Induction of Apoptosis by *Cordyceps militaris* through Activation of Caspase-3 in Leukemia HL-60 Cells

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*Cordyceps militaris* is a traditional herbal ingredient frequently used for tonic and medicinal purposes in eastern Asia. The hot water extract of its cultivated fruiting bodies demonstrated a potent cytotoxic effect against the proliferation of the human promyelocytic leukemia cell HL-60, with an IC_{50} of 0.8 mg/ml for a 12-h treatment. It induced the characteristic apoptotic symptoms in the HL-60 cells, including DNA fragmentation and chromatin condensation, occurring within 12—16 h of treatment at a dose of 1 mg/ml. The activation of caspase-3 and the specific proteolytic cleavage of poly (ADP-ribose) polymerase were detected during the course of apoptosis induction. These results indicate that the hot water extract of *Cordyceps militaris* fruiting bodies inhibited cancer cell proliferation by inducing cell apoptosis through the activation of caspase-3, and that the *Cordyceps militaris* extract may therefore have therapeutic potential against human leukemia.

Key words *Cordyceps militaris*; apoptosis; caspase-3; cytochrome c; HL-60; poly (ADP-ribose) polymerase (PARP)

Apoptosis is characterized by a series of distinct morphological changes including cell shrinkage, membrane blebbing, nuclear breakdown, and DNA fragmentation, and is essential for the maintenance of tissue homeostasis and the elimination of harmful cells. An apoptotic death stimulus activates caspases, the major executors of this process, either directly or via the activation of the mitochondrial death program. The mitochondria-mediated pathway involves an increase of mitochondrial permeability and the release of cytochrome c into the cytosol. Cytochrome c promotes the activation of caspase-3 in the cytosol, resulting in the cleavage of an inactive 32 kDa precursor into a 17 kDa and an 11 kDa subunit, which dimerize to form an active enzyme. During the progression of apoptosis, caspase-3 is one of the strongest candidates for the cleavage of 116 kDa native poly (ADP-ribose) polymerase (PARP) into an 86 kDa and a 36 kDa fragment. The cleavage of this PARP protein ultimately results in cellular, morphological, and biochemical alterations of apoptosis.

*Dong-Chong-Xia-Cao* in Chinese, which translates as “winter worm and summer grass,” is an entomogenous fungus that colonizes the larvae or pupae of insects. It includes many different genera, such as *Cordyceps*, *Paeclomyces*, *Torrubiella*, and *Podonectria*. The entomopathogenic mycelia naturally grow in the intestine of pupa or larva in the autumn, and fruiting bodies of the fungus protrude from the body in the following summer. The fungal fruiting body that is colonized by the caterpillar host are chemically similar, have similar pharmacological properties, and are traditionally dried and consumed together.

*Cordyceps sinensis* and *Cordyceps militaris* are representative insect-born fungi of the genus *Cordyceps* used for medicinal purposes in eastern Asian countries. The known active bioactive compounds of these *Cordyceps* species include cordycepin, ophicordin, a polysaccharide, galactomannan, and l-tryptophan. The recent scientific evaluation of *Cordyceps sinensis* has confirmed this efficacy of this extract in the treatment of cardiovascular and endocrine disorders, in immune enhancement, hepatic protection, and in the inhibition of tumor growth, and in relieving fatigue and stress.

*Cordyceps militaris*, together with *Paecilomyces japonica*, another entomopathogenic fungi, has been listed as a food additive in Korea since 2000, and is often used in different types of tonic drinks sold in Korea due to its folkloric activities, which have not been verified scientifically. Although some recent studies have reported its effectiveness in inhibiting angiogenesis, cancer cell growth, and inflammation, scientific evidences of the biological activities of *Cordyceps militaris* are still very limited compared to other frequently used insect-born fungi of the *Cordyceps* species, such as *Cordyceps sinensis*. It was previously reported by these authors that the hot water extracts of *Cordyceps militaris* of silkworm pupa (CMP) or silkworm larva (CML) exhibited an anti-proliferation effect on tumor cells as well as a life prolongation effect in mice bearing ascitic tumors. Moreover, both CMP and CML showed tumor growth inhibitory and immunomodulatory activities in ICR mice bearing sarcoma-180 solid tumors. Despite the fact that there are ample evidences of its antitumor activities *in vitro* and *in vivo*, the working mechanism of *Cordyceps militaris* remains unclear. Therefore, the purpose of this study was to investigate whether the hot water extract of *Cordyceps militaris* affects the apoptosis of human leukemia cells (HL-60) through the activation of caspase-3, which might explain one of its action mechanisms underlying the growth inhibition of cancer cells.

**MATERIALS AND METHODS**

**Preparation of Hot Water Extract of *Cordyceps militaris*** The fruiting bodies of *Cordyceps militaris* (500 g) were washed with distilled water and then boiled with 4 l of water at 100 °C for 6 h. The insoluble materials were removed through centrifugation at 10000×g for 30 min at 4 °C, and the resulting supernatants were freeze-dried. The dried hot water extract of *Cordyceps militaris* was dissolved in distilled water and was sterilized with a 0.45 μm syringe filter

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before it was used for cell culture.

**Cell Culture** The human myeloid leukemia cell line, HL-60, was obtained from American Type Culture Collection (ATCC). The cells were grown in 90% RPMI 1640 and 10% fetal bovine serum (GIBCO BRL, Grand Island, NY, U.S.A.), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were seeded at 5×10^5 cells/ml in a 100-mm petri dish, and were grown at 37°C under a humidified, 5% CO₂ atmosphere.

**Cell Viability Analysis** MTT assay was conducted, as described previously. The HL-60 cells were seeded at 1×10^6 cells/well in a 96-well plate, and were treated with the appropriate concentrations of the hot water extract of *Cordyceps militaris* for 12 h. Then, a 20-MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, St. Louis, MO, U.S.A.) solution (5 mg/ml) was added to each well and was incubated at 37°C for 4 h. The cell suspension was then centrifuged at 1000×g for 5 min, and the cells from each well were solubilized with 100 μl DMSO for optical density (OD) reading at 570 nm. The percent viability was calculated as (test OD/control OD)×100.

**Morphological Analysis** The HL-60 cells were treated with the hot water extract of *Cordyceps militaris* (1 mg/ml) for 16 h, were washed with ice-cold phosphate-buffered saline (PBS), pH 7.4, and were placed onto sterile microscope slides using a cytospin. The cells were fixed with ethanol and were stained with Giemsa. The morphology of the cells was examined using the inverted microscope (Axiovert 135, Carl Zeiss, Oberkochen, Swiss).

**DNA Extraction and Electrophoresis** The HL-60 cells (5×10^5 cells/ml), treated with various concentrations of the hot water extract of *Cordyceps militaris* for different periods, as described in the figure legends, were harvested and were washed with ice-cold PBS, pH 7.4. The cells were lysed with the digestion buffer (pH 7.5) containing 0.5% SDS, 25 mM Tris–HCl, 0.5% mg/ml proteinase K, and 5 mM EDTA at 55°C for 3 h. After extracting the cell lysates with phenol–chloroform (1:1) and chloroform, DNA was precipitated and was washed with 100% ethanol, and was stained with Ethidium bromide (Sigma, St. Louis, MO, U.S.A.) at 37°C for 30 min. Approximately 15 μg DNA was electrophoresed on a 1.5% agarose gel, and was visualized with ethidium bromide staining under UV light.

**Detection of Cytochrome c Release** Mitochondrial and cytosolic fractions were prepared by resuspending the cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 8 μg/ml aprotinin, and 2 μg/ml leupeptin; pH 7.4). The cells were made to pass through a needle (1 ml; 0.45×13 mm; 26 G×1/2) ten times. Unlysed cells and nuclei were pelleted through centrifugation at 1000×g at 4°C for 10 min. The supernatant was spun at 10000×g for 15 min at 4°C. This pellet was resuspended in buffer A, and represents the mitochondrial fraction. The supernatant was subjected to the next centrifugation at 100000×g for 1 h at 4°C, and the supernatant from the final centrifugation represents the cytosolic fraction. Both the mitochondrial and cytosolic fractions (20 μg protein) were electrophoresed on a 15% SDS-PAGE gel, and were then analyzed with the use of Western blot. The primary antibody was rabbit monoclonal anti-human cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and the secondary antibody was anti-rabbit peroxidase-conjugated secondary immunoglobulin G antibody (Santa Cruz Biotechnology).

**Analysis of Caspase-3 and PARP Cleavage** The HL-60 cells were treated with the hot water extract of *Cordyceps militaris*, as described in the figure legends. At indicated times, the cells were washed with PBS and were lysed (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 1% NP-40, and 10 μg/ml aprotinin). The cell lysates were centrifuged at 10000×g for 30 min at 4°C, and the protein content of the cytosolic fraction (supernatant) was measured by using a modified Bradford assay kit (Bio-Rad, San Diego, CA, U.S.A.). The cytosolic fraction (50 μg protein) was mixed with 5× sample buffer containing 0.5 M Tris–HCl (pH 6.8), 25% mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixture was boiled at 95°C for 5 min, and was subjected to 10% SDS-PAGE at a constant current of 20 mA. Following electrophoresis, the proteins on the gel were electro-transferred onto a nitrocellulose membrane (Amersham, Arlington Heights, IL, U.S.A.) with the use of a transfer buffer composed of 25 mM Tris–HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membrane was blocked with the use of the blocking solution containing 20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide. The blots were probed with the use of rabbit monoclonal anti-human caspase 3 (Upstate, Lake Placid, NY, U.S.A.) and an anti-PARP (Santa Cruz Biotechnology) antibody. Immune complexes were detected using the anti-rabbit peroxidase-conjugated secondary immunoglobulin G antibody (Santa Cruz Biotechnology), and were visualized with the use of ECL (electrochemiluminescence) Western blotting detection reagents (Amersham).

**RESULTS**

The inhibition of cell proliferation was observed following the treatment of the HL-60 cells with various concentrations of the hot water extract of *Cordyceps militaris* for 12 h. *Cordyceps militaris* inhibited cell proliferation in a dose-dependent manner (Fig. 1). The concentration of the hot water extract of *Cordyceps militaris* required for 50% growth inhibition (IC₅₀) at 12 h was calculated as 0.8 mg/ml.

The morphological analysis of the Giemsa-stained HL-60 cells indicated that they had undergone gross morphological changes of apoptosis (Fig. 2). After a 16-h exposure to the hot water extract of *Cordyceps militaris* at 1 mg/ml, the cells showed typical apoptotic changes, including cell shrinkage, chromatin condensation, and the loss of normal nuclear architecture. *Cordyceps-militaris*-induced DNA fragmentation was demonstrated by incubating the HL-60 cells with various concentrations of the hot water extract of *Cordyceps militaris* for 24 h (Fig. 3A). The DNA ladders became apparent at 0.5 mg/ml, and became stronger as the concentration of the extract of *Cordyceps militaris* was increased (Fig. 3A). When the cells were treated with 1 mg/ml *Cordyceps militaris* hot water extract for various periods (from 0 to 24 h), DNA lad-
ders were seen after treatment periods of 12 h or longer (Fig. 3B). Therefore, the hot water extract of *Cordyceps militaris* used in the current study induced DNA fragmentation in the HL-60 cells in a concentration- and time-dependent manner.

The effect of *Cordyceps militaris* on the mitochondrial cytochrome c release into the cytosol was detected through Western blot analysis. The mitochondrial cytochrome c was released into the cytosol fraction as early as within a 12-h treatment during the *Cordyceps-militaris*-induced apoptosis of the HL-60 cells (Fig. 4). The effects of the hot water extract of *Cordyceps militaris* on the cleavages of caspase-3 and the PARP protein in the HL-60 cells were also evaluated through Western blot analysis. The hot water extract of *Cordyceps militaris* (1 mg/ml) resulted in the proteolytic cleavage of native caspase-3 (32 kDa) into a 17 kDa and an 11 kDa subunit as early as within a 12-h treatment (Fig. 4). The treatment of the HL-60 cells with the 1 mg/ml hot water extract of *Cordyceps militaris* for various periods (0—24 h) caused a time-dependent proteolytic cleavage of a native PARP protein, with an accumulation of the 85 kDa fragment and the concomitant disappearance of the full length 116 kDa protein (Fig. 5).

**DISCUSSION**

Apoptosis is a mechanistically driven form of cell death that is either developmentally regulated or activated in response to specific stimuli or various forms of cell injury. In cancer biology, it is now evident that many cancer cells circumvent the normal apoptotic mechanisms to prevent their self-destruction. Therefore, it would be advantageous to shift the balance in favor of apoptosis over mitosis in cancer prevention and chemotherapy. The HL-60 cell line has been widely used as a model system for cancer cell apoptosis and for the investigation of the mechanisms of antileukemic as well as general antitumor agents for apoptosis induction.29)

In the current study, the *Cordyceps militaris* extract inhibited the growth of human myelocytic leukemia cells, and induced apoptosis. The hot water extract of *Cordyceps militaris* fruiting bodies showed cytotoxicity on the HL-60 cells with an IC$_{50}$ value of 0.8 mg/ml. Internucleosomal DNA
fragmentation is a biochemical hallmark, and chromatin condensation is one of the early morphological changes of apoptosis. These changes result from the proteolytic cleavage of various intracellular polypeptides, which is most often caused by a family of cysteine-dependent proteases called caspases. Since there are increasing evidences that altered mitochondrial membrane permeability is linked to apoptosis, the effect of Cordyceps militaris on the mitochondrial cytochrome c release into the cytosol was evaluated. The sequential activation of caspases upstream then downstream of the apoptosis signal pathway is required for the initiation and execution of apoptosis. Caspase-3 is a major downstream effector of apoptosis, and the caspase-3-mediated proteolytic cleavage of PARP and other substrates is a critical step leading to DNA fragmentation and chromatin condensation. All these major apoptotic events were detected in the Cordyceps-militaris-treated cells in the current study.

The results of the present study coincide with those of two recent investigations reporting the anti-proliferation activities of Cordyceps militaris. The growth inhibition of U937 leukemia cells treated with the aqueous extract of Cordyceps militaris was excited through the induction of apoptosis. The regulation of several major growth-regulatory gene products, such as the Bcl-2 family expression and the caspase protease activity, was associated with Cordyceps-militaris-induced apoptosis. Furthermore, Western blotting and RT-PCR revealed that treatment with the aqueous extract of Cordyceps militaris caused a dose-dependent inhibition of cyclooxygenase-2 as well as prostaglandin E2 accumulation.

Cordyceps sinensis, another species of Chinese caterpillar fungus, has been well recognized for its antitumor activities through its induction of apoptosis as well as the characteristic events of cell apoptosis induced by the activation of caspase-3 activity and the proteolytic cleavage of PARP. A polysaccharide fraction of 410 kDa isolated from Cordyceps sinensis induced apoptosis by inhibiting the phosphorylation of Bcl-2 and Bcl-XL, anti-apoptotic Bcl-2 family members known to sequester cytosolic pro-apoptotic proteins, such as Bax.

The active ingredients responsible for the cytotoxic and apoptotic effects of Cordyceps militaris have not been identified. Cordycepin (3'-deoxyadenosine), a metabolite of Cordyceps militaris, has been shown to inhibit the growth of various tumor cells. Both Cordyceps militaris and cordycepin have anti-inflammatory and anti-angiogenic activities, which are supported by their inhibitory activity on iNOS expression. It is generally believed that most polysaccharides from the Cordyceps fungus and other medicinal fungi exert an anti-tumor effect in vivo through immunomodulation rather than direct cytotoxicity. Some studies, however, have attributed the inhibitory effect of Cordyceps sinensis on several cancer cell lines, including human leukemia K562, to components other than polysaccharides. A group of sterols isolated from Cordyceps sinensis fruiting bodies and fungal mycelia has also been recognized as toxic compounds. Therefore, different ingredients, including sterols, polysaccharides, and alkaloids, might be associated with the growth inhibition and apoptosis induction of the Cordyceps militaris extract in the HL-60 cells. The active compounds responsible for the specific molecular signaling pathways for the induction of apoptosis, however, have yet to be identified.

In conclusion, it was demonstrated in this study that the hot water extract of Cordyceps militaris strongly inhibited the growth of HL-60 human promyelocytic leukemia cells by promoting cell apoptosis through sequential activations of caspase-3 and PARP. The more precise signaling pathway by which Cordyceps militaris triggers caspase-3 activation, cytochrome c release, and the other apoptotic phenomena described herein awaits further investigation. The apoptotic induction activity of the Cordyceps militaris fungus may imply a new line of mechanisms for the anti-tumor and other health promoting effects of the Cordyceps militaris herb, and suggests possible new opportunities for its application in cancer therapy and in the treatment of autoimmune diseases.

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REFERENCES