Compound K, a metabolite of ginseng saponin, induces apoptosis of hepatocellular carcinoma cells through the mitochondria-mediated caspase-dependent pathway

Xia Dong1, Hongbo Han1, Haizhou Shi1, Tingrui Wang1, Baoyun Wang1, Jing Zhao2

1Department of Gastrointestinal Surgery, Binzhou Central Hospital, Binzhou Medical College, Binzhou, China; 2Department of Gastrointestinal Surgery, Provincial Hospital of Shandong University, Jinan, China

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Abstract: Compound K (20-O-β-d-glucopyranosyl-20(S)-protopanaxadiol, CK), an intestinal bacterial metabolite of ginseng protopanaxadiol saponins, has been shown to possess the potential ability in preventing tumor development and suppressing cancer growth. However, the exact mechanism of its antitumorigenic effects is still not clear. The purpose of this project was to detect whether CK has an anticancer effect on hepatocellular carcinoma (HCC) cells, and to further explore the possible mechanisms. The Hep-G2 cells and xenograft in nude mice were used as models to study the anticancer effect of CK. Methylthiazolyldiphenyl-tetrazolium-bromide (MTT) assay showed that CK significantly inhibited the viabilities of Hep-G2 cells in dose- and time-dependent manners. In addition, flow cytometry analysis showed that CK induced apoptosis and cell cycle arrest in Hep-G2 cells, which possibly accounted for the antiproliferative effects of CK. Notably, the data in the present research indicated that CK upregulated the expression of p21Cip1 and p27Kip1, and downregulated the expression of cyclin D1 and cyclin-dependent kinase 4, causing a G0/G1 phase arrest, blocking cell cycle progression, and inducing apoptosis in the Hep-G2 cells, which was mediated by the mitochondrial pathway through a modulation of the ratio of Bcl-2 to Bax. In vivo studies showed that tumor volume, compared with control group, was reduced dramatically in CK-treated group. Therefore, the present study provides new insights that CK may be an potential agents in the prevention or treatment of HCC.

Keywords: Compound k, hepatocellular carcinoma, apoptosis, mitochondria-mediated

Introduction

HCC is known as a common and aggressive malignant tumor worldwide, and has been one of the main reasons of cancer-associated mortality. Despite the recent advances in the clinical management of HCC, the long-term prognosis of patients is poor [1, 2]. In China, estimated by the World Health Organization (WHO), HCC is usually secondary to liver cirrhosis, especially hepatitis B-induced liver cirrhosis. Besides, HCC is the fourth most common cancer, accounting for 11.6% of all new-onset malignant tumor in China, and is the second most fatal cancer with 15.97% of total cancer deaths [3, 4]. What’s the worse, HCC are particularly tend to recurrence and distant metastasis, which have a negative effect on survival rate. Previous data showed that the survival rate is evaluated at around 60% after 5 years for patients without clinical symptoms and deceases to 20% when distant metastases are discovered at the time of diagnosis [5, 6]. Apart from potentially surgical resection, chemotherapy, radiochemotherapy and interventional therapy may be applied at advanced stages of HCC but neither of these can be curative. Thus, there is a strong demand for new curative approaches to HCC. Chemotherapy plays an important role in the treatment of cancer, but it is limited to a significant extent by its toxicities, significant resistance to available chemotherapeutic agents and side effects, including thrombocytopenia, neutropenia and myelosuppression [7, 8]. One possible way to increase the efficacy of anticancer drugs and to decrease toxicities or side effects is to explore traditional medicines, especially from chinese medicinal herb.

CK is a novel ginseng saponin metabolite, formed from ginsenosides Rb1, Rb2 and Rc by
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the human intestinal bacteria deglycosylation [9, 10]. And it has demonstrated such diverse pharmacological actions as anti-inflammatory, cardiac protection [11], antiallergy, anti-diabetic, anti-angiogenesis [12], anti-aging, hepatoprotective [13] and neuroprotective effects [14]. Interestingly, Dozens of studies have confirmed that CK can inhibit proliferation and induce apoptosis in a variety of tumors such as gastric cancer, breast cancer, lung carcinoma, colon cancer and glioma [15]. For example, Ming's researches reported that CK could inhibit the metastatic in HCC through the NF-κB p65 nuclear export and the reduction of MMP2/9 expression, suggesting that CK may be a potential cytotoxic drug in the prevention and treatment of hepatocellular carcinoma [16]. Besides, Li Yang et al [17]. indicated that CK could enhance the anti-tumor effects of cisplatin against pulmonary adenocarcinoma cells in a p53-dependent manner, and provided strong rationale to develop CK as an adjuvant drug for cisplatin-based lung cancer intervention. However, the anticancer mechanisms of CK are not clearly clarified, especially in HCC. In the present study, to determine the potential of CK as a novel chemotherapeutic drug, we use human hepatocellular carcinoma cell line Hep-G2 and the model of human hepatocellular carcinoma xenograft in nude mice to clarify the mechanisms of CK-induced apoptosis, especially CK's possible roles on mitochondria-dependent pathway in human hepatocellular carcinoma cells.

Materials and methods

Chemicals and reagents

Cell culture chemicals were obtained from Invitrogen Company (Burlington, Ontario, Canada). CK was purchased from ApexBio Technology (Apexbio Technology LLC, Houston, TX, USA), MTT were obtained from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in phosphate-buffered saline (PBS). The antibodies against B-cell lymphoma (Bcl)-2, Bax, caspase-9, caspase-3, PARP, p21<sub>Cip1</sub>, p27<sub>Kip1</sub>, CyclinD1, Fas, and Fasl were purchased from Cell Signaling Technology (Beverly, MA, USA). Enhanced chemiluminescence (ECL) reagents, horseradish peroxidase-conjugated anti-rabbit immunoglobulin (IgG) and anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and treatments

Hep-G2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS: Hyclone, Logan, UT, USA), 2 mM L-glutamine, and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a 5% CO₂ humidified incubator. In all experiments, cells were used in logarithmic growth phase.

Measurement of cell growth and viabilities

The Hep-G2 cells were seeded at a density of 3000 cells/well into 96-well flat-bottom cell culture plates (Corning Incorporated, NY, USA) in medium containing 10% FBS, and the MTT assay was used to detect the effect of CK on cell growth. Subsequently, the cells were treated with 2.5, 5, 10 or 20 µmol/l CK for 48 h, whereas only DMEM medium was added for the control group. For time course, cells were incubated under similar conditions for variable times ranging from 24 h to 72 h. Finally, absorbance at 450 nm was detected by using an automated microplate reader (Bio-Rad Laboratories, Tokyo, Japan). All experiments were performed at least 3 times.

Cell morphological studies

The Hep-G2 cells were treated with 10 µmol/l CK or 0.1% dimethyl sulfoxide (control) for 48 h. The cells were then incubated with 10 µg/ml Hoechst 33342 and observed by fluorescence microscope (DMIRB; Leica, Wetzler, Germany).

Cell cycle assay by flow cytometric (FCM) analysis

The Hep-G2 cells (~1×10<sup>5</sup> cells/well) were seeded into a 6-well plate overnight at 37°C. The cells were washed once with PBS, the medium was replaced with fresh medium, and the cells were subsequently treated with various concentrations of CK (0, 5 and 10 µmol/l) for 48 h. Following treatments for the various durations, the cells were collected, fixed with 70% ethanol, incubated with 25 µg/ml ribonuclease A and stained with 50 µg/ml PI for 30 min at room temperature in the dark. Subsequently, the cell cycle data were detected
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**Cell apoptosis assay by FCM analysis**

Apoptotic rate of the Hep-G2 cells was analyzed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Briefly, a total of $1 \times 10^5$ Hep-G2 cells/well were seeded into six-well plates and cultured in DMEM at 37°C overnight. Subsequent to starvation for 12 h, the Hep-G2 cells were treated with different doses of CK (0, 2.5, 5, and 10 μmol/l) in complete medium for 48 h, digested with 2.5 mg/ml trypsin, washed twice with PBS and suspended with 300 μl binding buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The cells were then incubated with 2 μl Annexin V and 5 μl PI for 15 min at room temperature, and the distribution of viable, early apoptotic, late apoptotic and necrotic cells was calculated using a FACSCaliber flow cytometer (BD Biosciences), according to the manufacturer’s instructions. Cells that were negative for the Annexin V-FITC and PI were considered to be viable cells. The sum of the early and late apoptotic cells constituted the total number of apoptotic cells, which was presented as the percentage of the total cells.

**Western blot analysis**

The Hep-G2 cells were treated with CK at different concentrations (0, 2.5, 5, and 10 μmol/l, respectively), and the protein collections from the above treated cells were harvested subsequently in RIPA buffer (9.1 mM dibasic sodium
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phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium vanadate and 0.2 U/ml aprotinin) containing protease inhibitor cocktail (Santa Cruz Biotechnology; Santa Cruz, CA). Western blotting was used to detect the effect of CK on apoptosis related proteins. And protein concentrations were determined using the bicinchoninic acid protein assay kit (KenGen Biotechnology Co. Ltd, Nanjing, China). Clarified protein lysates (50 g) were resolved electrophoretically on denaturing SDS-polyacrylamide gels (10%), and transferred to nitrocellulose membranes. The membranes were then blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with the indicated specific primary antibodies for 3 h. Proteins were visualized with Horseradish peroxidase (HRP)-conjugated secondary antibodies. To corroborate equal loading, membranes were stripped and reprobed using an antibody specific for β-actin (1:1000; Abcam, Cambridge, MA). Finally, antigen-antibody complexes were detected using the ECL system, and digital images were captured with a FlourChem HD2 Imager system (Alpha Innotech, San Leandro, CA).

Hep-G2 tumor xenograft

Hep-G2 cells were implanted by subcutaneous injection into the left hind leg of 15 BALB/c nude mice (~3×10⁶/mouse, supplied by Experimental Animal Department of Binzhou Medical College, Shandong, China). Seven days later, 10 mice with size of ~100 mm³ were randomly distributed into two groups, i.e. control group (normal saline) and CK (10 mg/kg/day) group. Tumor volume (mean values and 95% confidence intervals) was measured at various times after the initial intraperitoneal injection. At the end of the experiment, mice were sacrificed and tumor weight was measured. All the mouse experiments were performed in accordance with Institutional Animal Care and Use Committee procedures and guidelines.

Statistical analysis

Data are presented as mean ± standard deviation. All experiments were repeated at least three times. Statistically significant differences between the experimental groups were determined using Student’s t-test, with P<0.05 defined as statistically significant.

Results

CK inhibits the cell viabilities in a dose- and time-dependent manner

As shown in Figure 1A, the MTT viability assay demonstrated that CK led to a dose-dependent inhibition of cell growth in Hep-G2 cells. At the concentrations ranging from 2.5 to 20 µmol/l, CK decreased the cell viabilities of Hep-G2 cells by 19.60%, 46.54%, 54.30% and 63.81% respectively. To discern the direct connection between the inhibition in cell viabilities and the induction of cell proliferation, we followed the course of proliferation over three days after the addition of CK. The MTT data showed that CK inhibited the cell proliferation in a dose- and time-dependent manner in Hep-G2 cells (Figure 1B).

Cell morphological assessment following treatment with CK

The morphological changes due to apoptosis occurring in the nuclei of the cells were observed under a fluorescence microscope. The untreated Hep-G2 cells exhibited a pale blue fluorescence, demonstrating an even pattern of distribution of the chromatin in the nucleolus (Figure 1C), whereas those treated with CK were shown to manifest brighter, granular, blue fluorescence and more apoptotic bodies (Figure 1D).

CK treatment lead to G0/G1 phase arrest in the Hep-G2 cells

Treatment with increasing concentrations of CK (5 and 10 µmol/l ) for 48 h resulted in an increase in Hep-G2 cells in the G0/G1 population, to 68.61±2.91% and 78.29±2.57%, respectively, compared with 50.40±1.49% in the control group (Figure 2). Besides, the number of Hep-G2 cells in the S and G2/M phases correspondingly decreased. Therefore, these results indicated that CK induced a G0/G1 cell-cycle arrest in a dose-dependent manner.

CK induces apoptosis of Hep-G2 cells in vitro

To detect the effect of CK on the cell apoptosis, Annexin-V/PI staining technique was carried out after cells were treated with CK. The per-
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The percentage of apoptotic cells was determined by cell flow cytometric analysis following PI staining. Compared to the control group, CK treatment leads to a moderate induction of apoptosis in Hep-G2 (1.20% untreated cells, 19.0% at 2.5 µmol/l group, 31.9% at 5 µmol/l group and 49.1% at 10 µmol/l group. A significant difference was observed between them (P<0.05, Figure 3). These data provided evidence that CK induced the Hep-G2 cells apoptosis in a dose-dependent manner.

The effect of CK on the expression of cell cycle proteins

Western-blotting assay showed that the expression of CDK4 and its downstream kinase, cyclin D1, in the Hep-G2 cells decreased significantly in a dose-dependent manner. By contrast, a marked reduction in the levels of p21<sup>CIP1</sup> and p27<sup>KIP1</sup> were observed in the Hep-G2 cells following treatment with various concentrations of CK (2.5, 5 and 10 µmol/l) for 48 h (Figure 4).

**CK induces apoptosis mainly through the mitochondria-mediated pathway**

There are two classic apoptotic pathways in human cells, namely death receptor-mediated apoptotic pathway and mitochondria-mediated apoptotic pathway [18]. Previous studies indicate that both pathways cross-talk and are involved in CK-mediated apoptosis of tumor cells [19]. In order to further reveal the apoptotic molecular mechanisms of HCC cells induced by CK, we detected the expression of total proteins of Hep-G2 cells dealt with various concentrations of CK (0, 2.5, 5.0 and 10.0 µmol/l) by using Western blotting assay. Following treatment for 48 h, We found that CK...
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Figure 3. CK induces cell apoptosis in Hep-G2 cells. Hep-G2 cells were treated with CK at different concentrations for 48 h and stained with Annexin V-propidium iodide. The percentage of apoptotic cells was measured by FACS can analysis. Untreated cells were used as controls. PI, propidium iodide. *P<0.05 vs. control group.

Figure 4. The effect of CK on cell-cycle regulatory proteins in Hep-G2 cells. Cells were treated with different concentrations of CK (0, 2.5, 5 and 10 µmol/l) for 48 h. The total protein was extracted and the expression of cell cycle-related proteins was analysed by western blot assay. Data are reported as mean ± S.D. of three separate experiments. A. The expression of cyclin D1, CDK4 were assessed by western blotting; B. The bar chart of cyclin D1/β-actin, CDK4/β-actin. C. The expression of p27Kip1, p21Cip1 were assessed by western blotting; D. The bar chart of p27Kip1/β-actin, p21Cip1/β-actin. β-actin was used as a protein loading control. CDK4, cyclin-dependent kinase 4. */#P<0.05 vs. control group; **/##P<0.01 vs. control group.

increased the expression levels of the proapoptotic proteins cleaved-caspase-9, cleaved-caspase-3 and Bax in the Hep-G2 cells, whereas it also lead to a reduction of the antiapoptotic
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There was significant difference between them (P<0.05). However, the expression of Fas and Fas-L were not changed evidently (P>0.05, Figure 5). Furthermore, the concentration at which CK inhibited the expression of the apoptosis-associated proteins was similar to that at which the cell growth was suppressed and apoptosis was induced. According to the above results, We demonstrate that CK could induce apoptosis mainly through the mitochondria-mediated internal pathway in Hep-G2 cells.


tumors from the nude mice treated with CK (10.0 mg/kg) or normal saline were assessed. As shown in Figure 5, the tumor proliferation was obviously inhibited in CK-treated groups. Compared with the control group, the tumor xenograft treated with CK (10 mg/kg) was significantly decreased in size. Correspondingly, the average tumor weight in CK-treated mice was also decreased by 49.4% (P<0.01) compared with that in the control mice (Figure 6). Throughout this study, CK had no major side effects on the mice and did not affect their weight (data not shown). All animals remained

Figure 5. The effect of CK on apoptosis-associated proteins in Hep-G2 cells. Apoptosis-associated proteins in Hep-G2 cells following treatment with CK for 48 h, as determined by western blot analysis. Data are reported as mean ± S.D. of three separate experiments. A. The expression of apoptosis-associated proteins were assessed by western blotting; B. The vertical axis represents the ratio of various apoptosis proteins to β-actin, β-actin was used as a protein loading control. Cyt c, Cytochrome c; Cas-3, Caspase-3; Cas-9, Caspase-9; Bcl, B-cell lymphoma; PARP, poly(ADP-ribose) polymerase. * and ** indicate P<0.05 and P<0.01 compared with control group, respectively.
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Discussion

An imbalance between cell proliferation and apoptosis may result in tumorigenesis, and one
taken, following treatment with CK for 48 h. In the present study, the effects of CK in inhibiting the growth of Hep-G2 cells and inducing cell apoptosis were detected via MTT assays, cell morphological studies and annexin V-PI double-staining assays, respectively. Then we sought to confirm the possible involvement of signaling pathways including death receptor-mediated apoptotic pathway and mitochondria-mediated apoptotic pathway. Our results indicate that CK inhibits the Hep-G2 cell growth in a dose- and time-dependent manner. Besides, the apoptotic morphology of the Hep-G2 cells treated with 10.0 µmol/l CK for 48 h was observed, the CK-treated cells were shown to manifest brighter, granular, blue fluorescence and more apoptotic bodies compared with those cells with pale blue fluorescence in the control group (Figure 1). In order to determine whether CK induces cell cycle arrest and cell apoptosis in Hep-G2 cells, proliferating Hep-G2 cells were treated with CK for 48 h at different concentrations. As shown in Figure 2, an increasing number of cells accumulated in the G0/G1 phase. In parallel, a reduced percentage of cells was observed in the S and G2/M phases. However, Hu's group have reported that CK induced cell cycle arrest in the G2 phase in human gastric cancer cells [19], which indicates that CK might induce different modes of cell cycle arrest in different cell lines. It is well documented that cell cycle progression is partly controlled by a family of protein kinase complexes in tumor cells, including CDKs and their downstream kinase, the cyclins [21-23]. For example, during the G0/G1 phase progression, cyclin D1 binds to CDK4 or CDK6, promoting the formation of the cyclin D1/CDKs complex, eventually leading to the G1-S phase transition [24]. Our present studies reported that treatment of the Hep-G2 cells with CK evidently decreased the expression of CDK4 and its downstream kinase, cyclin D1, in a dose-dependent manner. Besides, the over-expression of p27kip1 was confirmed to prevent the activation of CDKs and entry into the S-transition phase [25], and p21cip1 was revealed to exert a pivotal role in modulating the G1-S and G2 checkpoints [26, 27]. The present study revealed that a obviously increase in the protein expression levels of p27kip1 and p21cip1 in the Hep-G2 cells was observed following treatment with CK for 48 h. Taken together, these results indicated that CK induced G0/G1 phase arrest in the Hep-G2 cells by regulating several key proteins. Furthermore, an Annexin V-fluorescein isothiocyanate labeling assay found that CK could increase the percentage of cells positive for Annexin V. These results suggest that compound K induces apoptosis in Hep-G2 cells. Consistent with our studies, Chae's group [28] reported that pretreatment of NCI-H460 cells with CK enhanced γ-ray radiation-induced cell apoptosis, suggesting that CK possesses anticancer effect in lung cancer. As we all known, the apoptotic process is tightly regulated by a fine-tuned balance between proapoptotic and antiapoptotic genes. The expression levels of apoptosis related proteins, including caspases-3 and -9, Bax, Bcl-2 and PARP, were assessed to determine the possible mechanism of CK-induced apoptosis. In the present study, we found that compound K could not change the expression level of Fas and FasL. These results indicated that Fas-mediated extrinsic pathway may not be involved in the compound K-induced apoptosis in Hep-G2 cells. On the contrary, Zheng [29] suggested that compound K significantly inhibited cell proliferation and induces apoptosis in MHCC97-H cells through Fas-mediated caspase-dependent pathways in human HCC cells. In the intrinsic pathway, mitochondria play a central role in the commitment of cells to chemical-induced apoptosis [30]. Cytochrome c normally locates in the mitochondrial intermembrane space, where it serves as a transducer of electrons in the respiratory chain. CK-treatment lead to the release of cytochrome c into the cytosol. After release from mitochondria, cytochrome c binds to apoptosis protease activating factor 1, which activates caspase-3 and caspase-9. Then, caspase-3 and caspase-9 act together to activate the death signal and finally lead to a immediate procedure to apoptosis by DNA damnification [31]. Our results showed that CK upregulated Bax, caspase-9, caspase-3, and downregulated PARP, Bcl-2 in a dose-dependent manner. Therefore, we demonstrate that modulation of the mitochondria-mediated caspase-dependent pathway may be an important mechanism underlying the biological effects of CK. Different from our results, KIM et al [32], demonstrated that CK induced cell apoptosis via activations of CAMK-IV (Ca2+/calmodulin-activated protein kinase-IV) and AMPK signal transduction path-
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ways. These data indicate that the antitumor mechanism of CK may be diverse because of different types of tumors. Given our data showing the inhibitory effects of CK on cell proliferation and cell signaling pathway in Hep-G2 cells, we examined whether or not this panaxoside could exert anticancer effects in nude mice. In vivo studies, we found that CK treatment (10 mg/kg/mouse) could affect the tumor growth markedly in nude mice. The above data are of particular important because it is the first time that CK has been revealed to exert anti-cancer effect in a HCC xenograft model. Consist with our research, Hu et al also indicated that CK obviously inhibited the tumor formation of gastric carcinoma cells in nude mice [19].

In conclusion, by using several in vitro and in vivo studies, We demonstrated that CK, a ginseng saponin metabolite, could significantly induced Hep-G2 cells apoptosis via mitochondria-mediated caspase-dependent pathway. The elucidation of the mechanism of CK-induced cell apoptosis suggests that CK might be a promising experimental cancer chemotherapeutic and chemopreventive agent for human HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jing Zhao, Department of Gastrointestinal Surgery, Provincial Hospital of Shandong University, 324 Jingwuweiqi Road, Jinan 250021, Shandong, China. E-mail: laohl2010@sohu.com

References


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