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

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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 IC50 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 G1 phase arrest in cell cycle distribution followed by apoptosis. The G1 phase arrest was accompanied by down-regulation of cyclin D3, CDK6, and up-regulation of p21WAF-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 G1 phase and inducing apoptosis.

Keywords: ginsenoside, G1 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 (Cho 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 propanaxatriol (PT), based on hydroxyl groups attached to the ginsenoside skeleton (Shibata 2001). P-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, Rh2, 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, Rh2, 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), anti-pruritic (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
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). 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-p21 WAF-1/CIP-1 (DCS60), anti-phospho-CDK6 (DCS83) and anti–p21 WAF-1/CIP-1 (DCS60), anti-phospho-CDK2 (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-antisemiotic (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% CO₂ and subcultured prior to confluence. After the incubation, the 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 3-6×10⁴ cells per well. The cells were incubated and attached to the bottom of the plate in a humidified incubator (37°C, 5% CO₂) 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-well RTCA SP station equipped with the E-plate™ containing the cells was placed inside the CO₂ incubator and the collected data were transferred to the connected analyzer 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 1×10⁶ 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 sulfate 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 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 electroelastically transferred to PVDF membrane at 100 V for 1 h. The membrane was blocked with 5% skim milk in 0.1% 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
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, F2, 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.
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 IC50 values calculated by integrated software were 9.3 µM at 24 h and 12.7 µM at 72 h, respectively. IC50 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 G1 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 G1 phase cell cycle arrest.

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-G1 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 G1 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 G1 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 p21WAF-1/CIP1 plays a role in inducing G1 arrest. The protein level of p21WAF-1/CIP1 was increased after CK treatment as compared to vehicle control (Fig. 6). To evaluate CK-induced apoptosis, 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.
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 RTCA profile of Rh2 at 25 μM. CK is a structural isomer of Rh2. Most potent growth inhibitory effect was found in CK especially in PD-type ginsenoside Rh2. Rh2 is a structural isomer of CK. The growth inhibitory effect of Rh2 was reported to be caused by the G1 phase arrest and apoptosis in breast (Choi et al. 2009), lung (Cheng et al. 2005) and esophageal (Li et al. 2005) 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 G1 cell cycle arrest, while the growth inhibitory effect after cytostatic stage was due to apoptosis.

The G1 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 G1 phase seems to involve a down-regulation of cyclin D3 and CDK6 and up-regulation of p21WAF-CIP. The complex between cyclin D3 and CDK6 function as a regulatory subunit whose activity is required for the G1/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 G1 or G2 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 G1 phase was increased and the blockage of cell cycle in G1 phase was induced through inhibition of complex formation between cyclin D3 and CDK6 by activation of p21WAF-CIP. The expression level of p-Akt and p-Bcl-2, which are related with cell survival and apoptosis, 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 something 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 efficiency was mediated by the blockage of the cell cycle progression at the G1 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.

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