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Synergistic property of cordycepin in cultivated *Cordyceps militaris*-mediated apoptosis in human leukemia cells

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ABSTRACT

Cordyceps militaris is a well-known Chinese traditional medicinal mushroom frequently used for tonics and recently of a potential interest for cancer intervention. Here, we explored the cancer cell killing activity of the hot water extracts of *C. militaris* cultured mycelia (CM_{MY}) and cultivated fruiting bodies (CM_{FB}). We found that CM_{FB} exhibited a greater cytotoxic effect against various cancer cells over CM_{MY}. Apoptotic phenotypes including apoptotic body formation, DNA laddering, caspase 3 activation and cleavage of PARP proteins were induced by CM_{FB} treatment but only slightly induced by same concentration of CM_{MY} treatment in human HL-60 leukemia cells. Cordycepin in CM_{FB} (10.47 mg/g) is significantly higher (~15.2 times) than that of CM_{MY} (0.69 mg/g). Using isobolographic analysis, the synergy of cytotoxicity was observed across different combined concentrations of CM_{MY} and cordycepin. By complementing cordycepin into CM_{MY} to the level comparable with CM_{FB}, we observed that CM_{MY} (500 μg/ml) with cordycepin (4.8 μg/ml) induced apoptosis to a level similar to that induced by CM_{FB} (500 μg/ml). Together, our results suggest that cordycepin possesses a synergistic cytotoxic effect with *Cordyceps militaris*-mediated apoptosis in human leukemia cells and therefore explaining a better anti-proliferating activity of CM_{FB} over CM_{MY}.

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Introduction

Cordyceps militaris, like *Cordyceps sinensis*, belongs to *Ascomycetes* and they parasitize insects at the larval stage and gradually grow into fruiting body expanded outside the insect larvae or pupae (Buenz et al., 2005). This parasitic complex of fungus and insect has long been used for tonics and folk medicinal purposes, especially in East Asia (Stone, 2008, 2010). It is commonly used for anti-aging (Das et al., 2010; Ji et al., 2009; Li et al., 2010) and also has been used for the treatments of various diseases such as hypertension (Ahmed et al., 2012), arrhythmia (Yan et al., 2013), hepatitis (Niwa et al., 2013) and hyperglycemia (Kan et al., 2012; Lo et al., 2004, 2006). Interestingly, recent reports have suggested a potential anti-tumor activity (Das et al., 2010; Reis et al., 2013; Yang et al., 2006).

Due to the rarity of *C. sinensis*, *C. militaris* has been regarded as a substitute for *C. sinensis* (Das et al., 2010). Although pharmacologically active components remain largely unresolved, experimental results reveal that *C. militaris* has a similar chemical composition to *C. sinensis* including cordycepin (3′-deoxyadenosine), D-mannitol (cordycepic acid) and polysaccharides (Lim et al., 2012). It should be noted here that cordycepin, an inhibitor of RNA polymerases, is considered as one potential pharmacological ingredient of *Cordyceps* spp. for anti-tumor activity (Chen et al., 2008; Choi et al., 2011; Koc et al., 1996; Tuli et al., 2013).

Not until recently, it is not possible to cultivate *C. militaris* *in vitro*, especially in the form of fruiting body (Das et al., 2010;

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Shrestha et al., 2005). The availability of large quantity of cultivated *C. militaris* has allowed scientists to investigate its biologically active ingredients responsible for various medicinal usages (Das et al., 2010).

In this present study, both mycelia and fruiting bodies were produced from laboratory cultivation. The hot water extracts of mycelia (CM_{MY}) and fruiting bodies (CM_{FB}) were prepared for biological study. Our results showed that CM_{FB} exhibited a much greater cytotoxic activity against various cancer cell lines than that of CM_{MY}. To investigate the underlying mechanism(s) giving rise to differential cytotoxic abilities of CM_{FB} and CM_{MY}, induced cell-death pathways and corresponding chemical compositions were analyzed. Consistently, CM_{FB} also displayed a better apoptosis-inducing activity than that of CM_{MY}. HPLC analyses revealed that CM_{FB} contains a 15-fold higher concentration of cordycepin compared to that of CM_{MY}. The isobolographic analysis defined a synergistic interaction between cordycepin and CM_{MY}. The reconstitution experiments further showed that a comparable administration of cordycepin (4.8 μg/ml, <10% apoptotic cells induced) into CM_{MY} (500 μg/ml, <10% apoptotic cells induced) synergistically promoted the apoptosis-inducing capability to the level of CM_{FB} (500 μg/ml, ~80% apoptotic cells). In conclusion, our results suggested that the synergistic apoptosis-inducing ability of cordycepin with *C. militaris* extract might provide a plausible explanation for the greater anti-cancer activity of fruit bodies over mycelia.

Materials and methods

Cultivation of Cordyceps militaris

C. militaris was obtained from Mucho Biotech Co. (Taipei, Taiwan). The composition of culture medium for producing fruiting bodies was 70% (w/w) rice, 23% (w/w) silkworm chrysalis powder, 5% (w/w) sucrose, 1.5% (w/w) peptone and 0.5% (w/w) yeast extract. Thirty grams of the above components were dispensed into bottles containing 30 ml of water. The bottles were wrapped with polypropylene film before being autoclaved. Each bottle was inoculated with 3% (v/v) of *C. militaris*. The culture condition was 15–20 °C and 65% of humidity in a dark environment. When mycelia covered the entire bottle surface and proliferated to the bottom of the bottle, fruiting body development was stimulated by exposure light for 12 h a day at 85% of humidity and 20–25 °C. The formation of fungal orange buds was observed at the 14th day. When the fruiting body grew to a height of 8 cm, it could be harvested for extraction. The isolated mycelia from *C. militaris* for the bioassays were transferred to seed culture SDAY plates containing Sabouraud dextrose agar (SDA) and yeast extract by punching out about 6 mm diameter agar disks from culture grown on potato dextrose agar (PDA) plates and maintained at 25 °C in the dark for 14 days.

Extract preparation and HPLC analysis

The collected fruiting body or mycelium of *C. militaris* was dried at 50 °C and homogenized in liquid nitrogen with a pestle. One gram of sample powder was dissolved in distilled water with a ratio of solid: liquid of 1: 40 and vortexed for 30 s. The mixture was placed in a water bath with a constant temperature of 50 °C for 2 h and sonicated (power 150 W) for the first 30 min. The liquid phase was separated from the solid by centrifugation at 3500×*g* for 20 min and then filtered with 0.22 nm filter.

For HPLC analysis, the mobile phase was methanol: water (80:20, v/v) and the flow rate was 1.0 ml/min. For determination of adenosine, D-mannitol and cordycepin, a Purospher Lichro CART PP18 column (Merck) was used at 40 °C. The UV detection was at 260 nm, and the injection volume was 20 μl. The external standard method was applied. Identification of targeted components was compared with their retention time and spectrum against known standards.

Cell culture and MTT assay

The HCT116 colorectal and HL-60 leukemic cancer cell lines were a generous gift from Dr. L.F. Liu (Univ. Med. Den., NJ, USA). The PC-3 prostate and Huh7 liver cancer cell lines and BJ normal skin and WI-38 lung fibroblast cell lines were from Dr. T.-L. Shen (National Taiwan Univ., NTU, Taiwan). The other of all cell lines except HL-60 were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C and a 5% CO_2 atmosphere. The HL-60 cells were grown in RPMI Media 1640 (Gibco) medium.

Viability of control and treated cells was evaluated using MTT assay in triplicate. Cells of dimension 1×10^3 (per well) were seeded in 96-well plates containing 100 μl culture medium per well at 24 h before treatment. After 4 days of treatment with the appropriate concentrations of the hot water extract of *Cordyceps militaris*, the cells were incubated at 37 °C in 200 μl MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) solution (5 mg/ml) for 4 h. After removal of the medium and MTT, 100 μl of DMSO was added to each well for optical density (OD) reading at 570 nm using an ELISA reader (Model 680, Bio-Rad). The percent viability was calculated as (test OD/control OD) ×100.

Apoptotic bodies formation assay

For detection of apoptotic morphology and chromatin condensation, the cells were fixed after treatment with 100% methanol at −20 °C for 10 min and then washed twice with 1× PBS. The cells were stained with 10 μM of Hoechst 33342 (Sigma) in 1× PBS for 1 h and washed two more times with 1× PBS. Apoptotic morphological and nuclear changes were scored under an epi-fluorescence microscope (Nikon Eclipse 80i) with a CCD camera (Nikon DS-Ri1). Up to 200 stained cells per field were captured to examine the formation of apoptotic bodies.

DNA fragmentation assay

The fragmented DNA extract was prepared from the treated HL-60 cells. After 12 h treatment, the cells were lyzed with buffer A (1% NP-40, 20 mM EDTA, 50 mM Tris-HCl pH 7.5) and then subjected to centrifuge at 2500×*g* for 5 min to acquire the supernatant fraction containing the fragmented DNA. SDS (final conc. 1%) and RNase A nuclease (final 5 μg/μl) were added into the supernatant for 1 h at 56 °C prior to adding the proteinase K (final 2.5 μg/μl) at 37 °C for another 1 h. After ethanol precipitation, 1.5% agarose electrophoresis was performed to analyze the DNA fragments.

Immunoblotting analysis (Western blotting)

The CM_{MY} or CM_{FB} treated HL-60 cells were lyzed with 1× sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.02% bromophenol blue). The collected samples were resolved by SDS-PAGE on 8% gels, and then transferred to nitrocellulose membranes (Pall) at 330 mA for 2.5 h at 4 °C. The membranes were blocked with 5% non-fat milk in 1× TBS (Tris buffered saline, 50 mM Tris-HCl pH 7.4 and 150 mM NaCl) for 1 h at room temperature. The membranes were then washed two times with 1× TBS and incubated with the primary antibody against PARP (Cell Signaling), cleaved caspase 3 (Cell Signaling) or GAPDH (Cell Signaling) for 12 h at 4 °C. The membranes were then washed three times with 1× TBS containing 0.2% Tween 20 and then incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse

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