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# Pivotal Roles of Ginsenoside Rg3 in Tumor Apoptosis Through Regulation of Reactive Oxygen Species

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**Abstract.** *Background: Elevated production of reactive oxygen species (ROS) is observed in various cancer types and pathophysiological conditions. In cancer cells, ROS induce cell proliferation, genetic instability, and a malignant phenotype. Ginsenoside Rg3 is the main pharmacologically active component in ginseng and has been reported to have an antioxidant effect. To overcome lung cancer by regulating the ROS level, we investigated the antitumor effect and mechanism of Rg3 and its antioxidative property on Lewis lung carcinoma (LLC) cells. Materials and Methods: Inhibition of ROS was suppressed in LLC cells by Rg3 treatment, and these cells were used to investigate the antioxidant, antiproliferative, and antitumor effects in LLC cells. Results: ROS production was increased in cells grown in serum-containing media (conditioned media) compared to those grown in serum-free media. The high level of ROS induced LLC cell proliferation, but treatment with Rg3 (200 ng/ml) resulted in reduction of ROS, leading to inhibition of cell proliferation. Treatment with Rg3 significantly reduced cyclin and cyclin-dependent kinase expression in LLC cells. Additionally, Rg3 treatment significantly suppressed activation of mitogen-activated protein kinases and induced LLC cell apoptosis through activation of pro-apoptotic proteins and suppression of anti-apoptotic proteins. Conclusion: Taken together, these findings demonstrate the role of Rg3 in reduction of the intracellular ROS level, attenuation of proliferation via augmentation of cell cycle- and cell proliferation-associated proteins, and activation of apoptosis through regulation of apoptosis-associated proteins in LLC. These findings suggest that Rg3 could be used as a therapeutic agent in lung cancer.*

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Lung cancer is the most common malignancy of the respiratory system, accounting for 23% of all malignant cancer deaths (1). After diagnosis, the survival rate in patients with lung cancer is approximately 15% at 5 years (2). Conventional therapy for lung cancer generally includes surgery, chemotherapy, and radiotherapy but results in a low survival rate because of their toxic side-effects (3, 4). Therefore, it is important to improve the therapeutic efficacy of lung cancer treatment and to reduce the associated side-effects. In addition, novel anticancer agents are urgently needed. Several researchers have focused on natural products as cancer therapeutic agents with anti-aging, anti-inflammatory, and antitumor effects (5-8).

Ginsenoside Rg3, the major active component of ginseng, has shown various pharmacological activities including immunomodulatory, antioxidant, anti-inflammatory, anticancer, and anti-aging activities in several diseases and infections, including different types of cancer, metabolic diseases, and neurodegenerative diseases (9, 10). Several studies show that Rg3 exhibits anticancer activities, such as inhibition of cancer cell growth, invasion and metastasis; suppression of angiogenesis; and induction of apoptosis, in different types of cancer, including ovarian, lung, intestinal, prostatic, and gastric (11-17). In addition, Rg3 reduces tumor migration and metastasis by inhibiting epithelial–mesenchymal transition, which is an important factor in cancer metastasis (18).

Reactive oxygen species (ROS) are associated with cancer progression. ROS can both accelerate and delay tumor initiation and progression. ROS and oxidative stress result in

cancer cell growth and genetic instability but a high level of ROS causes considerable damage, leading to cancer cell death (19). In addition, up-regulation of antioxidant capacity against intrinsic oxidative stress can confer drug resistance in cancer cells (20). Therefore, redox-regulatory pathways and signaling mechanisms should be investigated in more detail. Recently, Chandel and Tuveson proposed two potential antioxidant therapeutic strategies (21). The first involves effective therapies that can directly inhibit the generation of or scavenge ROS. The second strategy involves the inhibition of antioxidant systems, inducing tumor cell death because of toxicity due to the consequently high level of ROS. However, this strategy should be able to differentiate clearly between normal and cancer cells.

In this study, we investigated the effect of ginsenoside Rg3, as an antioxidant, on tumor apoptosis of Lewis lung carcinoma (LLC) cells. In addition, we explored the mechanism underlying the effects of Rg3 on tumor growth and apoptosis.

## Materials and Methods

*Preparation of ginsenoside Rg3*. Ginsenoside Rg3 was purchased from Sigma Aldrich (St. Louis, MO, USA). Rg3 was dissolved in dimethyl sulfoxide, sterilized by filtration through a 0.45-μm pore filter (Millipore, Billerica, MA, USA), and stored at 4˚C until use.

*Cell culture*. Mouse LLC cells were kindly provided by the College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University (Republic of Korea). The cells were maintained in Dulbecco's modified Eagle's medium (4.5 g/l glucose) supplemented with 10% fetal calf serum, L-glutamine, and antibiotics (Biological Industries, BeitHaemek, Israel) at 37˚C with 5% $CO_2$ in a humidified incubator.

*Cell viability assay*. Exponentially growing LLC cells in 96-well plates (1,000 cells/well) at subconfluence were incubated with $H_2O_2$ (0-$10^{-14}$ M) for 24 h and different durations (0, 6, 12 and 24 h) in serum-free medium, and treated with Rg3 (0, 100, 200, 400, and 600 ng/ml) for different durations (0, 12, 24, and 48 h) in conditioned medium. Cell viability was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium to formazan by mitochondrial dehydrogenase. The formazan product was quantified by evaluating the absorbance of the reaction at 570 nm by using the SPECTROstar Nano® (BMG Labtech, Ortenberg, Germany)

*Western blot analysis*. The proteins of LLC cells treated with Rg3 (200 ng/ml) were extracted using RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA). The cell lysates were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% skim milk for 1 h at room temperature and then incubated overnight with primary antibodies at 4˚C. Antibodies against B-cell lymphoma 2 (BCL2), BCL2-associated X protein (BAX), cleaved poly(ADP ribose)polymerase-1 (PARP1), cyclin-dependent kinase 2 (CDK2), CDK4, cyclin D1, cyclin E, extracellular signal-regulated kinase (ERK), p38, c-JUN *N*-terminal kinase (JNK), and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA); and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA) were diluted in Tris-buffered saline in 0.2% Tween-20 (TBS-T). After the membranes were washed with TBS-T, the bound primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology). The bands were visualized using enhanced chemiluminescence reagents (Thermo Scientific, Rockford, IL, USA) in a dark room.

*Flow cytometric analysis for ROS generation, proliferation, and apoptosis*. LLC cells treated with $H_2O_2$ or Rg3 were harvested, fixed in 70% ethanol at –20˚C for 48 h, and then washed twice with cold PBS. To detect ROS production, dihydroethidium (DHE, 10 μM; Sigma Aldrich) was added to the cells and the cells were then washed thrice with PBS. The cells were then incubated with RNase and the DNA-intercalating dye, propidium iodide (PI) for cell-cycle analysis or with PI and annexin V (SysmexPartec GmbH, Gorlitz, Germany) for apoptosis analysis. ROS generation, cell-cycle distribution, and apoptosis were assessed with a Cyflow Cube 8 instrument (Partec, Munster, Germany). Data analysis was performed using the standard FSC Express (De Novo Software, Los Angeles, CA, USA).

*Statistical analyses*. Results are expressed as mean±standard error of the mean (SEM). All experiments were analyzed by Student's *t*-test, where differences with $p<0.05$ were considered significant.

## Results

*Effect of ROS on proliferation of LLC cells*. To investigate the difference between serum-free and conditioned media for ROS generation, ROS generation was confirmed by flow cytometry for DHE staining in cells from serum-free and conditioned media. ROS production was augmented in cells from conditioned medium compared to those in serum-free medium (Figure 1A). To explore whether ROS induced proliferation of LLC cells, the proliferative potential of LLC cells was assessed by flow cytometry for PI staining and by the MTT assay. Flow cytometry for PI staining indicated that conditioned medium-induced ROS generation resulted in a higher proliferative potential of LLC cells than did serum-free medium (Figure 1B). After treatment with $H_2O_2$ (0-$10^{-14}$ M) to verify the association between ROS and LLC cells, cell proliferation assay was performed. The proliferation of LLC cells was significantly increased at a low concentration of $H_2O_2$ ($10^{-10}$ M) compared with that in the control cells (Figure 1C). In addition, the proliferation of LLC cells was significantly increased in a time-dependent manner (0-24 h; Figure 1D). These results show that ROS induce proliferation of LLC cells.

*Ginsenoside Rg3 reduces LLC cell viability*. To explore whether Rg3, as an antioxidant, reduces LLC cell viability,

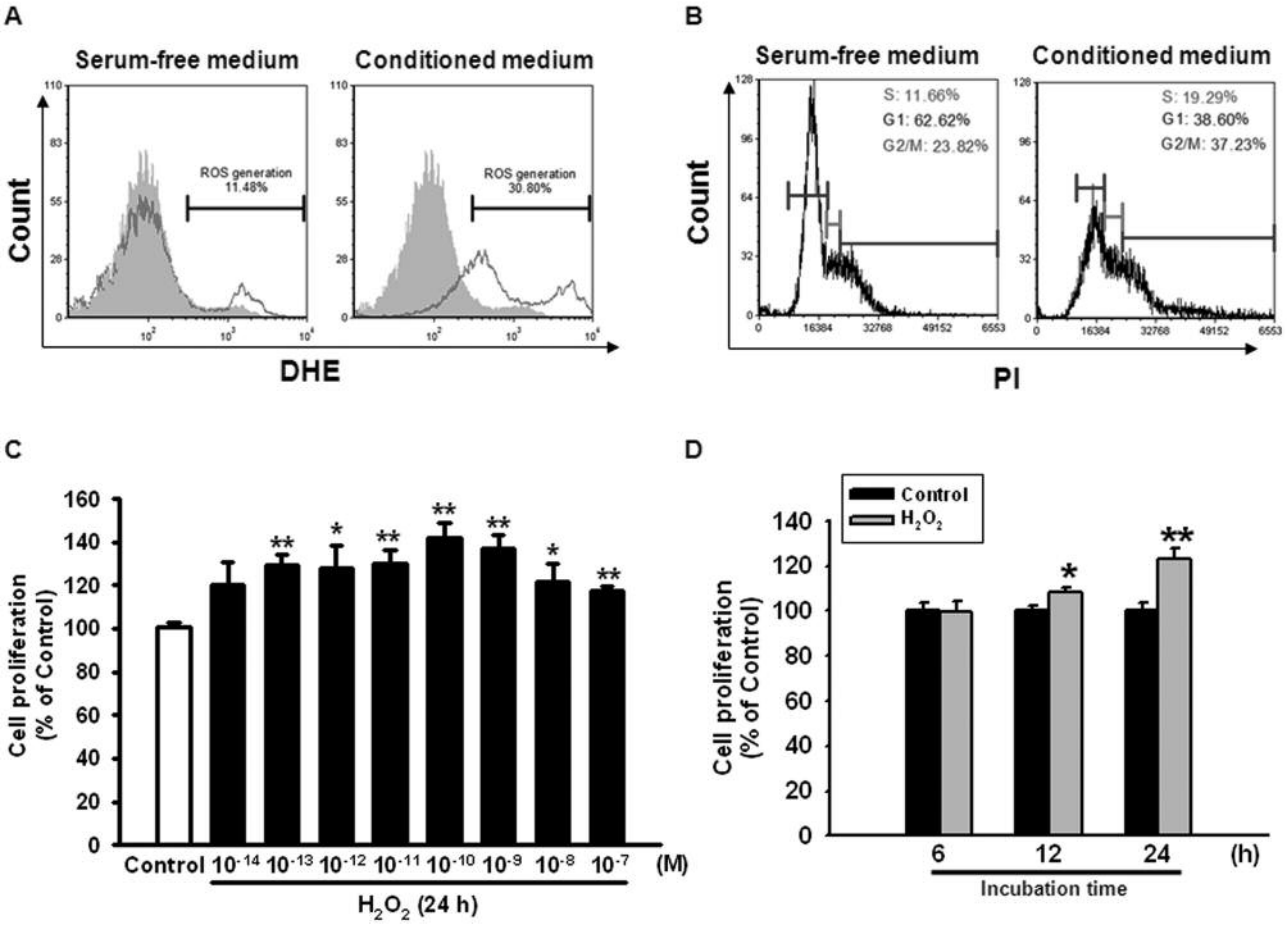


Figure 1. *The effect of reactive oxygen species (ROS) on proliferation of Lewis lung carcinoma (LLC) cells. A: Production of ROS was assessed by flow cytometry for dihydroethidium (DHE) staining in serum-free and serum-containing (conditioned) media. B: Cells in the S phase were measured using flow cytometry for propidium iodide (PI) staining in serum-free and conditioned media. C: Proliferation of LLC cells was evaluated after treatment with $H_2O_2$ for 24 h. D: Proliferation of LLC cells was assessed after treatment with $H_2O_2$ ($10^{-10}$ M) for the indicated times.*

an MTT assay was performed after treatment with Rg3 at different concentrations (0-600 ng/ml) for 48 h. Cell viability was significantly lower at an Rg3 concentration of 200 ng/ml compared to that of the control (Figure 2A). Moreover, cell viability was significantly lower after treatment for 24 h and 48 h (Figure 2B). The number of LLC cells was significantly reduced when they were treated with Rg3 (200 ng/ml) compared to control (Figure 2C). To confirm the antioxidant effect of Rg3 in LLC cells, ROS generation was assessed by flow cytometry for DHE staining after treatment of LLC cells with Rg3. ROS production by LLC cells was found to be reduced by treatment with Rg3 in conditioned medium (Figure 2D). These findings indicate that Rg3 reduces LLC cell viability *via* reduction of the ROS level.

*The mechanism underlying Rg3-mediated reduction of proliferation in LLC cells*. To verify whether Rg3 regulates cell proliferation-associated signaling pathways, these pathways in LLC cells were assessed by western blotting. On culture with conditioned medium, the percentage of LLC cells in the S phase decreased upon treatment with Rg3 (200 ng/ml) compared to that of control (Figure 3A). The expression of cell cycle-associated proteins CDK2, cyclin E, CDK 4 and cyclin D1 significantly decreased in LLC cells treated with Rg3 compared with untreated cells (Figure 3B and C). In addition, the activation of cell proliferation-associated mitogen-activated protein kinases (MAPKs), ERK, p38, and JNK, was significantly reduced in LLC cells treated with Rg3 compared to untreated cells (Figure 4). Our findings suggest that Rg3 reduces LLC cell proliferation through regulation of cell cycle-associated proteins and phosphorylation of MAPKs.

*Rg3 induces LLC cell apoptosis through activation of apoptosis-associated proteins*. To investigate whether treatment

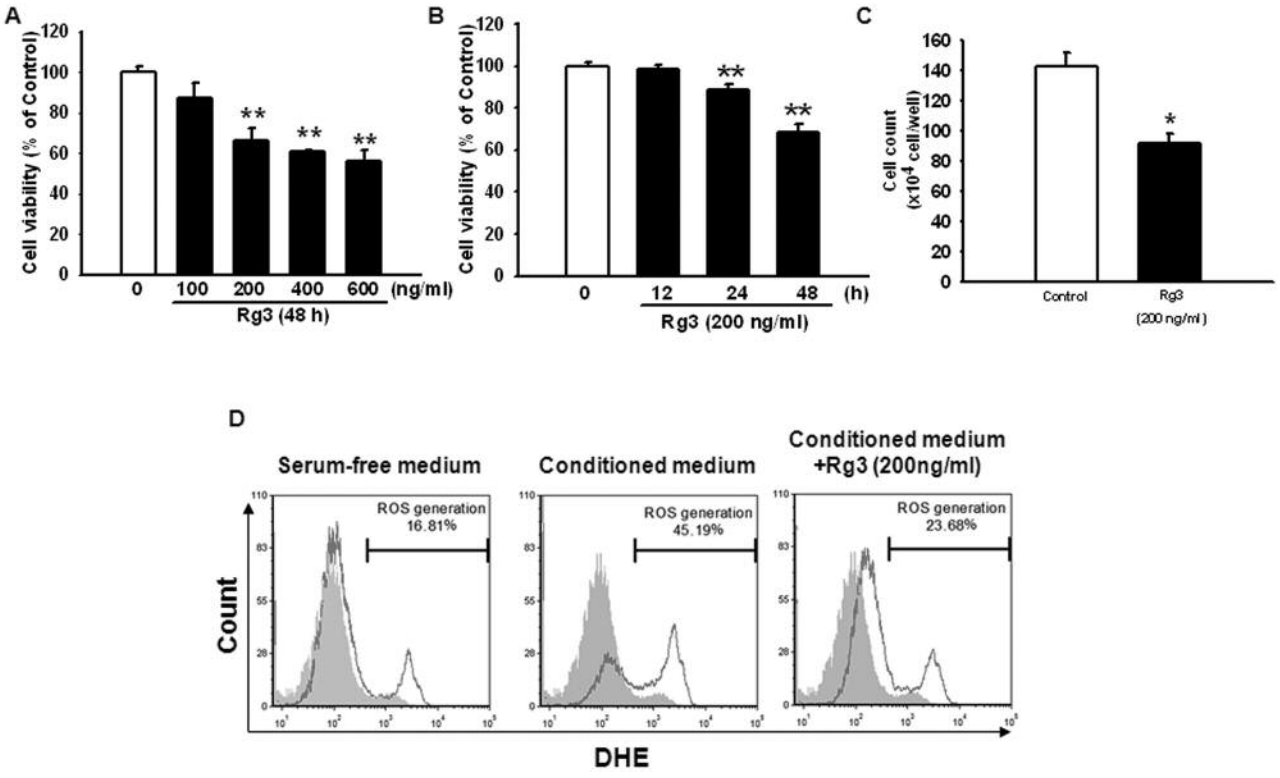


Figure 2. *The effect of ginsenoside Rg3 on proliferation of Lewis lung cancer (LLC) cells. Cell viability was assessed after treatment with Rg3 at different concentrations for 48 h (A) and after treatment with Rg3 (200 ng/ml) for the indicated times (B). C: After treatment with Rg3 (200 ng/ml) for 48 h, the number of cells was directly counted. D: Reactive oxygen species (ROS) generation was evaluated using flow cytometry for dihydroethidium (DHE) staining. Values are expressed as the mean±SEM. *p<0.05 and **p<0.01 vs. control.*

with Rg3 (200 ng/ml) leads to LLC cell apoptosis, flow cytometry for PI and annexin V double staining was performed to assess the apoptotic cell population. The apoptotic cell population was found to be significantly higher upon treatment with Rg3 than in the control (Figure 5A). Apoptosis-associated proteins were assessed by western blotting. The expression of the anti-apoptotic protein BCL2 significantly decreased in Rg3-treated cells compared to the control (Figure 5B). The expression of pro-apoptotic proteins BAX, PARP, and cleaved caspase-3 was significantly increased upon treatment with Rg3 compared to the control (Figure 5C-E). These results suggest that Rg3 promotes LLC cell apoptosis *via* the regulation of apoptosis-associated proteins.

## Discussion

ROS, which act as secondary messengers in cellular signaling, play important roles in several normal physiological and abnormal pathophysiological processes. The ROS level is increased in various cancer cells because they have higher metabolic rates compared to normal cells (22). The high level of ROS generation in cancer cells result in stimulation of proliferation, promotion of mutations, induction of genetic instability, and enhancement of drug resistance (23), indicating that regulation of ROS metabolism and pathways in cancer cells might be an effective strategy for overcoming various types of cancer (24). This study demonstrated that Rg3, as a natural antioxidant, suppressed proliferation and induced apoptosis of LLC cells through the reduction of the ROS level. Moreover, these anticancer effects also involved regulation of cell proliferation- and apoptosis-associated proteins.

Ginsenosides have antioxidant, anti-inflammatory, and antitumor properties (25). Ginsenoside Rg3, one of the most active ginsenosides, has been shown to exhibit anticancer properties through its anti-angiogenesis (26) and anti-metastasis (27) effects, activation of endoplasmic reticulum stress (28), promotion of senescence and apoptosis (29), suppression of epithelial–mesenchymal transition (18), inhibition of proliferation (17), and attenuation of migration (30). In particular, Rg3 has antioxidant activities (31). Alphalipoic acid, another natural ROS scavenger, was shown to increase cell-cycle arrest and apoptosis of breast cancer cells (32). The

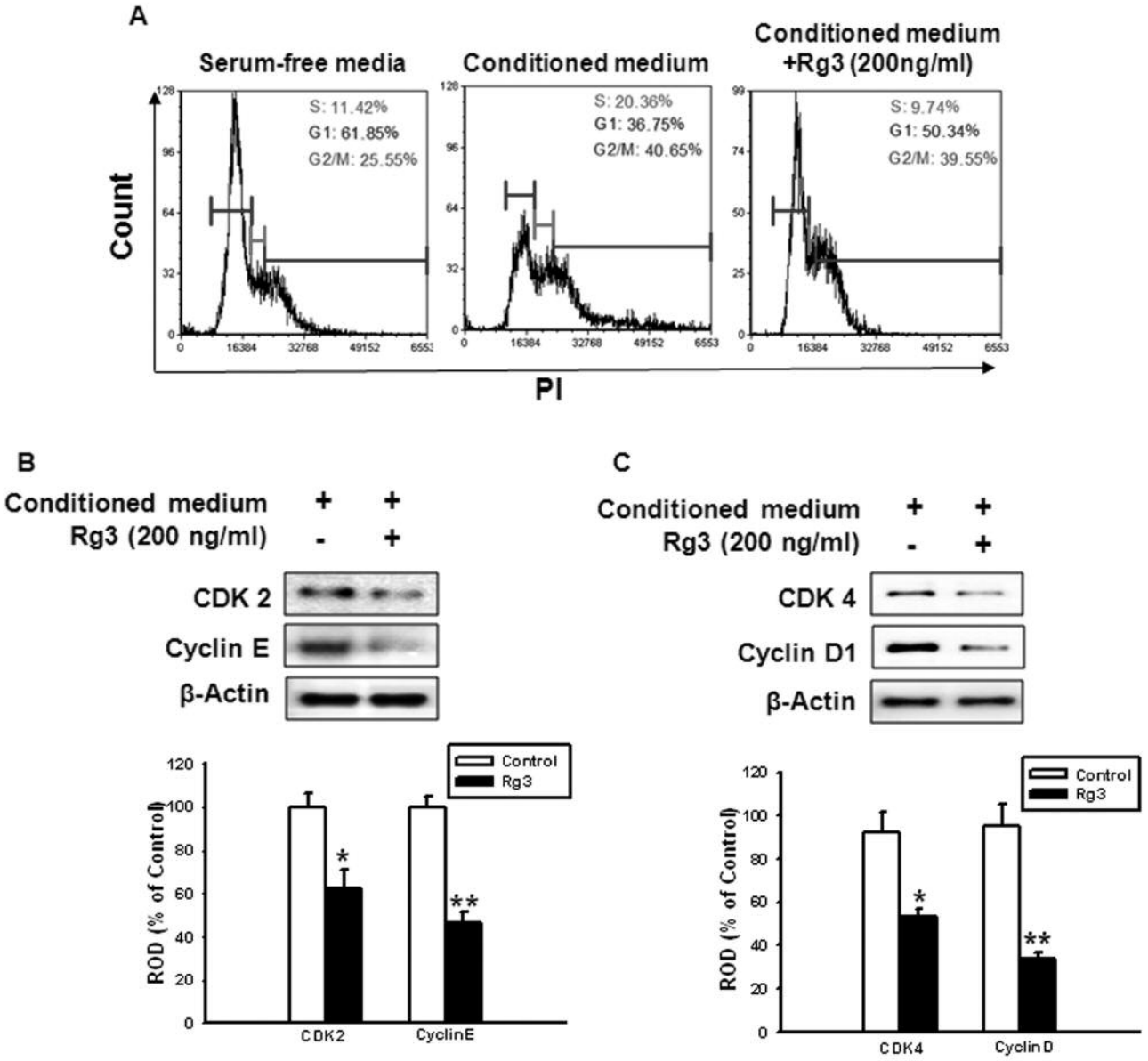


Figure 3. *Rg3 reduces Lewis lung cancer (LLC) cell proliferation through reduction of cell cycle-associated proteins. A: After treatment with Rg3 (200 ng/ml), cells in the S phase were assessed by flow cytometry for propidium iodide (PI) staining. After treatment with Rg3 in conditioned medium, the expression of cyclin-dependent kinase 2 (CDK2), cyclin E (C), CDK4 and cyclin D1 (D) was assessed by western blotting. The lower panel shows the expression levels of proteins normalized to that of β-actin. Values represent the mean±SEM. *p<0.05 and **p<0.01 vs. untreated LLC cells.*

apolipoprotein A1 mimetic peptide suppressed proliferation and tumorigenicity of epithelial ovarian cancer cells *via* induction of the antioxidant enzyme manganese-dependent superoxide dismutase (33). In this study, ROS generation was significantly increased in cells from conditioned medium compared to those cultured in serum-free medium, resulting in ROS-mediated promotion of LLC cell proliferation. In addition, we reveal that treatment with Rg3 reduced ROS production, thereby reducing LLC cell viability. Our findings suggest that Rg3 inhibits LLC cell viability *via* its antioxidant effect.

ROS-regulated signal pathways include pro-survival and pro-proliferative pathways, including ERK, JNK, and p38 MAPKs (34). Our results indicate that treatment with Rg3 inhibited LLC cell proliferation through suppression of cell cycle-associated proteins, including CDK2, cyclin E, CDK4, and cyclin D1, and cell growth-associated MAPKs,

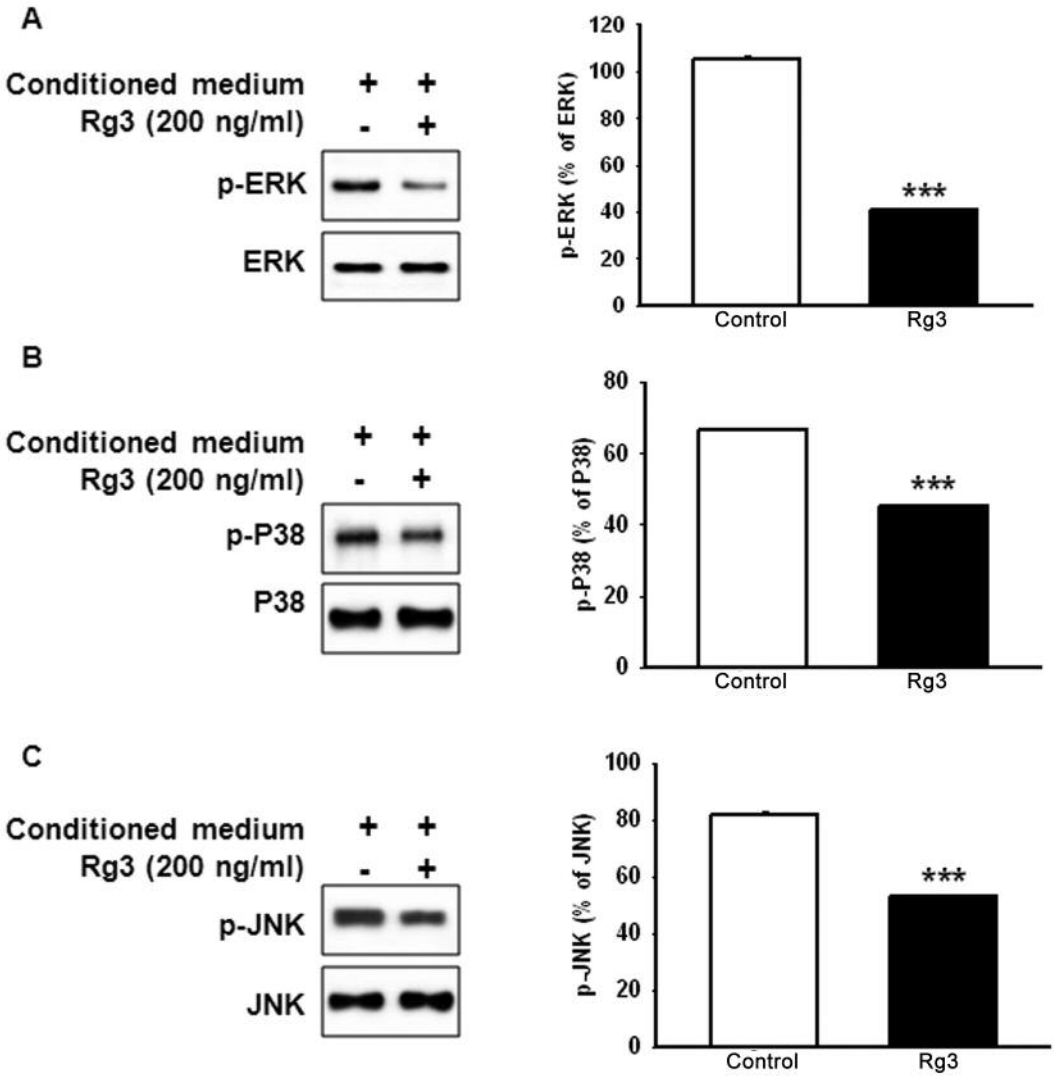


Figure 4. *Rg3 reduces the activation of cell proliferation-associated mitogen-activated protein kinases (MAPK). After treatment of Lewis lung cancer (LLC) cells with Rg3 (200 ng/ml) for 48 h in conditioned medium, the phosphorylation of extracellular signal-regulated kinases (ERK) (A), p38 mitogen-activated protein kinases (p38) (B), and c-JUN N-terminal kinases (JNK) (C) was assessed by western blot. The right panel shows the phosphorylation levels of ERK, p38, and JNK normalized to that of total ERK, p38, and JNK, respectively. Values represent the mean±SEM. ***p<0.001 vs. untreated LLC cells.*

including ERK, p38, and JNK. Rg3-induced MAPK de-activation was found to inhibit melanoma cell proliferation *via* reducing fucosyltransferase 4 expression (17). Moreover, our findings revealed that Rg3 induced LLC cell apoptosis *via* regulation of apoptosis-associated proteins, such as BCL2, BAX, PARP-1, and cleaved caspase-3. Rg3 was also found to induce human myeloma cell apoptosis by activating BAX and cleaved caspase-3 (35). Furthermore, Rg3 induced apoptosis of hepatoma cells through activation of the mitochondrial pathway (36). Taken together, these findings suggest that Rg3 suppresses cell proliferation and induces apoptosis of LLC cells through modulation of cell proliferation- and apoptosis-associated proteins.

In conclusion, this study demonstrates the anticancer effects of ginsenoside Rg3 through the regulation of ROS in LLC cells. Our data indicate that ROS were significantly reduced by the natural product Rg3, resulting in inhibition of LLC cell viability. In addition, our findings showed that Rg3 inhibited the expression of cancer cell proliferation-associated proteins and promoted activation of the apoptosis pathway, suggesting that Rg3 could be a potential chemopreventive agent for lung carcinoma.

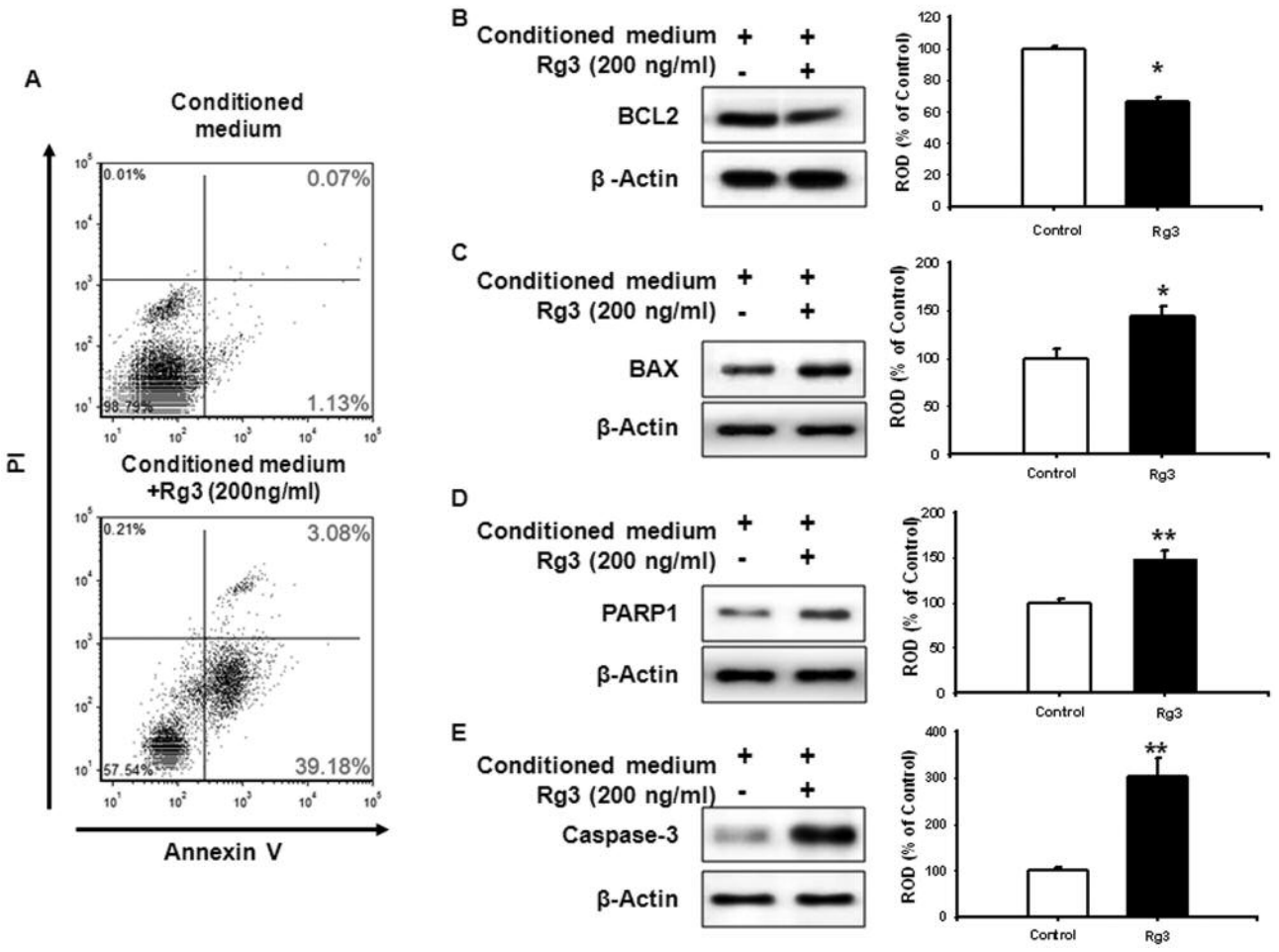


Figure 5. *Rg3 induces Lewis lung cancer (LLC) cell apoptosis through regulation of apoptosis-associated proteins. A: LLC cell apoptosis was measured using flow cytometry for propidium iodide (PI)/annexin V double staining. PI/annexin V double-negative cells were considered as live cells, PI-negative/annexin V-positive cells were considered as early apoptotic cells, and PI/annexin V double-positive cells were considered late apoptotic cells. After treatment with Rg3 (200 ng/ml) for 48 h in conditioned medium, the expression of B-cell lymphoma 2 (BCL2) (B), BCL-2-associated X protein (BAX) (C), cleaved poly [ADP-ribose] polymerase 1 (PARP1) (D), and cleaved caspase-3 (E) was assessed by western blotting. The right panel shows the expression levels of BCL2, BAX, cleaved PARP1, and cleaved caspase-3 normalized to that of β-actin. Values represent the mean±SEM. *p<0.05 and **p<0.01 vs. untreated LLC cells.*

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## Conflicts of Interest

The Authors have no conflicts of interest to declare in regard to this study.

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