Cordycepin induces apoptosis in human liver cancer HepG2 cells through extrinsic and intrinsic signaling pathways

LE-WEN SHAO¹, LI-HUA HUANG¹, SHENG YAN², JIAN-DI JIN³ and SHAO-YAN REN³

¹Nursing Department; Departments of ²Hepato-Biliary-Pancreatic Surgery and ³Infectious Disease, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, P.R. China

Received March 6, 2015; Accepted April 12, 2016

DOI: 10.3892/ol.2016.4706

Abstract. Cordycepin, also termed 3’-deoxyadenosine, is a nucleoside analogue from Cordyceps sinensis and has been reported to demonstrate numerous biological and pharmacological properties. Our previous study illustrated that the anti-tumor effect of cordycepin may be associated with apoptosis. In the present study, the apoptotic effect of cordycepin on HepG2 cells was investigated using 4',6-diamidino-2-phenylindole, tetraethylbenzimidazolylcarbocyanine iodide and propidium iodide staining analysis and flow cytometry. The results showed that cordycepin exhibited the ability to inhibit HepG2 cells in a time- and dose-dependent manner when cells produced typical apoptotic morphological changes, including chromatin condensation, the accumulation of sub-G1 cells and change mitochondrial permeability. A potential mechanism for cordycepin-induced apoptosis of human liver cancer HepG2 cells may occur through the extrinsic signaling pathway mediated by the transmembrane Fas-associated with death domain protein. Apoptosis was also associated with Bcl-2 family protein regulation, leading to altered mitochondrial membrane permeability and resulting in the release of cytochrome c into the cytosol. The activation of the caspase cascade is responsible for the execution of apoptosis. In conclusion, cordycepin-induced apoptosis in HepG2 cells involved the extrinsic and intrinsic signaling pathway and was primarily regulated by the Bcl-2 family proteins.

Introduction

Cordyceps militaris is a fungus that parasitizes Lepidoptera larvae and has been extensively used as a folk tonic food and crude drug (1). Cordyceps militaris has long been considered to have natural medicinal properties, such as anti-angiogenic, anti-tumor, anti-viral, anti-inflammatory and hypoglycemic effects (1-8).

Cordycepin, also termed 3’-deoxyadenosine, is a nucleoside analogue from C. militaris (9) and has been reported to demonstrate numerous notable biological and pharmacological properties, including immunological stimulation, anti-cancer and anti-viral effects (10-15), a stimulating effect on interleukin-10 production as an immune modulator (16) and preventing hyperlipidemia (17).

Apoptosis, also termed programmed cell death, is a key regulator of tissue homeostasis and is characterized by typical morphological and biochemical hallmarks, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (18). The mechanisms for foreign chemicals disrupting cell functions and causing tissue damage have been associated with the triggering of apoptosis signal transduction pathways, consisting of the intrinsic (mitochondrial) and extrinsic (death receptor) pathways (19).

Although cordycepin has been shown to have a cytotoxic effect on HepG2 cells, the detailed molecular mechanisms have not been well elucidated (17,19). In the present study, the mechanistic understanding of how cordycepin mediates apoptosis in HepG2 cells was investigated and the intrinsic and extrinsic apoptosis pathways were the main focus.

Materials and methods

Materials. Dried Cordyceps militaris was purchased from Cordyceps Garden Biotechnology Company (Fuzhou, China). The macroporous adsorption resin NKA-II was purchased from the Chemical Plant of Nankai University (Tianjin, China). Sulforhodamine B (SRB), 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were purchased from Sangon Biotech Co., Ltd. (Shanghai, China): Mouse monoclonal anti-Fas (1:3,000; catalog no. D198888); rabbit polyclonal anti-Fas ligand (1:3,000; catalog no. D262701); mouse monoclonal anti-Fas associated with death domain protein (FADD; 1:3,000; catalog no. D199671); rabbit polyclonal anti-caspase-8 (1:2,000; catalog no. D155240); rabbit polyclonal anti-caspase-9 (1:2,000; catalog no. D220078); rabbit polyclonal anti-caspase-10 (1:2,000; catalog no. D230078); rabbit polyclonal anti-activated caspase-3 (1:3,000; catalog no. D199671); rabbit polyclonal anti-activated caspase-7 (1:3,000; catalog no. D155240); mouse monoclonal anti-Fas associated with death domain protein (FADD; 1:3,000; catalog no. D199671); rabbit polyclonal anti-caspase-8 (1:2,000; catalog no. D155240); rabbit polyclonal anti-caspase-9 (1:2,000; catalog no. D220078); rabbit polyclonal anti-caspase-10 (1:2,000; catalog no. D230078).
Cell lines and culture. HepG2 cell lines were purchased from the Culture Center of the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences (Beijing, China). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich), and cultured at 37°C in 48 h in a humidified atmosphere consisting of 95% air and 5% CO₂.

Isolation and purification of cordycepin. A total of 150 g dried fruiting bodies of C. militaris were cleaned with distilled water, dried in a dark and ventilated place and crushed prior to soaking in 500 ml distilled water overnight at room temperature, and then boiled in distilled water for 3 h. Subsequent to filtration to remove debris, the filtrate (crude aqueous extract) was concentrated in a rotary evaporator (model no. 2690/2695; Waters Technologies, MA, USA) set at 254 nm, connected to a Cs420 Hardware (model no. 2690/2695; Waters Technologies, MA, USA). In addition, subsequent to being treated with various concentrations of cordycepin (0, 125, 250 and 500 µM) at 37°C for 48 h, the cells were collected and washed with phosphate-buffered saline (PBS). Cell pellets were fixed with 4% paraformaldehyde for 10 min and washed three times with PBS. The cells were then incubated with 5 µg/ml DAPI for 20 min. Subsequent to washing with PBS, the cells were observed under a fluorescence microscope (model no. Bx51; Olympus Corporation, Tokyo, Japan) and images were captured.

Assessment of apoptosis by flow cytometry. HepG2 cells cultured with or without cordycepin at 37°C for 48 h were harvested, washed with PBS and fixed with 70% cold ethanol at 4°C for 4 h. The fixed cells were washed and stained with a PI solution containing 20 µg/ml PI and 10 mg/ml RNase (Takara Biotech, Inc., Dalian, China) in PBS for 20 min in the dark. The stained cells were then analyzed by flow cytometry using fluorescence-activated cell sorting (FACS) and the FACScalibur system (BD Biosciences, Franklin Lakes, NJ, USA). In addition, subsequent to being treated with various concentrations of cordycepin (0, 125, 250 and 500 µM) for 48 h, the harvested HepG2 cells were washed in PBS and incubated in a freshly prepared JC-1 solution at 37°C for 20 min. Cell-associated fluorescence was also measured by FACS.

Western blot analysis. HepG2 cells were treated for 48 h with various concentrations of cordycepin (0, 125, 250 and 500 µM) and harvested and lysed in RIPA lysis buffer that contained 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. The cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane, and then blocked for 1 h with 5% fat-free milk in PBS with Tween-20 (PBST) at room temperature. The membrane was immunoblotted overnight with antibodies in PBST at 4°C. Subsequent to being washed with PBST, the membrane was detected by enhanced chemiluminescence (Kodak, Rochester, NY, USA). Equal loading was confirmed by probing with an antibody against β-actin (1:3000; catalog no. sc-8432; Santa Cruz Biotechnology, Inc.). Mitochondrial and cytosolic fractions were prepared in order to detect cytochrome c. Following treatment with the indicated concentrations of cordycepin for 48 h, HepG2 cells were harvested and disposed using the Cell Mitochondria Isolation kit, according to the manufacturer’s protocol. Finally, the mitochondrial pellet and cytosolic

D260010; rabbit monoclonal anti-caspase-3 (1:3000; catalog no. D120074); mouse monoclonal anti-B-cell lymphoma-2 (Bcl-2; 1:3000; catalog no. D19B682); rabbit polyclonal anti-Bcl-2-associated X protein (Bax; 1:3000; catalog no. D120073); mouse monoclonal anti-BiH3 interacting domain death agonist (Bid; 1:3000; catalog no. D19B911); and rabbit polyclonal anti-cytochrome c (1:2000; catalog no. D110006). Rabbit polyclonal anti-mouse IgG (1:3000; catalog no. sc-358920) and goat anti-rabbit IgG (1:3000; catalog no. sc-2768) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The Mitochondrial Membrane Potential Assay kit with JC-1, radioimmunoprecipitation assay (RIPA) lysis buffer and Cell Mitochondria Isolation kit were purchased from Beyotime Institute of Biotechnology (Haimen, China).

Antiproliferative activity assay of cordycepin. Various concentrations of cordycepin (0, 125, 250 and 500 µM) were inoculated into 96-well microplates when the cell concentration was adjusted to 1x10⁴ cells/ml. Cultures in triplicate were treated with cordycepin for 48 h and the control wells received only maintenance medium (DMEM). Cellular responses were colorimetrically evaluated by an SRB assay. Briefly, the cells were fixed with 25% trichloroacetic acid (Sigma-Aldrich) and washed and stained with 0.4% SRB. Subsequent to the cell-bound SRB being solubilized by the addition of 10 mM Tris-HCl, bound SRB was colorimetrically assessed using an ELISA microplate reader (MK3; Thermo Fisher Scientific, Inc.) at 490 nm. Cell growth inhibition was expressed as a percentage of the untreated control absorbance following the subtraction of the mean background absorbance. Compounds were considered to have potent growth inhibitory activity when the reduction in SRB absorbance was >25% compared with the untreated control cells. The half maximal inhibitory concentration (IC₅₀) values were calculated from the dose-response curves.

Assessment of apoptosis by DAPI staining. HepG2 cells were seeded into a 50 ml culture flask at a density of 1x10⁴ cells/ml for 24 h. Subsequent to treatment with or without cordycepin (0, 125, 250 and 500 µM) at 37°C for 48 h, the cells were collected and washed with phosphate-buffered saline (PBS). Cell pellets were fixed with 4% paraformaldehyde for 10 min and washed three times with PBS. The cells were then incubated with 5 µg/ml DAPI for 20 min. Subsequent to washing with PBS, the cells were observed under a fluorescence microscope (model no. Bx51; Olympus Corporation, Tokyo, Japan) and images were captured.
supernatant were separated using a centrifuge (model no. 5415C; Eppendorf, Hamburg, Germany).

Results

Isolation and purification of cordycepin. Two fractions from crude extract, termed A and B, were eluted using NKA macro-porous resin (data not shown). Fraction A was then purified on a HPLC preparative column followed by an analytical column using CH$_3$OH:H$_2$O = 15:85 (v/v) as the mobile phase. The retention time of fraction A was ~10.5 min, which is consistent with that of standard cordycepin (Fig. 1A). In addition, mass spectrometric analysis revealed that the m/z of fraction A was 252.1096 Da, which corresponds with the m/z of standard cordycepin (Fig. 1B).

Effect of cordycepin on HepG2 cell viability. Cordycepin exhibited the ability to inhibit the proliferation of HepG2 cells in a time- and dose-dependent manner (Fig. 2). The IC$_{50}$ values for HepG2 cells treated with cordycepin for 24, 48, 72 and 96 h were 735±3.67, 497.5±2.49, 385±1.93 and 307.5±1.54 µM, respectively.

Assessment of apoptosis by DAPI staining. The fluorescence microscopic examination with DAPI staining revealed that morphological changes, such as chromatin condensation, nuclear shrinkage or fragmentation and apoptotic body formation, occurred in HepG2 cells subsequent to being treated with cordycepin for 48 h (Fig. 3A).

Assessment of apoptosis by flow cytometry. The flow cytometric analysis with PI staining revealed that the cells treated with various concentrations of cordycepin for 48 h exhibited an apoptotic hypodiploid sub-G1 peak in a concentration-dependent manner (Fig. 3B). The flow cytometric analysis with JC-1 staining showed the respective green fluorescence intensity ratios, which indicated the apoptotic HepG2 cells, were 5.72, 18.58, 32.12 and 36.65%, respectively (Fig. 4).

Cordycepin-induced apoptosis signaling pathway. Cordycepin increased the expression levels of Fas and FADD, but had no effect on FasL expression. Additionally, the level of procaspase-8 decreased, while the level of cleaved caspase-8 was elevated (Fig. 5A). The expression of the Bcl-2 family proteins was also detected and the results showed that cordycepin enhanced the expression level of truncated Bid (tBid) and decreased the level of Bid, but had little effect on the levels of Bax and Bcl-2 (Fig. 5B). A decreased level of cytochrome c was detected in the mitochondrial fraction and an increased level was detected in the cytosolic fraction (Fig. 5C). Furthermore, the results also showed that HepG2 cells treated with cordycepin for 48 h underwent significant cleavage of procaspase-9 and procaspase-3 (Fig. 5C).

Discussion

As a natural compound, cordycepin plays a key role in cancer therapy and induces apoptosis in numerous cells by targeting molecules and pathways (20-22). However, studies investigating the induction of apoptosis by cordycepin through the extrinsic and intrinsic signaling pathways in HepG2 cell are currently available (21,22). The present study investigated the effect of cordycepin on HepG2 cells and the molecular pathway by which cordycepin induces apoptosis. Subsequently,
It was demonstrated that cordycepin induced apoptosis by the activation of caspase, interaction between Fas and FADD, and modulation of the protein levels of Bid and tBid. It is well known that mitochondria play a major role in stress-induced cell death pathways, and damage to mitochondria was associated with the release of proapoptotic factors, such as cytochrome c, from the mitochondrial intermembrane space into the cytoplasm. The release of cytochrome c initiates the caspase cascade, leading to the execution of apoptosis.

Cordycepin is known to induce apoptosis in HepG2 cells, and the mechanism involves the activation of caspases, the interaction between Fas and FADD, and the modulation of the protein levels of Bid and tBid. These findings suggest that cordycepin-induced apoptosis in HepG2 cells is mediated through a caspase-dependent pathway involving Fas-FADD and Bid. The release of cytochrome c from mitochondria is a crucial event in this process, as it leads to the activation of caspases and the execution of apoptosis.

**Figure 3.** Analysis of the effect of cordycepin on HepG2 cells by DAPI and PI staining. (A) Effect of cordycepin on HepG2 cells, as determined by DAPI staining. HepG2 cells were fixed and stained with DAPI and the nuclear morphology of the cells was then examined with a fluorescence microscope subsequent to treatment for 48 h with the indicated concentrations of cordycepin. Cytoplasmic shrinkage and nuclear condensation were observed in the cordycepin group, due to the increased fluorescent intensity compared with the control group. (B) Effect of cordycepin on the sub-G1 proportion of HepG2 cells, as determined by PI staining. HepG2 cells were fixed and stained with PI for flow cytometric analysis subsequent to treatment for 48 h with the indicated concentrations of cordycepin. The percentage of cells with a hypodiploid DNA content (sub-G1) represent those fractions undergoing apoptotic DNA fragmentation. DAPI, 4',6-diamidino-2-phenylindole; PI, propidium iodide.

**Figure 4.** Mitochondrial membrane potential, as assessed by JC-1 staining. HepG2 cells were fixed and stained with JC-1 staining for a flow cytometric analysis subsequent to treatment for 48 h at the indicated concentrations of cordycepin.

**Figure 5.** Western blot analysis of the signaling pathway involved in cordycepin-induced apoptosis. (A) Effects of cordycepin on the expression levels of Fas, FasL, FADD and caspase-8. (B) Effects of cordycepin on the expression level of the Bcl-2 family proteins. (C) Effects of cordycepin on the expression level of cyto c, caspase-9 and caspase-3. FasL, Fas ligand; FADD, Fas-associated death domain protein; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; Bid, BH3 interacting domain death agonist; t-Bid, truncated Bid; cyto C, cytochrome c.

**Figure 6.** Overview of the pathways involved in cordycepin-induced apoptosis in HepG2 cells. FADD, Fas-associated death domain protein; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; Bid, BH3 interacting domain death agonist; t-Bid, truncated Bid; cyto C, cytochrome c.
mitochondria with subsequent loss of mitochondrial membrane potential has been known to be the point of no return in apoptotic cascades (20,23). In the present study, it was found that cordycepin had an inhibitory effect on the viability of HepG2 cells by DAPI staining and could increase the fraction of sub-G1 cells by PI staining. The JC-1 assay also revealed that cordycepin-induced collapse of the mitochondrial membrane occurred in a concentration-dependent manner. All these findings demonstrated that cordycepin may induce apoptosis in HepG2 cells.

In mammalian cells, apoptosis occurs via either the extrinsic (receptor-dependent) or the intrinsic (mitochondria-dependent) pathway, with each involving caspase activation. Fas is a TNF-receptor that transduces the apoptotic signal into cells (23,24). FADD is an apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to activated Fas (25). FasL is a cytokine that binds to Fas, and it has been proposed that Fas-mediated apoptosis involves the interaction between Fas and FasL (26). In the present study, cordycepin increased the expression levels of Fas and FADD, without changing the level of FasL, and the expression of cleaved caspase-8 was elevated. Therefore, the present study hypothesizes that cordycepin may stimulate Fas/FADD signaling independently of FasL and subsequently activate caspase-8 in HepG2 cells.

Fas may induce caspase-mediated cleavage of p22 Bid into a major p15, and minor p13 and p11 product; the major proteolytic product p15 tBid allows the release of cytochrome c (27-29). The mitochondrial membrane potential was shown to be decreased by the JC-1 staining analysis and confirmed the increased cytosolic cytochrome c by western blotting. The activation cascade of caspase-9 and caspase-3, which are responsible for apoptosis execution, was detected. In particular, caspase-3 plays a central role in the execution of apoptosis and its activation requires the activation of initiator caspases, such as caspase-8 or caspase-9, in response to pro-apoptotic stimuli (30). Caspase-8 is crucial for triggering apoptosis via the death receptor pathway and its activation requires the binding of death ligands to death receptors (31). In addition, in the prior time course assay, the active forms of caspase-8 was detected notably earlier than that of caspase-3 and -9, which clearly demonstrated that caspase-8 may act as an upstream initiator and the extrinsic pathway was involved in cordycepin-induced apoptosis.

The Bcl-2 family proteins play an essential role in apoptotic progress, such as Bcl-2 protein (anti-apoptosis) and Bax protein (pro-apoptosis). These proteins are regulators of mitochondrial membrane permeability and intermembrane space protein efflux, according to the opposing fractions of the anti-apoptosis members and pro-apoptosis members (32,33). Bax accelerates programmed cell death and undergoes a conformational change that causes translocation to the mitochondrial membrane, leading to the release of cytochrome c, which then triggers apoptosis (34). Cordycepin enhanced the expression level of Bax and decreased the expression level of Bcl in the present study.

Cleaved Bid causes cytochrome c efflux from mitochondria, which in turn leads to the activation of caspase-9 and caspase-3 (35). In addition, other Bcl-2 family molecules, such as the proapoptotic protein Bax and anti-apoptotic protein Bcl-2, are also key regulators of apoptosis, which control the release of mitochondrial cytochrome c by modulating the permeability of the outer mitochondrial membrane (36). The upregulation of the ratio of Bax/Bcl-2 caused by cordycepin was confirmed by immunoblotting. The release of cytochrome c from mitochondria was also relevant to the dissipation of the mitochondrial membrane potential (ΔΨm) (37). In the present study, cordycepin treatment caused a significant decrease of ΔΨm in HepG2 cells and increased cytosolic cytochrome c. These findings indicated that activation of caspase-3 in cordycepin-induced apoptosis may result from direct caspase-8 cleavage and caspase-8-mediated caspase-3 activation through the intrinsic pathway, which is regulated by the Bcl-2 family proteins.

It has been shown that cordycepin is an analogue of adenosine (22). The possibility that the adenosine receptor is involved in the apoptosis of certain types of cancer cells, including ovarian (38), breast (39) and renal (40) cancer cells, has also been proposed. Thus, additional studies investigating whether cordycepin induces apoptosis through adenosine receptors in HepG2 cells may be valuable.

In conclusion, cordycepin-induced apoptosis in HepG2 cells may be initiated by the FADD mediated signal pathway and regulated by the Bcl-2 family proteins that can change the alternation of mitochondrial membrane permeability and cause the mitochondria mediated apoptosis signal pathway (Fig. 6). To the best of our knowledge, the present study is the first to report apoptosis induced by cordycepin in human liver cancer HepG2 cells.

References


