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Anti-tumor effect of *Cordyceps militaris* in HCV-infected human hepatocarcinoma 7.5 cells

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Cordyceps extract has been reported to have various pharmacological activities including an anti-cancer effect. We investigated the inhibitory effect of *Cordyceps militaris* on hepatitis C virus-infected human hepatocarcinoma 7.5 cells (J6/JFH1-huh 7.5 cells). The huh7.5 cells with or without HCV infection were treated with various concentrations of ethanol extract of *Cordyceps militaris* (CME) for 48 h and the cytotoxicity was measured by CCK-8 assay. Both J6/JFH1-huh7.5 cells and huh7.5 cells were highly susceptible to CME. To examine the molecular mechanisms of the inhibitory effect on huh7.5 cells, the effect of CME on cell apoptosis was measured using flow cytometry and the expressions of p53, Bim, Bax, PARP, (cleaved) caspase-9, and (cleaved) caspase-3 in huh 7.5 cells were detected by western blot assays. CME significantly increased early apoptosis and up-regulated the expression of Bim, Bax, cleaved PARP, cleaved caspase 9 and cleaved caspase-3. We also found the decrease of HCV Core or NS3 protein by CME in HCV-infected huh 7.5 cells.

***Keywords*:** *Cordyceps militaris*, human hepatocarcinoma 7.5 cells, HCV, apoptosis, Bax, PARP

Introduction

Hepatitis C virus (HCV) is a positive single-stranded RNA of the *Flaviviridae* family. The HCV genome encodes a large polyprotein that is divided into 10 proteins including Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. HCV primarily infects liver via CD81, SR-B1, claudin-1 or occludin, which is expressed on hepatocytes (Lauer and Walker, 2001). Therefore, HCV frequently causes chronic hepatitis, which leads to liver cirrhosis and hepatocellular carcinoma (Liang *et al.*, 2000).

Hepatocellular carcinoma (HCC) is the fifth most commonly occurring cancer in the world. HCC does not have a successful recovery rate for several reasons (Kim and Park, 1993; El-Serag and Rudolph, 2007), and the rate of HCC reappearance-free, 5-year survival after surgery is very low (Maluccio and Covey, 2012; Ahn *et al.*, 2013).

Besides the routine methods of surgery and chemotherapy, traditional plant or microbial-derived medicine is one of the major complementary and alternative treatments for various malignant tumors, including hepatocellular carcinoma (Zhai *et al.*, 2013; Ling *et al.*, 2014). People are also aware of the importance of prophylactic medications to prevent HCC.

A large number of edible mushrooms possess various biological activities including anti-tumor and anti-microbial effects (Yang *et al.*, 2011; Kim *et al.*, 2013; Kunjadia *et al.*, 2014). Among them, cordyceps mushrooms have potent pharmacological activities. The well-known active ingredients are cordycepin, nucleosides and polysaccharides (Yue *et al.*, 2013). *Cordyceps militaris* has a higher content of cordycepin than other kinds of Cordyceps and is successfully cultivated in Republic of Korea (Tuli *et al.*, 2013). Extracts of *Cordyceps militaris* reportedly have immunomodulatory, anti-inflammatory and antitumor activities (Park *et al.*, 2005; Lee *et al.*, 2006). We recently found that ethanol extract of *Cordyceps militaris* had an anti-viral effect in DBA2 mice infected with influenza virus, and the anti-viral mechanism of *Cordyceps militaris* was mediated by an immune-enhancing effect through IL-12 and the activation of natural killer cells (Lee *et al.*, 2014).

Various forms of cordyceps extract and compounds have a potent cytotoxic effect on human cancer cells including hepatocarcinoma cells (Wang *et al.*, 2005, 2014; Zhang *et al.*, 2008; Zhao *et al.*, 2011; Lee *et al.*, 2013; Sun *et al.*, 2014). However, the effect of cordyceps extract on HCV-infected human hepatocarcinoma cells was not clearly investigated, which is probably due to the lack of an HCV replication cell culture system. Recently, human hepatocarcinoma 7.5 cells have been developed as a culture system to study HCV life cycles, as well as for the development of anti-HCV drugs (Wakita *et al.*, 2005; Binder *et al.*, 2007).

p53 stimulates cellular apoptosis in response to DNA damage, which is actually mediated by the activation of Bax, a member of pro-apoptotic molecules in the Bcl-2 family (Lakin and Jackson, 1999; Wu and Deng, 2002). The Bcl-2 family proteins include both pro-apoptotic members (Bim, Bax, and Bad) and anti-apoptotic members (Bcl-2). The expression ratio between pro-apoptotic and anti-apoptotic Bcl-2 proteins controls mitochondrial-dependent apoptosis (Murphy *et al.*, 2000). In fact, the activation of Bax leads to the release of cytochrome C into cytosol, which stimulates the activation

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of caspase 9 and 3, the effector caspases in the mitochondrial apoptotic pathway (Adrain *et al.*, 1999; Cain *et al.*, 1999).

In this study, we investigated the cytotoxicity of *Cordyceps militaris* ethanol extract (CME) on human hepatocarcinoma cells (huh) 7.5 with or without HCV infection, and the anti-tumor effect of CME on huh 7.5 cells was precisely examined in the context of HCV infection. We found that the underlying molecular mechanism of the anti-tumor effect of CME was highly associated with the increased expression of Bax, bim, cleaved caspase 9, 3 and cleaved PARP in a p53-independent manner.

Materials and Methods

J6/JFH1 cDNA clone and establishment of infectious J6/JFH1 cell culture system

HCV genotype 2a-derived infectious HCV clone (pFL-J6/JFH) was provided by TMIN/Toray and Dr. Rice's laboratory through material transfer agreement (MTA 291, MTA 1464). Human hepatocarcinoma 7.5 cells (huh 7.5) were obtained from Apath, LLC, through material transfer agreement (MTA-1465) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin and streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO_2. HepG2 cells were obtained from ATCC and cultured in same conditions with huh 7.5. HCV replication was established in huh7.5 cells by transfecting the full length genome of J6/JFH1-derived HCV RNA. Stable HCV expression was detectable by Immunohistochemistry staining within 48–72 h in huh 7.5 cells. Supernatant from huh7.5 cells replicating J6/JFH1 were collected at various time points and the infectivity of supernatant was measured to provide MOI (multiplicity of infection). MOI 5 was used to infect naive huh7.5.

Specimen preparation of *Cordyceps militaris*

The mushroom of *Cordyceps militaris* (Hoengseong) was collected, cleaned, and extracted with 50% ethanol at room temperature. The extract was filtered, concentrated, sterilized, and dried. The main components of the extract are cordycepin, adenosine, urasil, and guanosine, which structurally belong to the nucleoside family (provided by Dong-a Pharm. Co., LTD). *Cordyceps militaris* ethanol extract (CME) was diluted with distilled water for treatment of huh 7.5 cells or HCV-huh7.5 cells.

Cell cytotoxicity

Cell cytotoxicity of CME in huh 7.5 cells, J6/JFH1-huh7.5 cells or HepG2 cells was assessed by Cell Counting Kit-8 (CCK-8, Dojindo) as previously described (Son *et al.*, 2015). Briefly, cells were seeded in 96-well plates at a density of 3×10^3 cells/well. After 24 h incubation, cells were treated with serial concentrations of CME (0, 25, 50, 100, 250, 500, 1,000 μg/ml) for 24 h or 48 h. Ten microliter of CCK-8 solution was added to each well and incubated for another 3 h. The absorbance of the reaction was measured using microplate reader (BMG Labtech) at 450 nm.

Cell apoptosis assay

Cell apoptosis was analyzed using the Annexin V-FITC Apoptosis Detection Kit (Roche) as previously described (Son *et al.*, 2015). Briefly, huh 7.5 cells were plated in 6-well plates at a density of 1×10^6 cells/ml and incubated for 24 h. Cells were treated with different concentrations (0, 100, 300 μg/ml) of CME for 24 h or 48 h. Cells were washed with cold PBS, re-suspended in 100 μl of Annexin-V-FLUOS buffer and Annexin V and PI solution were added. After 20 min incubation at room temperature in the dark, cells were analyzed by flow cytometry (EasyCyte guava, Merck Millipore).

Western blot analysis

Western blot assays were performed as previously described with modification (Son *et al.*, 2015). Briefly, huh 7.5 cells were treated with CME (100 and 300 μg/ml) for 48 h. Protein in cell lysates was measured using the Bradford assay, separated by electrophoresis, and transferred onto nitrocellulose membranes. Membranes were subsequently incubated with primary and secondary antibody, and the blots were visualized by enhanced chemiluminescent (ECL) detection solutions (GE Healthcare). Primary antibodies used in this study included Pro-apoptotic Sampler kit (Cell Signaling), anti-p53 Ab (clone BP53-12, Millipore), anti-β-actin Ab (Sigma), anti-Hepatitis C virus NS3 Ab (Abcam), and anti-Hepatitis C Virus Core 1b Ab (Abcam).

Immunohistochemical staining

The huh7.5 cells or J6/JFH1-huh7.5 cells plated in 96 wells were washed twice with PBS. Cells were fixed and permeabilized in 100% methanol for 30 min at -20°C. Cells were washed twice with PBS and once with 1 × PBS/0.1% Tween-20 and then blocked with 1% BSA, 0.2% Skim milk for 30 min at room temperature. 3% H_2O_2 was added to block endogenous peroxidase activity. Cells were stained with HCV-specific Core 1b monoclonal antibody (Abcam) diluted 1:2000 in 1 × PBS/0.1% Tween-20 and incubated with secondary antibody (Goat-α-mouse-HRP, Jackson Immuno Research) diluted 1:200 for 30 min at room temperature. DAB Substrate (DAKO, K3468; diluted 1 drop/ml as per manufacturer's instructions) was added for 5 min to detect positive spots.

Xenograft mouse model experiments

All animal experiments were conducted in accordance with recommendations in the National Research Council's Guide (IACUC) for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Experiments Committee of Duksung Women's University (permit number: 2014-015-007). Balb/c nude mice (female, 5 weeks old; Joong-ang Animal Experiment Company) were used as our xenograft animal model. Mice were housed individually on a 12-h day/12-h night cycle at 23–27°C and had free access to food and water. Mice were randomly divided into two groups: (1) drinking water group (n = 5): animals received oral administration of drinking water; (2) CME group (n = 5): animals received oral administration of CME (30 mg/kg). To produce tumors, each mouse was implanted with huh 7.5 cells (1×10^6 cells/animal), subcutaneously in the back next

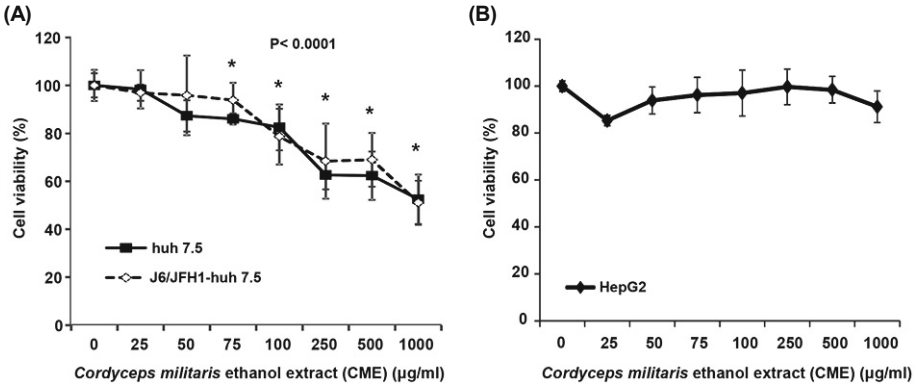

Fig. 1. Cell cytotoxicity of CME in huh7.5 cells, HCV-huh7.5 cells and HepG2 cells. The huh7.5 cells, HCV-huh7.5 cells or HepG2 cells were treated with various concentrations (0–1,000 μg/ml) of CME for 48 h, and cell viability was determined by CCK-8 assay. The results are presented as mean ± standard deviation (SD) for five independent experiments. (A) Cytotoxicity in huh-7.5 cells or HCV-huh7.5 cells (B) Cytotoxicity in HepG2 cells.

to the right hind leg. CME (30 mg/kg) or drinking water was then administered orally every day for 3 weeks. Twelve days later, the tumors were identified and then measured every three days with a Standard Caliper. Tumor volume was calculated as follows: tumor volume (mm^3) = [tumor length (mm) × tumor width $(mm)^2$] / 2. Once tumor volume reached 2,000 mm^3, animals were euthanized. To exclude the possibility of a general toxicity of CME, changes in body weight of fed as well as unfed animals were measured.

Statistical analysis

Data were processed using Microsoft Excel software and were expressed as mean ± SD. Comparisons of several means were performed using one-way or two-way analysis of variance followed by Fisher's exact test to identify significant differences between groups. *P* values of less than 0.05 were considered significant.

Results

Cell cytotoxicity of CME in huh 7.5, J6/JFH1-huh7.5, and HepG2 cells

To investigate the cytotoxic effect of CME in huh 7.5, J6/JFH1-huh7.5, and HepG2 cells, cells were treated with serially diluted CME (0, 25, 50, 75, 100, 250, 500, and 1,000 μg/ml) for 48 h and cytotoxicity was determined by Cell Count Kit-8. Figure 1A shows that CME from 75 μg/ml significantly decreased cell viability compared to untreated cells ($P < 0.0001$). However, there was little difference in cell viability between huh 7.5 or J6/JFH1-huh7.5 cells under treatment of CME. We also measured the cytotoxic effect of CME toward HepG2, which is another human liver cancer cell line, but not tumorigenic in xenograft animals. Figure 1B clearly shows that there is no cytotoxic effect in HepG2, which indicates that the cytotoxicity of CME toward huh 7.5 is selective.

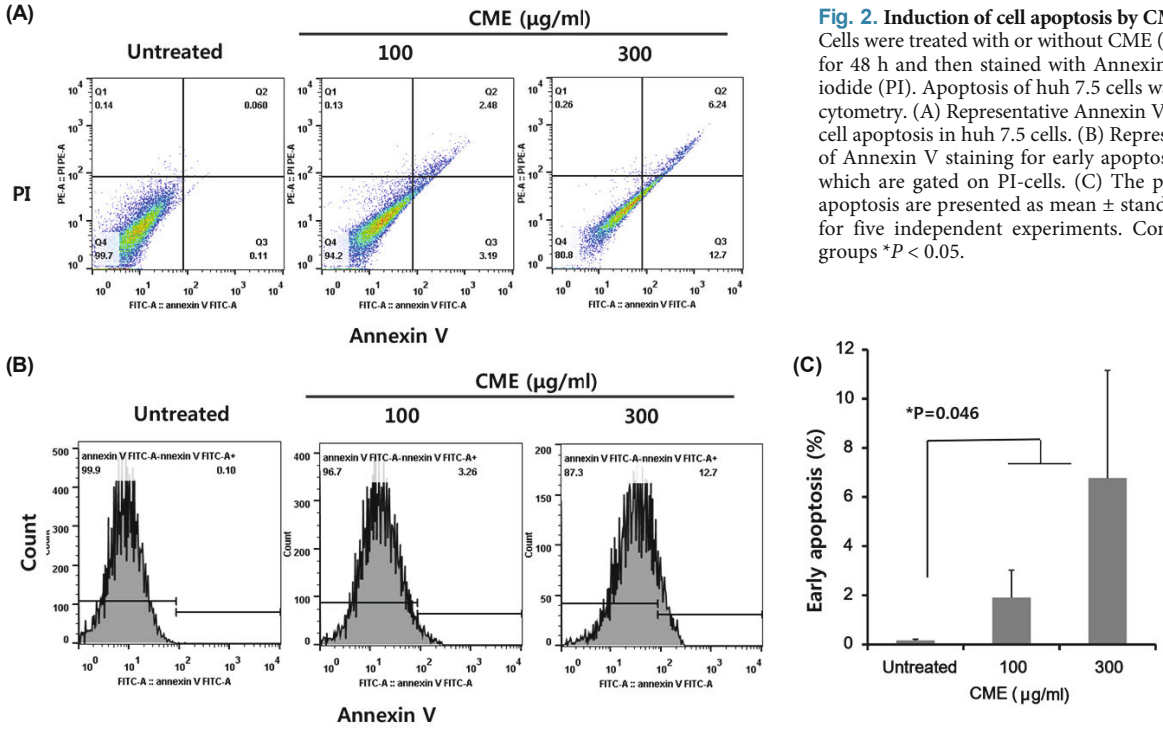

Fig. 2. Induction of cell apoptosis by CME in huh 7.5 cells. Cells were treated with or without CME (100 and 300 μg/ml) for 48 h and then stained with Annexin V and propidium iodide (PI). Apoptosis of huh 7.5 cells was analyzed by flow cytometry. (A) Representative Annexin V and PI staining for cell apoptosis in huh 7.5 cells. (B) Representative histogram of Annexin V staining for early apoptosis in huh 7.5 cells, which are gated on PI-cells. (C) The percentages of early apoptosis are presented as mean ± standard deviation (SD) for five independent experiments. Compared with other groups $^*P < 0.05$.

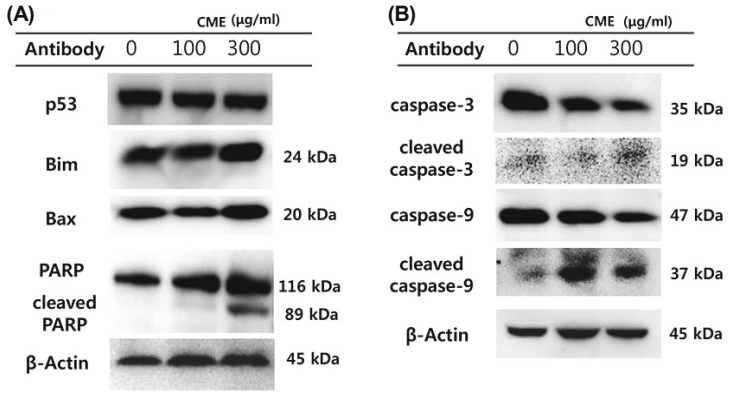

Fig. 3. Induction of mitochondrial-mediated apoptosis by CME in a p53-independent manner. Cells were treated with or without CME (0, 100, and 300 μg/ml) for 48 h and then western blot analyses for various apoptotic related molecules were performed. β-Actin served as the loading control. (A) Expression of p53, Bcl-2 family proteins (Bim, Bax) and PARP. (B) Expression of (cleaved) caspase-3 and -9 proteins.

Induction of cell apoptosis in huh 7.5 cells treated with CME

We next examined the effect of CME on cell apoptosis using Annexin V-FITC and propidium iodide (PI) staining. Annexin V staining is known to be positive for apoptotic as well as necrotic cells and PI staining is commonly used for detection of necrotic cells. Therefore, we specifically measured Annexin V^+ PI^- cells, which are only apoptotic cells, not necrotic cells. As shown in Fig. 2B and C, 100 or 300 μg/ml of CME induced 1.91% or 6.78% early apoptosis in huh 7.5 cells, respectively, compared to untreated cells ($p= 0.046$), which suggests that the induction of apoptosis is highly dose-dependent in the treatment of CME.

Increased expression of apoptotic-related proteins in huh 7.5 cells treated with CME

To investigate the molecular mechanism of cell apoptosis by CME, we examined the expression of p53, Bcl-2 family related apoptotic proteins, caspases, and poly (ADP-ribose) polymerase (PARP) using western blot analysis. The intrinsic (or mitochondrial) pathway of apoptosis is associated with various signaling molecules including p53, caspase activators and proapoptotic proteins (Wu and Deng, 2002). In Fig. 3A, the expressions of Bim, Bax, and cleaved form of PARP were increased in huh 7.5 cells treated with CME. However, the expression of p53 was not significantly changed in treated cells.

The release of cytochrome *c* from mitochondria induces the expression of caspase-9 and caspase-3. Activation of caspase 3 triggers the cleavage of PARP (89- and 24 kDa), which prevents DNA damage (Boulares *et al.*, 1999). Figure 3B shows that the expressions of caspase-3 and -9 were decreased in a dose-dependent manner, but the expressions of cleaved forms of caspase-3 and -9 were simultaneously increased, which indicates that caspase-3 and -9 were activated in huh 7.5 cells treated with CME.

Decreased expression of HCV Core and NS3 proteins in J6/JFH1-huh 7.5 cells treated with CME

We also tried to examine if CME directly affected HCV-associated protein. To do so, we used the HCV replication cell culture system, which was established in our laboratory

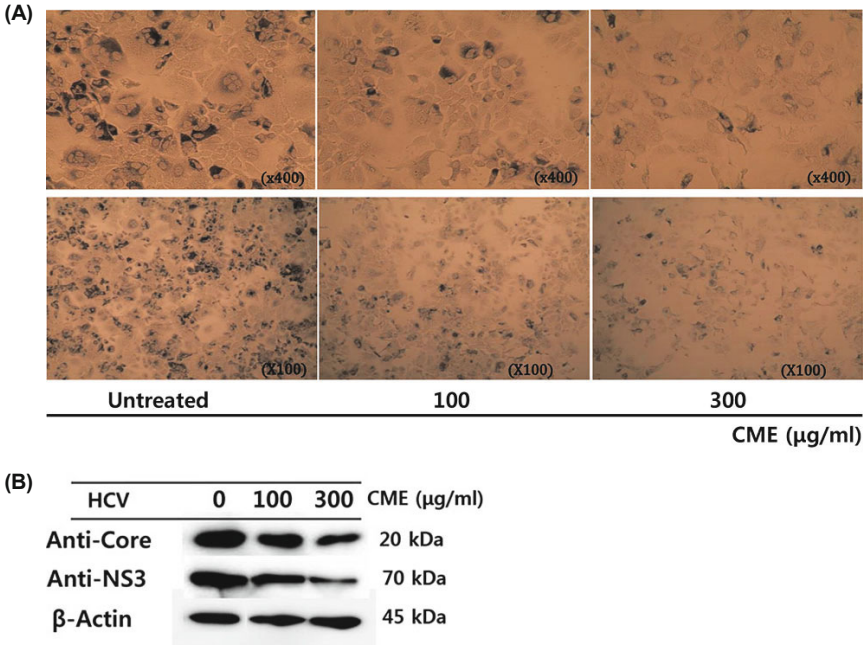

Fig. 4. Decreased expresssion of HCV Core and HCV NS3 protein by CME. Cells were treated with or without CME (0, 100, and 300 μg/ml) for 48 h. Immunohistochemical staining for HCV CORE protein expression and western blot analysis for HCV CORE and HCV NS3 proteins were performed. β-actin was served as the loading control. (A) Immunohistochemical expression of HCV CORE protein (dark spots) in cytoplasm of huh 7.5 cells. (B) Expression of HCV CORE and HCV NS3 proteins by western blot.

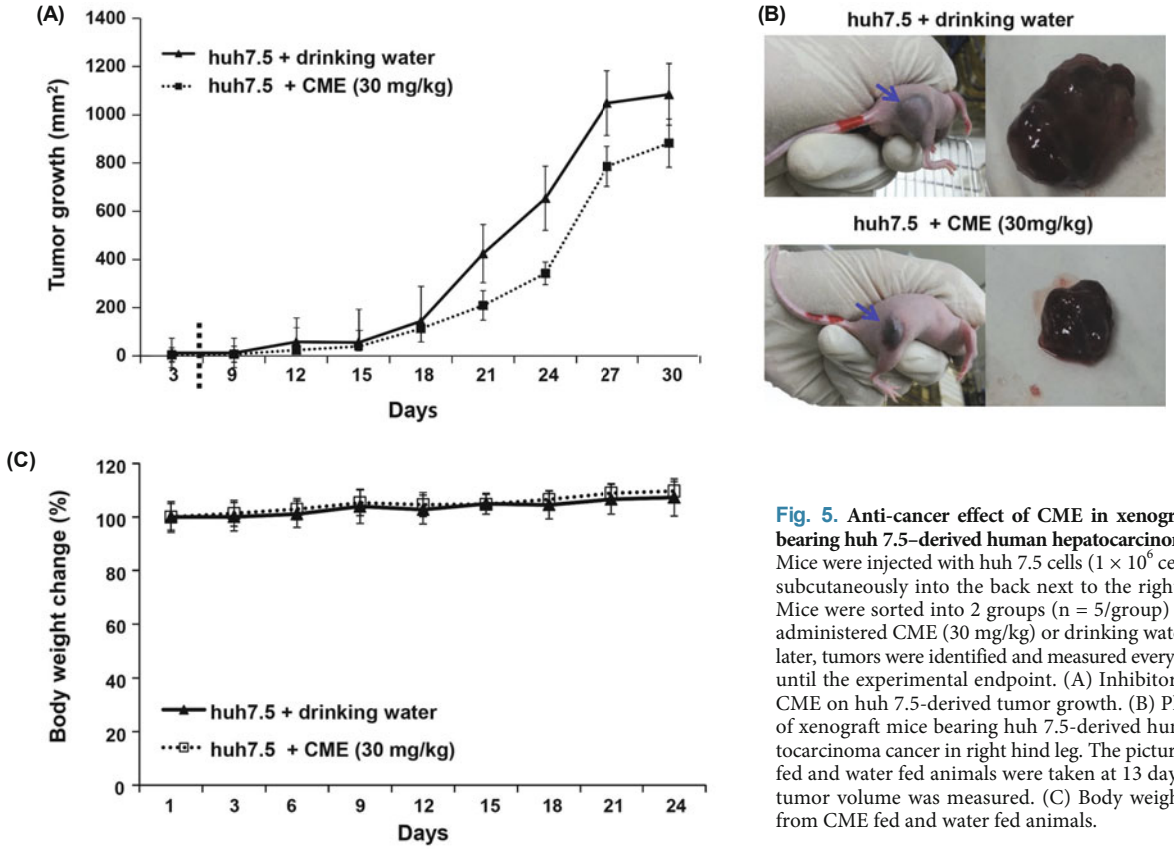

Fig. 5. Anti-cancer effect of CME in xenograft mouse bearing huh 7.5–derived human hepatocarcinoma cancer. Mice were injected with huh 7.5 cells (1×10^6 cells/mouse) subcutaneously into the back next to the right hind leg. Mice were sorted into 2 groups (n = 5/group) and orally administered CME (30 mg/kg) or drinking water. 12 days later, tumors were identified and measured every three days until the experimental endpoint. (A) Inhibitory effect of CME on huh 7.5-derived tumor growth. (B) Photograph of xenograft mice bearing huh 7.5-derived human hepatocarcinoma cancer in right hind leg. The pictures of CME fed and water fed animals were taken at 13 days after the tumor volume was measured. (C) Body weight changes from CME fed and water fed animals.

through MTA291, 1464, and 1465. Figure 4A shows that there are more dark spots when staining the cytoplasmic expression of HCV CORE protein in huh 7.5 cells treated with 100 μg/ml or 300 μg/ml of CME compared to untreated huh 7.5 cells. This result indicates that the effect of CME on huh 7.5 cells also associated with the expression of HCV CORE protein. For the quantitative analysis, we measured the expressions of HCV CORE or HCV NS3 protein using western blot. As shown in Fig. 4B, the expressions of both HCV CORE and HCV NS3 proteins decreased as the concentration of CME increased.

Anti-cancer effect of CME in xenograft mouse bearing huh 7.5 – derived human hepatocarcinoma cancer

To evaluate the anti-cancer effect of CME *in vivo*, mice were injected subcutaneously with huh 7.5 and the CME (30 mg/kg) or drinking water was orally administrated every day for 3 weeks. Figure 5A shows that continuous feeding of CME (30 mg/kg) inhibited the growth of huh 7.5 cell-derived tumors. However, the inhibitory effect of CME was not statistically significant. Figure 5B shows representative photographs of xenograft mice bearing huh 7.5 cells-derived human liver cancer. The pictures of the CME fed and water fed groups were taken at 13 days since the tumor volume was measured. The size of tumors from the CME fed mice was smaller than that from water fed mice. Figure 5C shows that oral administration of CME didn't cause a significant weight loss in CME fed animals. This result tells us that CME doesn't have a general toxicity.

Discussion

In this study, for the first time, we report the anti-tumor effect of CME in HCV-replicating human hepatocarcinoma (huh) 7.5 cells. First, CME was potently cytotoxic to huh 7.5 cells as well as HCV-huh 7.5 cells (Fig. 1A), but not to HepG2. This results somewhat agree with previous studies of similar species of cordyceps conducted in other human cancer cell lines including human hepatocellular carcinoma MHCC97H cells (Park *et al.*, 2005; Sun *et al.*, 2014; Wang *et al.*, 2014). In human hepatocellular carcinoma MHCC97H cells, the water extract of *Cordyceps cicadae* inhibited the growth of MHCC97H cells in a dose-dependent manner via G2/M phase cell cycle arrest (Wang *et al.*, 2014). Interestingly, Sun *et al.* (2014) group reported that Cordycepol C, a sesquiterpene compound from Cordyceps extract, induced apoptosis in HepG2, which disagree with our results in Fig. 2B. We consider the reasons for the differential cytotoxicity of CME in huh 7.5 vs HepG2 in terms of two aspects. First, polyssacharrides, steroidal compounds, and terpenes from cordyceps extracts have been reported to have apoptotic effect in HepG2 (Zhao *et al.*, 2011; Sun *et al.*, 2014), and these compounds are not neucleosides. However, the CME used in this study is a nucleoside-enriched fraction of *Cordyceps militaris,* which has cordycepin, adenosine, urasil, and guanosine as main ingredients. Therefore, neucleoside-enriched fraction might have a selective cytotoxicity toward huh 7.5 compared to other kinds of compound from cordyceps. On the other hand, Cordycepin, one of the nucleoside compounds, has shown to

inhibit the proliferation of HepG2 (Lu *et al.*, 2014). However, the cytotoxic effect of cordycepin in HepG2 was shown with concentration of 2,000 μg/ml, which is much higher compared to the concentration of CME used in this study. Therefore, we speculate that we might be able to observe the cytotoxic effect of CME in HepG2 if we used higher concentration of CME. However, we believe that using 2,000 μg/ml of cordycepin is not practical when it is considered for anti-tumor reagents.

We next explored whether the CME affected the cell cycle progression of huh 7.5 cells. However, we found that there was no significant change in cell cycle progression by CME (data not shown). This indicates that the cytotoxic effect of our CME in huh 7.5 cells was not mediated by changes in cell cycle progression, but by other cytotoxicity-related mechanisms. We further investigated the effect of CME on cell apoptosis of huh 7.5 cells. To date, the induction of cell apoptosis by extracts of *Cordyceps militaris* or major ingredients has mostly been reported in Hep G2 cells or Hep 3B cells (Zhao *et al.*, 2011; Lee *et al.*, 2013; Sun *et al.*, 2014). Lee *et al.* (2013) demonstrated that cordycepin treatment stimulated Hep3B human hepatocellular carcinoma cells to TRAIL-mediated apoptosis. Also, Sun's group reported that Cordycepol C caused poly(ADP-ribose)polymerase-1 (PARP-1) cleavage and triggered the loss of mitochondrial membrane potential ($\Delta\Psi_m$) in HepG2 cells in a time- and dose-dependent manner (Sun *et al.*, 2014). The huh 7.5 is a cell line that was selected from huh-7 cells for its increased permissiveness for HCV RNA replication (Blight *et al.*, 2002). The parent huh 7 is a well-differentiated, hepatocyte-derived carcinoma cell line that was originally taken from a liver tumor in a 57-year-old Japanese male in 1982 (Nakabayashi *et al.*, 1982). The huh 7.5 is commonly used to study liver cancer and its potential therapies. In fact, we found that the implantation of huh 7.5 cells into a xenograft mouse model successfully produced a solid human tumor (Fig. 5). HepG2 is a continuous cell line that was derived from the liver tissue of a 15-year-old Caucasian American male with well-differentiated hepatocellular carcinoma. However, HepG2 is not tumorigenic in xenograft mice according to American Type Culture Collection. Therefore, we consider huh 7.5 a better model to clarify the anti-tumor mechanism of *Cordyceps militaris* compared to HepG2.

Figure 2 shows that CME significantly induced early apoptosis in huh 7.5 cells. We further examined the underlying apoptotic mechanism of CME in huh 7.5 cells. We found that the apoptotic effect of CME in huh 7.5 cells was directly associated with induction of Bim, Bax, cleaved caspase-3, and cleaved caspase-9, which consist of key molecules in mitochondrial-mediated apoptosis (Fig. 3A and B). However, the CME-mediated apoptotic effect was not associated with a change in the expression of p53 protein (Fig. 3A), which is a critical marker in cell apoptosis. In fact, Sun *et al.* (2014) previously reported that Cordycepol C, a novel sesquiterpene from *Cordyceps* ophioglossoides induced cell apoptosis in hepG2 cells through a p53-independent, caspase-independent and Bax-dependent mitochondrial pathway. Our results showed that the nucleoside-enriched CME induced cell apoptosis in huh 7.5 cells via p53-independent, caspase-3, 9-dependent and Bim, Bax-mediated pathway. Interestingly, we also found activation of PARP protein (cleaved form; 89 kDa) in huh 7.5 cells treated with CME (Fig. 3A). As mentioned earlier, activation of caspase-3 is known to trigger cleavage of PARP, which halts PARP function (Boulares *et al.*, 1999).

Since huh 7.5 cells were optimized for HCV replication and HCV is one of the main factors of hepatocellular carcinoma, we investigated the anti-viral effect of CME in HCV-huh 7.5 cells. Figure 4A and B showed that there is significant decrease of both HCV CORE and HCV NS3 proteins in CME treated huh 7.5 cells. Recently, Ueda *et al.* (2014) reported that *Cordyceps militaris* exhibited an additive effect in terms of anti-HCV activity in combination with interferon-α (and/or ribavirin), which is commonly used in clinic. And they also found that the responsible compound for anti-HCV effect is cordycepin in their Cordyceps extract. In fact, the Ueda group used a subgenomic HCV replicon, which cannot properly propagate in cell culture system compared to our full-length HCV (J6/JFH1). This indicates that their replicon system could be is limited for characterization of anti-HCV effect. Therefore, we report for the first time that there is a strong correlation between the reduction of HCV protein expression and CME treatment in HCV-replicating cell culture system. We will further characterize the anti-HCV effect of CME on the HCV replication level and the responsible single compound for the anti-viral effect.

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