Ginsenoside 20(S)-Protopanaxadiol Suppresses Viability of Human Glioblastoma Cells *via* Down-regulation of Cell Adhesion Proteins and Cell-cycle Arrest

CHERYL WANDERI[1], EUNSOO KIM[1], SOYOUNG CHANG[1], CHULHEE CHOI[1,2] and KYUNGSUN CHOI[1]

[1]*Department of Bio and Brain Engineering,*
[2]*KAIST Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea*

Abstract. *Background: Pharmacologically active components of ginseng, particularly protopanaxadiol (PPD)-type ginsenosides, have potent anticancer effects, although their effects on highly malignant glioblastoma multiforme (GBM) have not been systemically evaluated. Identification of effective anticancer ginsenosides and further delineation of their mechanisms of action may provide valuable information that aids in the development of alternative or adjuvant therapy for malignant cancer. Materials and Methods: We examined the viability of human GBM U251-MG and U87-MG cells treated with structurally related PPD-type ginsenosides, including F2, Rh_2, compound K (C-K), and PPD. Results: Incubation with PPD, C-K, and Rh_2 significantly reduced the viability of U251-MG and U87-MG cells in a dose- and time-dependent manner. The cytotoxic effect of PPD was accompanied by reduced expression of cell adhesion proteins, including N-cadherin and integrin β1, which led to reduced phosphorylation of focal adhesion kinase. Furthermore, incubation with PPD reduced the expression of cyclin D1 and subsequently induced cell-cycle arrest at the G_1 phase. Conclusion: These results collectively indicate that PPD might provide a new strategy for treating malignant GBM, which is quite resistant to conventional anticancer treatment.*

Panax ginseng and other related plants have long been used for their medicinal value (1). The major pharmacological effects of ginseng are mediated by bioactive saponins and their metabolites, ginsenosides. Ginsenosides have a common steroid backbone and are classified as protopanaxadiols (PPDs) and protopanaxatriols (PPTs) according to the sugar moieties attached at the C-3, C-6, or C-20 positions of the tetracyclic triterpene backbone (2). Structure–activity relationship studies demonstrate strong anticancer activities of PPD-type ginsenosides with three or fewer sugar molecules, such as Rg_3 (two sugar residues at C-3), F2 (two sugar residues at C-3 and C-20), Rh_2 (one sugar residue at C-3) compound K (C-K, one sugar residue at C-20), and PPD (no sugar) (3). Upon oral ingestion, PPD-type ginsenosides are rapidly hydrolyzed by enteric anaerobic bacteria to C-K and PPD, which are absorbed through intestinal epithelia and found in the plasma.

Glioblastoma multiforme (GBM) is the most common type of primary malignant brain tumor in adults (4). Overall prognosis of GBM remains poor despite combined treatment that includes surgical resection, radiotherapy, and chemotherapy (5). Thus, the development of new effective treatment modalities is in high demand. PPD-type ginsenosides such as Rh_2, F2, and C-K have been shown to have *in vitro* and *in vivo* anticancer efficacy in various GBM models (6-9), suggesting that they may be promising anticancer or adjuvant agents in combination with conventional chemotherapy. This anticancer activity might increase with a reduction in the number of sugar moieties in the ginsenoside molecule (3), and it has been proposed that PPD with no sugar moiety might have improved activity against various human cancer types compared to other structurally related PPD-type ginsenosides (10, 11). Interestingly, the anticancer effect of PPD has not been studied in GBM models. Thus, in this study, we tested the *in vitro* anticancer properties and mechanism of action of PPD in comparison to other structurally related PPD-type ginsenosides, including C-K, Rh_2, and F2, in human GBM cells.

Correspondence to: Kyungsun Choi, Ph.D., Department of Bio and Brain Engineering, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 34141, Korea. E-mail: ccbioks@kaist.ac.kr

Key Words: Ginsenosides, protopanaxadiol, PPD, glioblastoma, cell adhesion.

Materials and Methods

Cell culture and reagents. Human glioblastoma U251-MG and U87-MG cells were maintained in Dulbecco's modified Eagle's medium (Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 incubator at 37˚C. Ginsenoside standards for Rh2(S), F2, C-K, and PPD were purchased from Nanjing Zelang Medical Technology (Nanjing, Jiangsu, China) at >98% purity, as previously described (12). Cells were incubated in the absence or presence of varying doses (0-40 μg/ml) of ginsenosides for following experiments. Rabbit polyclonal antibodies against integrin β1, phospho-JUN-N-terminal kinase (JNK; Thr183/Tyr185), JNK (p46 and p54), extracellular signal-regulated kinase (ERK), phospho-ERK (Thr202/204), focal adhesion kinase (FAK), phospho-FAK (Y397), protein kinase B (PKB), phospho-PKB (S473), X-linked inhibitor of apoptosis protein (XIAP), B-cell lymphoma 2 (BCL2), BCL-xL, Bcl-2-associated X protein (BAX), cyclin D1, and poly ADP ribose polymerase (PARP) were purchased from Cell Signaling Technologies (Beverly, MA, USA). A polyclonal antibody against N-cadherin was purchased from BD Bioscience (San Jose, CA, USA), and a polyclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-Aminobenzamide (inhibitor of PARP, 2 mmol/l) was purchased from Sigma (St. Louis, MO, USA), and z-VAD-fmk, (pan-caspase inhibitor, 10 μmol/l) was purchased from Calbiochem (La Jolla, CA, USA).

Cell viability assay. To determine the dose-dependent cytotoxicity of ginsenosides, cells were treated with increasing doses (0-40 μg/ml) of F2, Rh_2, PPD, or C-K for 24 h. To determine the time-dependent cytotoxicity of ginsenosdies, cells were treated with 40 μg/ml of F2, Rh_2, PPD, or C-K for varying time periods. Cell viability was determined by measuring absorbance at 450 nm with a microplate reader (Bio-Rad, Richmond, CA, USA) in cells treated with using WST-1 reagent (Nalgene, Rochester, NY, USA).

Immunoblot analysis. Cells were treated with PPD (40 μg/ml) for varying times periods. Cell lysates (20 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes, and these were probed with the antibodies listed above. The blots were developed by chemiluminescence (AbFrontier, Seoul, Korea).

Fluorescence-activated cells sorting (FACS) analysis. Cells were treated with 30 μg/ml of PPD, C-K, or F2 for 18 h. Cells were harvested and washed in cold phosphate-buffered saline, then fixed in 1 ml of cold 70% ethanol, and incubated at 4˚C for 15 min. After incubation, cells were centrifuged at 1,000 × *g* for 5 min, and cell pellets were re-suspended in 50 μl RNAse A (100 μg/ml) containing 300 μl propidium iodide (PI; 50 μg/ml). Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lake, NJ, USA). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis. Data are presented as the means±standard error of the mean (SEM). Comparisons between groups were determined by Student's *t*-test. Statistical significance was defined as $p \leq 0.05$. All experiments were repeated three times and at least in triplicates when applicable.

Results

PPD suppresses the viability of human glioblastoma cells in a dose- and time-dependent manner. PPD with no sugar moiety has been postulated to have the most potent anticancer effect compared with other structurally related PPD-type ginsenosides (10, 11). To test whether this also applies to GBM cells, we tested the anticancer effect of PPD and structurally related PPD-type ginsenosides including F2 (two sugar moieties), Rh2 (one sugar at C-3), and C-K (one sugar at C-20) (Figure 1A). U251-MG cells were treated with various PPD-type ginsenosides for 24 h, and cell viability was assessed (Figure 1B). Treatment with PPD induced dose-dependent cytotoxicity, with an half-maximal inhibitory concentration (IC_{50}) of ~33 μg/ml, whereas C-K had more robust dose-dependent cytotoxicity, with an IC_{50} of ~30 μg/ml. Rh_2 was significantly cytotoxic only at 40 μg/ml, whereas F2 had a marginally cytotoxic effect. All ginsenosides exhibited a time-dependent cytotoxicity at a concentration of 40 μg/ml (Figure 1C). We obtained similar results using U87-MG cells (data not shown). These data collectively indicate that PPD has a considerable *in vitro* cytotoxic effect on human glioblastoma cells that is comparable to that of Rh2, although this effect was slightly less potent than that of C-K. The potency of all ginsenosides studied was as follows: C-K > PPD and Rh_2 > F2.

To determine the cytotoxic mechanisms underlying the effects of PPD, we first tested the effect of various pharmacological inhibitors for caspases, PARP, and mitogen-activated protein kinases (MAPK). Contrary to our expectation, treatment with pharmacological inhibitors of these pathways did not inhibit ginsenoside-induced cytotoxicity, whereas the same treatments suppressed tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced cell death (data not shown). We observed consistent and striking dose- and time-dependent changes in cell morphology after treatment with PPD; these changes were characterized by a rounded configuration and gradual detachment from the substrate (Figure 1D). We next examined the involvement of integrin β1 and N-cadherin by immunoblot analysis. Treatment with PPD reduced the expression of integrin β1 and N-cadherin within 24 h of treatment (Figure 1E). Consistent with this, treatment with PPD reduced phosphorylation of FAK, an integrin-associated protein kinase responsible for regulating various signaling pathways involved in cell growth and proliferation (Figure 1F). To confirm that reduced expression of cell-adhesion proteins was a unique finding of PPD-induced cell death, we treated glioblastoma cells with a combination of TRAIL, a well-known inducer of apoptosis, and *N*-acetyl cysteine (NAC), a reactive oxygen species (ROS) scavenger. We had previously reported that the suppression of intracellular ROS by NAC dramatically enhanced TRAIL-mediated apoptotic cell death (13). As expected, combined treatment of TRAIL

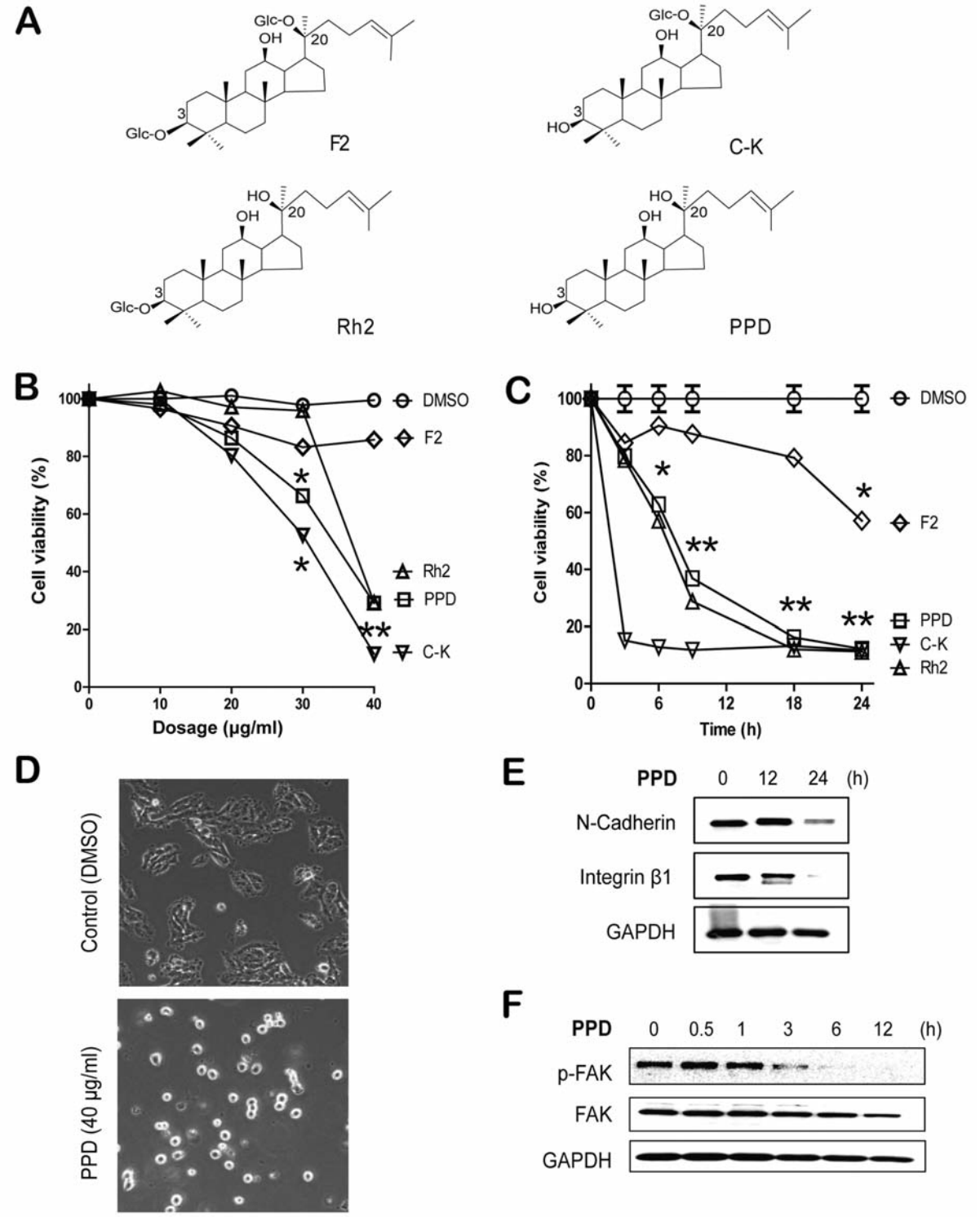

Figure 1. *Chemical structure of ginsenosides and their cytotoxic effects in human glioblastoma cells. A: Structure of ginsenosides F2, compound K (C-K), Rh2, and protopanaxadiol (PPD). B: Cells were treated with increasing doses (0-40 μg/ml) of F2, Rh2, PPD, or C-K for 24 h, and cell viability was determined by the WST-1 assay. C: Cells were treated with 40 μg/ml of F2, Rh2, PPD, or C-K for the indicated time periods, and cell viability was determined by WST-1 assay. D: Cells were incubated in the absence or presence of PPD (40 μg/ml) for 24 h, and phasecontrast images (×100) were taken. E, F: Cells were treated with PPD (40 μg/ml) for the indicated times periods. Cell lysates were examined by immunoblot analysis with antibodies against N-cadherin, integrin β1, phospho-focal adhesion kinase (FAK, Y397), FAK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as means±SD (n=3) and are representative of experiments carried out in triplicate. DMSO: Dimethyl sulfoxide. *p<0.05, **p<0.01 compared to untreated control.*

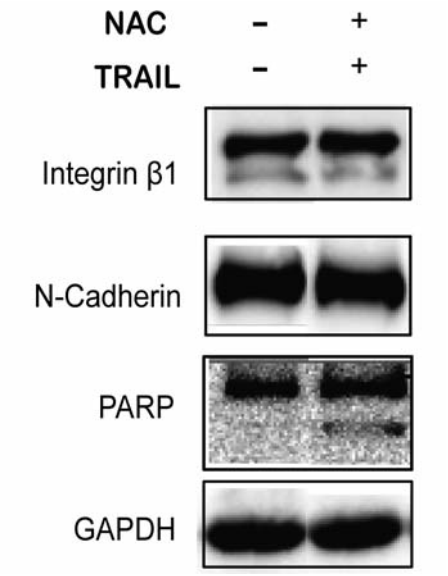

Figure 2. *Effect of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and N-acetyl cysteine (NAC) treatment on the expression of cell-adhesion proteins. U251-MG cells were incubated in the presence of NAC (5 mmol/l), for 1 h and then treated with TRAIL (100 ng/ml) for an additional 24 h. Cell lysates were subjected to immunoblot analysis with antibodies against N-cadherin, integrin β1, poly (ADP-ribose) polymerase (PARP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).*

and NAC induced significant apoptotic cell death and cleavage of PARP; however, the same treatment had no effect on the protein expression of integrin β1 and N-cadherin (Figure 2). This may indicate that inhibition of cell adhesion proteins is not a common consequence in apoptotic cell death. Taken together; these results clearly indicate that PPD induces cytotoxicity in human glioblastoma cells, which may be mediated by loss of cell adhesion.

To further delineate the molecular mechanisms responsible for PPD-induced cytotoxicity, we investigated the involvement of the signal transduction pathways associated with cell proliferation and survival. We observed that phosphorylation of PKB and ERK occurred within 15 min of stimulation but was not sustained up to 24 h after treatment (Figure 3A). Treatment with PPD induced phosphorylation of JNK as early as 1 h after treatment, which was sustained up to 24 h after treatment (Figure 3B). Next, we examined the effects of PPD treatment on anti-apoptotic and pro-apoptotic proteins. Treatment with PPD resulted in a time-dependent reduction in the expression of anti-apoptotic proteins, including XIAP, BCL2, and B CL-xL (Figure 3C). In contrast, PPD induced a time-dependent increase in the multi-domain pro-apoptotic BAX. Time-dependent proteolytic cleavage of PARP was also observed after treatment with PPD (Figure 3D). Taken together, these data indicate that PPD induces glioblastoma cell death by regulating the phosphorylation of various cellular protein kinases and expression of mitochondrial proteins involved in cell survival and cell death.

Ginsenoside PPD induces cell-cycle arrest. Various ginsenosides have been reported to induce cell cycle arrest in tumor cells (3). To investigate the effect of ginsenosides on glioblastoma cell cycle regulation, cells were incubated in the absence or presence of PPD, F2, or C-K for 18 h, and DNA content was analyzed by FACS after staining with PI. Treatment with PPD increased the cell population in the G_1 phase to 63.6%, whereas treatment with C-K increased the cell population to 67.7% compared with F2 (43.0%) and the control (47.8%). An increase in the G_1 cell population was accompanied by a concomitant reduction in the number of cells in the S-phase and G_2 phase (Figure 4A). To further investigate PPD-induced cell-cycle arrest at the G_1 phase, we evaluated the protein level of cyclin D1, which plays a key role in G_1 phase cell-cycle progression, and found it to be reduced after treatment of glioblastoma cells with PPD (Figure 4B). These data indicate that similarly to C-K, PPD induces cell-cycle arrest in glioblastoma cells at the G_1 phase.

Discussion

Structure–activity relationship studies of ginsenosides have indicated that the cytotoxicity of ginsenosides increases with a reduction in sugar moieties, supporting the potent anticancer effects of PPD in several cancer cell lines (11, 14). In this study, we aimed to investigate whether PPD would have an improved *in vitro* anticancer profile in comparison with C-K (one sugar moiety), Rh_2 (one sugar moiety), and F2 (two sugar moieties). We further revealed the detailed mechanism through which PPD induces cytotoxicity in glioblastoma cells. Intriguingly, we observed that PPD induced much stronger cytotoxicity and cell-cycle arrest compared to F2 but was slightly less cytotoxic compared to C-K against human glioblastoma cells. These results indicate that PPD may be a potent anticancer agent; however, its overall anticancer effect may be similar to that of the well-known anticancer ginsenoside C-K.

Although multiple modes of ginsenoside entry into the cell have been proposed, the exact mechanism remains elusive (15). A four *trans*-ring rigid steroid skeleton enables ginsenosides to traverse through the cell membrane freely or directly bind to G-protein-coupled receptors or steroid receptors on the cell membrane (15). It has been suggested that the hydrolytic cleavage of sugar moieties from ginsenosides may increase ginsenoside cytotoxicity (10, 11). However, we found that hydrolysis of C-K to form PPD may not increase the cytotoxicity of ginsenosides and that the differential effect might be due to the different uptake

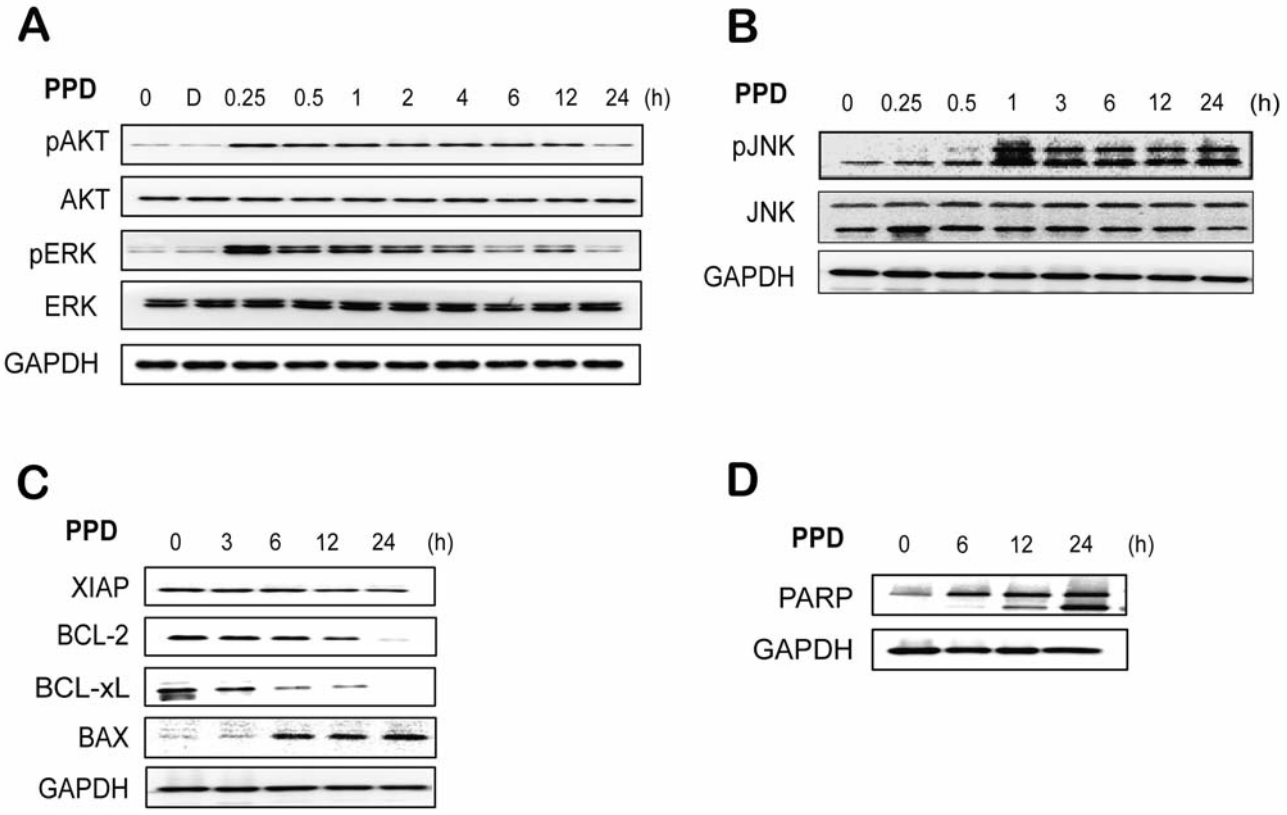

Figure 3. *Signaling pathways involved in ginsenoside protopanaxadiol (PPD)-induced cytotoxicity. U251-MG cells were incubated with PPD (40 μg/ml) for different time periods. Cell lysates were then subjected to immunoblot analysis with antibodies against phospho-protein kinase B (PKB), PKB, phospho-extracellular signal-regulated kinase (ERK)1/2 and ERK1/2 (D: DMSO) (A); c-JUN N-terminal kinase (JNK) and phospho-JNK (B); XIAP, B-cell lymphoma 2 (BCL2), BCL-xL and BCL2-associated X protein (BAX) (C); poly (ADP-ribose) polymerase (PARP); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (D).*

properties of these ginsenosides. PPD has the most favorable cellular uptake profile in MCF-7 breast cancer cells (16). Therefore, the cellular uptake of ginsenosides appears to vary depending on the types of tumor cells. Further work is needed to determine the exact mechanism through which C-K and PPD gain entry into cells and to identify the mechanism through which C-K could exert more rapid and robust cytotoxicity in glioblastoma cells compared with PPD.

The ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1 compose 80-90% of the total ginsenoside fraction found in ginseng. These so-called major ginsenosides contain relatively long sugar moieties and have poor membrane permeability, leading to poor bioavailability and pharmacodynamics (3). Minor ginsenosides such as F1, F2, C-K, Rh_1, Rh_2, Rg_2, Rg_3, and PPD are present at low concentrations in ginseng (3). Treatment of various cell lines with minor ginsenosides have demonstrated strong neuroprotective (17), antioxidative (18), and anticancer properties (3). Minor ginsenosides are produced by hydrolyzing the sugar moieties of major ginsenosides. After oral ingestion of ginseng, some major ginsenosides can be metabolized by intestinal bacteria into F2 and then further hydrolyzed to form C-K by step-wise cleavage of the sugar moieties (19). Further cleavage of the sugar moiety from C-K forms PPD (10). The structure–activity relationship of ginsenosides has not been fully elucidated. However, the pharmacological activity of ginsenosides increases with the reduction in the number of attached sugars moieties (3). The presence of sugar moieties is responsible for the reduction in the hydrophobicity of ginsenosides, which leads to less interaction with the cell membrane and reduced cellular uptake (3). Among the ginsenosides, PPD has been reported to have the most potent anticancer activity (3).

Despite its potential as an anticancer agent, the molecular basis of PPD action has not been extensively investigated. Liu *et al*. suggested that PPD may induce caspase-dependent and autophagic cell death in some glioma cells (20). We further elucidated the mode of action of PPD through the regulation of cell adhesion proteins and cell detachment. Integrin β1 has an important role in glioma biology (21) and has also been shown to control the proliferation,

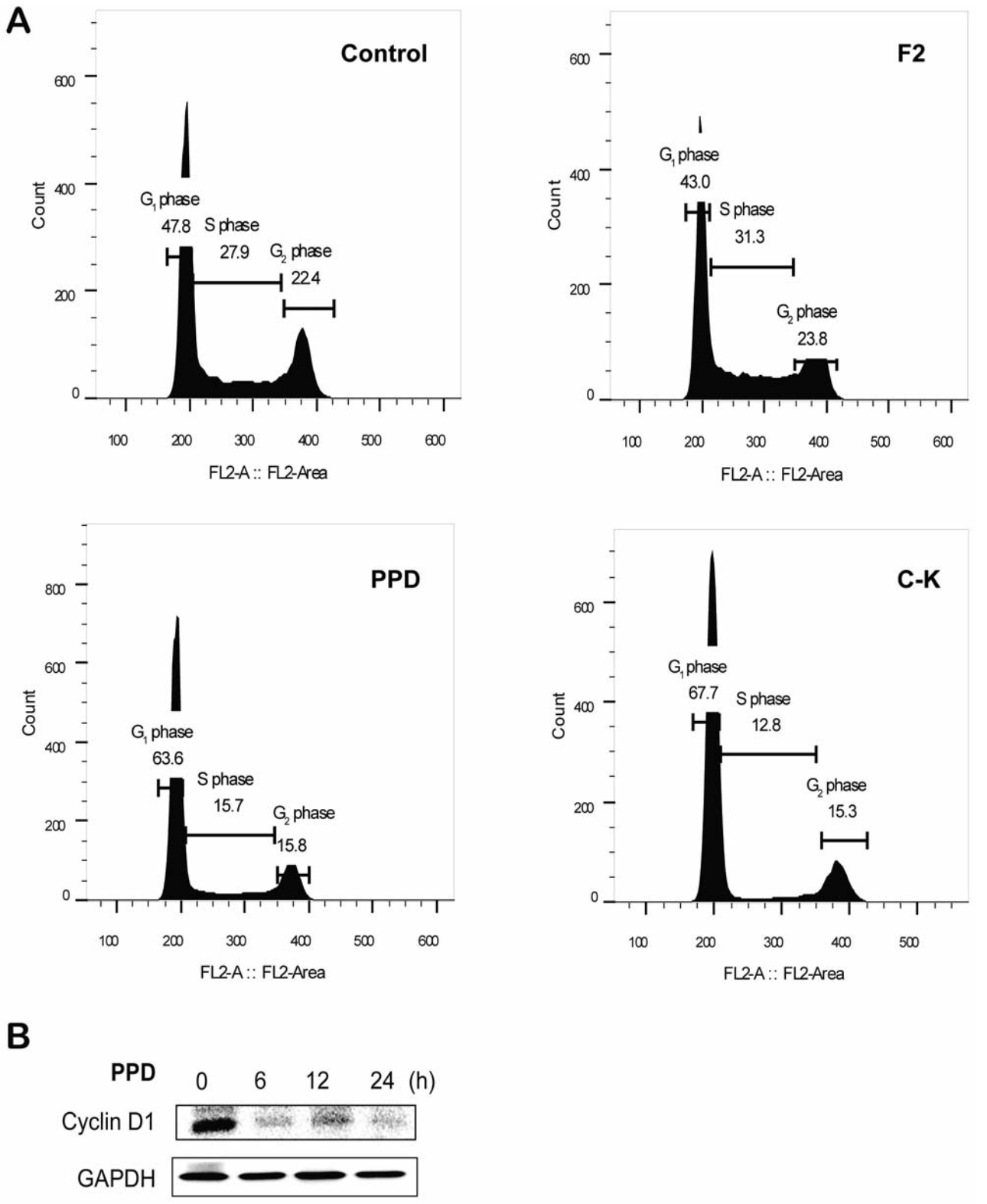

Figure 4. *Ginsenoside protopanaxadiol (PPD) arrests cell-cycle progression at the G_1 phase. A: U251-MG cells were treated with 30 μg/ml of PPD, compound K (C-K), or F2 for 18 h. Cell-cycle distribution was evaluated after staining with propidium iodide by flow cytometric analysis. B: Cells were treated with PPD (30 μg/ml) for the indicated time periods. Cell lysates were subjected to immunoblot analysis with antibodies against cyclin D1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The percentage of cells in each phase is indicated.*

differentiation, and migration of neural stem cells (15, 22). It has been suggested that N-cadherin adhesion promotes cell survival in a phosphoinositide-3 kinase/PKB-dependent manner and inhibits the mitochondrial apoptotic pathway (23). Cadherins can regulate cell survival indirectly through associations with integrins (23). The effect of cell adhesion pathways on triggering cell death has been well illustrated in anoikis systems.

During anoikis, loss of integrin adhesion coupled with cell-cycle arrest triggers the activation of cell death-related JNK pathways while inhibiting ERK and PKB pathways. Furthermore, inhibition of ERK and PKB pathways down-regulates the expression of pro-survival BCL2 family proteins *via* the phosphorylation of the transcription factor cAMP response element binding protein and induces proteolytic cleavage of PARP (24, 25). Similarly, we observed cell detachment coupled with increased phosphorylation of JNK, significant down-regulation of anti-apoptotic BCL2 proteins, and PARP cleavage. However, we also observed that PPD induced phosphorylation of PKB and ERK. It is possible that, upon entry to the cell, PPD may activate various signaling pathways simultaneously; however, only the activation of cell death pathways is sustained over long periods. Further studies are needed to investigate the molecular mechanisms responsible for PKB and ERK phosphorylation by PPD. Nevertheless, we observed cell death processes similar to anoikis in glioblastoma cells treated with PPD.

Acknowledgements

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2013R1A1A3009327), and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, (HI14C0042), Republic of Korea. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received December 21, 2015
Revised January 25, 2016
Accepted January 28, 2016