Food and Chemical Toxicology 60 (2013) 439–447

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Anti-cancer effect and apoptosis induction of cordycepin through DR3 pathway in the human colonic cancer cell HT-29

Seung Yuan Lee, Trishna Debnath, Si-Kwan Kim, Beong Ou Lim *

Department of Applied Biochemistry, College of Biomedical & Health Science, Konkuk University, Chungju, Republic of Korea

ARTICLE INFO

Article history:
Received 15 March 2013
Accepted 24 July 2013
Available online 11 August 2013

Keywords:
Apoptosis
Cordycepin
HT-29 cells
DR3

ABSTRACT

Cordycepin is known to have many pharmacological effects such as anti-tumorigenic, anti-inflammatory and anti-angiogenic activity. However, cordycepin induced apoptosis through the DR3 pathway in human colon cancer cells has not been studied. The effect of cordycepin on anti-proliferation was investigated in this study. Cordycepin significantly inhibited cell viability in a dose and time-dependent manner. Cordycepin increased sub G1 and G2/M phase arrest on HT-29 cells at the concentration of 100 μM, whereas cordycepin at 200 μM and 400 μM increased G1 phase arrest. Cordycepin induced apoptosis in HT-29 cells in a dose-dependent manner as detected by Hoechst and Annexin V-FITC staining. Intracellular ROS levels were higher in cordycepin treated cells as compared to control cells. The protein related to apoptosis was determined by antibody array. p53 and Bax expression increased treatment with cordycepin for 18 h. DR3, caspase-8, caspase-1, cleaved caspase-3 and cleaved PARP expression increased. These finding suggest that the cordycepin induces apoptosis through the DR3 pathway in human colon cancer HT-29. These findings suggest that cordycepin should be evaluated further as a therapeutic agent in human colon cancer.

1. Introduction

Colorectal cancer (CRC) is an important public health matter that has gradually increased to be the third most common cancer occurring in both men and women in the western world and in Korea (Hsu et al., 2012). CRC is the most common form of lower gastrointestinal cancer and can be attributed to common diseases influenced by many factors (Hu et al., 2011). Among genetic factors, the adenomatous polyposis coli (APC) gene plays a role in cancer development. Many studies have been working to develop conventional or complementary therapies for treating this cancer, including surgery, chemotherapy, radiation, nutritional therapy and physical rehabilitation. In spite of the many therapy factors, successful treatment has not yet been discovered and interrupted apoptosis occurs with drug resistance of cancer cells, which has become a blocking factor (Douillard et al., 2000). In addition, anti-cancer agents affect cytotoxicity in normal cells. Recently, natural products have been examined to confirm anti-cancer compounds and to explain mechanisms of cancer prevention through apoptosis (Ren et al., 2009).

Many studies have proposed that chemotherapy and irradiation inhibit cancer (target) cells primarily by the induction of apoptosis and arrest (Samowitz and Slattery, 2002). Generally, apoptosis is the process of programmed cell death, the occurrence of which involves many related genes that play key roles as regulators of homeostasis between cell death and proliferation (Debatin and Krammer, 2004; Lin et al., 2007). Apoptosis signaling is sorted according to two pathways: the intrinsic or mitochondria pathway, and extrinsic or death receptor pathway (Reyes-Zurita et al., 2011). Intrinsic pathways include Bcl-2 family members; pro-apoptotic proteins, Bax and anti-apoptotic proteins, Bcl-2 regulated apoptosis in mitochondrial. Extrinsic pathways including death receptors and a death domain-containing adaptor protein stimulated apoptosis via activating and binding seconds of ligands and death caspases (Ashkenazi and Dixit, 1998; Kurokawa and Kornbluth, 2009).

Extreme ROS generation, including that involved in oxidative stress, can induce dysfunction of cells, cell cycle arrest and apoptosis (Sauer et al., 2001). ROS also stimulates the pro-apoptotic Bcl-2 family protein (such as Bax and Bak), accordingly affecting mitochondrial membrane permeability and cell death (Kim et al., 2005; Ling et al., 2003; Zhang and Chen, 2004). DNA damage and stress signals may stimulate increasing p53 proteins, which have important functions: cell arrest, DNA repair and apoptosis. p53 also induces up-regulates the Bax and down-regulation of Bcl-2 during p53-related apoptosis (Martin and Elkon, 2004). Many studies suggest that the inactivation or defects of p53 is an important stage in the growth of colon cancer (Cho and Vogelstein, 1992).

Death receptors have a direct apoptotic pathway, transfer apoptosis signals (from death receptor) to death ligands, and

* Corresponding author. Tel.: +82 43 840 3570.
E-mail address: beongou@kku.ac.kr (B.O. Lim).

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.07.068

consequently play an important role in instructive apoptosis (Ashkenazi and Dixit, 1998).These receptors can activate death caspases within seconds of ligand binding, causing an apoptotic demise of the cell within hours (Ashkenazi and Dixit, 1998). Generally, DR3 regulate inflammation and autoimmune diseases: experimental autoimmune uveoretinitis, allergic lung inflammation and inflammatory arthritis (Bull et al., 2008; Calder and Wang, 2012; Meylan et al., 2008; Pappu et al., 2008). DR3 also activate apoptosis through initiator caspase-8, which then induces downstream molecular events either by activation of executioner caspase-3 or caspase-1, and regulate an NF-κB, anti-apoptosis protein, through TRADD, TRAF2 (Ashkenazi and Dixit, 1998; Gao and Abu Kwaik, 2000; Perry, 2000).

Caspases are expressed as inactive zymogenes (pro-caspases) that are activated in a cascade following pro-apoptotic stimuli (Wang et al., 2012).This cascade begins with autocatalytic activation of initiator caspases, which cleave and activate the effector caspases that, in turn, disassemble a cell. Caspase 8, an initiator caspase, plays a key role such as the extrinsic cell death pathway, and induces the apoptosis downstream of death receptors by activating self-cleavage and caspase 3. Activation of caspase 3 triggers the cleavage of poly (ADP-ribose) polymerase (PARP). Asp^{214}, Gly^{215}, and the in-PARP cleavage induce separation of DNA-binding motifs in NH_2 terminal region of catalytic domains, consequently, prevention of recruitment to a catalytic domain in damaged DNA sites. PARP was subsequently shown to be cleaved into 89- and 24 kDa fragments that contain the active site and the DNA-binding domain of the enzyme, respectively, during drug-induced apoptosis in a variety of cells (Boulares et al., 1999).

Cordycepin (3′-deoxyadenosine), an adenosine analog, is a major component of and was first isolated from *C. militaris*. Cordycepin is a derivative of the nucleoside adenosine only differing from the latter by the lack of oxygen in the 3′position of the ribose entity. Cordycepin has anti-inflammatory and anti-fungal activity, as well as decreases age-related oxidatives, improves antioxidant and anti-hyperglycemic effect (Kim et al., 2006; Lo et al., 2004; Ramesh et al., 2012; Sugar and McCaffrey, 1998). Cordycepin induces apoptosis in many cells, induces cell cycle arrest by targeting molecules and pathways (Lee et al., 2009, 2010).

Recently, many chemical agents with apoptotic activity but minimal toxicity have had potential as anticancer medicine. As a natural compound, cordycepin plays a key role in cancer therapy. However, there are no reports regarding the induction of apoptosis by cordycepin through DR3 pathway on HT-29. In this study, we demonstrated the effect of cordycepin on cancer cells and the molecular pathway by which it induces apoptosis in human colon cancer cell HT-29.

2. Materials and methods

2.1. Chemicals and reagents

2,7-Dichlorofluorescin diacetate (DCFH-DA). 1-(4.5-methylthiazol-2yl)-3,5-diphenylformazan (MTT), propidium iodide (PI), trypan blue and Ribonuclease A solution were purchased from Sigma (St.Louis, MO, USA), RPMI 1640 (Welgene Inc., Daegu, Korea), fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 g/mL) (GIBCO BRL, Life Technologies, Grand Island, NY). Annexin V-FITC (Enzo life sciences, Inc., USA), Hoechst 33342 Solution (BD briosciences, USA). Antibodies against TWEAK (DR3), caspase-8, caspase-1, cleaved caspase-3 and cleaved PARP were purchased from Millipore (Billerica, MA, USA). The polyclonal antibody against Bax and Bcl-2 was purchased from Santa Cruz (Santa Crus, CA, USA).

2.2. Isolation, identification and purity determination of cordycepin

All experiments were performed in accordance with the institutional guidelines of Konkuk University, Republic of Korea. The C. militaris used in this study was collected from the Chungju-city area and isolated by Dr. S.K. Kim (Konkuk University, Republic of Korea). To produce cordycepin, we incubated silkworm pupa for 90 days after inoculating with C. militaris until the fungus generated fruiting bodies. The fruiting bodies were then dried and subjected to isolation and purification of cordycepin. Cordycepin was isolated from an 80% methanol extract of C. militaris by BuOH partition chromatography, silica gel column chromatography (CHCl3: EtOAc: MeOH = 12:1:3), preparative HPLC [YMC-PAK C18 column (YMC, Kyoto), 20 × 250 mm, 5 μm, 260 nm, 13% aqueous methanol], and recrystallization in H2O. The isolated cordycepin was subjected to various spectrometric analyses for identification. The purity of the isolated cordycepin was determined by three different HPLC systems: silica gel column(4.6 × 250 mm, 5 μm, 260 nm, CHCl3-MeOH = 5:5), C18 column (Alltech, 4.6 × 250 mm, 5 μm, 220 nm, 13% Aq. MeOH; Alltech Associates, Deerfield, IL, USA), and carbamate column (Waters Xterra, 3.9 × 150 mm, 5 μm, 220 nm, 7% Aq. MeOH; Waters, Milford, MA, USA). Identification of the isolated cordycepin was achieved by spectrometric methods: 1H NMR (500 MHz; JEOL, Tokyo), 13C NMR (125 MHz, JEOL), LC–MS (LCQ DECA XP; Thermo Finnigan, Waltham, USA), and FAB–MS (JMS-700, MHz; JEOL). The spectral characteristics of cordycepin are as follows: 1H-NMR (500 MHz, DMSO-d6): 8.35 (1H, s, H-8), 8.14 (1H, s, H-2), 7.27 (2H, s, 6-NH2), 5.87 (1H, d, *J* = 2.5 Hz, H-1′), 5.65 (1H, d, *J* = 4.0 Hz, 2′-OH), 5.15 (1H, t, *J* = 5.7 Hz, 5′-OH), 4.57 (1H, m, H-2′), 4.35 (1H, m, H-4′), 3.69 (1H, m, H-5′), 3.52 (1H, m, H-5′), 2.25 (1H, m, H-3′), 1.92 (1H, m, H-3′); 13C-NMR (125 MHz, DMSO-d6): 148.83 (C-6), 152.37 (C-2), 156.00 (C-4), 139.02 (C-8), 119.05 (C-5), 90.75 (C-1′), 80.62 (C-4′), 74.54 (C-2′), 62.59 (C-5′), and 34.06 (C-3′). The molecular weight of the isolated compound was determined to be 251 by LC–MS measurements, which provided quasimolecular ion peaks at m/z 252.1 [M + H]+ and m/z 274.0 [M + Na]+ in the positive mode and at m/z 249.9 [M − H]− in the negative mode. The FAB mass spectrum in the positive mode revealed the same result. The purity of the isolated cordycepin was 100% as determined by three different HPLC systems.

2.3. Cell cultures

Human colon cancer HT-29 cells were obtained from the Korean cell line bank, seoul national university. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 lg/mL) (GIBCO BRL, Life Technologies, Grand Island, NY) in a humidified atmosphere with a 5% CO2 incubator at 37 °C.

2.4. Cell viability analysis

Cell viability of cordycepin was assessed by MTT assay. Briefly, cells were placed in 96-well culture plates at density of 1×10^4 cells/well in RPMI 1640 culture medium that contained 10% FBS and then incubated at 37 °C under 5% CO_2. After 24 h incubation, cells were treated with vehicle alone (2% DMSO) or cordycepin (25, 50, 100, 200 and 400 μM) for 6, 18 and 24 h. Next, MTT solution(5 mg/mL in Media) was added to each well of a 96-well plate and the samples were then incubated for 4 h at 37 °C. After washing, the formazan dye precipitates, which are proportional to the number of live cells, were dissolved in 100uL DMSO. The absorbance at 570 nm was then read using ELISA reader.

2.5. Light microscopic examination

HT-29 cells were plates in six-well plates at a density of 1×10^6 cells/mL. After 24 h incubation, the cells were treated with different concentration cordycepin. After 18 and 24 h, the cells were observed by light microscopy.

2.6. Cell cycle analysis

The cells were seeded at a density of 1×10^6 cells in 6-well plates, and cultured for 24 h in RPMI-1640. After culturing, the cells were treated with different concentrations of CM for 18 and 24 h. Next, the cells were collected and centrifuged at 13,000 rpm for 3 min, add 300 μL of cold PBS in pellets. The cells were fixed in ice-cold 70% ethanol and stored at 4 °C overnight. After centrifuged at 13,000 rpm for 5 min, add 1 mL of cold PBS in pellets and centrifuged at 13,000 rpm for 5 min. Before analysis, the cells were stained with a solution consisting of 500 μg/mL propidium, 10 μg/mL RNaseA. Following incubation at 37 °C for 30 min. After staining, the cells were sorted in a flow cytometry using Cellquest3.1 software (Becton-dickinson). For each sample, 1×10^4 cells were analyzed for cell cycle phase.

2.7. Hoechst 33342 staining

Morphology of apoptotic nuclear measured by Hoechst 33342 staining. Cells were seed in 96 well plates at a density of 1×10^4/well. After incubation, cells were treated with cordycepin. After treatment for 18 and 24 h, add 100 μL of the 2 μg/mL solution of Hoechst 33342 to each well at least 15 min before imaging. Apoptotic nuclear morphology was observed with a fluorescent microscope.