Direct Cytotoxicity of *Lentinula edodes* Mycelia Extract on Human Hepatocellular Carcinoma Cell Line

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***Lentinula edodes* mycelia (L.E.M.) is a dried powder extracted from shiitake mushrooms (*Lentinula edodes*). We previously demonstrated that it has immunomodulatory effects. In this paper, the direct cytotoxic effects of the polysaccharide-rich fraction of L.E.M. (L.E.M. ethanol precipitate; LEP) on HepG2 human hepatocellular carcinoma (HCC) cells were investigated. LEP directly killed the HepG2 cells efficaciously, but had only minor effects on normal rat hepatocytes and normal mouse dermal cells under the same conditions. Characteristic morphological changes associated with apoptosis such as shrinkage, rounding, and floating as well as chromatin condensation were confirmed; terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was positive as determined by fluorescence microscopy analyses. The caspase-3 and -8 death receptor pathway was found largely responsible for the apoptotic death of HepG2 cells treated with LEP. In conclusion, LEP can directly induce apoptosis of HepG2 cells, and thus may have potential chemotherapeutic applications for the treatment of HCC.**

Key words   *Lentinula edodes* mycelia; hepatocellular carcinom; apoptosis; caspase

In recent years, many edible mushrooms and mushroom products have been noted as sources for health food supplements and therapeutic agents against several types of cancer in humans.[1–3] Mushrooms have been shown to exert antitumor, antioxidative, antihypertensive, antihypercholesterolemic, and immunomodulatory effects.[4] According to numerous previous reports, these effects are dependent on the polysaccharides or polysaccharide–protein complexes included in these mushrooms. The bioactive components found in these fractions were thus considered biological response modifiers (BRMs).[5] Moreover, the polysaccharides or their complexes were confirmed cytotoxic or cytostatic to various cancer cell lines *in vitro*, while exerting low cytotoxicity in cultures of normal cells.[6,7] Indeed, studies revealed that mushrooms such as *Grifola frondosa*, *Poria cocos*, and *Antrodia camphorate* have apoptotic effects against gastric, breast, and hepatic carcinoma cells, respectively.[8–10]

Shiitake mushrooms (*Lentinula edodes*) are a common and traditional edible fungus consumed in Japan and China, as well as other parts of the world.[11] Lentinan, a compound possessing antitumor effects, was derived from shiitake mushroom fruiting bodies. Sia *et al.* showed that the aqueous extract of the shiitake mushroom increased apoptosis in the U937 (human lymphoma) monocytic cell line.[12] Fang *et al.* indicated that an ethyl acetate fraction from shiitake mushrooms induced apoptosis in human breast carcinoma cell lines.[13] This extract was approved for clinical use in Japan in 1985.

Lentinula edodes mycelia (L.E.M.) is a dried powder extracted from *L. edodes* with hot water before germination, and after culturing in a medium composed of bagasse and rice bran. It can be easily obtained in large amounts and more stably than fruiting bodies.[14,15] In addition, L.E.M. contains a wealth of polysaccharides, proteins, nucleic acids, and various physiological components. L.E.M. has been reported to have anti-viral activity, hepatoprotective effects, immunomodulatory effects, and antitumor activity *in vitro* and *in vivo*.[16] Mizoguchi *et al.*[17] reported that L.E.M. stimulates hepatic immune cells directly, and that these cells may be useful for immunotherapy against hepatic diseases. Sugano *et al.*[18] showed that a polysaccharide–protein fraction obtained from L.E.M. exhibited various antitumor activities. Our recent study[19] demonstrated that oral ingestion of L.E.M. suppresses B16 melanoma growth *via* mitigation of regulatory T cell-mediated immunosuppression in mice. However, these effects of L.E.M. are considered dependent on activation of the host immune system rather than direct cytotoxic effects on the cancer cells. Thus little is known about the direct cytotoxic effects of L.E.M. on cancer cells.

Hepatocellular carcinoma (HCC), one of the most common malignant diseases in the world, has one of the highest rates of morbidity and mortality among all cancers.[20–22] In this study, we investigated the direct cytotoxic effect of the polysaccharide-rich fraction (L.E.M. ethanol precipitate; LEP) obtained by precipitation of L.E.M. with 80% ethanol in water on HepG2 cells, a human HCC cell line. In addition, we assessed whether the death of HepG2 cells was due to apoptosis.

MATERIALS AND METHODS

Materials and Chemicals   The human HCC cell line HepG2 was purchased from ATCC. Modified Hanks Balanced Salts (H-2387) and ethylene glycol-bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (E-4378) were purchased from Sigma-Aldrich (St. Louis, Mo, U.S.A.). *N*-2-Hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) buffer (346-01373) and the Cell Counting Kit-8 (CCK-8) were purchased from Dojindo Laboratories (Kumamoto, Japan). CaCl2 (039-00475) and Hoechst33342 (2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole, trihydrochloride) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Collagenase S-1 was purchased from Nitta Zerachin Co., Ltd. (Osaka, Japan). The DeadEnd™ Fluorometric TUNEL System (G3250) was purchased from Promega (Tokyo, Japan). William's Medium E (12551-032), penicillin–streptomycin (15140-122), kanamycin sulfate, and CellEvent™ Caspase-3/7 Green Detection Reagent (C10423)

were purchased from Life Technologies™ (Osaka, Japan). The Caspase Inhibition Set II (218772) including a caspase-3 inhibitor II (Z-DEVD-FMK), caspase-8 inhibitor II (Z-IETD-FMK), and caspase-9 inhibitor I (Z-LEHD-FMK) were purchased from Merck (Tokyo, Japan).

Isolation and Preparation of Rat Hepatocytes Sprague-Dawley rats were purchased from SLC Japan and housed in a controlled environment (12-h light/dark cycle at 25°C) with free access to water and a standard chow diet before sacrifice. All conditions and the handling of animals in this study were conducted under protocols approved by Kobayashi Pharmaceutical Co., Ltd.

To obtain the rat primary hepatocytes, Sprague-Dawley rats were anesthetized with diethyl ether and their abdominal cavities opened. A cannula was inserted into the portal vein, fastened tightly with sutures, then fixed with portal clamps. The Perfusion Medium (modified Hanks Balanced Salts (500 mL) with HEPES (2.38 g) and EGTA (0.17 g)) warmed at 37°C was perfused at a flow rate of 20 mL/min through the inserted cannula, then the collagenase solution was perfused. After perfusion, the liver tissue was confirmed almost completely digested; each hepatic lobe was resected with scissors and moved to petri dish on ice. The obtained lobes were suspended in hepatocyte culture medium (William's Medium E (WE) with 10% fetal bovine serum, 1% kanamycin sulfate, 1% penicillin/streptomycin, 1 μM insulin, and 0.1 μM dexamethasone) and the suspended medium was purified using 100-, 80-, and 40-μm cell strainers in that order. The filtrate was separated by slow centrifugation (600 rpm, 4°C, 3 min). After the supernatant was removed, WE medium was added. This operation was repeated three times to obtain nearly homogeneous hepatic parenchymal cells (viability of rat hepatocytes, >80%).

Isolation and Preparation of Mouse Dermal Cells C57BL/6 mouse neonates were used as the sources for harvesting fresh epidermal cells. Dorsal skin was dissected from each animal and cut into 5–10-mm^2 pieces. These specimens were immersed into dispase solution (1000 U/mL; Godo Syusei, Tokyo, Japan) at 37°C for 1 h then separated into dermis and epidermis using fine forceps. The dermis was digested with collagenase (0.2 mg/mL; Roche Diagnostics, Mannnheim, Germany)-dispase (500 U/mL) at 37°C for 1 h then filtered through a 40-μm cell strainer. The dissociated cells were washed with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and cultured. These cells were defined as passage "0"; cells less than five passages were used for the experiments.[23]

Preparation of Mycelial Extracts from L.E.M. L.E.M. was inoculated into solid medium composed of bagasse and rice bran, and cultured at 25°C under 90% humidity for 12 weeks. After incubation, the medium was ground and extracted in 95°C hot water; L.E.M. was obtained by spray-drying (Figs. 1A, B). L.E.M. was dissolved in 80% ethanol in water, and the resulting precipitate, named LEP, was freeze-dried prior to use in the subsequent studies (Fig. 1C).

Cytotoxicity Assays HepG2 cells (5×10^3) were seeded in each well of a 96-well plate (SUMILON) with 100 μL of HepG2 culture medium (DMEM containing 10% FBS and 1% penicillin/streptomycin), and confirmed to adhere to the plate bottom within 1 d. The medium was replaced with 100 μL fresh incubation medium (DMEM containing 1% penicillin/streptomycin) and LEP was added into each well. The cells

were then incubated at 37°C for various time-periods. The viable cells were counted by CCK-8. The CCK-8 reagent (10 μL) was added into each well, and the reaction was allowed to proceed for ≤4 h. Absorption of the sample at 450 nm was measured against a background control by microplate reader (Multi Functional Microplate Reader GENios Plus, TECAN, Japan) and the cytotoxicity was calculated.

In rat primary hepatocytes, after 1-d incubation, the medium was exchanged for fresh hepatocyte culture medium (WE medium containing 10% FBS). The other conditions were the same as those used for HepG2 cells.

Chemical Staining To observe apoptotic nuclei, cells were washed three times with phosphate buffered saline (PBS) after incubation with LEP for 48 h, then incubated in Hoechst33342 (1 μg/mL) solution at 37°C for 30 min. Morphological changes were analyzed by fluorescent microscope within 1 h.

In situ DNA fragmentation was carried out by terminal deoxynucleotidyl transferase-dUTP nick-end labeling (TUNEL) technique according to the manufacture's technical bulletin. Briefly, cells were fixed with 4% formaldehyde in PBS at 4°C for 25 min then rinsed twice with PBS for 5 min. The cells were permeabilized in 0.2% Triton X-100 solution in PBS for 5 min then rinsed twice for 5 min. Equilibration buffer (100 μL) was added to the samples, which were allowed to equilibrate at room temperature for 10 min. Subsequently, the equilibration buffer was removed by blotting the samples with tissue paper, and aliquots (50 μL) of recombinant terminal deoxynucleotidyl transferase (rTdT) incubation buffer, including the equilibration buffer, nucleotide mix, and rTdT enzyme, were added to the samples and incubated in the dark in a humidified chamber at 37°C for 1 h. After rinsing with 2×SSC for 15 min to stop the reaction, the samples were rinsed twice with PBS. The samples were then embedded in mounting medium and observed by fluorescent microscope.

Caspase Inhibition Assay To investigate whether HepG2 cell death was dependent on caspases, the viability of cells incubated for 48 h with a combination of LEP and different caspase inhibitors was assessed using CCK-8. HepG2 cells (5×10^3) were seeded into each well of a 96-well black plate and incubated at 37°C for 1 d. The cells were then exposed to LEP at 500 μg/mL or vehicle alone (dimethyl sulfoxide (DMSO) diluted in medium), and a caspase-3 inhibitor II (Z-DEVD-FMK), caspase-8 inhibitor II (Z-IETD-FMK), and caspase-9 inhibitor I (Z-LEHD-FMK) were added into each well (50 μM each). After 2-d incubation at 37°C, cell viability was assessed by microplate reader. The test compound was dissolved in DMSO and diluted with the medium (final DMSO concentration, <1%).

Caspase-3 Activity Assay Further to investigate whether caspase-3 was activated in HepG2 cells exposed to LEP, caspase-3/7 activity was determined by CellEvent™ Caspase-3/7 Green Detection Reagent according to the manufacturer's protocol. In brief, HepG2 cells (5×10^3) were seeded into each well of a 96-well black plate and incubated at 37°C for 1 d. The cells were exposed to LEP at 500 μg/mL or to the medium for 2 d, and the fluorescence intensity was measured as the autogenic fluorescence intensity using a microplate reader (excitation and emission wavelengths, 485 and 535 nm, respectively). Next, Caspase-3/7 Green Detection Reagent (5 μM) was added into each well, and the fluorescence intensity was

A

a *Lentinus edodes*

← Fruit Body

← Mycelium

b

B

Culture Medium
(Bagasse and
defatted rice bran)

Inoculation | 2 weeks

Early stage in culture

Mycelium

Incubation | 10 weeks

Late stage in culture

Mycelium

Extraction
with hot water

Lentinus edodes mycelia
(L.E.M.)

C

L.E.M. 20g

Stir in 1L of D.W.
Celite 545 20g
Filtration by glassfilter

Residue | Supernatant

Add 4L of EtOH

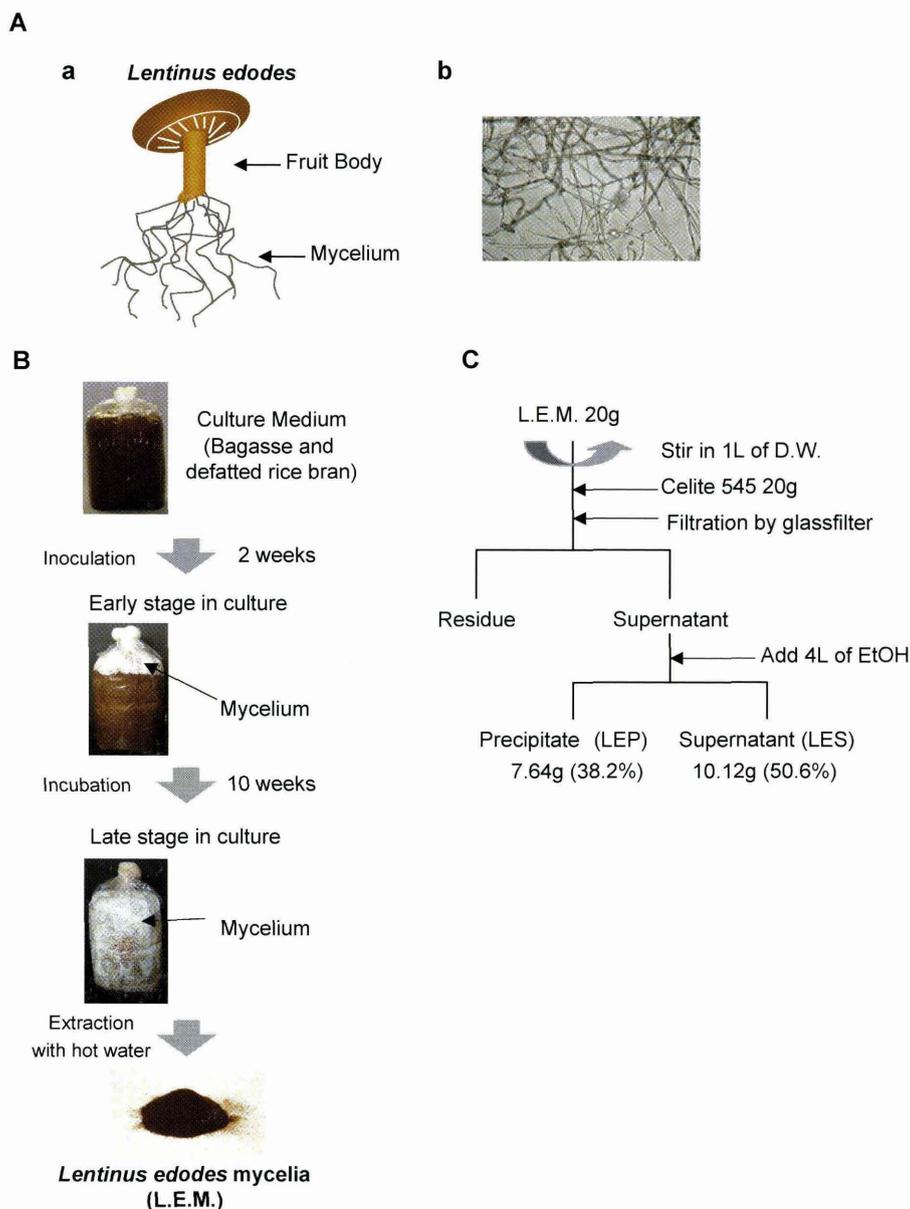Precipitate (LEP) | Supernatant (LES)
7.64g (38.2%) | 10.12g (50.6%)

Fig. 1. Morphology, Incubation Method, and Fractionation Scheme of L.E.M.

A: *Lentinus edodes* and morphology of L.E.M. (a) Position of fruit body and mycelium in *Lentinus edodes*. (b) Electromicrograph of L.E.M. B: The process and period of culture of L.E.M. with culture medium (bagasse and defatted rice bran). C: Schematic diagram showing fractionation of L.E.M. and yield of LEP.

measured after 2-h incubation at 37°C under the same conditions. Caspase-3 activity was calculated by subtracting the autogenic fluorescence intensity from that after the reaction. The test compound was dissolved in DMSO and diluted with the medium (final DMSO concentration, <1%).

Statistical Analyses Statistical analyses were performed using SPSS for Windows software package, version 14.0. For multiple-group analysis, the homogeneity of variance was assessed by Levene test. Parametric comparisons were examined by analysis of variance (ANOVA). If the results of the ANOVA were significant, then the significance of individual differences was evaluated by Bonferroni test.

RESULTS

Observation and Fractionation of L.E.M. The morphol-

ogy of the L.E.M. obtained by electron microscopy is shown in Fig. 1A-b. The L.E.M. was thereby confirmed to grow in filaments. LEP could be acquired from L.E.M. at a yield of 38.2% as an ethanol precipitate (Fig. 1C). The phenol–sulfuric acid method revealed that sugars including polysaccharides accounted for approximately 40% of the LEP (data not shown). In addition, LEP could be obtained from different lots of L.E.M. with reproducibility, and comparable results were confirmed in all experiments even if LEP derived from different lots of L.E.M. was used.

Influence of LEP on Cell Viability and Proliferation The influence of various concentrations (0, 10, 50, 100, 200, and 500 μg/mL) of LEP for different incubation times (0, 24, 48, and 72h) on the viability of normal rat hepatocytes without proliferative ability, normal mouse dermal cells with proliferative ability, and HepG2 cells is shown in Figs. 2A, B.
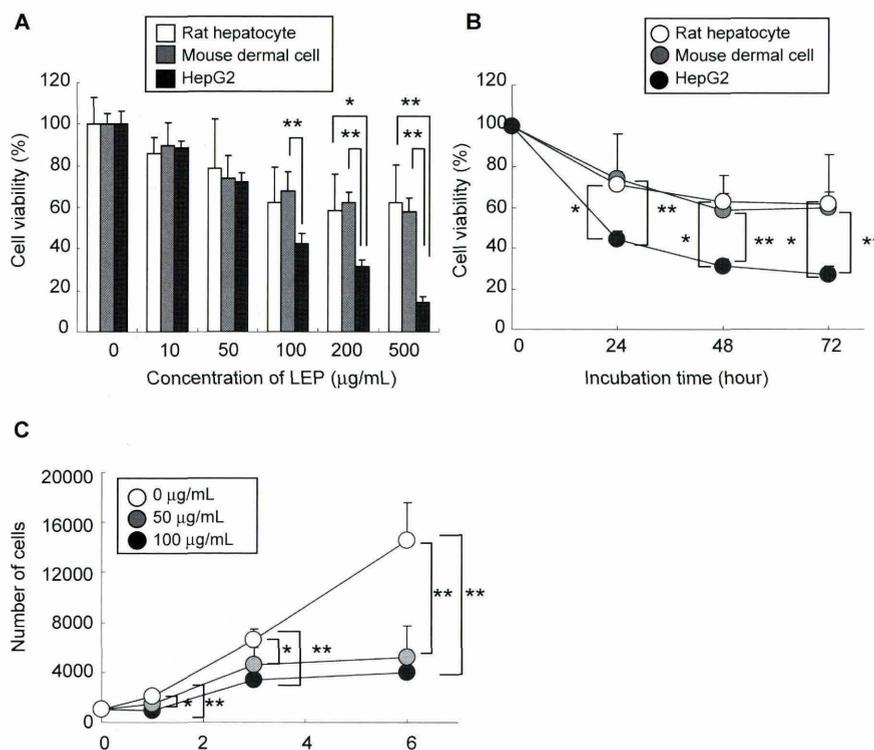
Fig. 2.   Effect of LEP on Rat Hepatocytes, Mouse Dermal Cells, and HepG2 Cells

A: Viability of rat hepatocytes, mouse dermal cells, and HepG2 cells treated with various concentrations (0, 10, 50, 100, 200, and 500 μg/mL) of LEP for 48 h. B: Viability of rat hepatocytes, mouse dermal cells, and HepG2 cells treated with LEP at 200 μg/mL for the indicated time-points (24, 48, and 72 h). C: Proliferation of HepG2 cells treated with LEP at 0, 50, and 100 μg/mL on days 0, 1, 3, and 6. Number of cells was measured by CCK-8; cell viability was determined relative to control (no LEP). Data are means±standard deviations of triplicate samples. *$p<0.05$; **$p<0.01$.

Figure 2A shows the viability of rat hepatocytes, mouse dermal cells, and HepG2 cells treated with LEP for 48 h. In these cells treated with LEP at 10, 50, 100, 200, and 500 μg/mL, viability was respectively 85.9%, 89.8%, and 88.4%, 78.5%, 73.5%, and 72.1%, 62.3%, 67.8%, and 42.2%, 58.4%, 62.4%, and 31.3%, and 62.1%, 57.8%, and 14.2%, respectively. The data indicate that LEP decreased the viability of all cell types in a concentration-dependent manner. However, the degree of cytotoxicity in HepG2 cells was consistently greater than that in rat hepatocytes and mouse dermal cells at all concentrations tested, and significant differences in the cell sensitivity were confirmed at the higher concentrations ($\geq$100 μg/mL) of LEP.

Figure 2B shows the viability of rat hepatocytes, mouse dermal cells, and HepG2 cells treated with LEP at 200 μg/mL for different time-periods. The viability of rat hepatocytes, mouse dermal cells, and HepG2 cells at 24, 48, and 72 h was respectively 73.9%, 70.9%, and 44.4%, 58.4%, 62.4%, and 31.3%, 59.9, 61.3, and 27.2%, respectively. The data indicate that LEP decreased the viability of all cell types in a time-dependent manner, and that the degree of cytotoxicity in HepG2 cells was significantly greater than that in rat hepatocytes and mouse dermal cells all time-points. No significant difference was observed between rat hepatocytes and mouse dermal cells. Thus the cytotoxicity of LEP to normal cells was not affected by their proliferative ability.

Figure 2C shows the proliferation of HepG2 cells treated with LEP at 0, 50, and 100 μg/mL on days 0, 1, 3, and 6. The proliferation rates of HepG2 cells treated with LEP at 50 and 100 μg/mL were significantly lower than those of normal HepG2 cells on all days. The data indicate that LEP inhibited the proliferation of HepG2 cells. These results suggest that LEP has potential to induce cell death and suppress growth of HepG2 cells *via* direct cytotoxic effects.

Cell Morphological Changes Induced by LEP Treatment To analyze changes in the cellular and nucleic shape of rat hepatocytes and HepG2 cells treated with LEP at 500 μg/mL for 48 h, a morphological observation was conducted using phase-contrast fluorescent microscopy. In the rat hepatocytes, no morphological changes in cell shape were found; similar morphology was observed with and without LEP. In addition, the shape of the nuclei was confirmed maintained after treatment with LEP (Fig. 3A).

On the other hand, in HepG2 cells, the control (no treatment with LEP) group cells showed a polygonal and cobblestone monolayer appearance. However, drastic morphological changes such as shrinkage, rounding, and floating were observed in these cells after treatment with LEP. Furthermore, the shape of the nuclei partially changed to small fragments exhibiting condensed chromatin, as opposed to the normal spherical nuclear shape of the control (Fig. 3B). These results suggest the possibility that LEP can induce apoptosis in HepG2 cells.

DNA Fragmentation Assay When apoptosis is induced in cells, DNA fragmentation, which occurs due to degradation of cellular DNA by endonucleases, can be detected. To determine whether the decreased viability and morphological changes in the HepG2 cells were the results of apoptosis, an *in situ* DNA-labeling TUNEL assay was performed after HepG2 cells were treated with 500 μg/mL of LEP for 48 h. Numerous

A   Rat Hepatocyte
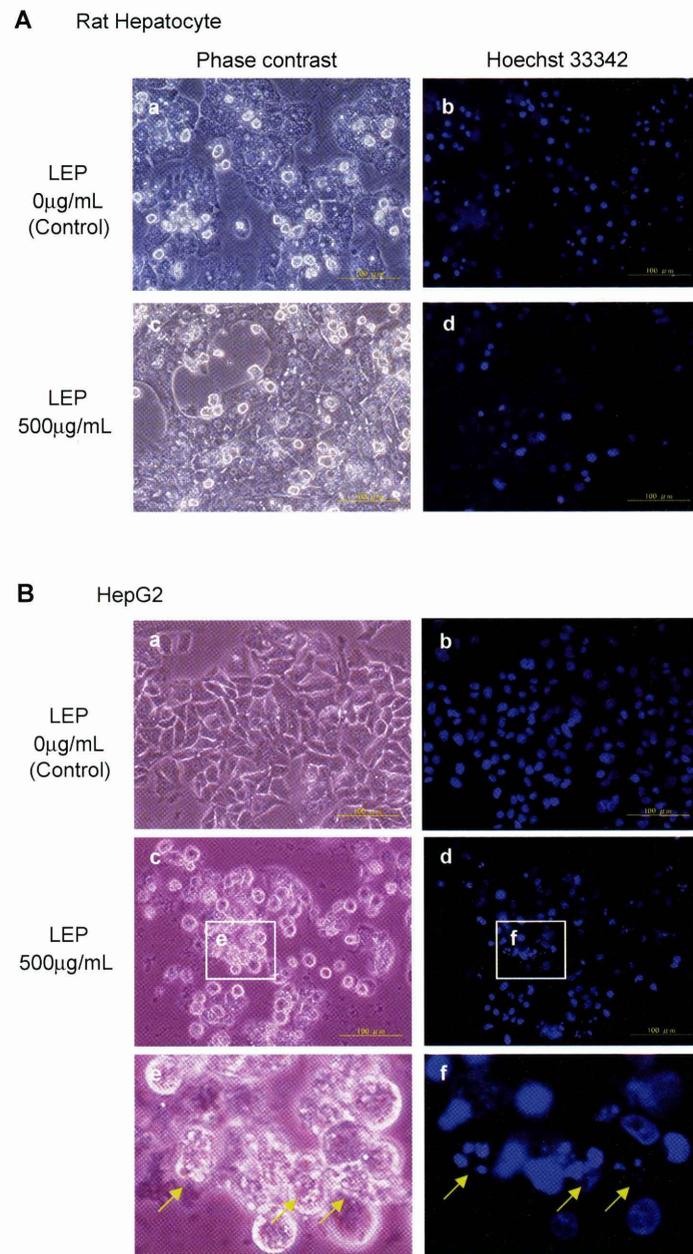

B   HepG2

Fig. 3.   Morphology of Rat Hepatocytes and HepG2 Cells Treated with LEP

A: Cell morphology (a, c) and nuclear morphology (b, d) of rat hepatocytes after treatment with 500 μg/mL of LEP for 48 h or after no treatment (control). B: Cell morphology (a, c) and nuclear morphology (b, d) of HepG2 cells after treatment with 500 μg/mL of LEP for 48 h or after no treatment (control). The marked areas in (c) and (d) are shown at higher magnification in (e) and (f), respectively. Yellow arrows indicate apoptotic cells (e) and small fragments exhibiting condensed chromatin (f). Nuclei were stained with Hoechst33342.

TUNEL-positive cells (apoptotic cells) could be observed in the HepG2 cells treated with LEP. However, few TUNEL-positive cells were observed in those not treated with LEP (Fig. 4A). In addition, the number of control and LEP-treated cells that were positive for TUNEL staining within a defined area (420×320 μm) was 1.7 and 6.7, respectively (Fig. 4B). These data suggest that LEP has potential to induce apoptosis in HepG2 HCC cells.

Effects of Caspase Inhibition on Cell Viability   To assess the importance of caspases on the death in HepG2 cells treated with LEP, we examined whether caspase inhibitors can improve viability of the cells. HepG2 cells were co-incubated with LEP at 500 μg/mL and with cell-permeable caspase-3, -8, and -9 inhibitors at 50 μM for 48 h, then the relative rates of

viability compared *versus* cells exposed to LEP were calculated. The relative viability in caspase-3 inhibitor-treated cells was significantly higher than in the cells treated with LEP alone, and that in caspase-8 inhibitor-treated cells also tended to be higher. However, the relative rate of viability in caspase-9 inhibitor-treated cells was not increased in comparison with cells treated with LEP alone (Fig. 5A).

We also investigated the enzyme activity of caspase-3 in HepG2 cells after treatment with different concentrations (0, 100, 200, 500, and 1000 μg/mL) of LEP for 48 h. The fluorescence intensity was remarkably increased when cells were treated with LEP at all concentrations. These data suggest that apoptosis induced by LEP was largely dependent on caspase-3 (Fig. 5B).
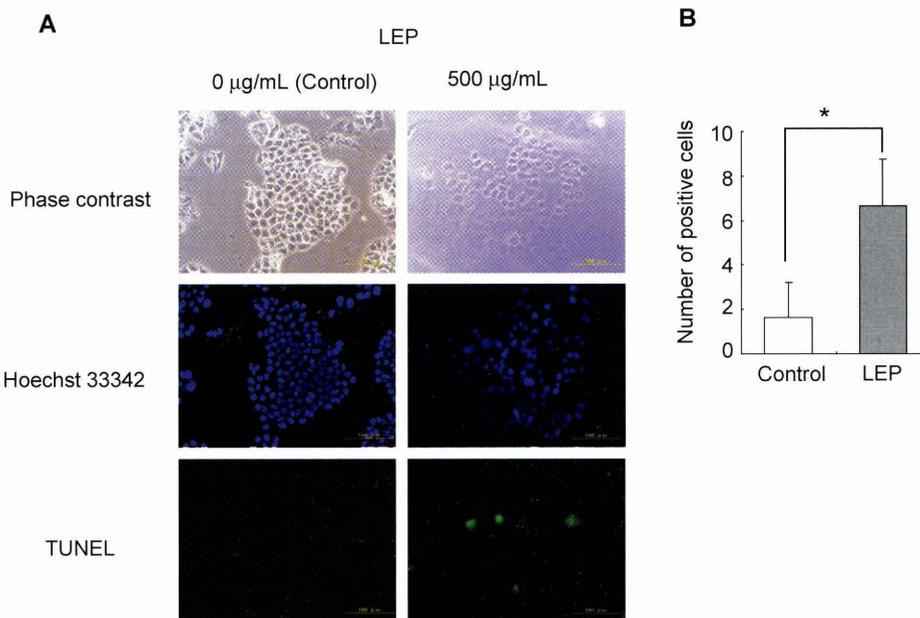
Fig. 4. TUNEL Assay in HepG2 Cells Treated with LEP

A: *In situ* DNA-labeling TUNEL assay in HepG2 cell after treatment with 500 μg/mL of LEP for 48 h (or after no treatment). TUNEL-positive cells were observed by fluorescence microscopy and were green in color. B: Number of TUNEL-positive HepG2 cells treated after LEP or vehicle treatment. Number of positive cells within a defined area were counted by fluorescent microscope. Data are means±standard deviations of triplicate experiments. *$p<0.05$.
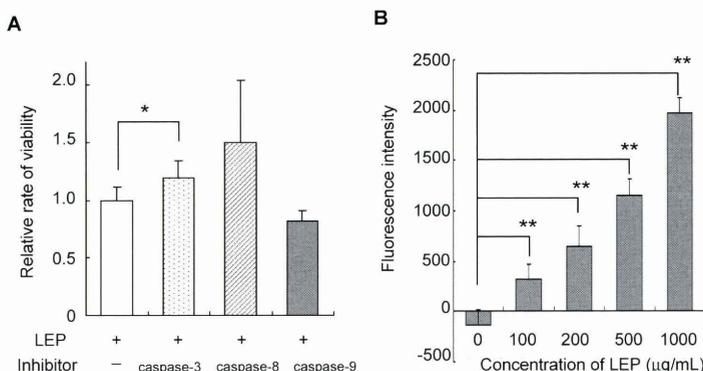

Fig. 5. Involvement of Caspases in Reduction in Cell Viability of HepG2 Cells Treated with LEP

A: Influence of caspase-3, -8, and -9 inhibitors on viability of HepG2 cells treated with 500 μg/mL of LEP for 48 h. Caspase-3 inhibitor significantly increased viability of HepG2 cells treated with LEP. B: Activity of caspase-3 in HepG2 cells after treatment with various concentrations (0, 100, 200, 500, and 1000 μg/mL) of LEP for 48 h. The fluorescence intensity measured by microplate reader is presented as caspase-3 activity. Data are means±standard deviations of triplicate experiments. *$p<0.05$; **$p<0.01$.

DISCUSSION

Numerous studies have demonstrated that compounds present in mushrooms can serve as BRMs, leading to immunomodulation and antitumor effects. As a result, these compounds have been drawing increasing attention. The ability to induce apoptosis is thought an important property of an antitumor substance.[24] In recent studies, it has been reported that various macromolecular polysaccharides and polysaccharide-protein complexes present in mushrooms can induce apoptosis in tumor cells.[6,7] Thus if whole or parts of the apoptotic mushrooms could be easily and abundantly obtained by artificial cultivation, they could be very useful as a BRM. We have already succeeded in mass-producing homogeneous L.E.M. and demonstrated that LEP, which can be obtained by fractionation of L.E.M., includes a high amount of polysaccharides with unique sequences.[15]

In the present study, we found that LEP directly killed HepG2 human HCC cells in a concentration- and time-dependent manner, with less pronounced effects on normal rat hepatocytes under the same conditions (Fig. 2). As shown in Figs. 3 and 4, morphological changes associated with apoptosis, such as cell shrinkage, rounding, and floating of the cell bodies, as well as condensed chromatin, were observed in the HepG2 cells treated with LEP; induction of apoptosis was confirmed by TUNEL *via* fluorescent microscopic analyses. These data suggest that the reduced HepG2 cell viability due to treatment with LEP was mainly caused by apoptosis.

Subsequently, to examine whether the cytotoxicity of LEP on HepG2 cells was caused by caspase-3, -8, and -9—known to be major effective caspases[10]—we measured changes in the viability of HepG2 cells treated with LEP in combination with different caspase inhibitors as shown in Fig. 5. Caspase-3 and -8 were thus found largely responsible for the apoptotic cell

death in this study. In addition, production of caspase-3 was confirmed increased in an LEP-dependent manner.

Although numerous effectors of apoptosis such as cell death ligands, decreases in survival signaling, and reactive oxygen species produced by various stresses such as UV rays, irradiation, and peroxides are widely known,[25–30] the mechanisms of apoptosis are dependent on two fundamental pathways: the mitochondrial pathway and the death receptor pathway, both of which finally lead to production of caspase-3, the "executioner" caspase that directly induces cell death.[31] The mitochondrial pathway is triggered by cytochrome c release from the mitochondria. Cytochrome c promotes activation of Apaf-1, leading to production of caspase-9.[32] On the other hand, the cell death receptor pathway is mediated by capase-8, which activates production of caspase-3.[31] In this study, the caspase-9 inhibitor did not improve the viability of HepG2 cells treated with the highest concentration of LEP at $500 \mu g/$ mL, whereas the caspase-3 and -8 inhibitors had a strong influence on cell viability. Therefore our results suggest that the cell death pathway might be the major pathway by which LEP induces apoptosis in HepG2 cells. The cell death pathway is dependent on the Fas/Fas ligand (FasL) system or tumor necrosis factor (TNF)-family system.[33,34] Because caspase-3 and -8 are activated by both systems, further investigations to elucidate the system by which LEP exerts its effects will therefore be needed.

The p53 tumor suppressor is known to play an important role in regulation of the normal cell cycle, and overexpression of the p53 protein can induce apoptosis.[35] HepG2 cells have functional p53 (wtp53), whereas another HCC cell line, Hep3B, lacks functional p53 (deleted p53).[36] We found that the viability of Hep3B cells treated with LEP was decreased to the same degree as was observed in the HepG2 cells (data not shown), suggesting that LEP did not induce apoptosis as a result of overexpression of p53 protein.

In conclusion, LEP fractionated from L.E.M. showed cytotoxicity to a human HCC cell line (HepG2 cells) and exhibited lower toxicity to normal rat hepatocytes. Morphological analysis and TUNEL staining indicated that the cytotoxicity of LEP was due to its induction of apoptosis. In addition, the apoptosis in HepG2 cells treated with LEP was thought attributable to the caspase-8 and -3 cascades. Our findings suggest that LEP may be useful for chemotherapeutic applications in the treatment of cancer. However, the active components of LEP leading to apoptotic death of HepG2 cells remain unidentified. Identification of active components and their mechanism of action will provide insights into the potential chemotherapeutic application of LEP against cancer.

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