Cordyceps sinensis Mycelium Extract Induces Human Premyelocytic Leukemia Cell Apoptosis Through Mitochondrion Pathway

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This study was to identify the signaling pathways for the induction of HL-60 cell apoptosis by *Cordyceps sinensis* mycelium extract (CSME). CSME at 25 μg/ml induced nuclear fragmentation and DNA degradation, two hallmark events of apoptosis, in the HL-60 cells within 12–24 hrs of treatment. Concomitantly, several major events in the mitochondrial signal pathway occurred, including the loss of MTP ($\Delta\Psi_m$), cytochrome *c* release into the cytoplasm, the decrease in Bcl-2 protein level, the translocation of Bax protein from cytoplasm into mitochondria, and the activation of caspase-2, -3, and -9, but caspase-8, the initiator caspase in the death receptor pathway, was not activated. These results suggest that CSME induces apoptosis in HL-60 cell through the mitochondrial pathway rather than the death receptor pathway. Exp Biol Med 232:52–57, 2007

Key words: *Cordyceps sinensis*; HL-60 cell; caspases; mitochondria; cytochrome *c*; Bcl-2 proteins

Introduction

Cordyceps sinensis (Cs), the Chinese caterpillar fungus, is a famous and highly valued medicinal fungus (mushroom) in Chinese herbal medicine. Cs has been used in China as a tonic and an herbal medicine for longevity, endurance, and vitality since ancient times, and its medicinal value has gained increasing worldwide attention in more recent years (1, 2). Cs has been shown to have multiple pharmacological activities, and one of the most notable is the antiproliferative activity on a variety of tumor cells (3, 4). Cs has also been used as an antitumor herb in Chinese medicine and as an adjuvant of chemo- and radiotherapy for treatment of various cancers (5). While immunomodulation has been generally considered as the most possible mechanism for the beneficial effects of Cs to cancer therapy, more recent studies have shown that Cs constituents have the ability to induce the apoptosis of malignant cells (6). For example, a sterol compound H1-A isolated from Cs fruiting body suppressed the proliferation of human mesangial cells and promoted apoptosis (7), and a fraction R obtained by supercritical CO_2 extraction of Cs has been shown to inhibit the growth and induce the apoptosis of colorectal and hepatocellular cancer cells (8).

Apoptosis plays an important role in developmental processes by eliminating unwanted cells so as to maintain homeostasis in healthy tissue. Deregulated cell proliferation and suppressed cell death together provide the physiological foundation for neoplastic progression (9). On the other hand, a wide variety of chemotherapeutic agents have been shown to cause the death of cancer cells by inducing apoptosis (10, 11). Two principal signal pathways have been established for the induction of apoptotic cell death, the death receptor pathway and the mitochondrial pathway (12). The death receptor pathway (also known as the extrinsic pathway) begins with the ligation of cell surface death receptors such as Fas at the plasma membrane, followed by the recruitment of the adapter protein Fas-associated death domain (FADD) and the activation of caspase-8, which activates the downstream effector caspases -3 and -7, and then -6 (13). The mitochondrion-mediated pathway (the intrinsic pathway) begins with the disruption of mitochondrial transmembrane potential (MTP) $\Delta\Psi_m$ and

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the release of apoptogenic factors (proteins) such as cytochrome *c* from the intermembrane space into the cytosol (14–16). These factors activate caspase-9, which in turn activates the executioner caspase-3. In this pathway, mitochondrion is the center of cell death control, and the mitochondrial membrane is the primary site of action by proapoptotic and antiapoptotic factors such as members of the Bcl-2 family of proteins.

We have shown previously that the ethyl acetate extract of Cs fungal mycelium (CSME) induced several characteristic symptoms in human premyelocytic leukemia HL-60 cells, including DNA fragmentation, chromatin condensation, the activation of caspase-3, and the specific proteolytic cleavage of poly ADP-ribose polymerase (17). In addition, CSME exhibited strong antitumor activity against B16 melanoma in C57BL/6 mice (18). Although a few studies have demonstrated the ability of Cs constitutes to induce apoptosis in malignant cells, the associated signal pathways still have not been characterized. The present study aims to detect the signal pathway mediating the CSME-induced apoptosis in the HL-60 cells and the involvement of some major Bcl-2 family proteins.

Materials and Methods

***Cordycpes sinensis* Fungal Mycelial Cultivation and Extract Preparation.** The Cs fungus used in this study was isolated from the fruiting body of a wild Cs organism collected on the high plateau at 4000–4500 m above sea level in the western Sichuan Province of China. The mycelium for the bioassays was obtained from liquid culture (at 25°C) after 7 days of cultivation, and was dried at 50°C. The dried fungal mycelium was extracted sequentially by petroleum ether and ethyl acetate (EtOAc), both at 1:10 w/v and room temperature (~24°C) for 24 hrs with constant shaking. The liquid phase was separated from the solid by filtration and concentrated by evaporation under vacuum. For the following bioassays, the EtOAc extract was redissolved in EtOH at 20 mg/ml, diluted with the culture medium, and then filter-sterilized. More details of these procedures have been given elsewhere (17).

Cancer Cell Culture and Drug Treatment. The HL-60 cell line used in this work was obtained from the American Type Culture Collection (Rockville, MD) and maintained on RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 25-cm^2 culture flasks at 37°C in humidified atmosphere with 5% CO_2. For the drug treatment experiments, the cells were harvested from the culture during the exponential growth phase and seeded into 6-well plates at 5×10^5 cells/well filled with fresh culture medium containing the CSME preparation or an equal volume EtOH as a control. The CSME dose was fixed in all tests at 25 μg/ml (final concentration in culture), which was chosen based on our previous study (17). After incubation under the normal culture conditions for selected time intervals, the cultures were harvested by centrifuging at 300 *g* for 3 mins in order for the various bioassays as follows. In the tests for the effect of specific caspase inhibitors on CSME-induced apoptosis, the inhibitors were added to the culture medium together with the CSME at a low concentration nontoxic to HL-60 cells according to the supplier's instructions (BioVision, Inc., Mountain View, CA).

Detection of Apoptotic Changes, Chromatin Condensation and DNA Fragmentation. For detection of apoptotic chromatin condensation, the HL-60 cells after drug treatment were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) solution for 10 mins and then washed with PBS solution. The cells were stained with 2 μ*M* Hoechst 33258 (Sigma, St. Louis, MO) in PBS for 10 mins and observed under a fluorescence microscope (19). For DNA fragmentation analysis, the DNA extract of the cells was prepared using miniscale apoptosis DNA isolation kit from Watson Biotechnologies, Inc. (Shanghai, China) according to the supplier's manual and was then loaded onto a 1.8% agarose gel.

Detection of MTP Disruption. The treatment-induced disruption of mitochondrial transmembrane potential $\Delta\Psi_m$ in cells was detected using the mitochondrion-specific probe chloromethyl-X-Rosamine (CMXRos, Mitotracker Red) from Molecular Probes, Inc. (Eugene, OR) according to the supplier's manual. Intact cells with a functional mitochondrial membrane and a normal $\Delta\Psi_m$ would take up and retain the dye within the mitochondria, where the dye molecules react with peptides and proteins to form an aldehyde-fixable conjugate exhibiting red fluorescence (20). For the assay, the cells were incubated in PBS containing 100 n*M* CMXRos for 30 mins and then fixed with 4% formaldehyde in PBS for 10 mins. Finally, the cells were rinsed twice with fresh PBS solution and observed under a fluorescence microscope.

Caspase Assays. Nine caspases (caspase-1, -2, -3, -4, -5, -6, -8, -9, and -10) were detected using the Caspase Fluorometric Substrate Set II Plus kit from BioVision Inc., which contains the 7-amino-4-trifluoromethyl coumarin–conjugated oligopeptide substrates for the specific caspases, according to the supplier's manual. The cells from each well of the 6-well culture plates were resuspended and incubated in 50 μl of ice-cold cell lysis buffer for 10 mins, then fed with 50 μl of 2× reaction buffer and 1 μl DL-dithiothreitol (DTT) solution. Each well was then fed with 5 μl of the specific caspase substrates (50 μ*M* final concentration) and incubated at 37°C for 2 hrs. The fluorescence was then detected with a fluorometer at 405 nm excitation and 520 nm emission. Treatment-induced change in caspase activities was represented by the fluorescence intensity of the treated cultures relative to that of the control.

Cytochrome *c*, Bcl-2, and Bax Assays. The HL-60 cells after the drug treatment were washed once with ice-cold PBS and then lysed in a lysis buffer (250 m*M* sucrose; 20 m*M* Hepes-KOH, pH 7.5; 10 m*M* KCl; 1.5 m*M* $MgCl_2$; 1 m*M* sodium EDTA; 1 m*M* sodium EGTA; 1 m*M* DTT; and

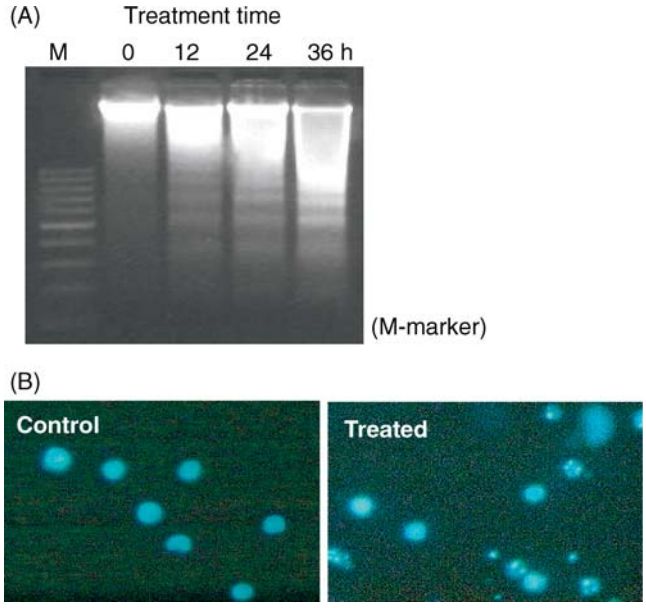

Figure 1. CSME-induced apoptotic damages in HL-60 cells. (A) DNA fragmentation and time dependence. (B) Chromatin condensation (cells stained with Hoechest 33258 and observed by fluorescence microscopy, 24 hrs treatment). Magnification: ×100. Color figure is available in the on-line version.

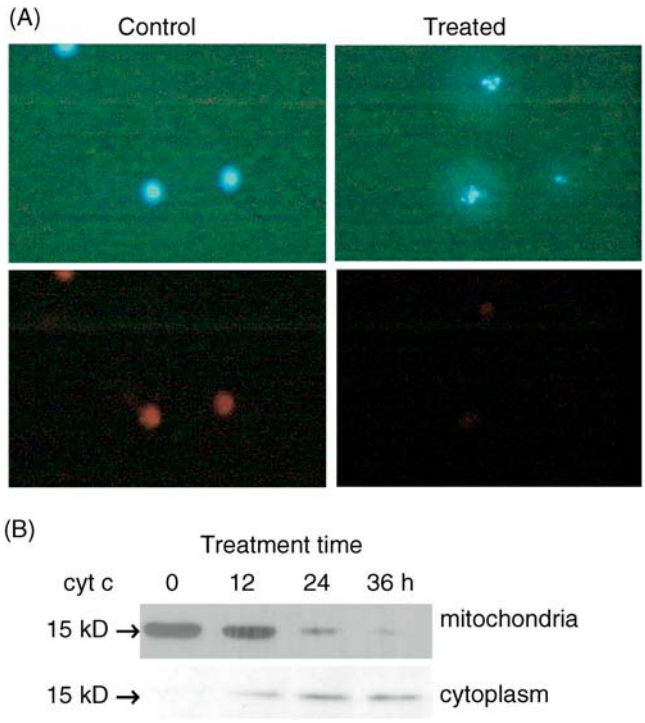

Figure 2. (A) CSME-induced changes in nuclear morphology (top row, cells stained with Hoechest 33258) and mitochondrial membrane potential (bottom row, cells stained with the membrane potential-sensitive dye CMXRos) in HL-60 cells (CSME at 25 μg/ml, 24 hrs treatment), both by fluorescence microscopy. Magnification: ×100. (B) Release of cytochrome *c* from mitochondria (decreasing) into cytoplasm (increasing) in HL-60 cells after CSME treatment (25 μg/ml) (results are representative of three separate experiments). Color figure is available in the on-line version.

0.1 m*M* phenylmethylsulfonyl fluoride, PMSF) (5×10^6 cells in 500 μl) with a homogenizer. The homogenate was then centrifuged twice at 750 *g* for 10 mins at 4°C, and the supernatant recovered was centrifuged at 14,000 *g* for 15 mins at 4°C. The supernatant (cytoplasmic extract) and pellet (mitochondrial extract) were applied to Western blotting analysis for cytochrome *c* (21).

Whole cell lysate was prepared using 150 μl extraction buffer consisting of 100 μl solution A (50 m*M* glucose, 25 m*M* Tris–HCl, pH 8, 10 m*M* EDTA, 1 m*M* PMSF) and 50 μl of solution B (50 m*M* Tris–HCl, pH 6.8, 6 *M* urea, 6% 2-mercaptoethanol, 3% SDS, 0.003% bromphenol blue). Bcl-2 in the whole cell lysate, and Bax expression levels in all above three extracts, were detected by Western blot with the primary antibodies, anti-Bcl-2, anti-Bax (BD Biosciences, San Jose, CA), and mouse anticytochrome *c* (Zymed Laboratories Inc., San Francisco, CA). The blots were developed by BM chemiluminescence Western blot kit (mouse/rabbit) (Roche, Mannheim, Germany).

Statistical Analysis. All treatments were performed in triplicate and the results expressed as their mean ± SEM. The statistical significance of treatment effects was evaluated by the Student's *t* test at a probability limit of $P < 0.05$.

Results

CSME-induced Apoptosis in HL-60 Cells. In the cultures treated by CSME at the selected dose of 25 μg/ml, DNA fragmentation (exhibited as ladders on an agarose gel, Fig. 1A) and chromatin condensation (Fig. 1B) could be observed in most of the cells within 24 hrs of treatment, which confirmed the CSME-induced apoptosis in the HL-60 cells as reported previously (17).

MTP Disruption and Cytochrome *c* Release. As expected, the intact cells from the control culture stained with the membrane potential-sensitive dye exhibited red fluorescence (Fig. 2A), which was virtually invisible (within 24 hrs) in the CSME-treated cells, indicative of the $\Delta\Psi_m$ loss in these cells. Concomitantly, the cytochrome *c* concentration gradually increased in the cytoplasm but decreased in the mitochondria of the CSME-treated cells at 12–36 hrs after treatment (Fig. 2B), indicative of the cytochrome *c* release from the mitochondrial membrane into the cytoplasm of the treated cells. These results suggest that MTP disruption was an early event during the induction of HL-60 cell apoptosis by CSME.

Changes in Bcl-2 and Bax Expression. After the CSME treatment, the expression level of Bcl-2 protein was downregulated in a time-dependent manner from 12 hrs to 48 hrs, while the Bax protein level showed no significant change in the whole cell lysates (Fig. 3A). The Bax level in the mitochondria showed a gradual increase and that in the cytoplasm showed a gradual decrease during 36 hrs of treatment (Fig. 3B), suggestive of Bax translocation from cytoplasm to mitochondria in the treated cells.

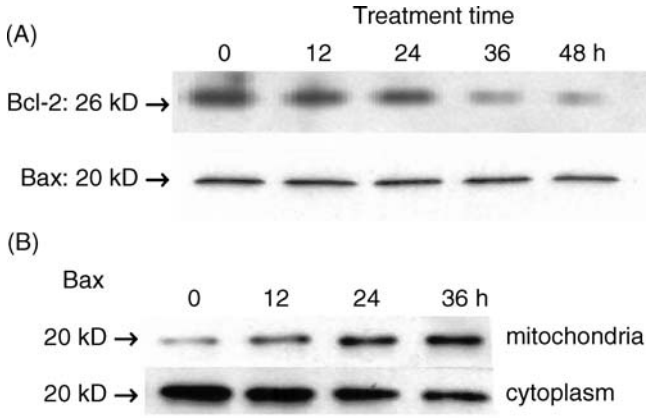

Figure 3. Changes in the expression levels of Bcl-2 and Bax proteins in HL-60 cells after CSME treatment (25 μg/ml), detected by Western blotting. (A) Bcl-2 and Bax protein levels in total cell lysates. (B) Bax protein levels in the mitochondria and cytoplasm. Results are representative of three separate experiments.

Involvement of Specific Caspases in CSME-induced Apoptosis. Compared with their activities in the untreated cells (at 0 hrs), caspase-2, -3, -4, and -9 activities in the cells showed notable activation or significant increases, and caspase-1 activity showed a slight decrease or suppression in 6–12 hrs after CSME treatment

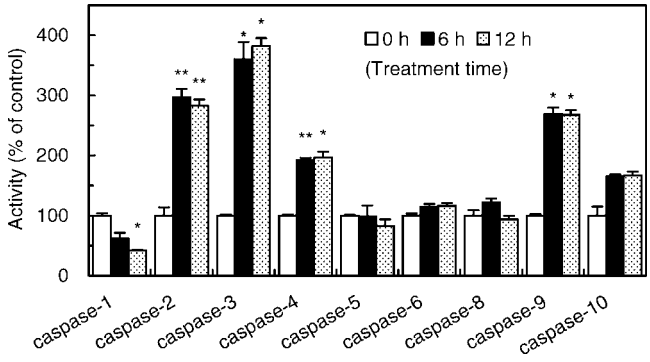

Figure 4. Activities of major caspases in HL-60 cells after CSME treatment (25 μg/ml) relative to those of untreated cells in the control (*, significant at $P < 0.01$; **, significant at $P < 0.05$ for the treatment-induced changes; error bars for SE, $n = 3$).

(Fig. 4). However, caspase-8 and the other caspases, -5, -6, and -10, were not activated after 12 hrs of treatment.

The specific inhibitors of caspase-2 (Z-VDVAD-FMK) and caspase-9 (Z-LEHD-FMK), and the general pan-caspase inhibitor (Boc-D-FMK) fed together with CSME to the HL-60 cell cultures all prevented the CSME-induced apoptotic damages, DNA fragmentation (Fig. 5A) and chromatin condensation (Fig. 5B, top row). The CSME-

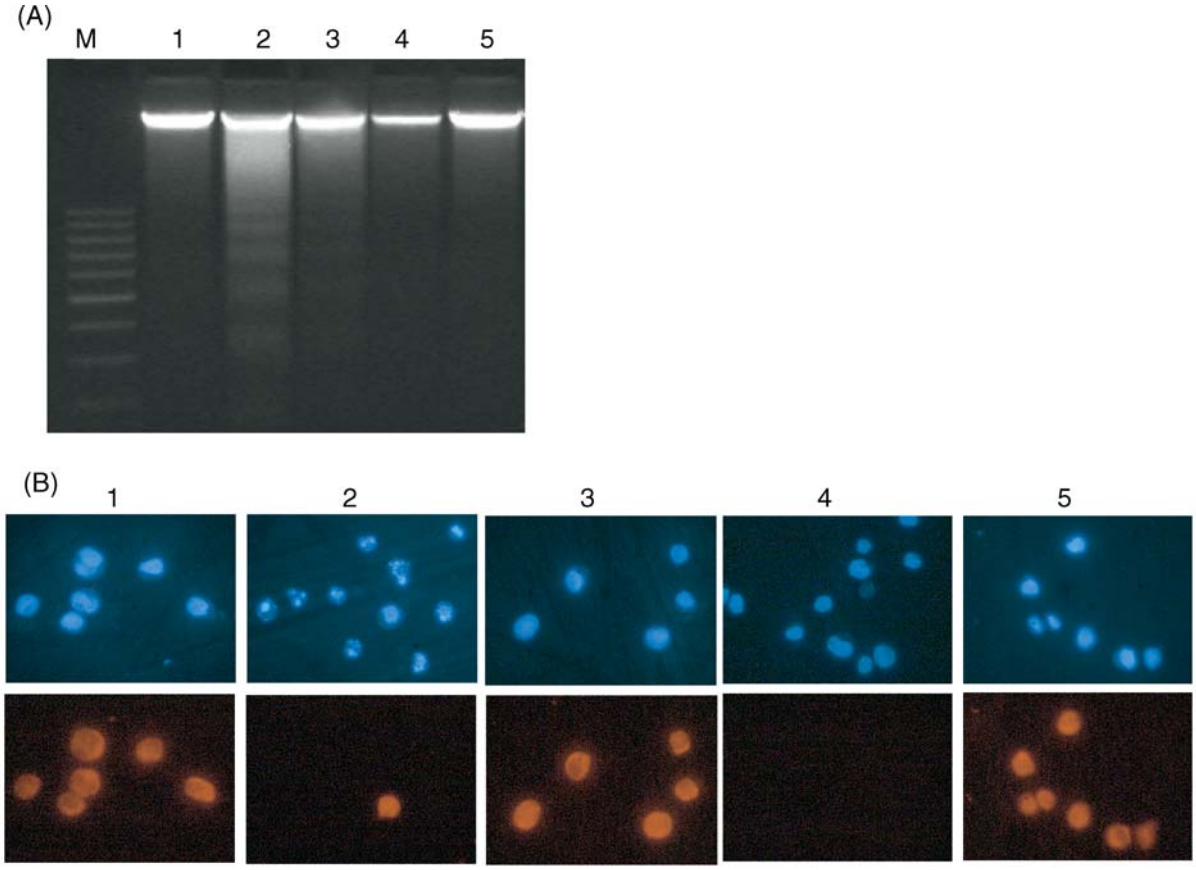

Figure 5. Effects of caspase inhibitors on CSME-induced DNA fragmentation (A) and MTP loss (B) in HL-60 cells (CSME at 25 μg/ml and all caspase inhibitors at 5 μ*M*, 12 hrs treatment). Lane 1, control; 2, CSME; 3, CSME+Z-VDVAD-FMK, caspase-2 specific inhibitor; 4, CSME+Z-LEHD-FMK, caspase-9 specific inhibitor; 5, CSME+Boc-D-FM, pan-caspase inhibitor; (B) top row: cells stained with Hoechest 33258, and bottom row: cells stained with CMXRos, both by fluorescence microscopy. Magnification: ×100. Color figure is available in the on-line version.

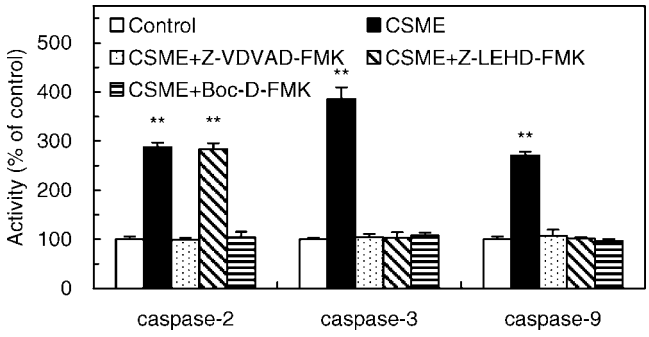

Figure 6. Effects of caspase inhibitors on CSME-induced caspase activities in HL-60 cells (CSME at 25 μg/ml and all caspase inhibitors at 5 μ*M*, 12 hrs treatment; error bars for SE, $n = 3$; **, significant at $P < 0.05$).

induced MTP disruption was prevented by both the caspase-2 inhibitor (Fig. 5B, lane 3) and the pan-inhibitor (Fig. 5B bottom row, lane 5), but not by the caspase-9 inhibitor (lane 4). These results suggest that activations of caspase-2 and -9 were essential for activating the CSME-induced apoptotic process in HL-60 cells, with caspase-2 activation occurring upstream and caspase-9 activation downstream to the MTP loss. Besides, the effective blockage of these CSME-induced changes by the pan-caspase inhibitor further confirmed the treatment-induced apoptosis in the cells.

On the other hand, the caspase-2 inhibitor blocked the CSME-induced activation of caspase-3 and -9, while the caspase-9 inhibitor blocked the CSME-induced activation of caspase-3 activity but not caspase-2 (Fig. 6). This suggests that the signal sequence was from caspase-2 down to caspase-9, and then caspase-3.

Discussion

The ethyl acetate extract of CSME at a low dose induced the apoptosis in HL-60 cells within 12–24 hrs of treatment. This is similar to the effect observed in our previous study (17), once again confirming the potent apoptosis-inducing capacity of the Cs mycelium extract. More importantly, the present study has detected the activation of several signal events in the mitochondrial pathway and the silence of some crucial events in the receptor pathway during the apoptosis induction by CSME in the HL-60 cells. The results strongly suggest that CSME-induced apoptosis in the HL-60 cells was executed *via* the mitochondrial pathway but not the death receptor pathway. In addition, the effective blockage of the CSME-induced $\Delta\Psi_m$ loss and caspase-3 activation by the specific caspase-9 inhibitor suggests that the activation of caspase-9 was downstream of MTP disruption but upstream of caspase-3 activation. Moreover, the blockage of CSME-induced apoptosis and $\Delta\Psi_m$ loss by the caspase-2 inhibitor suggests that caspase-2 played a pivotal role in the CSME-induced apoptosis, and its activation occurred upstream of the MTP disruption. This is in agreement with the fact that the activation of caspase-2 is required for the permeabilization of mitochondria induced by cytotoxic stress, as shown previously (22).

The mitochondrial pathway is regulated by several Bcl-2 family proteins, including the antiapoptotic member Bcl-2 and the proapoptotic member Bax (16). It has been shown that the downregulation of Bcl-2 mRNA and protein is involved in the regulation (or inhibition) of Bax translocation from cytoplasm to mitochondria and cytochrome *c* release during apoptotic cell death (14, 23). In a previous study (7), inhibition of the tyrosine phosphorylation of Bcl-2 and Bcl-XL was proposed as a possible mechanism for the apoptosis in mesangial cells induced by an H1-A compound isolated from Cs fruiting bodies. Our present study showed that the Bcl-2 protein expression was downregulated and the Bax protein was translocated from cytoplasm to mitochondria in the HL-60 cells after CSME treatment. This suggests that the Bcl-2 content decrease in the cells may be an essential condition for the Bax translocation and cytochrome *c* release during the apoptosis induction in the HL-60 cells by CSME.

In conclusion, our present study has identified the mitochondrion signal pathway mediating the apoptosis induction in HL-60 cells by CSME. However, some important issues remain unresolved about the apoptotic effect of CSME, particularly about which constituents of the medicinal fungus, pure compounds or complex mixtures, are responsible for its apoptotic effect, and how these constituents interact with the cancer cells to trigger the apoptosis signals. Resolving these issues will help establish the physiological mechanisms for the antitumor functions of a valuable and traditional medicinal fungus and may also help develop more effective herbal remedies from the fungal species for the treatment of malignant tumors.

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