Hepatoprotective Effect of Extracts from *Lentinus edodes* Mycelia on DimethylNitrosamine-Induced Liver Injury

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Mycelia of the edible mushroom *Lentinus edodes* (shiitake) were cultivated in a solid medium, and two fractions were obtained by hot-water extraction (L.E.M.) and then ethanol extraction followed by Sephadex LH-20 column chromatography (ESMe). The L.E.M. and ESMe were then examined for their hepatoprotective effect on dimethylnitrosamine-injured mice. Both fractions decreased the blood aspartate aminotransferase and alanine aminotransferase levels, partially inhibited the overaccumulation of collagen fibrils, and suppressed the overexpression of genes for α-smooth muscle actin and/or heat-shock protein 47 in the mice. Both fractions also inhibited the morphologic change and proliferation of isolated rat hepatic stellate cells (HSCs), which play a central role in liver fibrosis, in a dose-dependent manner and without cytotoxicity. The direct interaction between the extracts and HSCs appears to be important for the hepatoprotective activity. Polyphenols contained in both fractions are considered to be potential candidates for expressing the hepatoprotective effects. The finding of antifibrotic activity in extracts from an edible mushroom is expected to be helpful in the development of hepatoprotective agents with few side effects.

Key words: *Lentinus edodes*; hepatic stellate cell; antifibrotic effect; hepatoprotective effect; polyphenol

Liver fibrogenesis is a wound-healing process that occurs after chronic liver injury. It develops in a high percentage of patients with hepatitis virus infection. The development of hepatoprotective drugs that suppress liver fibrosis is very important because hepatitis C virus (HCV)- or hepatitis B virus (HBV)-infected patients develop liver cirrhosis and hepatocellular carcinoma with a relatively high probability through the fibrosis stage. Hepatic stellate cells (HSCs) are reported to be responsible for the development of liver fibrosis. During liver injury, HSCs are activated to transdifferentiate into myofibroblasts and overproduce extracellular matrix, leading to fibrogenesis. Oxidative stress stimulates the activation of HSCs, and substances with antioxidative activity, such as vitamin E, glutathione, and L-cysteine, inhibit HSC activation, thus suppressing liver fibrosis. Recently, many antioxidants have been found in plants including tea, fruit, and vegetables. The edible mushroom *Lentinus edodes* (shiitake) contains several compounds with physiologic activity, such as immunopotentiation, antiatherogenesis, and an anti-HIV effect. Mycelia of *L. edodes* can be cultured in a solid medium and the extract (L.E.M.) is commercially available as a nutritional supplement. In our previous study L.E.M. was shown to have antioxidant activity and to protect the liver from carbon tetrachloride-induced acute liver injury. We examined in this study whether L.E.M. and the further purified extract have a hepatoprotective activity against hepatotoxin-inducing liver fibrosis. When isolated HSCs are cultured on a plastic plate, cells spontaneously transform from the quiescent to the activated state. Here, using this in vitro activation model, we examined the direct effect of L.E.M. on HSCs. We found that L.E.M. suppress the development of liver fibrosis induced by dimethyl nitrosamine (DMN) and directly inhibit proliferation and morphological change of HSCs.

MATERIALS AND METHODS

Animals BALB/c mice and Sprague-Dawley rats were purchased from SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at 22 °C before the experiment. Hepatic injury in mice aged 6 weeks was elicited by the intraperitoneal administration of DMN (Sigma, St. Louis, MO, U.S.A.) at 10 mg/kg body weight for the first 3 consecutive days of the week for 4 weeks. After 4 weeks of treatment, the mice were anesthetized, and blood samples were taken from the orbital sinus. The animal experiments were conducted according to the ethical guidelines of Osaka University Graduate School of Pharmaceutical Sciences.

Histopathologic Staining The liver specimens were fixed in 10% formaldehyde and embedded in paraffin. Tissue block sections were mounted on slides and Elastica van Gieson (EG) staining was performed to analyze the extent of fibrosis.

Preparation of L.E.M. L.E.M. were prepared as previously reported. Briefly, *Lentinus edodes* mycelia were cultivated in a solid medium composed of sugar-cane bagasse and defatted rice bran. The medium with mycelia was incubated for digestion with mycelial enzymes in water at 30—55 °C, and then the incubation temperature was increased to 90 °C for inactivation of the enzymes and sterilization. The digest was filtered, lyophilized, and used as the L.E.M. preparation. The L.E.M. were then incubated in 50% ethanol for 3 d in the dark, and the soluble fraction was dried under a vacuum, yielding a brownish fraction (ES). The ES was dissolved in a small amount of ethanol and further purified on Sephadex LH-20 column chromatography. After elution with distilled water, the fraction containing polyphenols was obtained by elution with methanol. The eluate was concentrated under vacuum and lyophilized (ESMe). Both the L.E.M. and ESMe fractions were examined for their hepatoprotective activity.

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Isolation and Culture of HSCs  HSCs were isolated from male Sprague-Dawley rats aged 9 weeks by digesting the liver with Pronase-E (Merck Darmstadt, Germany) and collagenase (from Clostridium histolyticum type I; Wako Pure Chemical Co., Osaka, Japan), as previously described. Isolated HSCs were seeded at a density of 2 x 10^3 cells/cm^2 onto 24- and 96-well polystyrene culture plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) for the observation of morphology and proliferation assay, respectively. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Thermo Trace Ltd., Melbourne, Australia).

Assays  The proliferation of HSCs was measured using the Bromodeoxyuridine (BrDU) Cell Proliferation Assay kit (Merck). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using an assay kit (IATROZYME TA-Lq; Mitsubishi Kagaku Iatron Inc., Tokyo, Japan).

RT-PCR  Mouse liver was excised and homogenized after removing blood with phosphate-buffered saline. The total RNA was extracted from the liver homogenates using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). The gene expression of α-smooth muscle actin (α-SMA) was analyzed using the following primers: forward 5'-CAGGGAGTAATG-GTTGGAAT-3' and reverse 5'-CGTCGTATTCTGGTTGC-TGA-3'. Heat-shock protein 47 (HSP47) gene expression was analyzed using the following primers: forward 5'-CCA-TCGACAAGAACAGA-3' and reverse 5'-CTATATTTCCC-TTCCCCCACCAC-3'. β-Actin gene expression was analyzed using the following primers: forward 5'-CATCCCCCA-AAGTCTAC-3' and reverse 5'-CATACTTCATACATAC-TG-3'. RT was performed using 1 μg of total RNA sample with the BcaBEST RNA PCR kit (TaKaRa, Kyoto, Japan). The PCR conditions were: 1) 94°C for 1 min; 2) 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and 3) 72°C for 5 min.

Statistics  The data were analyzed for statistical significance using Student’s t-test.

RESULTS

Suppression of Liver Injury by L.E.M.  DMN is a well-known hepatotoxin, inducing hepatic necrosis and subsequent fibrosis. We first examined the hepatoprotective effect of L.E.M. on DMN-induced liver injury in mice. As shown in Fig. 1, after DMN treatment for 4 weeks, the activities of blood AST and ALT were increased 3.3- and 3.5-fold, respectively, compared with controls. The intraperitoneal administration of L.E.M. or ESMe (10 mg/kg body weight) significantly decreased the activities of AST and ALT. These results show that L.E.M. and ESMe suppress the hepatic inflammation caused by DMN treatment. Next, the accumulated collagen fibrils were observed in isolated liver specimens with EV staining. Figure 2 shows typical EV staining results, in which fibrous materials were stained red. In the control, hardly any red staining was observed in the pericentral area. In contrast, a considerable accumulation of fibrous materials was seen in liver injured by DMN. DMN treatment for 4 weeks was shown to cause a sufficient overaccumulation of collagen fibrils for evaluation of the antifibrogenic effect of the extracts. The administration of L.E.M. and ESMe appeared to suppress partially the collagen accumulation.

To confirm the antifibrotic effect of L.E.M. and ESMe, we next examined the gene expression of α-SMA and HSP47, which are markers of fibrosis, using RT-PCR analysis. As shown in Fig. 3, marked expression of the α-SMA and HSP47 genes was observed in the DMN-injured liver. L.E.M. significantly suppressed α-SMA gene expression, and ESMe clearly suppressed the expression of both the HSP47 and α-SMA genes. ESMe showed a stronger effect than L.E.M. on the suppression of fibrosis-related gene expression. These results indicate that L.E.M. and ESMe have protective activity against the liver injury and subsequent fibrosis induced by DMN.

Inhibition of HSC Activation  HSCs, one type of nonparenchymal liver cell, play a central role in the develop-
ment of liver fibrosis. We next examined whether L.E.M. have a direct effect on the activation of HSCs using monolayer-cultured cells. As shown in Fig. 4D, HSCs were activated during monolayer culture to transform into proliferating myofibroblast-like cells. The addition of L.E.M. and ESMe at 500 μg/ml clearly inhibited the activation (Figs. 4E, F). HSCs retained the spherical form that is characteristic of their quiescent state in the presence of L.E.M. and ESMe during 9 d of culture. In particular, ESMe completely suppressed the morphological change, indicating that effective factors were concentrated by the extraction with ethanol and methanol. We confirmed that L.E.M. and ESMe showed no cytotoxicity at doses up to 1 mg/ml (data not shown).

Figure 5 shows the effect of L.E.M. and ESMe on the DNA synthesis of cultured HSCs. HSCs were incubated with BrdU for 24 h in the presence or absence of the extracts, and the uptake was measured with ELISA using a specific anti-BrdU antibody. L.E.M. and ESMe inhibited the DNA synthesis of HSCs dose dependently. These results suggest that L.E.M. and ESMe have strong activity to maintain HSCs in a quiescent state, resulting in the suppression of liver fibrosis in vivo.

DISCUSSION

Recently, antioxidants derived from plants, such as (−)-epigallocatechin gallate (EGCG) and curcumin, have been reported to inhibit the proliferation of cultured HSCs. These compounds appear promising in the treatment or prevention of liver cirrhosis. In this study, we found that extracts from mycelia of shiitake mushroom had activity similar to that of EGCG and curcumin. Mycelia of L. edodes, the source of L.E.M., can be cultured on a large scale under completely controlled conditions inside a plant factory. Thus the seasons and external environment do not influence the supply of raw material. This gives L. edodes extracts an advantage over other plant-derived antioxidants.

The main components of the extracts were sugars, proteins, and polyphenolic compounds. Polyphenols have an antioxidative activity to scavenge oxygen free radicals, which are known to cause oxidative stress, affecting many cellular processes. Polyphenols have been proposed to protect against diseases, including cancer, cardiovascular disease, and neurodegenerative disorders. ESMe and L.E.M contained 13.73 and 3.34 g of polyphenols in 100 g extracts, respectively. When measured in a 1,1-diphenyl-2-picrylhydrazyl radical-scavenging assay, the antioxidation activity of ESMe was about 3-fold greater than that of the same amount of L.E.M. ESMe also had about 4-fold higher superoxide dismutase-like activity than L.E.M. (data not shown). There was a strong correlation between polyphenol content and antioxidant activity. In our preliminary experiments, several polyphenolic compounds were identified in the fraction with the highest antioxidant activity. Polyphenols are considered...
to be potential candidates for expressing hepatoprotective effects. Analysis of the activity of each compound is now underway.

α-SMA and HSP47 gene expression was used to mark fibrogenesis in this study. The expression of α-SMA is usually used as a marker of HSC activation. α-SMA expression increased remarkably in the injured mouse liver by DMN administration and decreased in response to L.E.M. and ESMe treatment. HSP47, known as the collagen-binding stress protein, is located in the endoplasmic reticulum and acts as a chaperone for procollagen biosynthesis. In this study, HSP47 expression increased markedly in the injured mouse liver. A significant change in its expression was not observed in mice treated with L.E.M. In contrast, ESMe, containing four times the concentration of polyphenols of L.E.M., markedly suppressed the expression. Further study will be necessary to clarify the different effects of L.E.M. and ESMe on HSP47 expression. It is well known that HSCs cultured in vitro on polystyrene plates change the phenotype from quiescent to myofibroblastic. HSCs proliferate and change the morphology accompanied by elongation of long processes. In this study, we showed that L.E.M. and ESMe act directly on HSCs to inhibit morphologic change and proliferation. Although further analysis of the activation marker, such as gene expression of α-SMA and transforming growth factor-β, is necessary to clarify the action of L.E.M. and ESMe on HSCs activation, the hepatoprotective effect appears to be derived in part from a direct interaction of the extracts with HSCs.

Since L.E.M. and ESMe had activities that decreased the blood AST and ALT levels in mice injured by DMN administration, these fractions appear to suppress liver inflammation in vivo. We found that L.E.M. and ESMe had protective activity for hepatocytes against hepatotoxic treatment (data not shown). Furthermore, the viability of cultured hepatocytes treated with bromobenzene, a well-known hepatotoxin, was significantly maintained in the presence of L.E.M. and ESMe (to be published elsewhere). We previously reported that tea catechins suppress cytoxin-induced apoptotic and necrotic cell death in isolated hepatocytes. The antioxidants, such as polyphenols, contained in L.E.M. and ESMe had a direct effect on hepatocytes and might inhibit the inflammatory response by suppressing necrotic cell death.

The development of antifibrotic agents with few side effects is important, because their concomitant use with an antiviral drug, such as interferon-α, could help prevent liver cirrhosis and hepatocellular carcinoma in HCV- or HBV-infected patients. Since the shiitake mushroom is a very popular food in Asia and the raw materials can be stably supplied by cultivation of the mycelia, the extracts are a promising candidate for antifibrotic agents.

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REFERENCES