Polygalacin D induces apoptosis and cell cycle arrest via the PI3K/Akt pathway in non-small cell lung cancer

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Received February 5, 2017; Accepted December 12, 2017

DOI: 10.3892/or.2018.6230

Abstract. Polygalacin D (PGD) is a bioactive compound isolated from *Platycodon grandiflorum* (Jacq.) and it has a similar structure to platycodin D, which is a well known anticancer agent. In the present study, we investigated the anti-proliferative effects of PGD using NSCLC cell lines. We evaluated the effects of PGD on proliferation, apoptosis and cell cycle arrest in A549 and H460 cells. PGD significantly induced apoptosis and nuclear condensation in both cell lines. Furthermore, PGD upregulated the cleavage of apoptotic proteins such as caspase-3, -9 and PARP. Additionally, treatment with PGD suppressed the expression of the IAP family of proteins including survivin, cIAP-1 and cIAP-2. Furthermore, PGD induced G0/G1-phase arrest in both cell lines. After treatment with PGD, the expression of TIMP-1, CDK2, cyclin A and cyclin E was reduced at the protein level. In addition, PGD blocked the PI3K/Akt pathway by inhibiting the phosphorylation of GSK3β, Akt and the expression of PI3K. Our results indicated that the anti-proliferative properties of PGD may result from the regulation of the PI3K/Akt pathway, which plays a critical role in cell survival and growth.

Introduction

Lung cancer is the leading cause of cancer-related deaths in both men and women and its incidence has been increasing over the past few decades (1,2). Lung cancer is clinically classified into small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). It is estimated that over 80% of lung cancer cases are NSCLC (3). NSCLC is further divided into squamous carcinoma, adenocarcinoma and large cell carcinoma based on histological classification. In addition, the classification of NSCLC into subtypes using genetic markers has great importance in relation to the selection of the therapeutic procedure (4). Squamous cell carcinoma is mainly located in the large airways such as the bronchial tree. Large cell carcinoma may occur in any part of the lung and its growth and metastasis are aggressive (5,6). Chemotherapy is the primary treatment option in most cases of lung cancer (7,8). However, treatment with traditional drugs such as cisplatinum is not considered ideal due to its adverse effects and drug resistance (9). Therefore, research has focused on discovering new chemical drugs. Conversely, natural medicine has certain advantages, such as a wide variety of compounds with low toxicity, that could serve as a source of new drugs.

Platycodon grandiflorum (Jacq.) A. DC. (*P. grandiflorum*) is a well known medicinal plant in East Asian countries. *P. grandiflorum* has been reported to be a plant that contains rich triterpenoid saponins such as platycodin D, D2, D3, deapioplatycodin D, D2, polygalacin D (PGD) and platyconic acid A (10). PGD is classified as a triterpenoid saponin and is one of the main chemical components of *P. grandiflorum*. PGD has a very similar structure to Platycodin D, a well known anticancer agent (11-14). Based on this relationship, we hypothesized that PGD would also have anticancer effects. However, there are only a few studies concerning the anticancer effects of PGD. In the present study, we investigated the effects of PGD on NSCLC using the adenocarcinoma cell line A549 and the large cell carcinoma NCI-H460 cell line.

Materials and methods

Reagents. RPMI-1640, fetal bovine serum (FBS), penicillin and streptomycin were purchased from HyClone Laboratories Inc., (Logan, UT, USA). Bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 4',6-diamidino-2-phenylindole (DAPI) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TIMP-1 (mouse, monoclonal, sc-365905), Cdk2 (mouse, monoclonal, sc-6248), cyclin A (rabbit, polyclonal, sc-751), cyclin E (rabbit, polyclonal, sc-198), P-GSK3β (mouse, monoclonal, sc-81495),

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Key words: NSCLC, apoptosis, cell cycle arrest, PI3K/Akt pathway

GSK3β (mouse, monoclonal, sc-81462), PI3K (rabbit, polyclonal, sc-602) and β-actin (mouse, monoclonal, sc-47778) were all purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA). Cleaved PARP (rabbit, polyclonal, #9541s), survivin (rabbit, monoclonal, #2808s), Cdk4 (rabbit, monoclonal, #12790s), cyclin D (rabbit, monoclonal, #2978s), p-Akt (rabbit, monoclonal, #4058s) and Akt (rabbit, polyclonal, #9272s) were purchased from Cell Signaling Technology (Beverly, MA, USA). Caspase-3 (active, rabbit, polyclonal, ALX-210-807-c100), caspase-9 (active, rabbit, polyclonal, ALX-210-816-c100), cIAP-1 (rat, monoclonal, ALX-803-335-c100) and cIAP-2 (rat, monoclonal, ALX-803-341-c100) were purchased from Enzo Life Sciences (Farmingdale, NY, USA) and diluted 1:1,000. Primary antibodies were diluted 1:1,000. Peroxidase-conjugated secondary antibodies (rabbit, monoclonal, sc-2357; mouse, polyclonal, sc-2005; rat, polyclonal sc-2006; dilution 1:2,000) were purchased from Santa Cruz Biotechnology. An enhanced chemiluminescent (ECL) kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Preparation of PGD. For the preparation of PGD dried roots, *P. grandiflorum* (5 kg) underwent extraction three times with methanol at room temperature for seven days. Concentration of the solvent gave a brown syrupy extract (1.4 kg) which was suspended in water and then partitioned successively with ethyl acetate (63 g) and n-butanol (130 g). The n-butanol layer was suspended in H_2O (2 liters) and poured into a Diaion HP-20 column (Φ = 5.0x100 cm; Mitsubishi Chemicals Corp., Tokyo, Japan), which was stabilized with H_2O. The column was washed with H_2O (2 liters) and then eluted with MeOH (5 liters). Polygalacin D2 (PD2) was prepared from the extract of *P. grandiflorum* for experimental procedures using our method (15).

Cell culture and proliferation assay. Human lung cancer cells NCI-A549 and H460 were obtained from the Korean Cell Line Bank (Seoul, Korea) and grown in RPMI-1640 containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. BEAS-2B, a human bronchial epithelial cell line, was purchased from the American Type Culture Collection (CRL-9609; ATCC, Manassas, VA, USA) and maintained in bronchial epithelial cell basal medium (BEBM) containing 1% penicillin/streptomycin. The cell lines were maintained in a humidified incubator, with an atmosphere of 5% CO_2 at 37˚C. The effect of PGD on cell proliferation was determined using an MTS assay. The cells ($2x10^3$/well) were seeded in 96-well culture plates and incubated for 24 h. Various concentrations (0-40 μM) of PGD were added and the cells were incubated for 48 h. The optical density (OD) of each culture well was assessed at 490 nm using a microplate reader (Titertek Multiskan; Flow Laboratories, North Ryde, NSW, Australia). Cell proliferation was calculated using the following formula: (mean absorbance value of treated cells/mean absorbance value of untreated cells) x 100.

DAPI staining. Cell nuclear morphology was assessed by fluorescence microscopy following DAPI staining. A549 and H460 cells were treated with PGD for 48 h. The cells were washed with phosphate-buffered saline (PBS), fixed with ice cold 70% ethanol, stained with DAPI and incubated for 5 min at 37˚C, in the dark. The cells were then washed with PBS and visualized using a fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany).

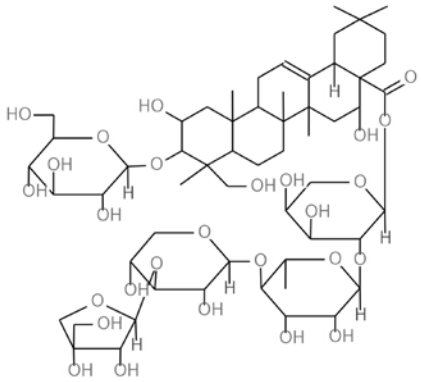

Figure 1. Structure of PGD.

Detection of apoptosis. The degree of apoptosis was assessed using Muse cell analyzer (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. A549 and H460 cells were seeded in 6-well plates (10^5 cells/well) and treated with PGD (0, 10, 20 and 40 μM) for 48 h. The harvested cells were washed with 1 ml of PBS, resuspended in 150 μl FBS free media and 150 μl of the Muse™ Annexin V Dead Cell kit (EMD Millipore) was added. Then, the cells were incubated at room temperature (RT) for 20 min in the dark. The samples were assessed with the Muse cell analyzer (EMD Millipore).

Cell cycle analysis. A549 and H460 cells were seeded in 6-well plates (10^5 cells/well) and treated with PGD (0, 10, 20 and 40 μM) for 48 h. The harvested cells were washed with 1 ml PBS and fixed with ice-cold 70% EtOH over 3 h. After fixation, the cells were washed with PBS and suspended in 200 μl PBS. Muse™ Cell cycle reagent (200 μl; EMD Millipore) was then added and the cells were incubated at RT for 30 min in the dark. The samples were assessed with the Muse cell analyzer (Merck Millipore).

Western blotting. Cell extracts were subjected to SDS gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (PVDF; EMD Millipore). The membrane was blocked with 5% skim milk for 1 h and probed with primary antibodies. After a series of washes, the membrane was further incubated with a secondary antibody conjugated with horseradish peroxidase (HRP). The proteins were then supplemented with ECL prime western blotting detection reagents (GE Healthcare Life Sciences, Little Chalfont, UK). The bands were evaluated using the ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare Life Sciences).

Statistical analysis. The statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Scheffe's test for multiple comparisons. Data are presented as the means ± standard deviations (n=5). All calculations were performed using SPSS statistics 23 software (SPSS Inc.,

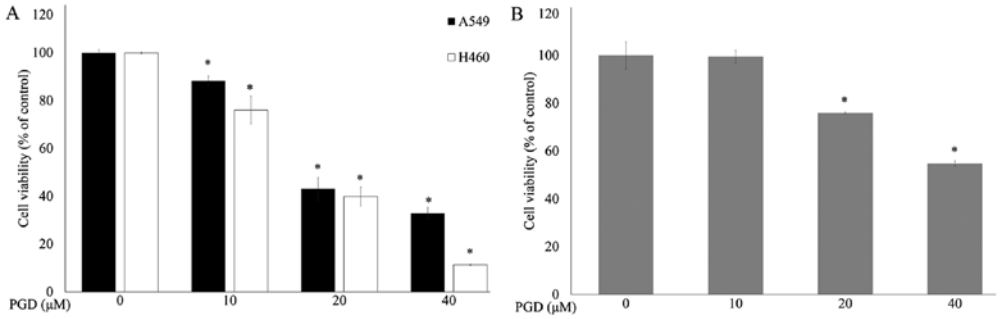

Figure 2. Effect of PGD on cell proliferation in (A) A549 and H460 cancer cell lines and (B) normal airway bronchus cell line BEAS-2B. Cell viability was evaluated with the MTS assay after 48 h of treatment with various concentrations of PGD. *P<0.05 compared to the non-treated group. Significant differences between the treated groups were determined using analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons. Values represent the means ± standard deviations of duplicate determinations from three separate experiments.

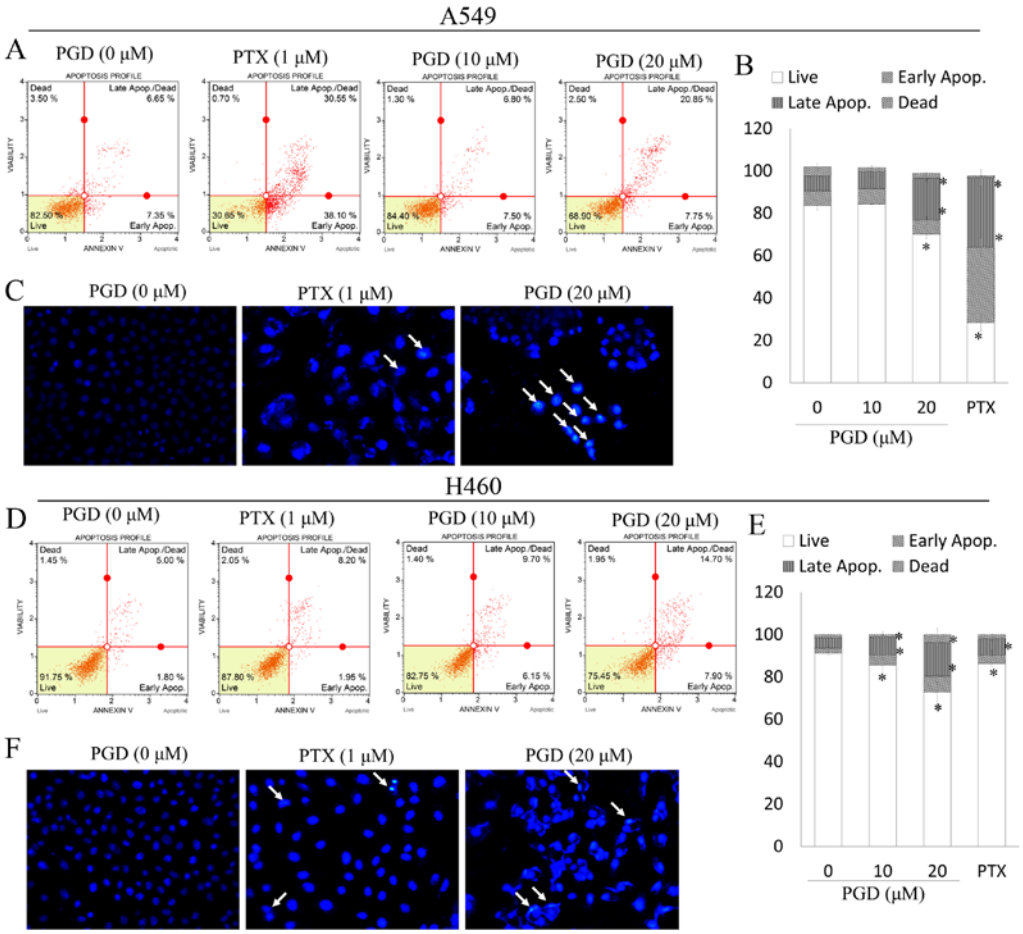

Figure 3. Effect of PGD on apoptosis and nuclear condensation in A549 and H460 cancer cells. (A, B, D and E) The apoptosis rate after 48 h of treatment with PGD at the indicated concentrations and 1 μM PTX (clinical anticancer reagent) was assessed using flow cytometry. (C and F) Nuclear condensation after 48 h of treatment with PGD was visualized using DAPI staining. Similar results were obtained in three independent experiments and the results of one representative experiment are presented. *P<0.05 compared to the non-treated group. Significant differences between treated groups were determined using Student's t-test. Values represent the means ± standard errors of duplicate determinations from three separate experiments.

Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

PGD inhibits the proliferation of NSCLC cell lines. MTS assays were performed to determine the effects of PGD (Fig. 1) on the proliferation of lung cancer cell lines. Cancer cells were treated with increasing concentrations of PGD. As shown in Fig. 2A, PGD inhibited the growth of the treated cell lines in a dose-dependent manner. The IC_{50} concentrations of PGD were 26.49±1.45 μM in the A549 cells and 20.52±0.30 μM in the H460 cells. Treatment with PGD at 40 μM reduced cell viability below 70% in normal airway bronchus cell line (Fig. 2B). Additional experiments were performed using PGD under 20 μM.

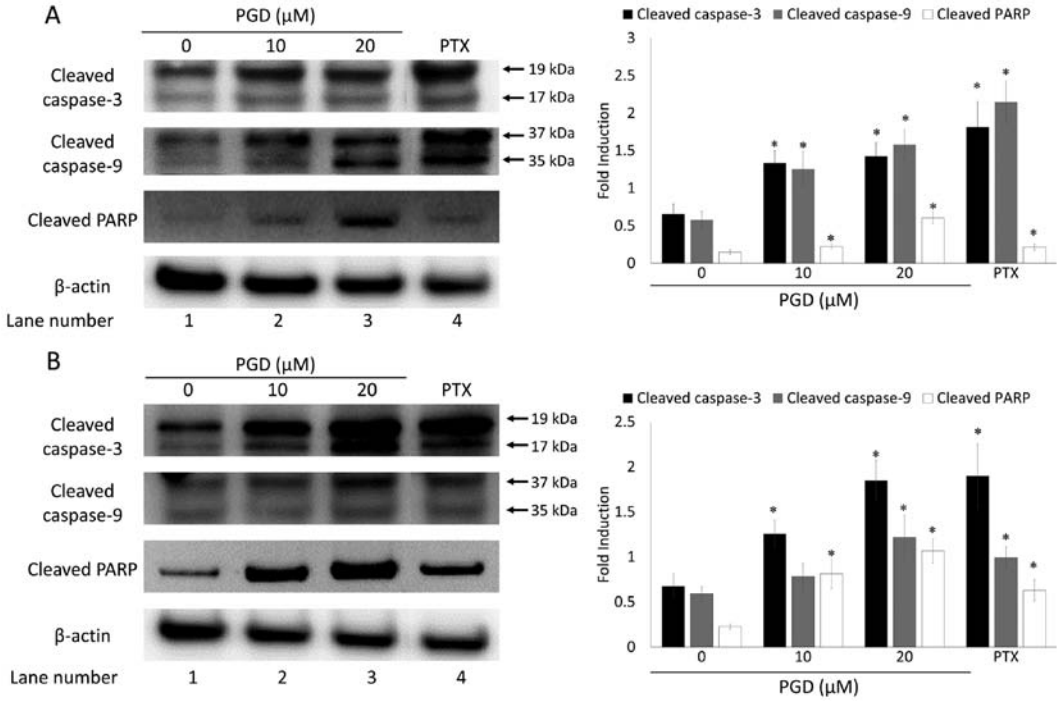

Figure 4. Effects of PGD on the expression of apoptosis-related proteins in (A) A549 and (B) H460 cells. The cells used for western blot analysis were incubated with PGD at the indicated concentrations and 1 μM PTX (clinical anticancer reagent) for 48 h. Representative western blots of at least three separate experiments are displayed. *P<0.05 compared to the non-treated group. Significant differences between the treated groups were determined using analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons. Values represent the means ± standard deviations of duplicate determinations from three separate experiments.

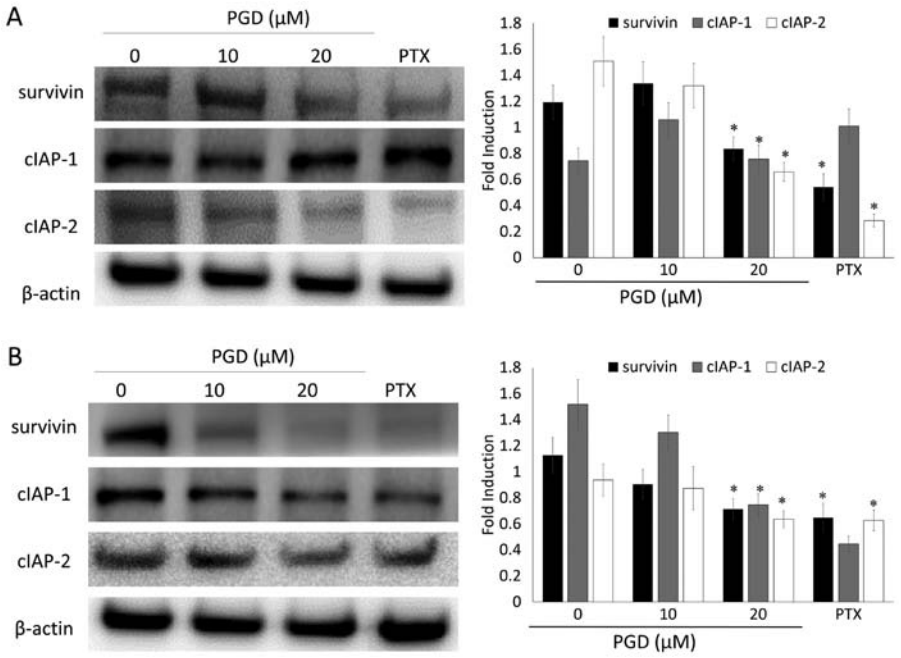

Figure 5. Effects of PGD on the expression of the IAP family of proteins in (A) A549 and (B) H460 cancer cells. The cells used for western blot analysis were incubated with PGD at the indicated concentrations and 1 μM PTX (clinical anticancer reagent) for 48 h. Representative western blots of at least three separate experiments are presented. *P<0.05 compared to the non-treated group. Significant differences between the treated groups were determined using analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons. Values represent the means ± standard deviations of duplicate determinations from three separate experiments.

PGD induces apoptosis of NSCLC cell lines. To determine whether the decreased viability of A549 and H460 cells occurs as a result of apoptosis, we investigated the apoptotic effects of PGD in NSCLC cell lines. In the present study, pemetrexed (PTX) was used as a control reagent to assess the apoptotic effects of PGD. PTX has been widely used in the clinical treatment of non-small cell lung cancer. As displayed in Fig. 3C and F, after treatment with PGD, nuclear condensation

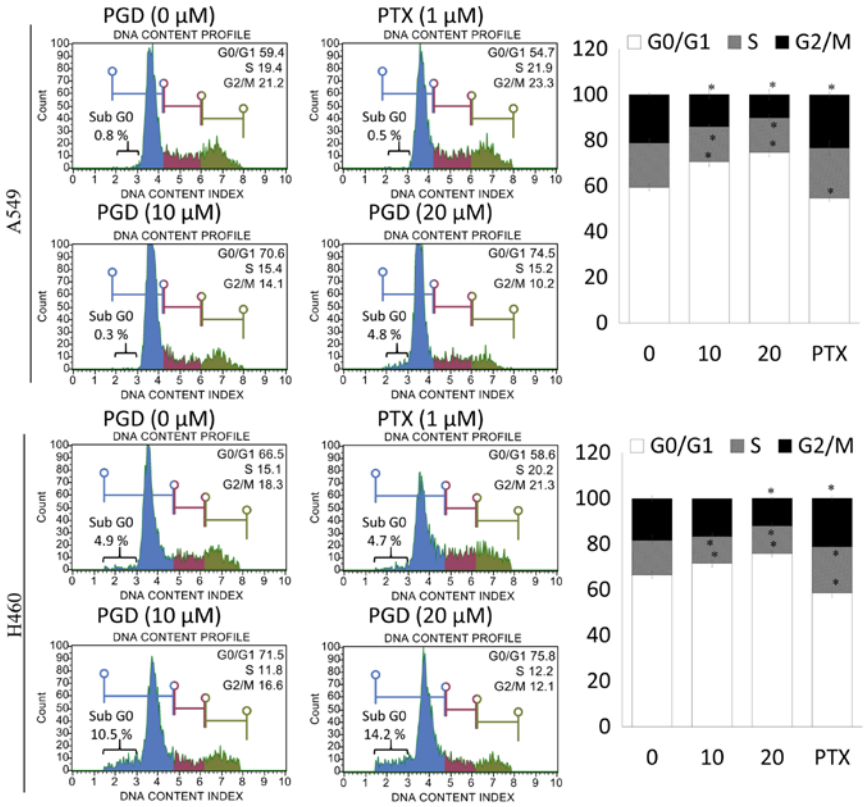

Figure 6. Effects of PGD on cell cycle progression in A549 and H460 cancer cells. The distribution of the cell cycle after 48 h of treatment with PGD at the indicated concentrations and 1 μM PTX (clinical anticancer reagent) was assessed using flow cytometry. *P<0.05 compared to the non-treated group. Significant differences between treated groups were determined using Student's t-test. Values represent the means ± standard errors of duplicate determinations from three separate experiments.

(white arrows) was observed in A549 and H460 cells. In addition, treatment with PGD increased the proportion of early and late apoptotic cells (Fig. 3A, B, D and E). Collectively, PGD-inhibited proliferation of NSCLC cells was due to cell apoptosis.

PGD regulates the expression of apoptosis-related proteins in NSCLC cells. To elucidate the mechanism by which PGD induced apoptosis, the expression levels of proteins involved in the apoptosis pathway were examined using western blot analysis. The results indicated that treatment with PGD induced the cleavage of caspase-3 and -9, as well as PARP in both A549 and H460 cells (in lanes 2 and 3 compared with lane 1; Fig. 4). The expression levels of cleaved caspase-3 and -9, as well as PARP were markedly increased when compared with those in non-treated cells.

PGD inhibits the IAP pathway in NSCLC cells. To further investigate the effects of PGD on the expression of the IAP family of proteins which play a significant role in intrinsic programmed cell death, NSCLC cells were treated with various concentrations of PGD and then, the expression levels of the IAP family of proteins including survivin, c-IAP-1 and c-IAP-2 were determined using a western blot assay. The expression of survivin, c-IAP-1 and c-IAP-2 was decreased, notably at the highest concentration of PGD, compared with that in non-treated cells. The expression of c-IAP-2 in A549 cells and the expression of survivin and c-IAP-2 in H460 cells was decreased to undetectable levels (Fig. 5). As a result, PGD may exert its apoptotic effects by regulating the apoptotic proteins and the IAP family of proteins.

PGD induces cell cycle arrest in NSCLC cells. To determine whether the effects of PGD on the inhibition of NSCLC cell-proliferation were related to cell-cycle arrest, the number of cells in the G0/G1, S and G2/M phases were assessed by flow cytometry. Treatment with PGD inhibited the progression of cell cycle in A549 and H460 cells. As displayed in Fig. 6, the percentage of cells in the G0/G1 phase was significantly increased in PGD-treated A549 and H460 cells compared with non-treated cells. Furthermore, treatment with PGD significantly increased the percentages of the sub G0 phase in both cell lines. In addition, PGD decreased the percentage of cells in the S and G2/M phases.

PGD regulates the expression levels of proteins related to cell cycle progression in NSCLC cells. To evaluate the underlying mechanism of the effects of the cell cycle-arrest of PGD, the expression levels of proteins involved in cell cycle progression were determined using western blot analysis. As displayed in Fig. 7, PGD reduced the expression levels of proteins related to the progression of the cell cycle such as TIMP-1, Cdk2, Cdk4, cyclin A, D and E in both cell lines. In addition, PGD reduced the phosphorylation of GSK3β (Fig. 8), which is also involved in cell cycle progression. These results indicated that the inhibitory effects of PGD on cell growth via cell cycle arrest were induced as a result of a decrease in the expression of proteins related to cell cycle progression.

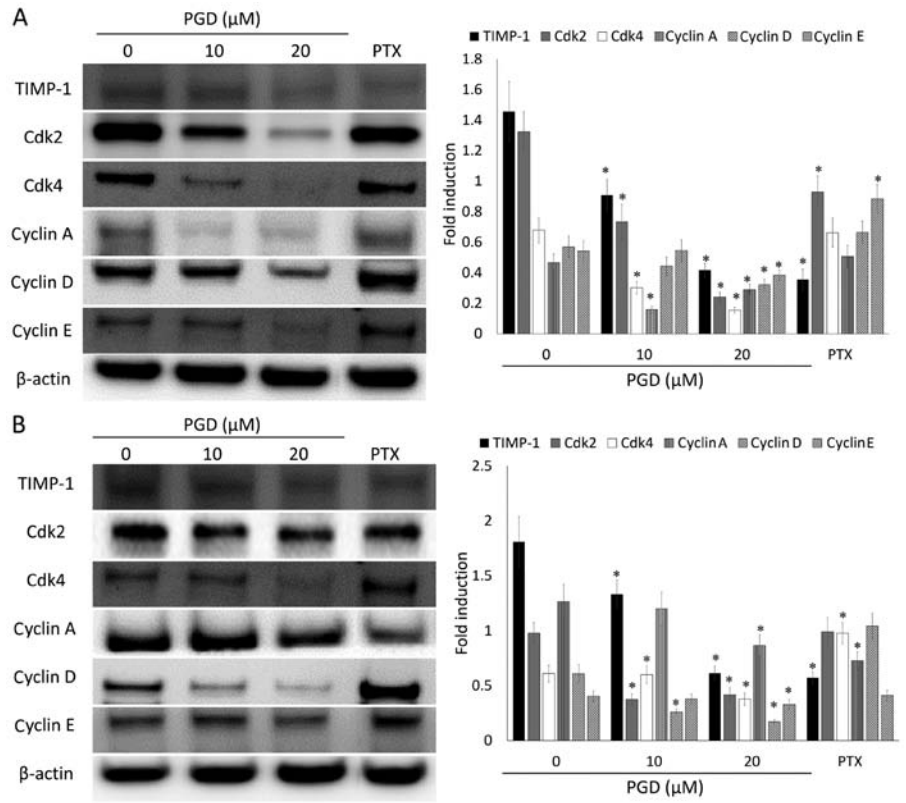

Figure 7. Effects of PGD on the expression of cell cycle progression-related proteins in (A) A549 and (B) H460 cancer cells. The cells used for western blot analysis were incubated with PGD at the indicated concentrations and 1 μM PTX (clinical anticancer reagent) for 48 h. Representative western blots of at least three separate experiments are presented. *P<0.05 compared to the non-treated group. Significant differences between the treated groups were determined using analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons. Values represent the means ± standard deviations of duplicate determinations from three separate experiments.

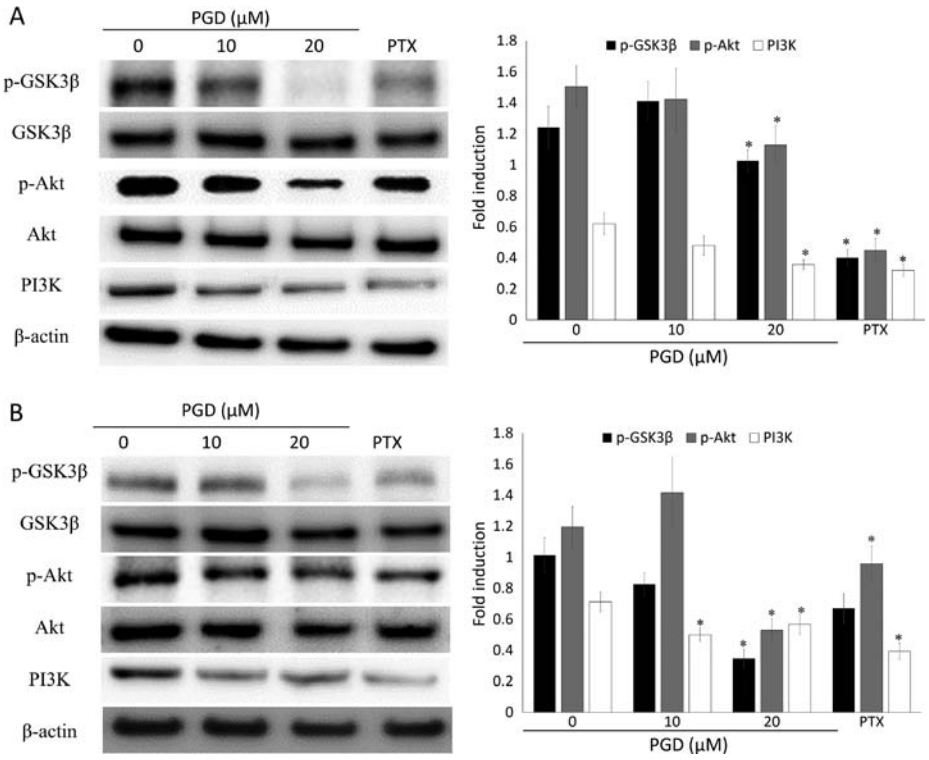

Figure 8. Effects of PGD on the PI3K/Akt pathway in (A) A549 and (B) H460 cancer cells. The cells used for western blot analysis were incubated with PGD at the indicated concentrations and 1 μM PTX (clinical anticancer reagent) for 48 h. Representative western blots of at least three separate experiments are presented. *P<0.05 compared to the non-treated group. Significant differences between the treated groups were determined using analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons. Values represent the means ± standard deviations of duplicate determinations from three separate experiments.

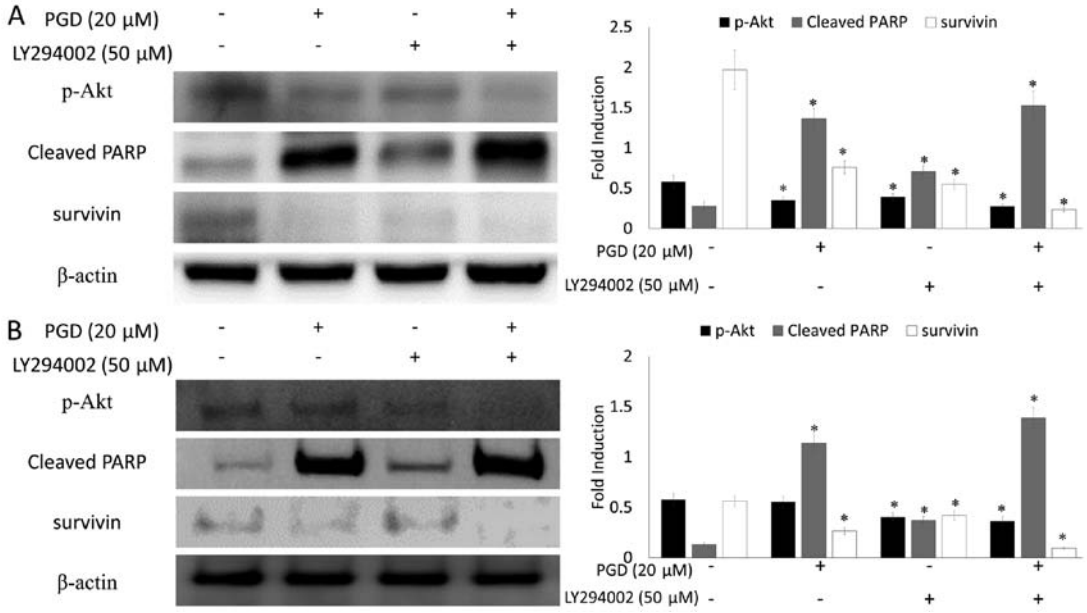

Figure 9. Pharmacological inhibition of Akt on the expression of survivin and cleavage of PARP in PGD-treated (A) A549 and (B) H460 cells. The cells used for western blot analysis were pretreated with or without PI3-kinase inhibitor LY294002, and then further incubated in the presence or absence of 20 μM PGD for 48 h. Representative western blots of at least three separate experiments are displayed. *P<0.05 compared to the non-treated group. Significant differences between the treated groups were determined using analysis of variance followed by Scheffe's test for multiple comparisons. Values represent the means ± standard deviations of duplicate determinations from three separate experiments.

PGD inhibits the PI3K/Akt signaling pathway. Previous research reported that the PI3K/Akt signaling pathway plays a critical role in cell survival. To confirm whether the inhibitory activity of PGD on cell growth was related to the PI3K/Akt signaling pathway, western blot analysis was performed. Treatment with PGD reduced the phosphorylation of Akt, while total Akt remained the same (Fig. 8). In addition, the expression of PI3K was reduced at the highest concentration of PGD compared with the non-treated group. In addition, as displayed in Fig. 9, co-treatment with LY294002 (the inhibitor of PI3K) decreased the expression of survivin and enhanced cleavage of PARP.

Discussion

In the present study, we examined the potential inhibitory effects of PGD, a triterpenoid saponin contained in *P. grandiflorum*, which has a similar structure to platycodin D, on cell proliferation in NSCLC cells such as A549 and H460 cell lines. Structure-activity relationships of Platycodon saponins provide important information regarding their bioactive roles (16) and it is expected that they have similar biological activities. However, the anticancer activity of PGD and its mechanisms still remain unclear. As shown in Results, PGD significantly reduced cell viability via the induction of apoptosis and cell cycle arrest. In addition, our finding indicated that PGD inhibited cell survival via regulation of the PI3K/Akt signaling pathway.

Apoptosis is one of the major goals of chemopreventive agents which inhibit the overall growth of cancer cells. Caspases are well-defined proteases related to apoptosis (17). Activation of caspase-3 and -9 induces the cleavage of PARP, eventually resulting in cell apoptosis. IAPs are a group of structurally-related proteins that block apoptosis either by binding and inhibiting caspases or through caspase-independent mechanisms (18). Notably, cIAPs are positive regulators of cell proliferation (19) and expression of cIAPs is associated with advanced disease stage, poor patient prognosis and squamous cell carcinoma (20-22). Survivin is one of the members of the IAP family and is known to play a critical role in cancer cell progression (23). As shown in Fig. 2, PGD significantly reduced cell viability in a dose-dependent manner and revealed IC_{50} values of 26.49±1.45 μM and 20.52±0.30 μM in A549 and H460 cells, respectively. Furthermore, PGD significantly induced apoptosis, which was accompanied by DNA damage, cleavage of caspase-3 and -9, as well as PARP and a decrease in the expression levels of the IAP proteins such as survivin, cIAP-1 and cIAP-2 (Figs. 3-5). These results indicated that the antiproliferative effects of PGD on the NSCLC cell lines were induced by activation of apoptosis-related proteins such as caspase-3 and -9 as well as PARP and inhibition of the IAP family of proteins such as survivin, cIAP-1 and cIAP-2.

In addition, the inhibitory effects of PGD on cell growth were related to the arrest of cell cycle progression. Our data revealed that treatment with PGD significantly reduced the proportion of cells in the S and G2/M phases, followed by an increase in the proportion of cells in the G0/G1 phase. These results were in agreement with the findings of the proliferation assay. Tissue inhibitor of metalloproteinases-1 (TIMP-1) was initially characterized as an endogenous inhibitor of matrix metalloproteinases (MMPs) (24). TIMP-1 is frequently overexpressed in several types of human cancers and promotes cell proliferation (25-27). Cell cycle progression is regulated by cyclins and cyclin-dependent kinases (CDKs). Specifically, cyclin D1 and CDK4 play pivotal roles in the cell cycle transition from the G1 to the S phase (28,29). Overexpression of cyclin D1 and CDK4 has been observed in various cancer cells and was strongly associated with the risk of tumor

progression and metastasis (30,31). CDK2 is a protein kinase essential for the G1/S transition and its activity is limited in the G1/S phase (32). CDK2 forms a complex with cyclin E and this complex leads to the progression of the G1 phase to the S phase (33). The CDK2/cyclin E complex also induces an increase in cyclin A expression, which facilitates G1 to S-phase transition. Cyclin A binds to CDK2 instead of cyclin E and this CDK2/cyclin A complex initiates DNA replication, which is required for the progression to the S phase (34). PGD reduced the expression of TIMP-1, CDK2, cyclin A and E and these results were related to the effects of cell cycle arrest of PGD at the G0/G1 phase.

Finally, we investigated the anticipated mechanism that is involved in the aforementioned effects of PGD. The PI3K/Akt pathway is a well-known essential pathway involved in cell proliferation, cycle progression, metastasis, survival and apoptosis and it is one of the most important oncogenic targets in almost all kinds of cancers (35,36). Furthermore, this pathway increases the resistance to DNA damage-induced apoptosis (37). In addition, increased phosphorylation levels of GSK-3β, which is a known downstream target, lead to the expression of cyclin D and cMyc, which play a role in the G1/S-phase check point of cell cycle progression and migration (38,39). Therefore, PGD-induced G1 arrest may be attributed to its influence on GSK-3β and upstream Akt and PGD-induced apoptosis may be attributed to the inhibition of Akt phosphorylation.

In conclusion, the anti-proliferative effects of PGD on NSCLC cells may be due to modulation of the PI3K/Akt pathway, which subsequently leads to apoptosis induced by the activation of apoptotic proteins, a decrease in the expression of the IAP family of proteins and growth inhibition induced by cell cycle arrest regulated by CDK2/4, cyclin A, D and E. Based on our findings, PGD exhibits a potential role as a new therapeutic agent for the treatment of NSCLC.

Acknowledgements

The present study was supported by Wonkwang University in 2017.

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