International Journal of Biomedical and Pharmaceutical Sciences ©2012 Global Science Books

Ginsenoside Compound K Induces Cell Cycle Arrest and Apoptosis in Human Colon Cancer Cells

Yun Ju Jeong[1*] • Hyun Ju You[1] • Geun Eog Ji[1,2]

[1] Department of Food and Nutrition, Research Institute of Human Ecology, Seoul National University, Shillim-dong, Kwanak-ku, Seoul 151-742, Korea
[2] Research Institute, BIFIDO Co., Ltd., 688-1, Sang-oan-ri, Hongcheon-gun, Gangwon-do 250-804, Korea

Corresponding author: * **geji@snu.ac.kr**

ABSTRACT

Compound K (CK) is one of the principal metabolites of ginseng in human body which has been reported to exert diverse pharmaceutical activities including anticarcinogenic and antitumor effects on different lineages of cancer cells. However, the effect and mechanism of CK on colon cancer cells are not fully understood. In the present study the screening process was conducted with 12 different ginsenosides and metabolites, which showed CK was the most potent growth inhibitory compound against HT-29 colon cancer cells. The IC_{50} value of CK was 12.7 μM at 72 h. Cellular responses and growth pattern was analyzed simultaneously after CK treatment by Real Time Cellular Analysis (RTCA) method. CK treatment at specific concentration and time-point represented characteristic cytostatic stage in growth profile of HT-29 cells, and flow cytometric analysis showed that CK induced G_1 phase arrest in cell cycle distribution followed by apoptosis. The G_1 phase arrest was accompanied by down-regulation of cyclin D3, CDK6, and up-regulation of $p21^{WAF-1/CIP1}$, and apoptosis was evidenced by inactivation of p-Bcl-2 and p-Akt. These results demonstrated that CK caused growth inhibition of HT-29 cells by blocking cells in G_1 phase and inducing apoptosis.

Keywords: ginsenoside, G_1 arrest, CK, compound K,HT-29, Colon cancer
Abbreviations: CDK, cyclin dependent kinase; **CI**, cell index; **CK**, compound K; **PD**, protopanaxadiol; **PT**, protopanaxatriol; **RTCA**, real time cell analysis

INTRODUCTION

Ginseng (the root of *Panax ginseng* C. A. MEYER; family Araliaceae) has been widely used as a traditional medicine and reported to have various pharmaceutical activities including hypoglycemic (Attele *et al.* 2002), hepatoprotective (Lee *et al.* 2005), antiallergic (Choo *et al.* 2003) and anticarcinogenic effects (Choo *et al.* 2008; Park *et al.* 2009). Ginsenosides belonging to the dammarane triterpene saponin in ginseng are the principal components responsible for these biological activities, and so far more than 30 ginsenosides are identified from ginseng extract and its metabolites.

Several studies have shown the structure-activity relationship of ginsenosides on diverse physiological activities (Liu *et al.* 2003; Zhou *et al.* 2006). Ginsenosides are generally divided into two categories, protopanaxadiol (PD) and protopanaxatriol (PT), based on hydroxyl groups attached to the ginsenoside skeleton (Shibata 2001). PD-type ginsenosides such as Rg3, Rh2, Compound K (CK) and PPD have been reported to show potent antitumor effect resulting from apoptosis on several cancer cell lines (Rg3 against HT-29 human colon cancer cell line (Lee *et al.* 2009) and B16 murine melanoma cell line (Chen *et al.* 2008); Rh2 against Caco-2 colon cancer cell line (Popovich and Kitts 2004); CK against HL-60 human leukemia cell line (Cho *et al.* 2009) and PPD against human fibrosarcoma HT1080 cell line (Li *et al.* 2006)). Major ginsenosides with various sugar moieties such as Rb1, Rb2, Rc, Rd, Re and Rg1 comprise more than 80% of total ginsenosides (Son *et al.* 2008). However, ginsenoside metabolites with less sugar moiety had tendency to show greater cytotoxic and antitumor effect compared to the ginsenosides with more sugar residues (Zhou *et al.* 2008; Noh *et al.* 2009).

Sugar moieties of ginsenosides are removed during fermentation, resulting in the conversion of glycones to aglycones (Chi and Ji 2005; Chi *et al.* 2005). Absorption of ginsenosides in human body mainly takes place in the intestinal tract after microbial biotransformation of glycone ginsenosides such as Rb1, Rb2, Rc, Re and Rd to their metabolites such as Rh1, Rh2 and CK (Tawab *et al.* 2003). Since the intestinal microbial composition differ individually, the degree of absorption and biological effects of ginsenosides were considered different depending on the composition of the individual's intestinal microbial flora (Tawab *et al.* 2003). In the lumen of colon, ingested ginsenosides are present as mixture of their intact forms and hydrolyzed forms. Therefore, we firstly screened 12 major ginsenosides in ginseng and their metabolites together using colon cancer cell line and normal colon cell line to find out the most effective growth inhibitory compound against colon cancer cells and to investigate the structure-activity relationship between major ginsenosides and metabolites.

CK is the minor ginsenoside in ginseng extract and metabolized by hydrolyzing sugar moieties of the major ginsenosides such as Rb1, Rb2, Rc and Rd in colonic environment. Among various ginsenosides, CK has received attention for its several pharmaceutical activities such as enhancing insulin secretion (Han *et al.* 2007), hypoglycemic (Yoon *et al.* 2007), hepatoprotective (Lee *et al.* 2005), antipruritic (Shin and Kim 2005) and anticarcinogenic (Cho *et al.* 2009) effect. However, the mechanism of anticarcinogenic effect of CK in colon cancer cells is not fully understood. In the present study, by using non-invasive real-time cellular analysis (RTCA) method, we monitored the cellular status and growth pattern of HT-29 colon cancer cells simultaneously for 72 h after the treatment of different concentrations of CK, and found out the characteristic pattern in growth profile at specific concentration and time point of CK treatment. Based on these findings including the screening process which demonstrated that CK was the most

Received: 12 May, 2010. Accepted: 7 July, 2011.

Original Research Paper

Table 1 The abbreviations and full names of treated ginsenosides.

Type	Abbreviation	Full name
Protopanaxa diol type	CK	20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol
	Rh2	3-O-β-D-glucopyranosyl-20(S)-protopanaxadiol
	F2	3-O-β-D-glucopyranosyl-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol
	Rd	3-O-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol
	Rc	3-O-[β-D-Glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-[α-L-arabinofuranosyl-(1-6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol
	Rb1	3-O-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol
	Rb2	3-O-[β-D-Glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-[α-L-arabinopyranosyl-(1-6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol
Protopanaxa triol type	PPT	Protopanaxatriol
	Rh1	6-O-β-D-glucopyranosyl-20(S)-protopanaxatriol
	Rf	6-O-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20(S)-protopanaxatriol
	Rg2	6-O-[α-L-rhamnopyranosyl-(1-2)-β-D-glucopyranosyl]-20(S)-protopanaxatriol
	Re	6-O-[α-L-rhamnopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-β-D-glucopyranosyl-20(S)-protopanaxatriol

potent growth inhibitory compound, we investigated the mechanism of CK treatment in colon cancer cells focusing on the cell cycle arrest and induced-apoptosis.

MATERIALS AND METHODS

Chemicals and reagents

All the ginsenosides used in this study (**Table 1**) were purchased from Chengdu Cogon Biotech Co., Ltd (Chengdu, China). Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless further specified. The materials used for cell culture including media, serum, antibiotic solutions and related reagents were obtained from GIBCO® products (Invitrogen Life Technologies, Carlsbad, CA, USA). The anti-cyclin D3 (DCS22), anti-CDK4 (DCS156), anti-CDK6 (DCS83) and anti–$p21^{WAF-1/CIP-1}$ (DCS60), anti-phospho-Bcl-2 (p-Bcl-2) and anti-phospho-Akt (p-Akt) were purchased from Cell Signaling Technology, Inc. (Danvers, USA). Enhanced chemiluminescence detection system used SuperSignal® West Dura Extended Duration Substrate purchased from ThermoScientific (Rockford, U.S.A).

Cell culture

Human colorectal adenocarcinoma HT-29 (KCLB 30038) cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 2% of fetal bovine serum (FBS) and 1% of antibiotic-antimycotic (AA) solution. FHC human normal colon cells (ATCC CRL-1831™) were maintained in 45% of Ham's F12 medium and 45% of DMEM with 25 mM HEPES, 10 ng/mL cholera toxin, 5 μg/mL insulin, 5 μg/mL transferrin, 100 ng/mL hydrocortisone and 10% of FBS. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and subcultured prior to confluence. After the incubation, cells were harvested and hemacytometer count was performed with trypan blue dye exclusion methods.

Cell viability

To estimate the effect of CK on the cell viability of human cancer cells and normal cells, MTT assay was used. The cells were seeded in 96-well plates at $3\text{-}6\times10^3$ cells per well. The cells were incubated and attached to the bottom of the plate in a humidified incubator (37°C, 5% CO_2) for 24 h. Then, the medium was replaced with FBS-free medium to synchronize the growth phase of cells. After 24 h, the medium was replaced with 2% FBS and various concentrations of test compounds were treated to each well. Test compounds were dissolved in DMSO and were diluted with culture medium to the final concentration prior to treatment. After incubation with test compounds for 72 h, 20 μL of the MTT solution (5 mg/mL) were added to each well and plates were incubated at 37°C for 2-4 h. The absorbance at 570 nm was recorded using a microplate reader (Bio-Rad Laboratories, Philadelphia, PA, USA).

Real time analysis of cell growth pattern

Dynamic monitoring of cell attachment, proliferation and growing patterns were conducted using real time cellular analysis (RTCA) with xCELLigence RTCA system (Roche Diagnostics, Mannheim, Germany) and 96-well E-plate™ (ACEA Biosciences, San Diego, CA, USA). The 96× RTCA SP station equipped with the E-plate™ containing the cells was placed inside the CO_2 incubator and the collected data were transferred to the connected analyzer outside under the control of integrated software (Zhu *et al.* 2006). Trypsinized and resuspended HT-29 cells were adjusted to the appropriate concentration, and 100 μL of the cell suspension was added to the wells of the E-plate™, which was monitored every hour. Approximately 24 h after seeding, the cells were treated with CK and monitored for 72 h. The cell-electrode impedance readout was displayed as an arbitrary unit called Normalized Cell Index (CI). As cell numbers and/or cell adhesion to contact area increase, CI values tend to increase in correlation.

Cell cycle analysis

CK dissolved in DMSO was serially diluted with culture medium to 10 and 20 μM concentration, and was treated to HT-29 cells. Cells were harvested by centrifugation, washed with cold PBS and fixed with ice-cold 70% ethanol. The cells were stored at -20°C before analysis. For the cell cycle analysis the cells were washed with PBS twice and RNA was removed by incubation with 1.4 U/mL RNAse A in PBS at 37°C for 30 min. After incubation, the supernatant was removed and 300 μL of PBS containing 0.1 mg/mL of PI was added in the dark. DNA content of cells stained with propidium iodide was measured on a FACSCalibur™ flow cytometry system (BD Biosciences, San Jose, CA, USA) within 1 h by using software (BD Biosciences).

Protein preparation and Western blot analysis

HT-29 cells stabilized in culture media for 24 h were treated with 30 and 40 μM CK. Cells (approximately 1×10^6 /dish) were harvested after 24 h from treatment, washed with ice-cold PBS and lysed for 30 min in protein extraction buffer. The cell lysates were centrifuged at 35,000 × *g* for 10 min at 4°C to obtain the supernatant, which was used for protein preparation and western blot analysis. Protein concentrations were determined using Lowry method (Lowry *et al.* 1951). For Western blot analysis, 40 μg of protein was equally loaded and separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE at 90 V) for 1 h. The gels were equilibrated in transfer buffer (diluted ten times with transfer buffer made with Tris base 58.38 g/L, glycine 29 g/L, 10% SDS 37 mL, and D.W 1 L at pH 8.3), and electrophoretically transferred to PVDF membrane at 100 V for 1 h. The membrane was blocked with 5% skim milk in 0.1% TBS-T buffer (diluted ten times with TBS-T buffer made with 0.2 M Tris 24.2 g, 1.37 M NaCl 80 g, D.W 1 L, and Tween 20 1 mL) for 1 h and incubated overnight at 4°C with specific primary antibodies. After washing 3 times with TBS-T, the membrane was incubated with horseradish

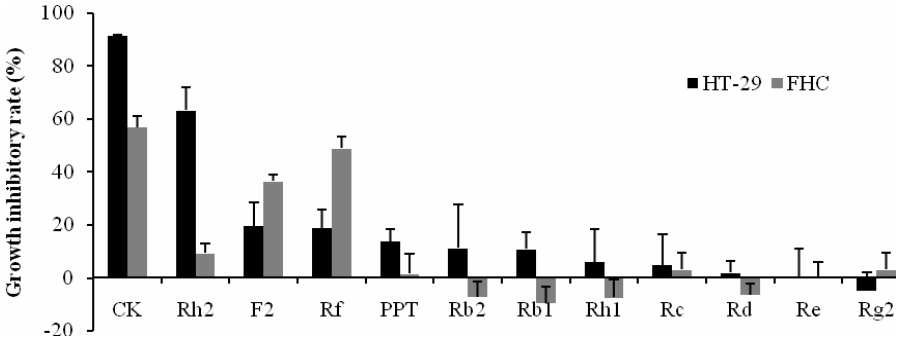

Fig. 1 Growth inhibitory effects of 12 ginsenosides (CK, Rh2, F2, Rf, PPT, Rb2, Rb1, Rh1, Rc, Rd, Re and Rg2) on human colon cancer cells (HT-29) and normal colon cell (FHC). The cells were incubated with 50 μM of experimental compounds for 72 h. The data expressed as the mean ± SD (*$p < 0.05$ compared to the vehicle control).

peroxidase conjugated secondary antibodies for 1 h at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system.

Statistical analysis

Data are presented as the mean±SD. Multiple comparison among groups was made by one-way ANOVA with *post hoc* Duncan's test using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered significant.

RESULTS

Growth inhibitory effect of CK

The growth inhibitory effects of different ginsenosides on HT-29 colon cancer cells and FHC normal colon cells were assessed by MTT assay. Twelve ginsenosides (CK, Rh2, F2, Rf, PPT, Rb2, Rb1, Rh1, Rc, Rd, Re and Rg2) were treated to each cell line at 50 μM concentration for 72 h. The growth inhibitory rate (%) was represented as relative ratio of growth inhibition compared to vehicle control (0.1% (v/v) DMSO). As shown in **Fig. 1**, CK, Rh2, Rf and F2 showed growth inhibitory effects on both cell lines. Compared to Rf and F2, the viability of FHC normal colon cells was higher than that of HT-29 cells when treated with CK and Rh2. In HT-29 colon cancer cells, CK showed the most potent growth inhibitory effect with 91.4% and Rh2 showed 68.1% growth inhibition. As shown in **Fig. 2**, CK inhibited the growth of HT-29 cells in a dose-dependent manner.

Effects of CK on cell cycle arrest and apoptosis in HT-29 cells

For the monitoring of simultaneous cellular status and the demonstration of growth pattern, RTCA was carried out in the presence of 2% FBS with 10, 25, 50, and 75 μM CK. The treatment with 25 μM CK showed the cytostatic profile

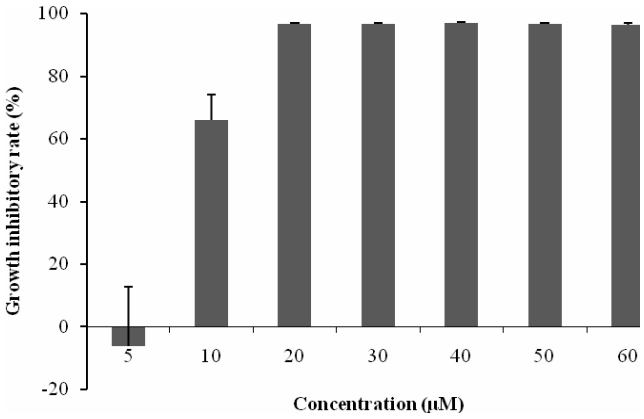

Fig. 2 Growth inhibitory effect of CK on human colon cancer cells (HT-29). The viability of HT-29 cells was measured by MTT assay at various concentrations (5, 10, 20, 30, 40 and 50 μM) of CK after 72 h from treatment. The data were expressed as the mean ± SD (*$p < 0.05$ compared to the vehicle control).

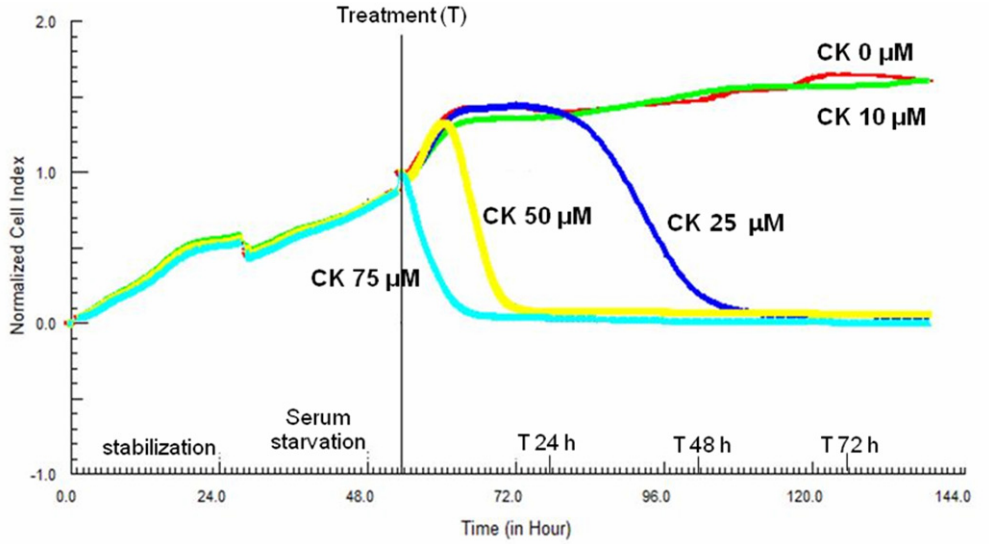

Fig. 3 RTCA profiles after the treatment of CK on HT-29 cells. HT-29 cells were seeded in the wells of 96-well E-plate™ and were treated with different concentrations of CK (10, 25, 50 and 75 μM) and vehicle control (DMSO 0.1% (v/v)). The viability of the cells were continuously monitored by RTCA system for 72 h and displayed as normalized CI values which was recorded every 30 min. Each trace at each concentration was an average of three replicates.

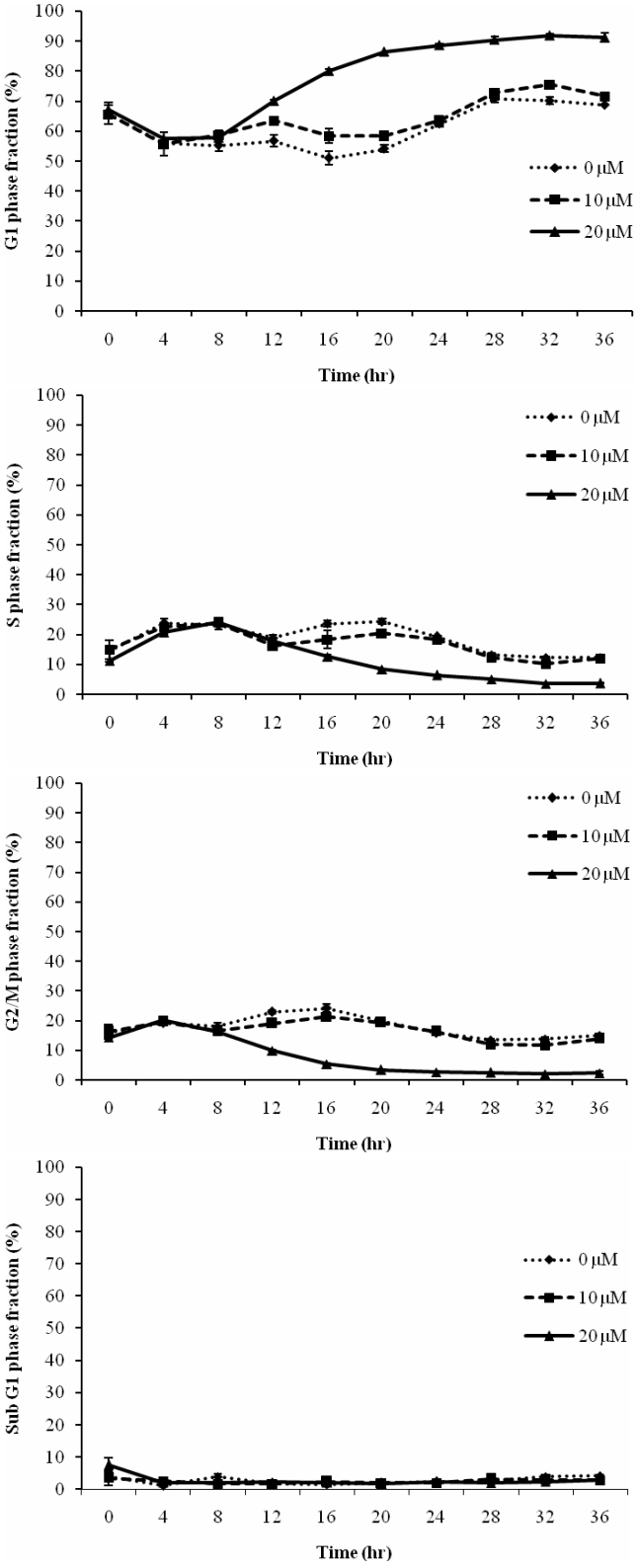

Fig. 4 Cell cycle analysis in 10 and 20 μM CK treated HT-29 cells up to 36 h compared to the control. The harvested cells were stained with propidium iodide and DNA content was analyzed by flow cytometry. The percentage of each phase fraction was assessed by counting DNA contents below 2N. After 36 h treatment 10 and 20 μM of CK, HT-29 cells demonstrated G_1 phase cell cycle arrest. The data were expressed as the mean ± SD.

from 8 to 32 h after treatment, followed by the loss of viability (**Fig. 3**). Cells treated with 50 μM CK maintained growth until 6 h, but thereafter lost viability as shown in the profile of 25 μM CK. In 75 μM CK group, the cells were detached from the surface of culture well and died immediately after treatment. The time-dependent IC_{50} values calculated by integrated software were 9.3 μM at 24 h and 12.7 μM at 72 h, respectively. IC_{50} value was increased as the incubation time was increased. To investigate the relationship between cytostatic/growth inhibitory effects of CK and the distribution of cell phases in cell cycle, FACS analysis with PI staining was conducted using HT-29 cells in the presence of CK at 10 or 20 μM. As shown in **Fig. 4**, 20 μM CK induced the accumulation of cells in G_1 phase after 12 h whereas control group and 10 μM CK group showed normal flow of cell cycle. In this regard, it was suggested that the cytostatic stage by CK treatment was related to the G_1 phase cell cycle arrest.

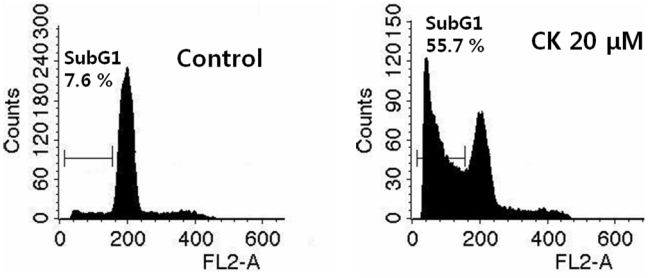

Fig. 5 Increase of the cells in sub-G_1 phase after 72 h treatment with 20 μM CK in HT-29 cells. Cells were cultured in the absence or presence of 20 μM CK for 72 h. The percentage of apoptotic cells was assessed by counting the cells with DNA contents below 2N.

To figure out whether the loss of viability and the growth inhibitory effect after prolonged treatment with CK were related to the induction of apoptotic event, HT-29 cells were incubated for 72 h in the presence of 20 μM CK and were analyzed by FACS. The sub-G_1 region of cell cycle was increased up to 55.7% compared to 7.6% in the vehicle control (**Fig. 5**), which suggested that the loss of viability after prolonged treatment with 20 μM CK was due to the induced-apoptosis following G_1 arrest.

Effects of CK on proteins involved in cell cycle arrest and apoptosis in HT-29 cells

Based on the results above, we investigated the expression of the G_1 phase-associated cell cycle regulatory molecules such as cyclin D3, CDK4 and CDK6 in HT-29 cells (**Fig. 6**). CK treatment resulted in decrease in the protein expression of cyclin D3 and CDK6. The cyclin dependent kinase inhibitors $p21^{WAF-1/CIP1}$ plays a role in inducing G_1 arrest. The protein level of $p21^{WAF-1/CIP1}$ was increased after CK treatment as compared to vehicle control (**Fig. 6**). To evaluate CK-induced apopotosis, we examined the expression of p-Bcl-2 which acts as suppressor of programmed cell death. After 24 h from treatment, CK showed dose-dependent down-regulation in the level of p-Bcl-2 (**Fig. 7**). Moreover, treatment with CK decreased the levels of p-Akt (**Fig. 7**), which is known to promote cell survival.

DISCUSSION

More than 30 kinds of ginsenosides have been identified from ginseng and its metabolites. In the present study, we assessed and compared the growth inhibitory effects of major ginsenosides and their metabolites on human colon cancer cells. Related to their anticancer activity, there have been several reports that the cytotoxic effects of ginsenosides on cancer cells were related to the structural differences including the type of dammarane backbone, the number of sugar moieties and the variety of substituted residues. For example, PD-type ginsenosides such as 25-OH-PPD, PPD, Rh2 and CK showed greater cytotoxic effects than PT-type ginsenosides such as Rb1, Rb2 and PPT on prostate and lung cancer cell line (Wang *et al.* 2007; Li *et al.* 2009). However, the structure-activity relationship of major ginsenosides and their metabolites regarding growth inhibitory effect against colon cancer cells has not been studied yet. In

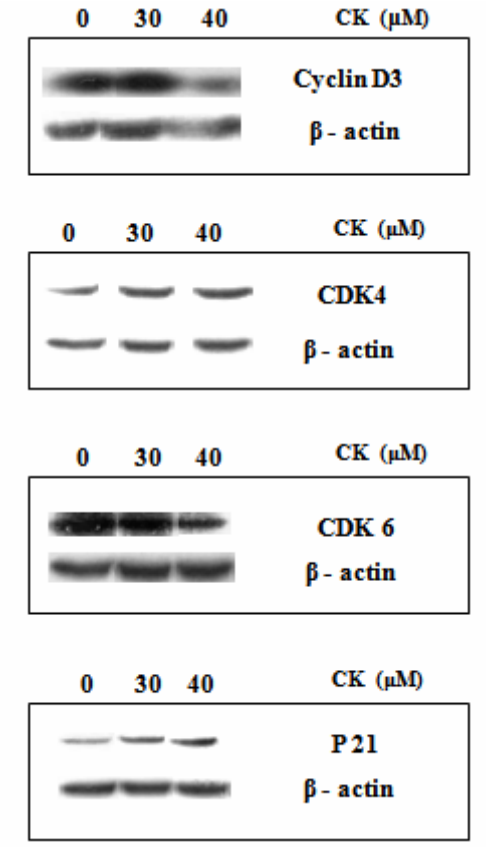

Fig. 6 Western blot analysis for cyclin D3, CDK4, CDK6 and p21$^{WAF-1/CIP1}$ protein expression in CK treated HT-29 cells. Cells were treated with 0, 30 and 40 μM CK for 24 h. β-Actin blotting was used as internal control.

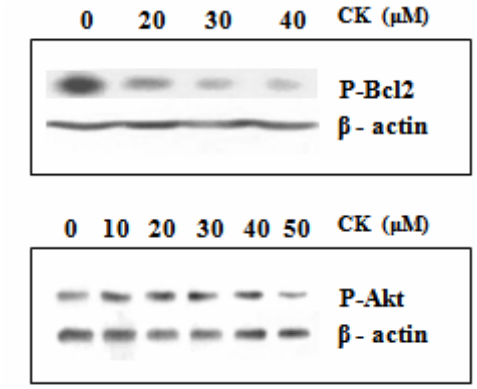

Fig. 7 Western blot analysis for p-Bcl-2 and p-Akt protein expression in CK treated HT-29 cells. Cells were treated with 0-50 μM CK for 24 h. β-Actin blotting was used as internal control.

colon cancer cells, PD-type ginsenoside CK exerted the most potent growth inhibitory effect, followed by the other PD-type ginsenoside Rh2. Rh2 is a structural isomer of CK. RTCA profile of Rh2 at 25 μM was very similar to that of CK especially in cytostatic stage which was not shown in other ginsenosides' profiles (data not shown). The growth inhibitory effect of Rh2 was reported to be caused by the G_1 phase arrest and apoptosis in breast (Choi *et al.* 2009), lung (Cheng *et al.* 2005), and esophageal (Li *et al.* 2005) carcinoma cell lines. In leukemia cells, CK was also reported to exert growth inhibition via G_1 phase cell cycle arrest or apoptosis (Kang *et al.* 2005; Cho *et al.* 2009).

Present study showed that CK induced a real-time cellular change from cytostatic stage to the ensuing loss of viability in HT-29 colon cancer cells. Based on our result and other studies (Kang *et al.* 2005; Cho *et al.* 2009), we supposed that this cytostatic profile was due to cell cycle arrest. FACS analysis confirmed that the cytostatic pattern in the RTCA profile was related with the G_1 cell cycle arrest, while the growth inhibitory effect after cytostatic stage was due to apoptosis.

The G_1 phase arrest induced by CK appears to follow the p53 independent pathway, since the p53 gene is inactive in the HT-29 cells due to a mutation (te Poele and Joel 1999). In this study, the arrest of cell cycle progression at the G_1 phase seems to involve a down-regulation of cyclin D3 and CDK6 and up-regulation of p21$^{WAF-1/CIP1}$. The complex between cyclin D3 and CDK6 function as a regulatory subunit whose activity is required for the G_1/S transition of cell cycle (Meyerson and Harlow 1994; Lin *et al.* 2001). p21, which binds and inhibits cyclin-CDK complex, plays an essential role in growth arrest and leads to G_1 and G_2 or S-phase arrest (Gartel and Tyner 2002). In Kim *et al*. (2009) study, CK did not show cell cycle arrest HT-29 colon cancer cells since their experimental concentration of treated CK was probably too high. In the present study 25 μM CK showed cytostatic pattern, whereas 50 μM CK displayed immediate decrease of viability. After the treatment of CK, the region of sub G_1 phase was increased and the blockage of cell cycle in G_1 phase was induced through inhibition of complex formation between cyclin D3 and CDK6 by activation of p21$^{WAF-1/CIP1}$. The expression level of p-Akt and p-Bcl-2, which are related with cell survival and apotosis, were also down-regulated. In response to the various stimuli, the increased level of p-Akt expression promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of several targets including Bad, c-Raf, and caspase-9. In intrinsic apoptosis pathway, p-Bcl-2 exerts a survival function through inhibition of mitochondrial cytochrome c release and can be a marker for mitotic events (Soucie *et al.* 2001). Even though the changes of upstream and downstream regulatory molecules of Bcl-2 and Akt need to be further investigated, our findings showed somewhat detailed molecular mechanism involved in the pro-apoptotic effects of CK in HT-29 cells.

In conclusion, CK showed the most potent growth inhibitory effect against colon cancer cells among 12 ginsenosides and its metabolites in human colon cancer cells. The efficacy was mediated by the blockage of the cell cycle progression at the G_1 phase and induction of apoptosis. The mechanism of the inhibition of tumor cell growth was via regulation of cyclin dependent kinase inhibitors and pro-apoptotic agents.

ACKNOWLEDGEMENTS

This work was supported by the Small and Medium Business Administration (S1072365, Korea).

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