Compound K-induced apoptosis of human hepatocellular carcinoma MHCC97-H cells in vitro

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Abstract. An intestinal bacterial metabolite of ginseng protopanaxadiol saponin, 20-O-(\(\beta\)-D-glucopyranosyl)-20(S)-protopanaxadiol (compound K), has been reported to induce apoptosis in a variety of cancer cells. However, the precise mechanisms induced by compound K in human hepatocellular carcinoma (HCC) cells remain unclear. In order to examine possible apoptotic mechanisms, we investigated the anticancer effect of compound K in MHCC97-H. MTT assay showed that compound K inhibited the proliferation of MHCC97-H cells with a relatively low toxicity in normal hepatoma cells. Cell cycle progression and cell staining showed an increase in apoptotic sub-G1 fraction. Treatment of MHCC97-H with compound K also induced a reduction in mitochondrial membrane potential (\(\Delta\psi\)m) and DNA damage. Further study showed that compound K upregulated Fas, FasL, Bax/Bcl-2 ratio and downregulated pro-caspase-9, pro-caspase-3 in a dose-dependent manner, and it also inhibited Akt phosphorylation. These results suggest that compound K significantly inhibits cell proliferation and induces apoptosis in MHCC97-H cells through Fas- and mitochondria-mediated caspase-dependent pathways in human HCC cells.

Introduction

Human hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world (1), and has a marked increase in younger-aged groups (2). The occurrence of tumor is induced by the imbalance between cell proliferation and apoptosis. Apoptosis is a fundamental cellular event during development and is critical for the cytotoxicity induced by anticancer drugs (3); it can be initiated by extracellular and intracellular signals that trigger a complex mechanism of pro-apoptotic proteases and mitochondrial changes and is the integration of multiple survival and death signals that determine whether a cell is to survive or undergo apoptosis. Previously, it was reported that compound K could induce apoptosis in cancer cell lines (4,5). However, the detailed molecular mechanism and crosstalk between these apoptosis-related signals remains largely unknown.

Ginseng radix, the root of Panax ginseng C.A. Meyer, is frequently used as a traditional medicine in Asian countries. It is used worldwide for preventive and therapeutic purposes (6). In recent years, ginsenosides, extracted from ginseng radix, have become a research hotspot for their wide range of biological and pharmacological activities (7). However, ginsenosides are considered a prodrug, and are rarely absorbed into the blood from the gastrointestinal tract (8). The really active components is 20-O-(\(\beta\)-D-glucopyranosyl)-20(S)-protopanaxadiol (compound K or M1; Fig. 1).

Compound K is a novel ginseng saponin metabolite, formed from ginsenosides Rb1, Rb2 and Rc by the human intestinal bacteria deglycosylation (9,10). It has been identified and purified after giving ginseng extract in humans and rats, and detected as one of the major metabolites after oral administration (11,12). Thus, compound K was speculated to be the major form of protopanaxadiol saponin absorbed by the intestine. Moreover, compound K has previously been found to possess chemopreventive and chemotherapeutic potential, including cardiac protection (13), antitumor and anticlastogenic activity induced by benzopyrene (16), and antitumor activity in cisplatin-resistant pulmonary adenocarcinoma cells (17) and reversing multidrug resistance in tumor cells (18).

In the present study, we specifically selected MHCC97-H cell line as the experiment model, as it is a highly metastatic HCC cell line, and this may provide more insight into some cases than normal HCC. The main focus of this study was to test the hypothesis that compound K inhibits the growth of
MHCC97-H cells through induction of apoptosis and possible apoptosis signal pathways.

Materials and methods

Materials. Compound K (purity >98%) was prepared and identified as previously described (19). HCC cell line MHCC97-H and normal human hepatocyte cell line chang-liver were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Propidium iodide (PI) was obtained from Bio Basic Canada Inc. (Toronto, Canada). Bisbenzimide (Hoechst 33258), acridine orange (AO), ethidium bromide (EB), methyl thiazolyl tetrazolium (MTT) and monoclonal mouse anti-β-actin antibody were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Mitochondrial membrane potential (Δψm) detection kit was purchased from Nanjing KeyGen Biotech, Co., Ltd. (Nanjing, China). Antibodies against procaspase-9, pro-caspase-3, cleaved-caspase-8, Fas, FasL, p-Akt, Akt, Bax, Bcl-2, horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and treatment. MHCC97-H cell line was cultured in Dulbecco’s modified Eagle's medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C in a water-saturated atmosphere of 5% CO2 in air. Compound K was prepared in dimethyl sulphoxide (DMSO) at a concentration of 50 mM as a stock solution. Prior to use, the stock solution was diluted to the required concentration immediately. Control cells were treated with equivalent amount of DMSO without compound K.

Cell viability assay. Cell viability was examined by MTT assay. Briefly, cells were plated in 96-well plates at a density of 5x10^3 cells/well in a 200 µl medium, and then treated with compound K or DMSO for a predetermined period. Then, 200 µl of MTT (0.5 mg/ml) was added to each well and the plates were incubated for an additional 4 h at 37°C. The medium was replaced with DMSO to dissolve the formazan produced from MTT by viable cells. The optical absorbance at 570 nm was proportional to the percentage of cell viability. Fifty percent inhibitory concentration value (IC50) was calculated using Log-Probit regression analysis in SPSS 13.0.

Morphological study with fluorescence microscope. Exponential growth phase MHCC97-H cells seeded in 6-well plates were treated with 50 µM compound K or 0.1% DMSO for 48 h, harvested with 0.25% trypsin and resuspended in DMEM medium. Cells (1x10^6) were washed and resuspended in PBS, followed by observation of the morphological changes. In addition, some cells were incubated with 10 µg/ml Hoechst 33258 for 10 min in the dark. Furthermore, 25 µl of cell suspension was mixed with 1 µl of dye mixture containing 100 µg/ml AO and EB in PBS. Thereafter, cell morphology was visualized immediately under a fluorescence microscope (Leica DM IRB).

Cell cycle analysis. Cells were treated with compound K for 24 h and were harvested and suspended in 1:1 (v/v) mixture of PBS and 0.2 M Na2HPO4-0.1 M citric acid (pH 7.5). Then, cells were fixed with 100% ice-cooled ethanol at 4°C for 4 h, centrifuged at 800 x g for 5 min and washed twice, resuspended with PBS (1x10^6 cells/ml). Subsequently, 1x10^6 cells were stained with 10 µg/ml PI containing 100 µg/ml DNase-free RNase A in the dark for 1 h at 37°C. Then 20,000 cells were measured using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data were analyzed with ModFit LT 3.2 software (Becton-Dickinson).

Mitochondrial membrane potential assay. Cells treated with 50 µM compound K for 48 h and control cells were incubated with 5,5',6,6'-tetrachloro-1',3',3'-tetraethyl-benzimidazolocarbocyanine iodide (JC-1) (0.1 µg/ml) for 15 min. After washing with PBS, the cells were suspended with PBS and observed with fluorescence microscope as previously described (20). The green fluorescence from JC-1 monomer and the red fluorescence from the aggregated form of JC-1 were visualized.

Single-cell gel electrophoresis (SCGE). The comet assay was performed as previously described (21,22). Briefly, cells were suspended in 1% (w/v) low-melting point agarose and pipetted on to slides which were precoated with a layer of 1% (w/v) normal melting-point agarose (warmed to 37°C before use). The agarose was allowed to set at 4°C for 10 min, and the slides were then immersed for 1 h at 4°C in a lysis solution. Slides were placed in single rows in a 30 cm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA, pH 13.0 (unwinding solution) and kept at 4°C for 40 min. Electrophoresis was performed for 30 min in unwinding solution at 30 V (1 V/cm) and 300 mA. Finally, the slides were washed 5 min for 3 times in 0.4 M Tris (pH 7.5, 4°C) and stained with EB.

Western blot analysis. Cells were washed and suspended in lysis buffer on ice for 10 min. Lysates were cleared by centrifugation at 12,000 x g for 10 min at 4°C. The total protein, as determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), was mixed with 4X loading buffer and pre-heated. Equal amounts of cell extracts were resolved by SDS-PAGE, and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked and incubated 1 h with appropriate primary antibody. Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody.
Final detection was performed with ECL™ western blotting reagents (Amersham, Livingston, NJ, USA).

**Statistical analysis.** Data are provided as the mean ± standard deviation and intergroup differences were analyzed using the Student's t-test. Statistical analysis was conducted by SPSS 13.0.

**Results**

**Compound K inhibits MHCC97-H cell proliferation.** To evaluate the cytotoxicity of compound K by MTT assay, MHCC97-H cells were treated with increasing concentrations of compound K for 6, 12, 24 and 48 h. As shown in Fig. 2A, cell viability was significantly reduced from 25-100 µM. In the same concentration, cell inhibitory rate is proportioned to the exposure time. Thus, compound K exhibited a dose and time-dependent decrease in MHCC97-H proliferation. In contrast, normal hepatocyte chang-liver showed relatively strong resistance to compound K, with 50% inhibitory concentration (IC$_{50}$) of 71.3±3.7 µM, whereas the IC$_{50}$ against MHCC97-H was 49.8±2.5 µM for 48 h.

**Morphological changes of MHCC97-H cells after exposure to compound K.** In order to verify compound K-induced inhibition, we examined the changes of cell morphology after compound K exposure. As shown in Fig. 3A, cells treated with 50 µM compound K for 48 h showed the typical appearance of apoptotic cells, such as nuclear fragmentation, cell shrinkage and apoptotic bodies under the phase-contrast microscope. In addition, with Hoechst 33258 staining, the blue emission light in apoptotic cells was much brighter than in the control cells (Fig. 3B). By comparison, the control cells were less bright blue and more homogeneous. With AO/EB staining, different cells (alive, apoptotic or necrotic) were clearly differentiated by different colors (Fig. 3C). These results showed that the compound K-treated cells exhibited morphological changes indicating apoptosis, including chromatin condensation and nuclear fragmentation.

**Effect of compound K on the proportion of apoptotic MHCC97-H cells.** To determine the effect of compound K on the proportion of MHCC97-H cells, apoptosis and cell cycle distribution were evaluated by flow cytometer. As shown in Fig. 4, MHCC97-H cells were treated with compound K at the concentrations of 25 µM (Fig. 4B), 50 µM (Fig. 4C), and 75 µM (Fig. 4D) for 24 h, and then analyzed for cell cycle progression by flow cytometer. Sub-G1 fraction was detected from 13.92±0.14 to 45.61±7.78% in MHCC97-H cells, while only 0.62±2.12% in the control group (P<0.05; Fig. 4A and E). As shown in Fig. 4E, compound K induced cell cycle arrest at the G0/G1 phase in MHCC97-H cells as well as a progressive
decline of S phase. These results revealed that compound K induced apoptotic cell death concurrent with cell cycle arrest in MHCC97-H cells.

**Disruption of Δψm by compound K.** To investigate the loss of Δψm during apoptosis induced by compound K, cells were stained with JC-1 and monitored with a fluorescence microscope. JC-1 forms monomer and emits green fluorescence when Δψm is depolarized (common in apoptosis), while JC-1 aggregates and emits red fluorescence at a highly polarized Δψm. As shown in Fig. 5, JC-1 was accumulated in intact cells where it displayed red fluorescence indicating a high potential. In contrast, JC-1 was poorly accumulated in compound K-treated cells, which displayed only green or weak red fluorescence, indicating low membrane potential. These results strongly support the hypothesis that, after exposure to compound K, an initial interaction with redox-active iron takes place in the mitochondrial membrane compartments, resulting in destabilization of their membranes and Δψm is disrupted.

**Tail-DNA is induced by compound K in SCGE.** SCGE was employed to investigate the DNA damage induced by compound K in MHCC97-H cells. As shown in Fig. 6A, the comets resulting from exposure to compound K differed from control. Relatively undamaged cells gave comets consisting of a compact head without tail, indicating double-stranded DNA, while the comets originating from cells exposed to compound K (lower panel) had a distinct head with a tail, indicating the induction of DNA damage by compound K.

As compared with appropriate control, the mean tail-DNA is presented in Fig. 6B. Compound K evoked more increase in tail-DNA at concentrations as compared with the control. The increase of tail-DNA is positively proportioned to the concentration of compound K. These results indicate that DNA induced by compound K is closely associated with apoptosis.

**Effect of compound K on the expression of apoptosis-related proteins.** Apoptosis is characterized by a well-organized sequence of cellular events, resulting in the activation of the
Fas/FasL and caspases cascade. In the group of apoptosis-related proteins, Fas/FasL, PI3K/Akt, caspase-8 and Bax/Bcl-2 are very important molecules for their regulatory role during apoptosis. We examined these proteins in MHCC97-H with compound K for 48 h.

As shown in Fig. 7, compound K not only significantly upregulated the expression of Fas/FasL and cleaved-caspase-8, but also decreased downstream proteins pro-caspase-9, pro-caspase-3 in a dose-dependent manner. In addition, compound K gradually inhibited Akt phosphorylation and increased Bax/Bcl-2 ratio. These results indicated that compound K-induced apoptosis may occur through Fas- and mitochondria-mediated caspase-dependent pathways.

Discussion

Apoptosis is required for proper tissue homeostasis. Defects in apoptosis signaling pathways contribute to carcinogenesis. Previous studies demonstrated that compound K may be the active metabolite responsible for the anticarcinogenic effects of ginseng saponin. This prompted us to investigate the effects of compound K in more detail. Although previous studies have reported that compound K exhibits a broad range of impor-
Our previous study revealed that compound K can induce apoptosis in HCC (25). In the present study, we sought to demonstrate the mechanisms of apoptosis induced by compound K. We found that compound K could inhibit the cell proliferation of MHCC97-H in a dose- and time-dependent manner with a relatively low cytotoxicity to normal hepatocytes, and increased sub-G1 phase in MHCC97-H cells. In addition, both phase contrast microscopy and fluorescence staining showed the typical appearance of apoptosis in compound K-treated cells. The results of SCGE showed the DNA damage in compound K is positively correlated with the drug concentration. These results suggest that compound K induces apoptosis in MHCC97-H cells. Moreover, the western blot analysis and Δψm of mitochondrial membrane results revealed apoptosis induced by compound K is through the caspase-dependent extrinsic and intrinsic pathways (26).

In the extrinsic pathway, Fas and FasL are widely recognized as key regulators of the apoptosis signal transduction pathway. Fas is a type I transmembrane receptor protein and Fas ligand is a type II transmembrane protein. The ligation of Fas and FasL results in receptor trimerization followed by the binding of the adaptor molecule, Fas-associated death domain (FADD) to the cytoplasmic domain of the receptor (27,28). Then, FADD activates caspase-8, which triggers the caspase cascade through biding and cytochrome c (29). Finally, caspase-3 and several other effector pro-caspases were considerably activated (30) and caused DNA damage. In the present study, we found that compound K can upregulate the expression level of Fas and FasL in a dose-dependent manner. These results indicated that Fas-mediated caspase-dependent pathway is involved in the compound K-induced apoptosis in MHCC97-H cells.

In the intrinsic pathway, mitochondria play a central role in the commitment of cells to chemical-induced apoptosis (31). Cytochrome c normally resides in the mitochondrial intermembrane space, where it serves as a transducer of electrons in the respiratory chain. Compound K can disrupt the Δψm of mitochondrial membrane, leading to the release of cytochrome c into the cytosol (32). After release from mitochondria, cytochrome c binds to apoptosis protease activating factor 1 (Apaf-1), which activates caspase-9 and downstream caspase-3. Then, caspase-9 and caspase-3 act together to destroy the death signal and finally lead to a unilaterial process to apoptosis (33,34) by DNA damage. In our experiments, we found that compound K induced the loss of Δψm, downregulated pro-caspase-9 and pro-caspase-3, in a dose-dependent manner. These data indicated that the mitochondria-mediated caspase-dependent signal pathway is involved in the compound K-induced apoptosis in MHCC97-H cells.

The Akt kinase regulates the balance between survival and apoptosis factors. It is activated by binding with phospholipids. Activated Akt (p-Akt) promotes cell survival by inhibiting apoptosis through phospho-inactivation of several targets, including Bad and caspase-9 (35). In the present study, we found that phosphorylation of Akt at Ser 473 was suppressed by compound K (Fig. 8A). The inhibition of Akt is likely to be involved in compound K-mediated growth inhibition of MHCC97-H cells. In addition, compound K promoted pro-apoptotic protein Bax expression, and decreased anti-apoptotic protein Bcl-2 expression in MHCC97-H cells, leading to a decrease of Bax/Bcl-2 ratio. The ratio between pro- and anti-apoptotic proteins determines the susceptibility of cells to an apoptotic death signal (36). Thus, p-Akt, Bax and Bcl-2 were also involved in compound K-induced apoptosis in MHCC97-H cells.

Taken together, the present study demonstrated that compound K, a ginseng saponin metabolite, significantly inhibited cell proliferation and induced apoptosis in MHCC97-H via Fas- and mitochondria-mediated caspase-dependent pathways (Fig. 8). The relatively low toxicity in normal hepatocytes and high activity in HCC MHCC97-H cells suggest that compound K might be a promising experimental cancer chemotherapeutic and chemopreventive agent for human HCC (37).

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