Induction of Apoptosis in Human Lung Carcinoma A549 Epithelial Cells with an Ethanol Extract of *Tremella mesenterica*

Nan-Yin Chen,¹² Hsi-Huai Lai,³ Tai-Hao Hsu,¹⁵ Fang-Yi Lin,¹
Jian-Zhi Chen,¹ and Hui-Chen Lo²⁵

¹ Department of Bioindustry Technology, Da-Yeh University, 112 Shan-Jiau Rd., Datsuen, Changhua, 51505, Taiwan
² Department of Food Nutrition, Chung-Hwa University of Medical Technology, 89 Wen-Hwa 1st St., Jen-Te Hsiang, Tainan Hsien, 71703, Taiwan
³ Department of Food Science and Technology, Chung-Hwa University of Medical Technology, 89 Wen-Hwa 1st St., Jen-Te Hsiang, Tainan Hsien, 71703, Taiwan
⁴ Department of Bioscience Technology, Chang-Jung Christian University, 396 Chung Jung Rd., Sec.1, Kway Jen, Tainan, 71101, Taiwan
⁵ Department of Medical Education and Research, Changhua Christian Hospital, 135 Nanhsiao Street, Changhua, 50006, Taiwan

Received November 29, 2007; Accepted January 28, 2008; Online Publication, May 7, 2008

*Tremella mesenterica* (TM) is a common food and folk medicine widely used in several Asian countries as a tonic for the lungs. In the present study, we compared the effects of extracellular polysaccharides (EPS), intracellular polysaccharides (IPS), and ethanol extract (EE) of *Tremella mesenterica* on the induction of apoptosis into human lung carcinoma A549 epithelial cells. The EE, but not the EPS or the IPS, almost completely inhibited the growth of A549 cells. The results of Annexin V-FITC/PI staining and flow cytometric analysis indicated that the percentage of Annexin V⁺/PI⁻ cells in EE-treated cells increased to 32.8%. The results of further investigation showed a disruption of mitochondrial transmembrane potential (ΔΨm), the production of reactive oxygen species (ROS), and the activation of caspase-3 protein in EE-treated cells. These findings suggest that EE can decrease cell viability and induce apoptosis in A549 cell lines by activating a mitochondrial pathway.

Key words: *Tremella mesenterica*; apoptosis; mitochondrial transmembrane potential; reactive oxygen species; caspase-3

Apoptosis, also called programmed cell death, is a type of cell extinction regulated in an orderly manner by a series of signal cascades in certain situations, and it is an important physical process involved in regulating growth, development, and immune responses. Induction of apoptosis in tumor cells with drugs or phytochemicals might prove to be an important approach in therapy for cancer and immune-system diseases. Recently, much attention has been devoted to the anticancer activity of edible mushrooms and their constituents. Increasing data show that polysaccharides and polysaccharide-peptides derived from mushrooms can have a cytotoxic or cytostatic effect on various tumor cell lines *in vitro* by inducing cell apoptosis. Moreover, it has been found that some organic solvent extracts from mushrooms, such as ethanol extract of *Lentinula edodes*,⁵ and ethylacetate extracts of *Antrodia camphorata*,⁶ can induce various forms of carcinoma cell apoptosis.

Many studies have shown that the *Tremella* species possesses a wide range of biological activities, including cytokine-stimulating, anti-diabetic, anti-inflammatory, vascular-stimulating, hypocholesterolemic, antiallergic, and hepatoprotective effects.⁷ It has been reported that *Tremella* polysaccharides significantly inhibited cancer-cell DNA synthesis and growth in mice, but this phenomenon was not observed *in vitro*, indicating that the antitumor effect might occur by activating immune responses in the host.⁸ Limited information regarding the antitumor mechanisms of the polysaccharides and mycelium of TM is available. In our previous study, it was found that the EPS of TM from submerged cultures...
with various carbon sources stimulated the production of cytokine (IL-6 and TNF-α) and nitric oxide (NO) in RAW 264.7 macrophages.9)

Besides on immunomodulating effect, Tremella, according to the record in the best-known Chinese book of medical herbs, "Bencao Gangmu," possesses antitussive and antiasthmatic effects, and is good for the lungs.10) A549 cell lines, a type of human lung carcinoma epithelial cells, are commonly used in the study of antitumor cell models. To evaluate the antitumor activity of TM, in this study the effects of the induction of apoptosis into A549 cell lines by EPS, IPS, and EE of TM were compared.

Materials and Methods

**Microorganisms and cell lines.** *Tremella mesenterica* (TM) BCRC 36028 and human lung carcinoma (A549) (BCRC60074) cell lines were purchased from the Bioresources Collection and Research Center (BCRC) (Hsinchu, Taiwan). TM was maintained on yeast malt agar (YMA) plates. A549 cells were grown in 90% Ham’s F12K medium (Biological Industries, Kibbutz Beit Haemek, Israel) with 10% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml). The cell line was cultured at 37 °C in a humidified incubator containing 5% CO₂.

**Submerged cultures of** *Tremella mesenterica*. TM seed cultures in the flask were obtained from 500-ml Erlenmeyer flasks filled with 200 ml of YM media. The sterilized media were inoculated with liquid 7-d-old precultures (2.5% vol/vol), and were incubated at 25 °C for 7 d on a rotary shaker at 150 rpm. Fermenter cultures were obtained in 5 liter fermenters (Bio-top BTF-600T, Taichung, Taiwan) in 3 liter of medium. The fermenters were sterilized by autoclaving for 30 min at 121 °C. The sterilized media were inoculated with 150 ml (cell number adjusted to about 1.5 × 10⁹ yeast-like cells/ml) 7-d-old seed cultures. The media consisted of the following components (in g/l): xylose, 20; yeast extract, 3; malt extract, 3; peptone, 5.

**Preparation of samples.** The flow chart for the preparation of EPS, IPS, and EE samples from the submerged cultures of TM is shown in Fig. 1. The supernatant of the TM fermentation broth was mixed with 4 volumes of 95% ethanol and left overnight at 4 °C to precipitate the crude EPS. Next, the precipitated EPS was collected by centrifugation at 9,000 rpm for 15 min and lyophilized. The biomass was washed twice with distilled water and collected for extraction of IPS. The extraction procedure for IPS was the same as aforementioned, except that prior to ethanol precipitation, the washed biomass was extracted with 100 vol. of 121 °C distilled water for 30 min. The precipitate obtained from the hot-water-treated biomass was collected for extraction of EE. This precipitate was extracted with 50% (v/v) ethanol at 70–80 °C for 8 h. Then the supernatant was collected by centrifugation at 9,000 rpm for 10 min, dried with a freeze-dryer to remove residual ethanol and then lyophilized.

The EPS and IPS samples were directly dissolved in a plain F-12 medium. The solutions were sterilized using a 0.22-µm filter and further diluted with the plain culture medium to the defined concentrations, as indicated. The preparation of EE was slightly modified from the method used by Ho et al.12) Plain F-12 medium was used
to dissolve the EE extract as stock solutions of 4 mg/ml for 8 h at room temperature with continuous shaking. The insoluble material was removed by centrifugation. The supernatant was sterilized using a 0.22-μm filter and further diluted with the plain culture medium to the defined concentrations, as indicated.

**Cell viability assay.** Viability of the A549 cells was determined by MTT assay. Briefly, 8 × 10^5 cells/well of these cells was seeded into 96-well microtiter plates. The next day, the indicated concentrations of EPS, IPS, and EE were added into the cells and co-cultured for 24, 48, and 72 h. These tests were repeated 3 times. At the end of the treatment, 20 μl of 5 mg/ml MTT (Sigma, St. Louis, MO) was added to each well, and the plates incubated for 2 h at 37 °C. Finally, dimethyl sulfoxide (DMSO) (200 μl) was added to each well, after which optical absorbance at 570 nm was read on a scanning multi-well spectrophotometer plate reader. The cell viability was expressed as the optical density ratio of the treatment to control.

**Determination of apoptosis rate.** An Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA), was used to detect early apoptotic activity according to the manufacturer’s instructions, with slight modifications. The cells were seeded at 4 × 10^5 cells/ml per well in 6-well plates. After treatment with 1 mg/ml of EE, the cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 100 μl of binding buffer. A total of 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) were added and the mixture was incubated for 30 min in the dark. Finally, 400 μl of binding buffer was added to the cells, the mixture were analyzed with a Flow cytometer (Becton Dickinson Co., San Jose, CA), using an FITC signal detector (FL1), and by PI staining with a phycocyanin emission signal detector (FL2). The apoptotic percentage of 10,000 cells was determined, after which all the experiments reported in this study were performed 3 times. The data were analyzed using WinMDI 2.8 software (Scripps Institute, La Jolla, CA) for calculation of percentage cells with apoptosis per group.

**Measurement of ΔΨm.** The cells were seeded at 4 × 10^5 cells/ml per well in 6-well culture plates. After treatment with EE for 24 h, the cells were collected from the culture dish by gentle scraping, washed twice with PBS, isolated by centrifugation (1,200 rpm, 5 min), and resuspended in 1 ml of PBS, then incubated with rhodamine 123 (Rh123) (final conc. 10 μM) for 15 min at 37 °C in the dark. After staining, the cells were washed once with PBS and immediately analyzed by flow cytometry to examine rhodamine-123 fluorescence.

**Measurement of intracellular ROS.** The cultured cells were loaded with 2 μl of 2',7'-dichlorofluorescein diacetate (DCFH-DA) (20 μM final) for 30 min for the indicated times (2–8 h), washed with PBS twice, detached from the culture dish by gentle scraping, isolated by centrifugation (1,200 rpm, 5 min), resuspended in PBS, and subjected to flow cytometry.

**Western blot analysis.** The cells were seeded at 1 × 10^6 cells/ml in 35-mm cell culture dishes. After treatment with EE for the indicated times, the cells were washed with ice-cold PBS, harvested using a rubber policeman, and sedimented by centrifugation (1,200 rpm for 5 min). A buffer consisting of 50 μl of ice cold RIPA (150 mM sodium chloride, 50 mM Tris–HCl, pH 7.4, 1 mM ethylene diamine tetra acet acid, 1% sodium deoxycholic acid, and 0.1% sodium dodecyl sulfate) with freshly added protease inhibitors (1 mM sodium orthovanadate, 1 mM phenyl methyl sulfonyl fluoride, 5 μg/ml of leupeptin, 1 μg/ml pepstatin, 1 mM sodium fluoride, and 1 μg/ml of aprotinin) and a pipet was added and transferred to a microcentrifuge tube. The cell suspensions were immediately frozen at −80 °C for Western blot analysis. The cell suspension was homogenized by sonication (2 × 10 s, using a Sonicator 3000, Misonix E2-101, Misonix Inc., Farmingdale, NY) before analysis. The cellular protein concentration was measured with a BioRad protein assay kit (Bio-Rad Laboratories, Munich, Germany), using bovine serum albumin as the standard. An aliquot was saved for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Each sample of 15 μl (containing a total of 50 μg of protein) was run on 12.5% SDS–PAGE, then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Pall Co., Ann Arbor, MI). The membrane was blocked for 2 h at room temperature with a blocking reagent (Tris-buffered saline, TBS, containing 5% non-fat milk), incubated for 1 h at 4 °C with a rabbit anti-caspase-3 antibody (1:1,000) (abcam, Cambridge, UK), washed 3 times with TBS containing 0.05% (v/v) Tween-20, incubated with horseradish peroxidase-conjugated second antibodies (dilution, 1:5,000) for 1 h, and washed 4 times with gentle shaking. After the final washing, the membranes were developed with an ECL substrate and exposed to X-ray film (Kodak, Rochester, NY). All the experiments reported in this study were performed 3 times, and the results were reproducible.

**Statistical analysis.** All data are expressed as means ± SD. ANOVA was used to evaluate difference among multiple groups. If significance was observed between the groups, Dunnett's test was used to compare the means of two specific groups. p < 0.05 was considered to be significant.

**Results**

**Effects on cell viability**
To determine the effects of EPS, IPS, and EE on cell
viability, the viability of the treated A549 cells was measured by MTT assay. When the cells were exposed to EPS, IPS, and EE at a concentration of 1 mg/ml for 24, 48, and 72 h, it was found that growth of the cells was slightly inhibited by the EPS and IPS treatments (the cell viability ratio of the treatment to untreated control was 85.0-78.3% for EPS and 69.2-63.9% for IPS). On the contrary, EE (1.4-1.2%) almost completely inhibited the growth of A549 cells during the testing period (Fig. 2). When these cells were exposed to various periods and concentrations of EE, their viability was significantly inhibited in a dose- and time-dependent manner (Fig. 3). In order to compare the effect of EE on human normal cells, the cell viability of human peripheral blood mononuclear cells (PBMC) was determined by the trypan blue dye-exclusion method. When PBMC were exposed to EE at various concentrations (0, 0.125, 0.25, 0.5, and 1 mg/ml) in triplicate for 24 h, cell viability was 82.0 ± 0.4, 91.1 ± 5.4, 89.4 ± 1.1, 86.0 ± 3.3, and 81.2 ± 1.4% respectively, and there were no significant differences statistically. This result indicates that there was no effect of EE on normal human cell PBMC.

**Effects on apoptosis rate**

To investigate the effects of EPS, IPS, and EE on the induction of apoptosis in A549 cells, Annexin V-FITC/PI staining and flow cytometric measurement were used to quantify the percentages of apoptosis in the total cell population. As shown in Fig. 4, after incubation with EPS, IPS, and EE at a concentration of 1 mg/ml for 24 h, no significant differences in the percentages of early apoptosis cells (Annexin V+PI−) were observed as compared to controls, approximately 20% of the cells induced by EE were detected by measuring the absorbance at a wavelength of 570 nm. The cell survival was expressed as the optical density ratio of the treatment to control. The data represent the mean ± SD of three independent experiments.

**Effects of EE on cell morphology**

After incubation with EE (0.5 mg/ml) for different time intervals (12, 24, 32, 40, and 46 h), the cells were examined by phase contrast microscopy (Olympus, Tokyo, Japan) for evidence of morphological apoptosis induced by EE (Fig. 5). The control cells showed a typical polygonal and intact appearance (Fig. 5A), whereas the EE-treated cells displayed morphological changes with preapoptotic characteristics, such as cellular shrinkage (Fig. 5B-D), rounding (Fig. 5E), and poor adherence, as well as floating round shapes (Fig. 5F).

**Effects of EE on ΔΨm**

The alteration of ΔΨm is a crucial step occurring in cells undergoing apoptosis. Flow cytometry of the Rhodamine 123 stained cells was performed to assess ΔΨm in the human lung cells. The cells were treated for 24 h for mitochondrial studies. As indicated in Fig. 6, as compared to controls, approximately 20% of the cells shifted leftward, indicating that EE treatment induced a disruption of ΔΨm.

**Induction of production of ROS**

To determine whether an ROS is involved in EE-
induced apoptosis in A549 cells, ROS production was examined at indicated time points after EE treatment. As shown in Fig. 7, the observed ROS levels reached a maximum at about 6 h (49.6%, as compared to 0 h) in the cells after exposure to EE, but subsequently decreased.

**Effects of EE on activation of Caspase-3 protein**
Caspase family proteases are key factors in apoptosis. To evaluate further the mechanism of EE-induced apoptosis in A549 cells, the expression of the Caspase-3 protein involved in apoptosis was examined by Western blot analysis. As shown in Fig. 8, it was observed that cleavage of procaspase-3 (32 kDa) into the active form of Caspase-3 (17 kDa) occurred at about 40 h, and higher expression levels of Caspase-3 occurred at 46 h in cells after treatment with EE (0.5 mg/ml). Active 17 kDa Caspase-3 was absent from the control cells (0 h).

**Discussion**
In this study, the effects of EPS, IPS, and EE of TM on the induction of apoptosis in A549 cells were compared. As mentioned above, some studies have reported that mushroom-derived polysaccharide or poly-
saccharide-peptide has an ability to induce various forms of tumor-cell apoptosis.2–4) However, it was observed here that EE, but not EPS or IPS, of TM almost completely inhibited the growth of A549 cells during the testing period (Fig. 2). Furthermore, it was found that EE induced a significant dose- and time-dependent inhibition of cell viability (Fig. 3). Such conditions were also observed on Annexin V-FITC/PI staining and flow cytometric analysis (Fig. 4). These results suggest that polysaccharides obtained from TM do not show direct action inhibiting cancer cell growth by a cytotoxic effect. Gu and Belury5) obtained similar results, reporting that a maitake-D fraction (active component, /C12-glucan) and other /C12-glucans purified from Coriolus versicolor and Ganoderma lucidum did not show any inhibitory effects on human prostate cancer PC-3 cells or murine skin carcinoma CH72 cells. On the basis of these results and our previous work,7,8) the hypothesis is supported that polysaccharide obtained from mushrooms exerts its anti-tumor effects via a systemic effect on immunity instead of direct action inhibiting tumor cell growth.7,8) On the other hand, we should not overlook the fact that a mushroom might contain several different bioactive compounds with diverse biological activities. Differences in mushroom samples or the methods used in test sample preparation, and even different culture conditions for the same mushroom, might affect the composition and bioactivity of test samples. The different characteristics of cell lines used in the study might also be reasons contradiction results have been obtained as between studies.

Morphological changes in apoptotic characteristics, such as cellular shrinkage, rounding, poor adherence, and floating round shapes of EE-treated cells, were also observed by phase-contrasted microscopy (Fig. 5). These findings prompted us to evaluate further the changes in /C12m and in the production of ROS in the cytosol, since these two events are often associated with apoptosis.15,16) Mitochondria are sensitive to changes in the redox state of cells. Maintenance of mitochondrial membrane integrity is a dynamic process. Therefore, mitochondrial dysfunctions, including mitochondrial permeability transition, changes in /C12m, production of

Fig. 6. Effect of EE on /C12m in A549 Cells.
A549 cells (4 x 10⁶ cells/ml) treated with EE for 24 h were harvested and stained with Rhodamine 123 to determine the mitochondrial membrane potential: A, control untreated cells; B, 1 mg/ml of EE-treated cells. M1 indicates the number of cells with low Rh123 fluorescence.

Fig. 7. Effect of EE on Reactive Oxygen Species Generation in A549 Cells.
The cells were treated with 1 mg/ml of EE for different time intervals (2, 4, 6, and 8 h). ROS levels were measured by DCFH-DA (20 μM) flow cytometrically.

Fig. 8. Caspase-3 Activation in A549 Cells (1 x 10⁶ cells/ml) Treated with EE (0.5 mg/ml) at Indicated Time Intervals Detected by Western Blotting with /C12-Actin as Internal Control.
ROS, and release of cytochrome c and caspase proteins into the cytosol, can contribute to apoptosis.\textsuperscript{17)} Flow cytometry of the Rh123 staining experiments showed disruption of $\Delta \Psi_m$ in the EE-treated cells (Fig. 6), indicating that the mitochondrial apoptotic death-sIGNAL pathway plays a pivotal role in EE-induced apoptosis in A549 cells. Furthermore, the ROS levels reached a maximum at about 6 h in the cells after exposure to EE, but subsequently decreased (Fig. 7). These results indicate that ROS production is an early phase in apoptosis induced by EE. Ling et al.\textsuperscript{18)} also found that human H460 lung cancer cells exposed to bortezomib, a proteasome inhibitor, at 0.1 $\mu$m showed induction of apoptotic cell death starting at 24 h, with increasing effects after 48–72 h of treatment. After 3–6 h, an elevation in ROS generation were observed in a time-dependent manner.

Activation of the caspase cascade is a well-known molecular mechanism of the induction of apoptosis. Caspase-3, a key factor in apoptosis execution, is the active form of procaspase-3. The latter can be activated by various molecules, such as Caspase-8, Caspase-9, Caspase-10, granzyme B, and so on.\textsuperscript{17)} Further examination of the expression of the Caspase-3 protein (Fig. 8) showed that the cleavage of procaspase-3 (32 kDa) into the active form of Caspase-3 (17 kDa) occurred at about 40h; moreover, higher expression levels of Caspase-3 were clearly observed at 46 h in the cells after treatment with EE. Generally, the caspase family of proteases can be activated through two pathways, death-receptor-mediated and mitochondrion-mediated.\textsuperscript{17)} From these data, one may conclude that EE induces A549 cell apoptosis by activating the mitochondrial pathway.

In conclusion, this study indicates that exposure to EE, but not EPS or IPS, of TM can decrease cell viability and induce apoptosis in A549 cell lines. This induction of apoptosis was closely associated with increasing generation of ROS, a significant drop in $\Delta \Psi_m$, and activation of the Caspase-3 protein. Further investigations to determine the bioactive compounds of EE are currently in progress.

Acknowledgment

The authors wish to express appreciation to Dr. Cheryl Rutledge, Department of English, Da-Yeh University, for editorial assistance.

References