Available online at www.sciencedirect.com

SCIENCE DIRECT

Life Sciences 75 (2004) 2911–2919

Life Sciences

www.elsevier.com/locate/lifescie

Induction of HL-60 apoptosis by ethyl acetate extract of Cordyceps sinensis fungal mycelium

Qiaoxia Zhang[a,b], Jianyong Wu[b,*], Zongding Hu[a], Duan Li[b]

[a]College of Chemical Engineering, Tianjin University, Tianjin, China
[b]Department of Applied Biology and Chemical Technology, the Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

Received 15 March 2004; accepted 3 May 2004

Abstract

The cultivated mycelium of a Cordyceps sinensis (Cs) fungus was sequentially extracted by petroleum ether, ethyl acetate (EtOAc), ethanol and water. The EtOAc extract showed the most potent cytotoxic effect against the proliferation of human premyelocytic leukemia cell HL-60, with an ED_{50} V 25 Ag/ml for 2-day treatment. The EtOAc extract induced the characteristic apoptotic symptoms in the HL-60 cells, DNA fragmentation and chromatin condensation, occurring within 6–8 h of treatment at a dose of 200 Ag/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 suggest that the Cs mycelium extract inhibited the cancer cell proliferation by inducing cell apoptosis.

Keywords: Cordyceps sinensis; Mycelium extract; Cytotoxicity; Apoptosis; HL-60

Introduction

Apoptosis or programmed cell death is a normal physiological process serving to eliminate unwanted cells and maintain homeostasis in healthy tissue. Tumor growth is regulated by the balance between cell

* Corresponding author. Tel.: +852 27666687; fax: +852 23649932.
E-mail address: bcjywu@polyu.edu.hk (J. Wu).

0024-3205/$ - see front matter
doi:10.1016/j.lfs.2004.05.029

proliferation and apoptosis. Deregulated cell proliferation and suppressed cell death together provide the underlying platform for neoplastic progression (Evan and Vousden, 2001). It has been suggested that the loss of apoptotic control in favor of cell proliferation is responsible for prostate cancer initiation and progression (Tu et al., 1996). In turn, one essential strategy for cancer therapy is to target the lesions that suppress apoptosis in the tumor cells. It has been found that most cancer chemotherapy drugs exert cytotoxic effects on malignant cells by inducing apoptosis (Kaufmann and Earnshaw, 2000).

In the search for new cancer therapeutics, the herbs being used in traditional medicines for cancer treatment are promising candidates. Cordyceps sinensis (Cs), the Chinese caterpillar fungus, has been widely used as a tonic food and herbal medicine since ancient times. Although Cs has been mainly used in traditional medicine to protect and strengthen the lung and kidney, it has shown remarkable antitumor activities in several in vitro and in vivo studies (Yamaguchi et al., 1990; Bok et al., 1999; Huang et al., 2000). Cs has also been recommended for cancer prevention and treatment or as an adjuvant drug of cancer chemotherapy (Zhu et al., 1998; Ji, 1999). In most cases, the antitumor effects of Cs and its beneficial effect to cancer therapy have been attributed to its health-protecting and immonomodulatory functions. Recently, a sterol compound H1-A isolated from Cs has been shown to inhibit autoimmune disease in MRL lpr/lpr mice, and promote apoptosis and to suppress the proliferation of human mesangial cells (Yang et al., 1999, 2002).

Wild Cs herb is a complex of a fungal fruiting body and a caterpillar. It is a rare natural species and cannot meet the increasing demand for tonic and medicine uses. Therefore, the cultivated mycelium of Cs fungal species produced by liquid fermentation has become widely used as a substitute of the wild Cs herb. In the present study, we examined the cytotoxic effects of Cs mycelium on HL-60 (human premyelocytic leukemia) cells in culture and the characteristic events of cell apoptosis induced by the mycelium extract, chromatin condensation, DNA fragmentation, activation of caspase-3 activity and proteolytic cleavage of poly ADP-ribose polymerase (PARP). The HL-60 cell line has been widely used as a model system for testing anti-leukemic as well as general anti-tumor agents (Guth et al., 1995) and in studies on induction and mechanisms of cancer cell apoptosis (Kaufmann, 1989; Studzinski, 1999).

Materials and methods

Cultivation of Cordyceps sinensis fungus

The Cs fungus was isolated from the fruiting body of a wild Cs herb collected on the high plateau of 4000–4500 m above sea level in the western Sichuan Province of China. This fungus was stored at 4 °C on slants of potato-dextrose-agar medium and subcultured every 6 months. The mycelium for bioassays was obtained from liquid culture on a medium containing 10 g/l peptone, 40 g/l glucose, 3 g/l yeast extract, 0.5 g/l $MgSO_4$ and 1 g/l KH_2PO_4, in Erlenmeyer flasks on a shaking incubator at 260 rpm and 25 °C. The mycelium was harvested by filtration after 7 days of cultivation, and dried at 80 °C to constant weight.

Preparation of mycelium extracts

The dried fungal mycelium (10 g) was ground into a fine powder with an electrical mill and then extracted sequentially from non-polar to polar solvents by petroleum ether (PE), ethyl acetate

(EtOAc), ethanol (EtOH) and water (1:10 w/v for all solvents) as illustrated by Fig. 1. The extraction by all solvents except water was performed in Erlenmeyer flasks shaking on an orbital shaker at 260 rpm and at room temperature (about 24 °C) for 24 h, and for water extraction, the solid was stirred in boiling water on a hot plate for 3 h. The liquid phase was separated from the solid by filtration and concentrated by a rotary evaporator to dryness, and then redissolved in EtOH at 20 mg/ml for the bioassays.

Cell culture

The HL-60 cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The cell culture was 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. All cells to be tested in the following assays had a passage number of 3–6.

MTT assay

HL-60 cells in exponential growth stage were harvested from culture by centrifuging at 180 g for 3 min, and resuspended in fresh medium at a cell density of 1×10^5 cells/ml. The cell suspension was dispensed into a 96-well microplate at 50 μl/well, to which an equal volume of complete medium containing the extract being tested was added, and maintained in a CO_2 incubator with 5% CO_2 at 37 °C. After 2 days of treatment, 50 μl of PBS solution containing 1 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-

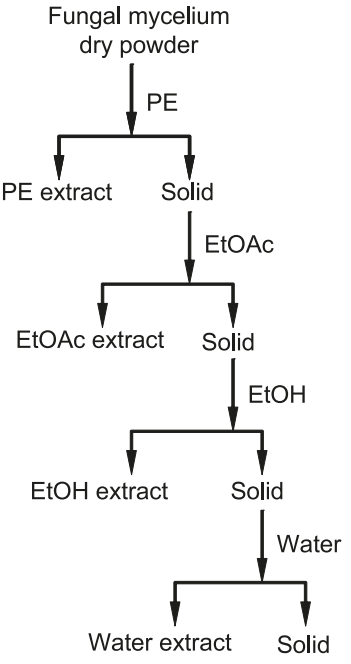

Fig. 1. Flow chart of the procedure for preparation of various extracts of Cs fungal mycelium (PE = petroleum, EtOAc = ethyl acetate, EtOH = ethanol).

2,5-diphenyl tetrazolium bromide] (Sigma Cat. M2128) was added to each well, and further incubated for 4 h. The cell suspension was then centrifuged at 720 g for 5 min, and the cells from each well were solublilized with 100 μl DMSO for optical density reading at 570 nm.

DNA fragmentation analysis

The cells in the culture flasks were treated with fungal extracts during exponential growth. The DNA was extracted from the cells using mini-scale apoptosis DNA isolation kit from Watson Biotechnologies, Inc. (Shanghai, China) according to the supplier's manual, and then 10 μl of the extract was loaded onto a 1.8% agarose gel.

Caspase-3 activity assay

The cells were seeded in 6-well plates at 5×10^5 cells/well and treated with mycelium extracts at selected doses and duration, and harvested by centrifuging at 300 g for 3 min. The cells were resuspended and incubated in 200 μl DL-dithiothreitol (DTT) solution for 1 min on ice, and the suspension was then spun down at 1800 g for 15 min to collect the supernatant for assay. Caspase-3 activity of the supernatant was detected with an assay kit from Roche (Mannheim, Germany) according to the supplier's manual. In brief, the anti-caspase-3 coating solution was added to a microtiter plate (MTP) at 100 μl per well, and the MTP was tightly covered with an adhesive foil cover and incubated at 37 °C for 1 h. After removal of the coating solution, the MTP was treated by 200 μl of the blocking buffer, and then loaded with the sample supernatant at 10 μl per well and incubated at 37 °C for 1 h. The MTP was then washed thoroughly with the incubation buffer, and loaded with the substrate solution at 100 μl per well and incubated at 37 °C for 2 h. The fluorescence of the substrate solution was measured with a luminescence spectrometer at 405 nm excitation and 520 nm emission.

Analysis of PARP cleavage

The cleavage of poly ADP-ribose polymerase (PARP) was determined by Western blotting. After treatment of cells in the culture flasks with the mycelium extract, each 10^6 cells were lysed in 150 μl extraction buffer consisting of 100 μl solution A (50 mM glucose, 25 mM Tris–HCl, pH 8, 10 mM EDTA, 1 mM PMSF) and 50 μl of solution B (50 mM Tris–HCl, pH6.8, 6M urea, 6% 2-mercaptoethanol, 3% SDS, 0.003% bromphenol blue). The suspension was centrifuged at 10,000 rpm at 4 °C for 5 min, and the supernatant (10 μl each sample) was loaded onto 10% polyacrylamide gel and then transferred to microporous polyvinylidene difluoride (PVDF) membrane. Western blotting experiment was done using rabbit polyclonal anti-PARP antibody and BM chemiluminescence Western blot kit (mouse/rabbit) both from Roche (Mannheim, Germany) according to supplier's manual.

Statistics

All treatments were conducted in triplicate and the results represented as their mean ± standard error (SE). The statistical significance of all treatment effects was evaluated by the Student's *t* test at a probability limit of $p < 0.05$.

Results

Inhibition of cell growth by various mycelium extracts

PE, EtOAc and EtOH extracts all exhibited significant and dose-dependent inhibition of HL-60 cell growth within 2 days of treatment, while the water extract did not show a consistent effect on cell growth (Fig. 2). Of the four extracts, the EtOAc extract exhibited the most potent growth inhibition of HL-60 cells, with an estimated ED_{50} (dose required for 50% inhibition) of 25 μg/ml. The most effective EtOAc extract was used exclusively in the following studies on the induction of HL-60 cell apoptosis by the Cs fungal mycelium.

Chromatin condensation and DNA fragmentation in HL-60 cells

The change of nuclear morphology in the HL-60 cells after treatment with 200 μg/ml EtOAc extract for 8 h was observed under fluorescence microscopy (Fig. 3). Chromatin condensation, a specific and distinct feature of apoptotic cells, was found in the majority of treated cells.

Another characteristic event of cell apoptosis is the fragmentation of genomic DNA into integer multiples of 180 bp units producing a characteristic ladder on agarose gel electrophoresis. This event was observed in the HL-60 cells within 4–5 h after treatment with EtOAc extract at 200 μg/ml (Fig. 4) (also at 50 μg/ml, data not shown). Alternatively, this event could be induced by the extract at a much lower concentration for a longer period of treatment (5–10 μg/ml for 5 days, data not shown).

Activation of caspase-3 and cleavage of PARP

Fig. 5 shows the dose- and time-dependent activation of caspase-3 in the HL-60 cells induced by EtOAc extract treatment. Significant increase in caspase-3 activity was induced with 50–100 μg/ml extract treatment for 5 h or with 200 μg/ml extract within 2–3 h. Fig. 6 shows that the 113 kD PARP protein in normal cells was cleaved into 89 kD fragments in 4–5 h after treatment of the cells with

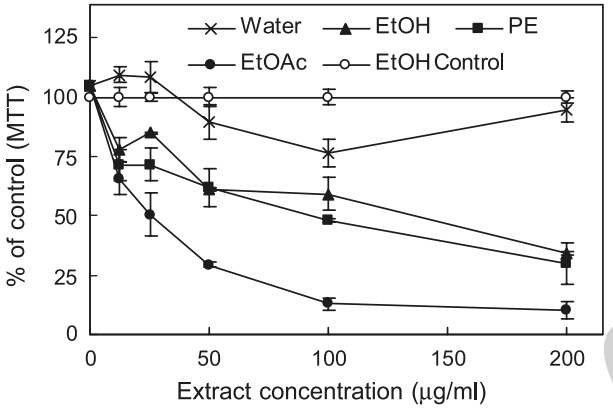

Fig. 2. Effect of various fungal mycelium extracts on HL-60 proliferation after 2 days of treatment.

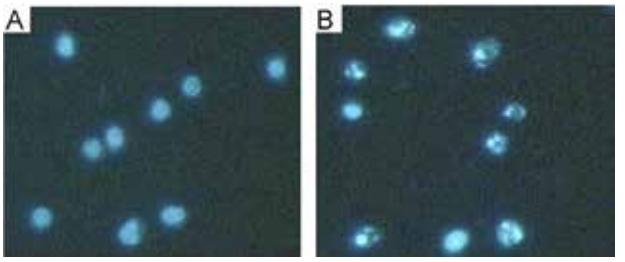

Fig. 3. Nuclear morphology of HL-60 cells in the control culture (A) and the culture treated with 200 μg/ml fungal mycelium EtOAC extract for 8 h (B). Cells were stained with Hoechest 33258 and observed under fluorescence microscopy at ×100 magnification.

EtOAc extract at 100–200 μg/ml. PARP is a nuclear enzyme involved in DNA repair process and its cleavage by caspase-3 during apoptosis disables the DNA repair.

Discussion

Cells undergoing apoptosis experience many biochemical and morphological changes in cascades (Studzinski, 1999; Kaufmann and Earnshaw, 2000). Internucleosomal DNA fragmentation is a biochemical hallmark, and chromatin condensation one of the early morphological changes of apoptosis. These changes result from the proteolytic cleavage of various intracellular polypeptides which is mostly caused by a family of cysteine-dependent proteases called caspases. The sequential activation of

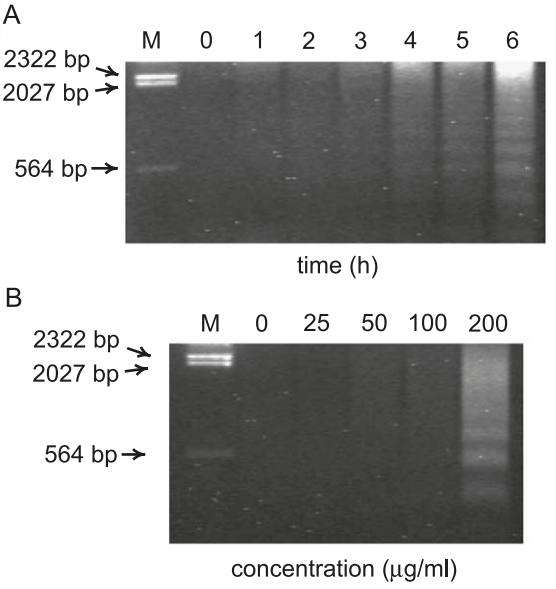

Fig. 4. Induction of DNA fragmentation in HL-60 cells by mycelium EtOAc extract. A: Time course of genomic DNA gel electrophoresis after treatment with 200 μg/ml extract; B: Genomic DNA gel electrophoresis after 5-h treatment with extract at various concentrations. Molecular size markers were run on the left lane.

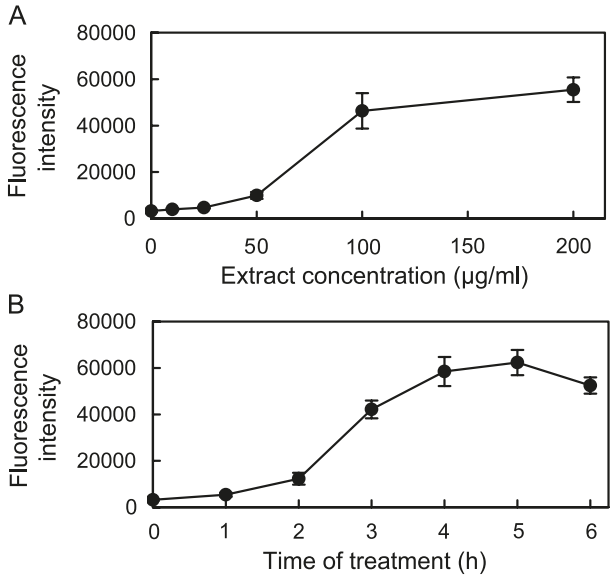

Fig. 5. Increase of caspase-3 activity in HL-60 cells after treatment with mycelium EtOAc extract, A: 5-h treatment with extract at various concentrations and B: the time course of caspase-3 activity after treatment with extract at 200 Ag/ml.

caspases from upstream to downstream of the apoptosis signal pathway is thus required for the initiation and execution of apoptosis. Caspase-3 is a major downstream effector of apoptosis, and caspase 3-mediated proteolytic cleavage of PARP and other substrates is a critical step leading to the subsequent DNA fragmentation and chromatin condensation. All these major apoptotic events were detected in the EtOAc extract treated cells. Therefore, the results confirmed that apoptosis in the HL-60 cells was induced by the mycelium extract treatment and the involvement of caspase-3 mediated PARP cleavage in the apoptosis process.

The EtOAc extract of Cs fungus mycelium has shown potent cytotoxicity on the HL-60 cells with an ED_{50} V 25 Ag/ml. At the United States National Cancer Institute, ED_{50} b 20 Ag/ml is used as a benchmark of active crude extracts suitable for screening cancer drugs from plants and herbs (

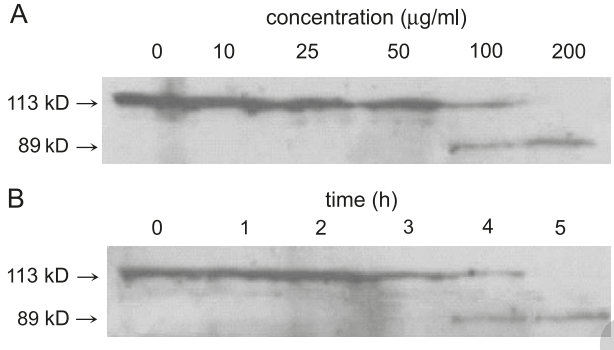

Fig. 6. Proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) in HL-60 cells after treatment with mycelium EtOAc extract, A: 5-h treatment with extract at various concentrations; B: the time course of PARP after treatment with extract at 200 Ag/ml.

and Pezzuto, 1991). Therefore, the Cs mycelium extract may be a promising source of antitumor compounds deserving further screening and activity studies. Although we still have not identified the active ingredients responsible for the cytotoxic and apoptotic effects, our qualitative chemical analysis indicated that the EtOAc extract contained ergosterols, glycosides and polysaccharides but no alkaloids, organic acids, amino acids or peptides. It is generally believed that most polysaccharides from Cs and other medicinal fungi exert an antitumor effect in vivo through immunomodulation rather than direct cytotoxicity. Some studies also have attributed the inhibitory effect of Cs on several cancer cell lines including a human leukemia K562 to components other than polysaccharides (Kuo et al., 1994). So far the only proven toxic compounds isolated from Cs fruiting bodies and fungal mycelia are a group of sterols (Bok et al., 1999; Yang et al., 2002). Therefore, sterols are the most possible ingredients responsible for the growth inhibition and apoptosis induction of the EtOAc extract in the HL-60 cells.

In conclusion, the present work has shown that the Cs fungal mycelium extracts cause strong growth inhibition of HL-60 human premyelocytic leukemia cells perhaps by promoting cell apoptosis. However, the active ingredients responsible for the observed effect and the specific molecular signaling pathways for induction of apoptosis remain to be identified. The apoptotic induction activity of Cs fungus may imply a new line of mechanism for the antitumor and other health effects of Cs herb and possible new opportunities for its application in cancer therapy and treatment of autoimmune diseases.

Acknowledgements

This work was financially supported by grants from the Hong Kong Polytechnic University (G-YD28 and ASD fund), and the Areas of Excellence Scheme established under the University Grants Committee of the Hong Kong SAR (AoE/B-10/2001 and AoE/P-10/01).

References

Bok, J.W., Lermer, L., Chilton, J., Klingeman, H.G., Towers, G.H., 1999. Antitumor sterols from the mycelia of Cordyceps sinensis. Phytochemistry 51 (7), 891–898.

Evan, G.I., Vousden, K.H., 2001. Proliferation, cell cycle and apoptosis in cancer. Nature 411, 342–348.

Huang, B.M., Chuang, Y.M., Chen, C.F., Leu, S.F., 2000. Effects of extracted Cordyceps sinensison steroidogenesis in MA-10 mouse Leydig tumor cells. Biological and Pharmaceutical Bulletin 23, 1532–1535.

Ji, Y.B., 1999. Pharmacological Actions and Applications of Anitcancer Traditional Chinese Medicines (150Cordyceps sinensis (Berk) Sacc). Heilongjiang Ke-Xue-Ji-Shu Chu-Ban-She, Ha'erbin, China (in Chinese), 494–501.

Kaufmann, S.H., 1989. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Research 49, 5870–5878.

Kaufmann, S.H., Earnshaw, W.C., 2000. Induction of apoptosis by cancer chemotherapy. Experimental Cell Research 256, 42–49.

Kuo, Y.C., Lin, C.Y., Tsai, W.J., Wu, C.L., Chen, C.F., Shiao, M.S., 1994. Growth inhibitors against tumor cells in Cordyceps sinensis other than cordycepin and polysaccharides. Cancer Investigation 12, 611–615.

Studzinski, G.P., 1999. An overview of apoptosis. In: Studzinski, G.P. (Ed.), Apoptosis: a Practical Approach. Oxford University Press, Oxford, UK.

Suffness, M., Pezzuto, J.M., 1991. Assays related to cancer drug discovery. Dey, P.M., Harborne, J.B. Methods in Plant Biochemistry vol. 6. Academic Press, London, pp. 71–133.

Suh, N., Luyengi, L., Fong, H.H., Kinghorn, A.D., Pezzuto, J.M., 1995. Discovery of natural product chemopreventive agents utilizing HL-60 cell differentiation as a model. Anticancer Research 15 (2), 233–239.

Tu, H., Jacobs, S.C., Borkowski, A., Kyprianou, N., 1996. Incidence of apoptosis and cell proliferation in prostate cancer: Relationship with TGF-b and bcl-2 expression. International Journal of Cancer 69, 357–363.

Yamaguchi, N., Yoshida, J., Ren, L.J., Chen, H., Miyazawa, Y., Fujii, Y., Huang, Y.X., Takamuta, S., Suzuki, S., Koshimura, S., Zeng, F.D., 1990. Augmentation of various immune reactivities of tumor-bearing hosts with an extract of Cordyceps sinensis. Biotherapy 2, 199–205.

Yang, L.Y., Chen, A., Kuo, Y.C., Lin, C.Y., 1999. Efficacy of a pure compound H1-A extracted from Cordyceps sinensis on autoimmune disease of MRL lpr/lpr mice. The Journal of Laboratory and Clinical Medicine 134, 492–500.

Yang, L.Y., Huang, W.J., Hsieh, H.G., Lin, C.Y., 2002. H1-A extracted from Cordyceps sinensis suppresses the proliferation of human mesangial cells and promotes apoptosis, probably by inhibiting the tyrosine phosphorylation of Bcl-2 and Bcl-XL. The Journal of Laboratory and Clinical Medicine 141, 74–83.

J.S. Zhu, G.M. Halpern, K. Jones, 1998. The scientific rediscovery of an ancient Chinese herbal medicine: Cordyceps sinensis. Journal of Alternative and Complementary Medicine 4, 289–303 (I); 429–457 (II).