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G_1 Phase Arrest of the Cell Cycle by a Ginseng Metabolite, Compound K, in U937 Human Monocytic Leukamia Cells

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We recently reported that the ginseng saponin metabolite, compound K (20-O-β-D-glucopyranosyl-20(*S*)-protopanaxadiol, IH901), inhibits the growth of U937 cells through caspase-dependent apoptosis pathway. In this study, we further characterized the effects of compound K on U937 cells and found that, in addition to apoptosis, compound K induced the arrest of the G_1 phase. The compound K treated U937 cells showed increased p21 expression; an inhibitory protein of cyclin-cdk complex. The up-regulation of p21 was followed by the inactivation of cyclin D and the cdk4 protein, which act at the early G_1 phase, and cyclin E, which acts at the late G_1 phase. Furthermore, compound K induced the activation of JNK and the transcription factor AP-1, which is a downstream target of JNK. These findings suggest that the up-regulation of p21 and activation of JNK in the compound K treated cells contribute to the arrest of the G_1 phase.

Key words: Compound K, U937 cell, Apoptosis, G_1 phase, p21, AP-1, JNK

INTRODUCTION

Recently, ginseng saponin metabolites, formed by intestinal bacteria, were identified in human and rat subjects after the oral administration of a ginseng extract. (Hasegawa *et al.*, 1996). 20-O-(β-D-Glucopyranosyl)-20(*S*)-protopanaxadiol (compound K, IH-901; Fig. 1), which is one of the major intestinal metabolites, was detected in urine and blood samples after administering the ginseng saponins to rats. We recently reported that compound K showed cytotoxicity by inducing apoptosis through the caspase dependent pathway *via* the disruption of mitochondria (Kang *et al.*, 2004). It has been reported that compound K inhibits the glucose uptake by tumor cells (Hasegawa *et al.*, 1995a), reverses the multidrug resistance in tumor cells (Hasegawa *et al.*, 1995b), has chemo-preventive activity against chemical carcinogens (Lee *et al.*, 1998), and exhibits an *in vivo* anti-metastatic effect (Hasegawa *et al.*, 1997). Various reports have suggested that the cell cycle arrest leads to cell growth inhibition and/or apoptosis. The cell cycle is subject to numerous surveillance mechanisms, which are responsible for arresting the cells during unfavorable conditions and/or tissue damage. The surveillance operates primarily at major points of restriction, known as checkpoints, which occur during the G_1 and G_2 phases of the cell cycle. Cell progression is usually modulated by at least four factors, which are related to the cdk (cyclin dependent kinase) activity. These factors are (i) altered cdk levels; (ii) changed cyclin expression, with

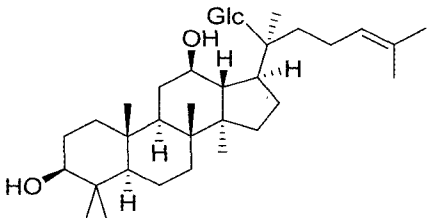

Fig. 1. The structure of compound K [20–O-(β-D-glucopyranosyl)-20(*S*)-protopanaxadiol]

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which the cdks interact; (iii) the regulation of cdk kinase activity through reversible phosphorylation; and (iv) inhibitory proteins (cdk inhibitors, CKIs), which interact reversibly with the cdks to inhibit the cdk activity. Among the cdk inhibitors, p21 has been shown to inhibit virtually all the cdk-cyclin complexes tested *in vitro* (Xiong *et al.*, 1993). The mitogen activated protein kinase (MAPK) pathway is regulated by a variety of extracellular stimuli in numerous cell types and it takes part in a wide range of cellular programs, including proliferation, differentiation, and movement (McCubery *et al.*, 2000; Pages *et al.*, 1991; Robinson and Cobb, 1997; Widmann *et al.*, 1999). In particular, *c*-jun *N*-terminal kinase (JNK), stress-activated protein kinase, has been linked to the cell cycle. The activated JNK phosphorylates Ser-130 of p21 and stabilizes this protein, resulting in the arrest of cell growth (Ikezoe *et al.*, 2004; Zada *et al.*, 2003; Bunnett *et al.*, 2001). Therefore, this study investigated whether compound K affects the cell cycle distribution through the arrest of the cell cycle and whether it involves the JNK pathway.

MATERIALS AND METHODS

Preparation of compound K

Compound K was prepared by the incubation of the protopanaxadiol type ginsenosides with *Bacteroides* JY-6, a human intestinal bacterium, sub-cultured in a general anaerobic medium for 24 h at 37°C. The incubated medium was extracted with butanol. The supernatant was concentrated *in vacuo* and was processed using silica gel column chromatography with $CHCl_3$-MeOH-H_2O (65:35:10, v/v). The isolated compound K was characterized using mass spectroscopy and ^1H-and ^{13}C-nuclear magnetic resonance (NMR) spectrometry. Compound K was dissolved in DMSO, with an ultimate concentration of less than 0.2%.

Cell culture

U937 cells (a human monocytic leukemia cell line) were cultured at 37°C in 5% CO_2 in RPMI 1640 (Gibco BRL, Gaithersburg, MD) containing 2 mM glutamine, 10% heat-inactivated fetal calf serum, penicillin (100 units/mL) and streptomycin (100 mg/mL). This cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Cell cycle analysis

Flow cytometric analysis of the DNA content was performed to assess the cell cycle phase distribution (Nicoletti *et al.*, 1991). At several intervals samples with 1 $\times 10^6$ cells were harvested after the treatment with compound K and the cells were fixated for 1 hour at 4°C in 1 mL of 70% ethanol. The cells were then washed twice with phosphate buffered saline (PBS) and were incubated in the dark with 100 μg/mL propidium iodide and 100 μg/mL RNAse A at 37°C for 30 minutes. Finally the cells were analyzed using FACSCalibur flow cytometry (Becton Dickinson, Mountain View, CA). The effect on the cell cycle was assessed by measuring the changes in the distribution of the cell cycle phase, which was assessed by generating histograms using the Cell Quest and Mod-Fit programs.

Western blot analysis

At several intervals the cells were harvested after the treatment with compound K and washed twice with PBS. The harvested cells were then lysed on ice for 30 minutes in a lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at 13,000×g for 15 minutes. The supernatant was collected and the protein concentrations were determined. The cellular proteins were boiled for 5 minutes and 40 μg of the protein was electrophoresed in 10% SDS-polyacrylamide gel. The blots were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA), which was then incubated with the primary rabbit polyclonal anti-cyclin D, -cyclin E, -cdk2, -cdk4, -p21, -phospho JNK1/2 (54 kDa JNK/46 kDa JNK), and -JNK1/2. The gel was further incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase-conjugates (Pierce, Rockford, IL), and exposed to X-ray film. The protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

The nuclear extracts of the U937 cells were prepared as follows. The cells (2×10^7) were treated with 1 mL of a lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 1% NP-40) on ice for 4 minutes. After 10 minutes of centrifugation at 3,000 × g, the pellet was resuspended in 50 mL of an extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF), incubated on ice for 30 minutes, and centrifuged at 13,000 × g for 5 minutes. The supernatant was then harvested as nuclear proteins extract and stored at -70°C after determining its protein concentration. The oligonucleotides containing the AP-1 consensus sequence (Santa Cruz Biotechnology, Santa Cruz, CA) were annealed, labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, and used as probes. The probes (50,000 cpm) were incubated with 6 μg of the nuclear extracts from U937 cells in an ultimate volume of 20 μL of 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT with 1 μg of poly (dI-dC), as described previously (Kim *et al.* 1998). The

reaction mixture was incubated at 4°C for 30 minutes. The binding products were resolved on a 5% polyacrylamide gel and ultimately visualized by autoradiography.

RESULTS

Compound K induces G_1 arrest of the cell cycle in U937 human monocytic leukemia cells

In order to examine the effect of compound K on the cell cycle distribution, the U937 cells were treated with compound K at 30 μg/mL (50% growth inhibitory concentration to U937 cells) for the indicated periods and the distribution of cell cycle was analyzed using flow cytometry. Compound K induced the accumulation of cells at the G_1 phase as indicated in the increase of 58% after 24 h compared with 41% of control (Fig. 2A). This increase was retained for 72 h (Fig. 2B). This finding indicates that compound K inhibits the growth of U937 at the G_1 phase.

Compound K suppresses the expression of cyclin-cdk at the G_1 phase of the cell cycle

The expression of the cell cycle regulators was examined to clarify the mechanisms underlying the compound K induced G_1 arrest in U937 cells. This study analyzed the expression of cyclin D and cdk4, which are active in the early G_1 phase (Fujita *et al.*, 2002). The cyclin D and cdk4 protein levels in the U937 cells decreased dramatically after 12 h and 6 h, respectively, after the treatment with compound K (Fig. 3A and B). In addition to the observations of the cyclin D and cdk4 component, the expression of cyclin E and cdk2, which is active in the late G_1 phase, was examined (Chiariello *et al.*, 2000). As shown in Fig. 3C and D, the cyclin E expression level was reduced after 6 h after the treatment with compound K, but the cdk2 expression level remained unchanged. This suggests that the decreased expression of the cyclin and/or cdk by compound K leads to the arrest of the G_1 cell cycle.

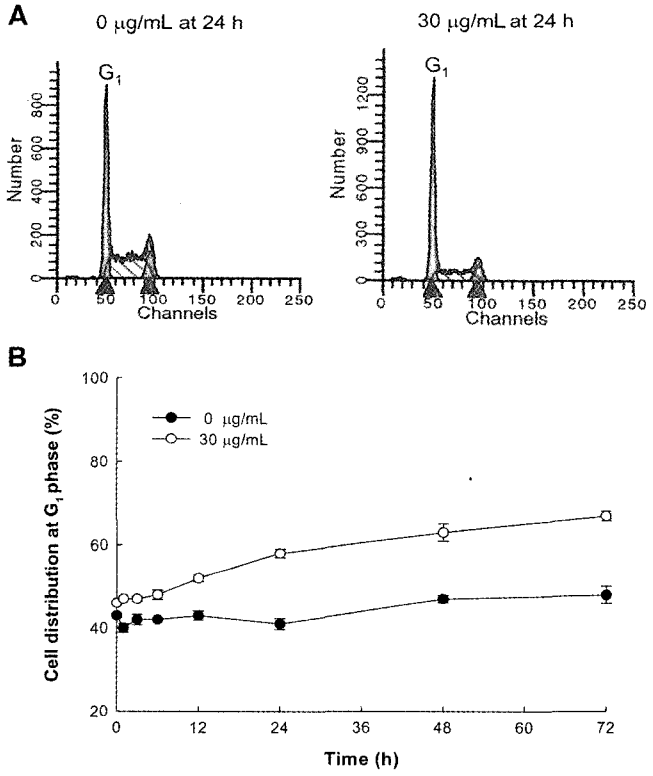

Fig. 2. Effect of compound K on the distribution of the G_1 phase in U937 cells. U937 cells were incubated with 30 μg/mL of compound K for the indicated periods. The cell cycle distribution at G_1 phase was determined after 24 h (A) and after indicated periods (B) using flow cytometric cell cycle analysis.

Compound K induces the expression of p21, independently of p53 in U937 cells

The mechanism of cdk activity inhibition frequently involves the induction of the cdk inhibitory protein, p21, as well as its interaction with the specific cdk/cyclin complex at the G_1 cell phase. Therefore, this study investigated whether compound K induces p21 in U937 cells. As shown in Fig. 4, compound K markedly up-regulated the p21 protein level after 3 h and this increased p21 protein level persisted for 12 h. It has been reported that p53 regulates a DNA damage-triggered G_1 checkpoint. Also the induction of p53 results in the transcription activation of p21 (Slebos *et al.*, 1994; Hartwell and Kastan 1994). This is known as a mechanism where p53 regulates the G_1 checkpoint for DNA damage. However, since U937 cells are a p53 mutation (Sugimoto *et al.*, 1992), it is possible that the p21 induction caused by compound K is p53 independent. Therefore, these results suggest that the arrest at the G_1 phase of the compound K treated cells, involves the decreased expression of the cyclin and/or cdk *via* induction of p21.

Compound K induces activation of JNK and AP-1

It has been reported that the activation of p54 and p46 kDa of *c*-jun *N*-terminal kinase 1 and 2 (JNK1/2), which is known as a stress-activated protein kinase, is linked to the cell cycle (Ikezoe *et al.*, 2004; Zada *et al.*, 2003; Bunnett *et al.*, 2001). In order to examine the effect of compound K on JNK1/2 activation, the phosphorylation status of 54 kDa JNK/46 kDa JNK (JNK 1/2) was analyzed using western blotting with the antibody of phosphorylated JNK 1/2. As shown in Fig. 5A, the phosphorylation of JNK1/2 by compound K occurred after 30 minutes and was sustained for 6 h. The total amount of JNK was the same in each lane. This suggests that compound K activated the JNK pathway. Therefore, the results of recent studies were examined for the effect of compound K on the DNA binding activity of the AP-1 transcription factor, which is a

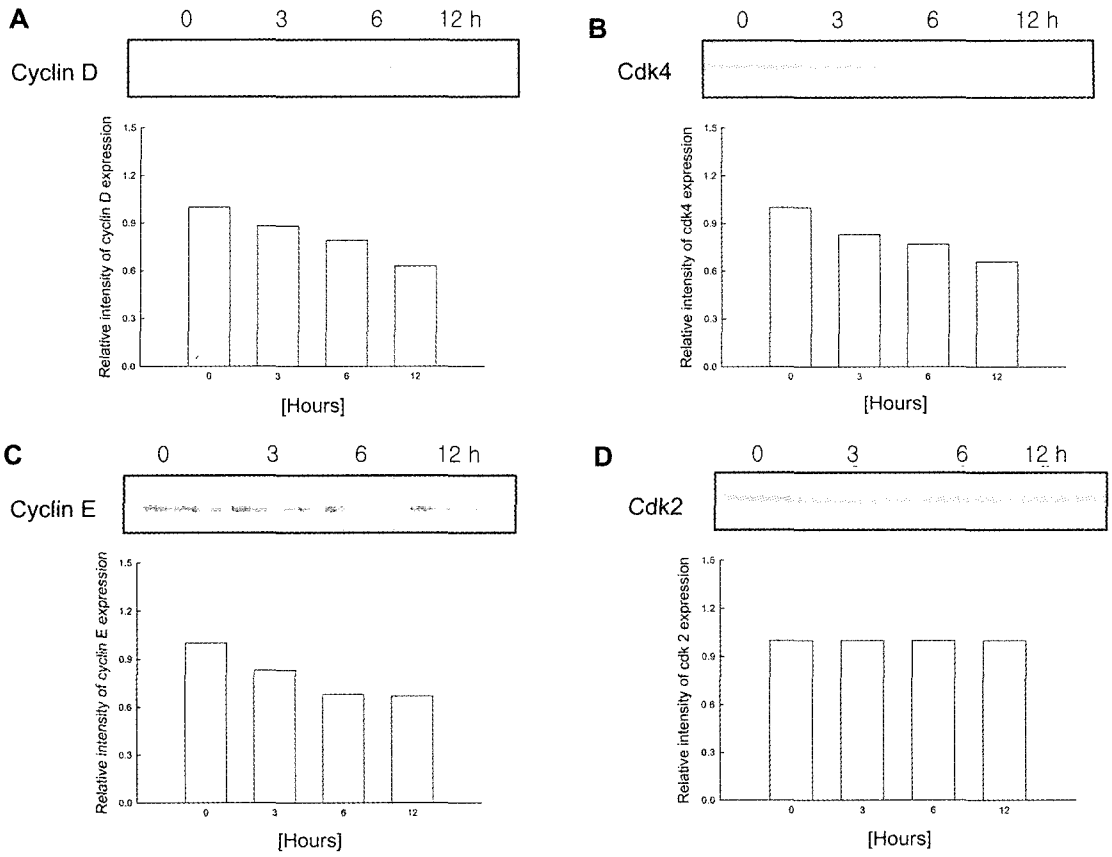

Fig. 3. Effect of compound K on the expressions of cyclin and cdk at the G_1 phase in U937 cells. The whole-cell lysates were prepared from the U937 cells cultured with 30 μg/mL of compound K after the indicated periods, and subjected to Western blot analysis to determine the cell cycle regulatory proteins, cyclin D, cdk4, cyclin E, and cdk2.

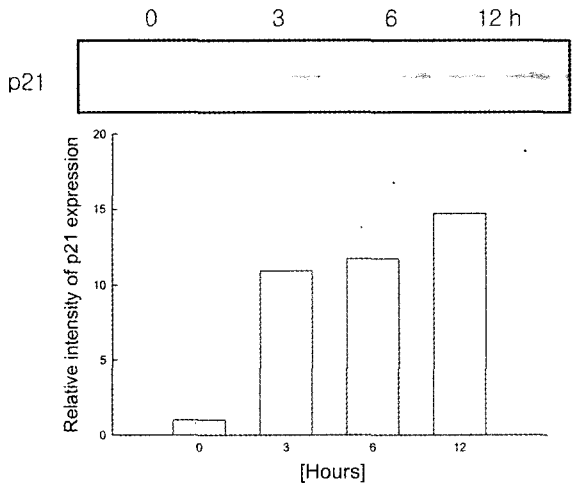

Fig. 4. Effect of compound K on the expression of p21 at the G_1 phase in U937 cells. The whole-cell lysates were prepared from the U937 cells cultured with 30 μg/mL of compound K after the indicated periods, and subjected to Western blot analysis for the p21 protein.

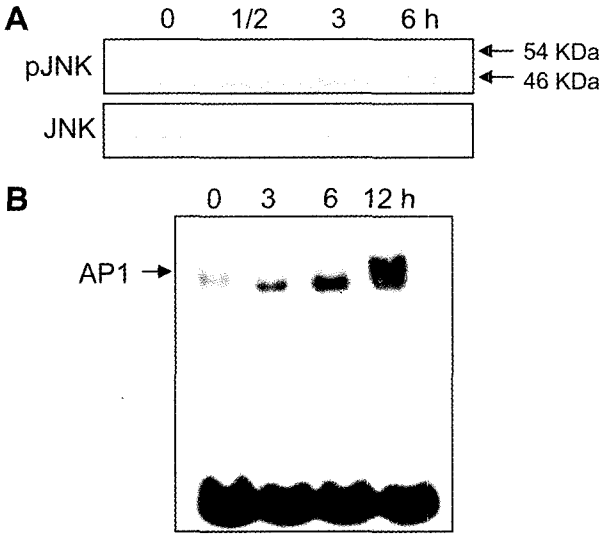

Fig. 5. Effect of compound K on the activation of JNK and AP-1 in U937 cells. The cells were treated with 30 mg/ml of compound K after the indicated periods. (A) The cell lysates were prepared and Western blot analysis was performed using anti-phospho-JNK1/2 and anti-JNK1/2 antibodies. (B) The DNA binding activity of AP-1 was detected using the EMSA method. The arrow indicates a specific DNA-protein complex of AP-1.

downstream target of JNK1/2 (Karin, 1995). As shown in Fig. 5B, the DNA binding activity of AP-1 was increased by the treatment with compound K, suggesting that

compound K induces the activation of the JNK/AP-1 signal pathway.

DISCUSSION

Some studies have reported that constituents of *Panax ginseng*; panaxytriol, polyacetylenic compounds, ginsenodside-Rs_3 and Rh_2, inhibits cell proliferation *via* the cell cycle arrest, exerting their effects at the G_1 and /or G_2 stage (Kim *et al.,* 2002; Moon *et al.,* 2000; Kim *et al.,* 1999; Oh *et al.,* 1999). However, there are no reports on the cytotoxic mechanism of ginseng saponin metabolites formed by the intestinal bacteria in respect to the cell cycle. In this study, compound K, which is one of ginseng saponin metabolites, induced the arrest of the cell cycle at the G_1 phase in U937 cells.

The cell cycle arrest induced by DNA-damaging agents usually occurs via the p53-dependent (Slebos *et al.,* 1994; Hartwell and Kastan, 1994) and/or the cdk inhibitory proteins, p21, independently of the p53 status (Demers *et al.,* 1994; Strasser *et al.,* 1994; Sheikh *et al.,* 1994). The arrest at the G_1 phase induced by compound K appears to occur *via* the latter pathway, because the p53 gene is inactive in the U937 cells due to a mutation (Sugimoto *et al.,* 1992). In this study, compound K dramatically induced the up-regulation of p21. This induction was associated with the decrease in cyclin D, E, and cdk4 expression. Additionally, it was found that compound K activated JNK, the transcription factor, AP-1, and it induced p21 expression, resulting in cell cycle arrest. Recent studies have shown that activation of the JNK/c-Jun/AP-1 signal pathway induced the p21 activation, resulting in growth arrest (Ikezoe *et al.,* 2004; Yang *et al.,* 2004). In conclusion, compound K induces the arrest of the G_1 phase *via* the induction of p21 and activates the JNK/AP-1 pathway.

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