (9). These results suggest that Rg1 may be the main candidate for angiotherapy due to its potential to induce wound healing and tissue regeneration.

We verified whether the inhibitory effect of Rg3 was related to cell apoptosis. Observation of morphological changes in the cells, flow cytometry, TUNEL assay and expression of Bax or Bcl-2 indicated that Rg3 led to apoptosis (Figs. 2 and 3).
Apoptosis or programmed cell death is a common type of cell death, and is one of the principal mechanisms involved in tissue homeostasis (39-41). This physiological 'cell suicide' program is essential for diverse cellular processes, particularly for the elimination of damaged, infected and redundant cells. Apoptosis is induced by a disparate variety of pathways that can be further divided into intrinsic and extrinsic apoptotic pathways. The general methods for effectively detecting apoptotic cells are the TUNEL assay and flow cytometry (42,43).

We conducted a TUNEL assay using an *in situ* Cell Death Detection kit. As expected, positive-stained cells (green) were highly detected by DNA fragmentation labeling of the terminal end of nucleic acids. Cells treated with Rg3 showed morphological changes ranging from rounding shape to shrinkage. Furthermore, the level of Bax expression was largely detected in the cells, but that of Bcl-2 was not. Collectively, these results indicate that Rg3 induced the death of U87MG cells through apoptosis.

The relationship between GBM and Rg3 was uncertain, therefore we investigated whether Rg3 conducted molecular mechanisms during apoptosis of the cells.

Rg3 was previously reported to exert anticancer activity through various molecular pathways including Wnt/β-catenin signaling (44), the caspase-dependent signaling cascade (45) and the mitochondrial pathway (46) in different cell lines. Therefore, we examined the involvement of the caspase cascade in the effects of Rg3 treatment. The intracellular cysteine enzymes mediating the caspase cascade are well defined as a family of proteins that are major executors of apoptosis processes (17,18). Although the caspase cascade has been shown to activate apoptosis, the roles of the individual caspases remain uncertain. The results of the present study showed that the caspase cascade was not involved in the apoptosis signal induced by Rg3 (Fig. 4).

We demonstrated that MAPK signaling may be related to Rg3-induced anticancer activity. MAPK cascades are the main signaling pathways involved in various cellular responses including proliferation, survival, inflammation and differentiation (21,47). The key factors involved in these cascades are MAPK/ERK, SAPK/JNK, and p38-MAPK. Moreover, the MAPK/ERK signaling cascade is well known to occur in response to Raf and MEK in cancer progression and cancer growth (23,25,48). The MAPK ERK signaling cascade starts with the phosphorylation and activation of MEK by Raf, which is followed by the phosphorylation and activation of ERK by MEK (22,49). MEK plays a specific dual role in phosphorylation of tyrosine and activation of threonine residues on ERKs 1

and 2. However, the relationship between JNK and p38 MAPK and their involvement in cancer signaling pathways is less clearly established.

As shown in Fig. 5, pretreatment of PD98059 on the U87MG cells showed maintained cell viability markedly better than other inhibitor pretreatment groups compared with control. MEK signaling has relevance to Rg3-induced apoptosis, while other inhibitors of MAPKs had only a slight effect on the viability of U87MG cells. We indicated if the MEK signaling pathway could be controlled in living systems, cancer could be also controlled in the same system. The results of the present study suggest that efficient cancer treatment can inhibit the MEK signaling pathway.

ROS occur naturally in organisms as side-products from the homeostatic intracellular signaling and as a part of the defense mechanism of the immune system. Moreover, ROS have been found to directly injure cells and to play an important role in the physiological and pathological processes of diseases including ischemia, tissue damage, endocrine dysfunction and cancer (26,50,51). To accelerate oxidative stress to exert cell damage, antioxidant systems and capacity must fail and decline. ROS are neutralized by antioxidant enzymes such as superoxide dismutase (SOD), glutation peroxidase (GSHPx), glutation reductase (GR) and CAT (52-54). Moreover, antioxidant enzyme imbalances induce the arrest of cell proliferation and growth, leading to cell cycle arrest being switched on by activated p53 proteins, resulting in apoptosis (55,56).

We found that antioxidant enzyme systems have significant control over molecular signaling pathways and the mechanism of Rg3-induced apoptosis in U87MG cells. As shown in Fig. 6A-C, cells that were treated with Rg3 displayed decreased viability. However, the group subjected to antioxidant enzyme pretreatment showed sustained cell viability, similar to the results observed in the control group. CAT is a tetrameric heme containing an enzyme that degrades hydrogen peroxide into oxygen and water (53,57). N-acetylcysteine and N-acetyl-L-cysteine (NAC) are thiols that act as mucolytic agents and are derived from sulfhydryl groups in cells and scavengers that interact with the ROS of free radicals (58,59). These are the main parts of the enzymatic and protective system against ROS, and are known to be indispensable to ROS neutralization under oxidative stress conditions (56).

To confirm that the antioxidant enzyme system exerts an effect on the molecular mechanism during Rg3-induced apoptosis, we stained the cells with DCFH-DA (Fig. 6D). The results revealed that Rg3-induced apoptosis signaling was regulated by CAT, and that this association was triggered by the antioxidant enzyme system.

There have been few studies on Rg3 for the treatment of GBM, and there have been few investigations of its molecular mechanism in such treatments. The results of our present study indicated that Rg3 has a greater inhibitory effect on the U87MG human glioblastoma cell line, and that its apoptotic mechanism was regulated through MEK and ROS. We expect Rg3 to be the major candidate for natural treatment of GBM, as well as other types of cancer. Further studies using various cancer cells should be conducted to verify the anticancer effects of Rg3 and its molecular mechanism on these cells.

Acknowledgements

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