JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Hericium erinaceus (Lion's Mane) Mushroom Extracts Inhibit Metastasis of Cancer Cells to the Lung in CT-26 Colon Cancer-Tansplanted Mice

Sung Phil Kim,[†] Seok Hyun Nam,*[,‡] and Mendel Friedman*[,§]

[†]Department of Biological Science and [‡]Department of Molecular Science and Technology, Ajou University, Suwon 443-749, Republic of Korea

[§]Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710, United States

ABSTRACT: This study investigated the antimetastatic activity of four *Hericium erinaceus* edible mushroom extracts using CT-26 murine colon carcinoma cells as an indicator of inhibition of cell migration to the lung. Hot water (HWE) and microwaved 50% ethanol (MWE) extracts of *H. erinaceus* strongly elicited cancer cell death through apoptosis and inhibited metastasis of cancer cells to the lungs by 66% and 69%, respectively. HWE and MWE reduced the expression of matrix metalloproteinases MMP-2 and MMP-9 in cells and their activities in culture media. Urokinase-type plasminogen activator (u-PA), another extracellular matrix (ECM)-degrading proteinase, also showed decreased protein expression. In CT-26 cells, HWE and MWE down-regulated extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) phosphorylations. The reduced phosphorylations seem to cause reduction of activity of the MMPs, thereby blocking migration and invasion of cells. Dietary administration of HWE and MWE reduced the formation of tumor nodules in the lung by about 50% and 55%, respectively, and prevented increases in lung weight caused by cancer cell metastasis. These results demonstrate the effectiveness of HWE and MWE as beneficial antimetastatic agents, targeting their upstream signaling molecules for mediating the expression of the ECM-degrading proteinases. Acidic and alkaline extracts were not bioactive. Bioactivity seems to be related to composition. *H. erinaceus* edible mushrooms have the potential to serve as a health-promoting functional food.

KEYWORDS: *Hericium erinaceus mushrooms, CT-29 murine colon cancer, lung tumors, antimetastasis mechanism, health-promoting food, functional food*

INTRODUCTION

The edible medicinal *Hericium erinaceus* mushroom is called "Yamabushitake" in Japan or "Houtou" in China and is also known by the common name Lion's Mane. In a previous study,[1] we evaluated the following four extracts of the fruitbody (sporocarp) of *H. erinaceus* mushroom for the ability to induce apoptosis (programmed cell death) in U939 human monocytic leukemia cells: hot water extract (HWE), microwaved 50% ethanol extract (HWE), acidic extract (ACE), and alkaline extract (AKE). The first two extracts (HWE and MWE) were bioactive, and their activity could be explained by a mechanism governing suppression of the cell proliferation pathway that involves activation of mitochondria-mediated caspase-3 and caspase-9 enzymes. The acid and alkaline extracts exhibited low activity. In a second study,[2] we investigated the composition and antitumor effects of the same four extracts in tumor-bearing mice. Gas chromatography/mass spectrometry analysis revealed the presence of 40 characterized compounds in HWE, 27 in MWE, 16 in ACE, and, and 13 in AKE. The first two extracts contained high levels of bioactive β-glucans. The observed tumor regression was associated with changes in several cancer biomarkers. The results seemed to suggest that induction of natural killer (NK) activity, activation of macrophages, and inhibition of angiogenesis all contributed to mechanism of reduction in tumor size. In a third study,[3] we found that administering HWE and MWE to mice infected with a lethal dose of the virulent foodborne pathogen *Salmonella* Typhimurium protected them against liver damage and mortality via stimulation of the immune system.

To place our results described below in proper perspective, we will first mention in chronological order related reported aspects of *H. erinaceus*. (a) A polysaccharide isolated from *H. erinaceus* up-regulated functions mediated by macrophages in RAW264.7 cells such as production of nitric oxide (NO) and expression of cytokines (IL-1β and TNF-α).[4] (b) A polysaccharide from *H. erinaceus* fruiting body increased apoptosis by the cancer drug doxorubicin in human hepatoma cells.[5] (c) A mouse diet with added *H. erinaceus* mycelia induced anti-inflammatory effects via increasing the level of plasma glutathione and reduction of hepatic oxidative stress.[6] (d) *H. erinaceus* extracts suppressed pro-inflammatory genes through inhibition of NF-κB and JNK activity.[7]

The main objective of this study was to extend these studies further by investigating the potential of HWE and MWE to inhibit metastasis of the CT-26 murine colon carcinoma cells to

Received: February 28, 2013
Revised: April 23, 2013
Accepted: May 2, 2013
Published: May 13, 2013

lung cells. To our knowledge, this is the first report on *H. erinaceus*-induced antimetastasis of cancer cells.

MATERIALS AND METHODS

Materials. DMEM, phosphate-buffered saline (PBS), fetal bovine serum (FBS), and other cell miscellaneous cell culture reagents were purchased from Hyclone Laboratories (Logan, UT, USA). Matrigel Matrix was purchased from BD Bioscience (Bedford, MA, USA). Hematoxyline, eosin Y, lipopolysaccharide (LPS), and other chemical reagents of analytical grade were from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Primary antibodies against ERK1/2, pERK1/2, JNK1, pJNK1/2, p38, and p-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against MMP-2, MMP-9, JNK2, and u-PA were the product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse β-actin antibody and HRP-conjugated anti-IgG antibody were obtained from Millipore (Billerica, MA, USA) and Sigma-Aldrich, respectively.

Preparation of *H. erinaceus* Extracts. The dried fruit body of *H. erinaceus* was obtained from Forest Environment Science Laboratory, College of Agriculture and Life Science, Kyungpook National University, Daegu, Korea. The fruit body was ground into powder using a mill and then passed through a 40-mesh sieve. The powder was extracted with 20-fold weight of following solvents as previously reported by Kim et al.:[8] boiling in pyrogen-free water for 3 h (HWE); microwaving in ethanol (50% v/v) at 60 W for 3 min using a focused microwave-associated Soxhlet extractor (Prolabo, Paris, France; MWE); acidic extract (ACE) and alkaline extract (AKE) by boiling in 1% HCl and 3% NaOH for 2 h, respectively (internal negative controls). After extraction, solid materials were removed by centrifugation at 3000*g* for 30 min. To the recovered supernatants was then added a 4-fold volume of ethanol to precipitate polysaccharides at 4 °C for 24 h. The precipitates were dissolved in deionized water and dialyzed in a tube (Sigma-Aldrich) with a cutoff molecular weight of 12 kDa against the same solvent. All extracts were lyophilized to powders. The use of a dialysis tube with a size of 12 kDA allowed the collection of the high molecular weight bioactive polysaccharide and polysaccharide protein complexes (β-glucans). Analytical data for each extraction fraction were previously reported by Kim et al.[1]

Cancer Cells and Mice. The CT-26 mouse colon carcinoma cell line from the American Type Tissue Culture Collection (Manassas, VA, USA) was cultured in DMEM supplemented with 10% heat-inactivated FBS containing penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Pathogen-free female BALB/c mice (6 weeks old), weighing 20−25 g, were purchased from Orient Bio (Seongnam, Korea). The mice were housed in a stainless steel cage under a 12 h light/dark cycle with a temperature range of 20−22 °C and relative humidity of 50 ± 10%. The mice were fed pelletized commercial chow diet and sterile tap water ad libitum during the entire experimental period. The mice were then randomly divided into groups (n = 10). Each group was fed a standard diet supplemented with MWE and HWE and a control without supplementation.

Experimental Lung Metastasis and Treatment. The protocol of the mice studies was approved by the Ethics Committee for Animal Care and Use, Ajou University, Republic of Korea. All experiments were performed in compliance with the relevant laws and institutional guidelines. A pulmonary colonization assay was conducted as described by Son et al.[9] Briefly, BALB/c mice were intravenously transplanted with 1×10^5 cells of CT-26 cancer cells in 200 μL of PBS into the tail vein. The mice were divided into five groups (n = 10) and then subjected to an intraperitoneal (ip) injection of each mushroom extract (10 mg/kg mouse) once a day for 2 weeks. Control group mice were administered the same volume of PBS only. Mice were sacrificed by CO_2 inhalation at the end of the treatments for the excision of lung.

Cell Cytotoxicity Assay. Cell cytotoxicity was assessed by MTT staining following the method of Mosmann.[10] Briefly, the CT-26 cells were seeded into a 96-well plate at a density of 1×10^5 cells/well and cultured for 24 h at 37 °C humidified air with 5% CO_2. The cells were then treated with 1, 50, 100, 500, and 1000 μg/mL of *H. erinaceus* mushroom extracts for 48 h. After treatment, cells were stained by adding MTT. The resultant intracellular chromogen formazan products were solubilized with DMSO. Absorbance of the chromogen was read in a microplate reader (model 550, Bio-Rad, Hercules, CA, USA) at 570 nm and a reference wavelength of 655 nm. Cell cytotoxicity was expressed by the following formula:

$$\%\ \text{cytotoxicity} = 100 \times (1 - \text{absorbance of extract} - \text{treated/absorbance of PBS} - \text{treated cells})$$

Migration assay. The migration assay was conducted following the method of Choi et al.[11] with some modifications. In brief, CT-26 cells were seeded at a density of 1×10^4 cells/mL in a 6-well plate. After reaching overfluence, the center of the culture dishes was scratched once with a 200 μL yellow tip. Cells were washed three times with serum-free medium and incubated for 20 h with *H. erinaceus* mushroom extracts (500 μg/mL). After incubation, images of each well were examined under a microscope (model D50, Olympus, Tokyo, Japan) and photographed. The degree of cell spreading and directional migration of the cells was then compared.

Invasion Assay. Invasiveness of cancer cells into stromal cells was quantified by using BD BioCoat GFR matrigel invasion chamber with transmembrane inserts of 8.0 μm pores as described by Choi et al.[11] CT-26 cells were seeded onto matrigel-coated transmembrane inserts at a density of 1×10^5 cells/well, followed by addition of *H. erinaceus* mushroom extracts (500 μg/mL). After 48 h of incubation, the membrane inserts were washed three times with PBS and fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4 °C overnight. After another gentle washing with PBS, the fixed cells were treated with ice-cold methanol for permeabilization, followed by washing for 5 min. The inner side of invasion chambers was then wiped out. Subsequently, the cells were stained in hematoxylene solution for 2 min and destained in PBS for 10 min. The membrane inserts were cut, mounted on glass slide, and observed under a microscope. After random selection of six fields of each membrane, the total number of pores and the number of cancer cells that had migrated through, thus being trapped in the pores, were counted. The average invasion rate was calculated according to the following formula:

$$\%\text{invasion} = 100 \times \text{total number of trapped cells} / \text{total number of pores in a field}$$

Western Blot Analysis of Cell Proteins. CT-26 cells were lysed and extracted with RIPA buffer (50 mM Tris Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA, pH 7.4). Protein concentrations were determined according to the Bradford method using a Bio-Rad Protein Kit. Bovine serum albumin (BSA) was used as standard. Cell extracts containing proteins (30 μg) were separated on 10% polyacrylamide gels and electrophoretically transferred onto a nitrocellulose membrane (Millipore). After blocking with 5% skim milk, provided that the 3% BSA (fraction V) was used with phosphorylated proteins-blotted membranes, the gel was incubated with each primary antibody, followed by HRP-conjugated anti-IgG antibodies. Blots were developed using the ECL detection kit (Pierce, Rockford, IL, USA). The intensity of separated protein bands was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film, Tokyo, Japan). At least three separate replicates were determined for each experiment.

Gelatin Zymography of Matrix Metalloproteinases (MMPs). Gelatin zymography was conducted following the method described by Birkedal-Hansen and Taylor[12] with some modification. CT-26 cells were seeded into a 6-well culture plate at a density of 1×10^5 cells/well and incubated for 24 h in serum-free DMEM with *H. erinaceus* mushroom extracts (500 μg/mL). After incubation, medium was harvested and protein concentration determined by the Bradford method (Bio-Rad). Proteins (20 mg) were separated on 10% SDS−polyacrylamide gels containing gelatin (1 mg/mL). The gels were washed twice for 30 min in 2.5% Triton X-100 at room temperature to

remove all traces of SDS and incubated in activation buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.5) for 24 h at 37 °C. Then, the gels were stained with 0.2% Coomassie Brilliant Blue, destained, and dried.

Histology and Assessment of Tumor Nodules. For histological analysis, mice were sacrificed by CO_2 inhalation at the end of the treatments for the excision of lung. Excised lung was fixed with 4% paraformaldehyde in PBS overnight at 4 °C. The tissues were rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sliced into 4 μm sections and mounted onto glass slides. The sections were dewaxed using xylene, rehydrated with ethanol and water, and stained with H&E. Pulmonary nodules were counted in six blindly chosen random fields under the microscope at 100× magnification, and nodule density was recorded.

Statistical Analysis. Results are expressed as the mean ± SD of three independent experiments. Significant differences between means were determined using the Statistical Analysis Software package SAS (Cary, NC, USA): $p < 0.05$ is regarded as significant.

RESULTS

Growth Inhibition of CT-26 Cells. To determine whether HWE and MWE are cytotoxic, we evaluated cell viability using the MTT assay. As an internal control, we also tested two extracts (ACE and AKE) that did not induce NO production in the RAW264.7 murine macrophage cell line.[8] Figure 1 shows

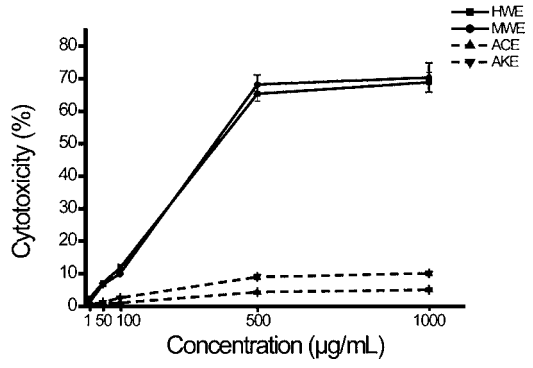

Figure 1. Cytotoxic effects of the four *H. erinaceus* extracts on murine colon carcinoma CT-26 cells. The cells were plated at a density of 1×10^5 cells/well in a 96-well microplate and then treated with various concentrations of each mushroom extract for 48 h. After incubation at 37 °C, cell death was determined using the MTT assay. ACE and AKE were used as the internal negative controls selected among the previously reported *H. erinaceus* extracts. Cytotoxicity was calculated according to the formula shown under Materials and Methods.

that treatment with HWE or MWE decreased cell viability in a concentration-dependent manner, whereas ACE and AKE were not cytotoxic. Overall, MHE treatment was observed to induce slightly more, but significantly different, CT-26 cell cytotoxicity than HWE treatment (42% vs 39% inhibition at 1 mg/mL concentration). As described in our previous paper,[1] growth inhibition of CT-26 cells by HWE and MWE would result from apoptotic cell death.

Inhibition of CT-26 Cell Migration and Invasion. To determine whether HWE and MWE can suppress the migration of CT-26 cancer cells, the cell movements were observed at 20 h after treatment with *H. erinaceus* mushroom extracts. In the presence of each mushroom extract (500 μg/mL), HWE- and MWE-treated cells showed a reduced formation of lamellipodia and a decrease in collective cell migration compared with vehicle-treated or ACE- and AKE-treated controls (Figure 2). These results show that HWE and MWE suppressed the migration and invasion of cancer cells.

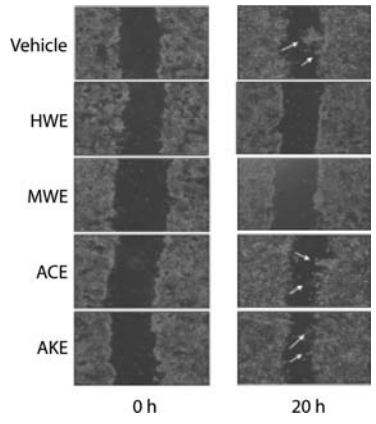

Figure 2. Inhibitory effects of the four *H. erinaceus* mushroom extracts on cancer cell migration. CT-26 cells were cultured to overconfluence in a 6-well plate, and wounds were made by scratching once with a 200 μL micropipet tip. After three washings with serum-free medium, the cells were treated for 0 or 20 h with *H. erinaceus* extracts (500 μg/mL). Vehicle indicates treatment of PBS alone. ACE and AKE were used as the internal negative controls selected among the previously reported *H. erinaceus* extracts. After incubation, images of each well were examined under a microscope (100× magnification). White arrows indicate cells migrating off from the line of wound.

The Matrigel invasion assay was used to determine whether HWE and MWE can block cancer cell invasion. Figure 3 shows that HWE and MWE extracts induced significant decreases in the invasion of CT-26 cells compared with the vehicle-treated or ACE- and AKE-treated controls.

Expression of Proteins Related to Invasion in CT-26 Cells. To determine the molecular mechanism underlying the anti-invasive activity of each mushroom extract, the effect of the extracts on the activation of MMP-2, MMP-9, three MAPKs, and u-PA was assessed in CT-26 cells. MMP-2 and MMP-9 gelatinases are known to degrade the extracellular matrix (ECM) in a process associated with colorectal cancer progression, invasion, angiogenesis, and metastasis.[13] This observation shows that the blocking of MMP-2 and MMP-9 activities could suppress the metastasis of cancer cells into other organs through cell invasion. Gelatin zymography depicted in Figure 4A indicated that HWE and MWE treatments suppressed the activities of MMP-2 and MMP-9 in the conditioned media of CT-26 cells compared with those of the internal controls, ACE and AKE. Western blot analysis shows that MMP-2 and MMP-9 expressions is suppressed by HWE and MWE treatments and that changes in the activity levels of MMP-2 and MMP-9 coincided with each protein expression level (Figure 4B). The serine proteinase urokinase type plasminogen activator (u-PA) is known to be present in cellular structures at the leading edge of migrating cells that are involved in adhesion, migration, invasion, and metastasis.[14] It was observed that HWE and MWE could also down-regulate the expression of u-PA (Figure 4B).

Western blot analysis also shows that ERK and JNK phosphorylation is suppressed by about 50% and 30% following treatment with HWE and MWE, respectively (Figure 5). In addition, HWE and MWE treatments suppressed the p38 gene MAPK phosphorylation by >50%. A related study reported that the treatment with ERK and JNK inhibitors, but not with a p38 inhibitor, resulted in a significant reduction in CT-26 cell invasion.[9] This finding supports our view that HWE and MWE treatments probably resulted in reduction in cell viability,

dx.doi.org/10.1021/jf400916c | J. Agric. Food Chem. 2013, 61, 4898−4904

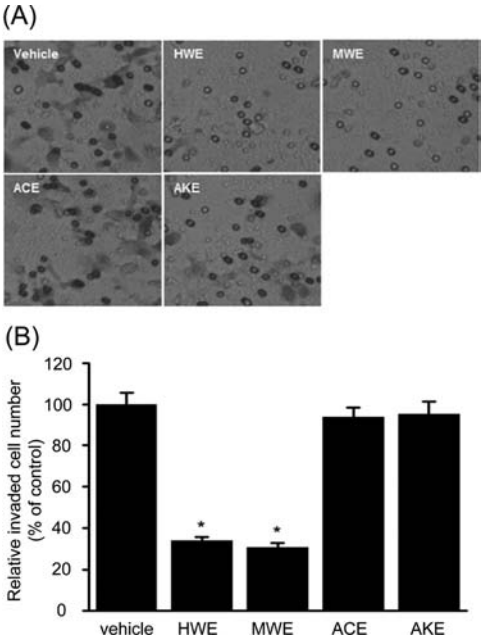

Figure 3. Inhibitory effects of the four *H. erinaceus* mushroom extracts on endometrial cancer cell invasion. (A) CT-26 cells (1×10^5 cells) were cultured in a 12-well plate with an invasive chamber of 8 μm pores. After 48 h of culture with addition of *H. erinaceus* extracts (500 μg/mL), the inner sides of transwell membrane inserts were fixed and stained in hematoxylene solution. Migrated cancer cells trapped in the pores were observed under a microscope (100× magnification). The cell photographs are representatives of membranes of the chamber insert. (B) To evaluate the modulatory effects of four mushroom extracts on cancer cell migration, the average invasion rate was calculated according to the formula described under Materials and Methods. Results are expressed as the mean ± SD. Bars with asterisks are significantly different between the groups at $p < 0.05$ as compared with the vehicle value.

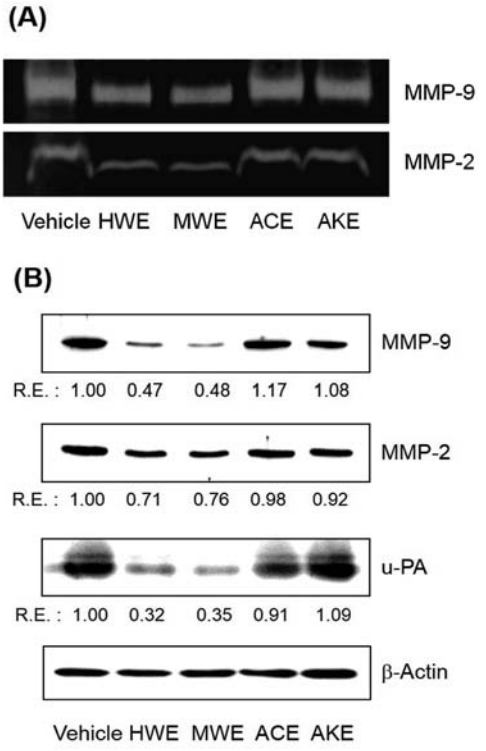

Figure 4. Effects of four *H. erinaceus* mushroom extracts on activation and expression of cell invasion-related proteins including MMP-2, MMP-9, and u-PA. CT-26 cells were treated for 24 h in serum-free D-MEM with each *H. erinaceus* mushroom extract (500 μg/mL). ACE and AKE are used as internal controls. (A) The conditioned medium was recovered, and gelatin zymography was conducted as described under Materials and Methods. (B) The prepared proteins (30 μg) were separated by SDS–polyacrylamide gel electrophoresis, and Western blot analysis was carried out with the indicated primary antibodies. Protein bands were visualized using the ECL detection system. β-Actin was used as a control for constitutively expressed cellular protein. Each protein was expressed as a relative expression (RE) value calculated from the target protein/β-actin expression. The figure represents three independent experiments.

migration, and invasion mainly through suppression of ERK and JNK activation.

Inhibition of Lung Metastasis in Vivo. The present study also investigated whether HWE and MWE could inhibit spontaneous lung metastasis of colon cancer in mice. We found that the number of pulmonary tumor nodules increased greatly in the lungs of mice intravenously injected with CT-26 cells alone (Figure 6); however, ip injection of HWE and MWE (10 mg/kg mouse) for 2 weeks significantly decreased the formation of tumor nodules by about 50% and 55%, respectively. By contrast, ACE and AKE showed slight, but not significant, reductions compared with the vehicle-treated control.

Table 1 shows that the increased weight of the lungs induced by CT-26 cancer cell metastasis is also considerably reduced as a result of HWE and MWE treatment. Body weight, an indicator of toxicity,[15] was not changed, however, by the injection of CT-26 cells and/or HWE or MWE treatment.

Histopathological analysis revealed large nodule areas in lung sections from mice injected with CT-26 cells alone. By contrast, much smaller nodule areas are apparent in mice administered HWE and MWE by ip injection (Figure 7).

DISCUSSION

The progression (metastatic spread) of tumors from one organ to another is reported to be a major cause of poor clinical outcome in cancer patients.[9,16] High rates of cancer-caused mortality are associated with metastatic spread of tumor cells from the original site to the lung. Extensive efforts have made to define the mechanism of metastasis and its inhibition.[17,18] Here, we will briefly mention reported mechanistic aspects of the inhibition of metastasis by naturally occurring compounds that may be relevant to the theme of the present study.

The metastasis and invasion of tumors is a complex, multistep process that involves degradation of the surrounding extracellular matrix, migration to and proliferation of the cells at a secondary site, and stimulation of angiogenesis (blood flow) to that site.[16] Studies designed to define the mechanism of metastasis and its inhibition indicate that expression of matrix proteinases (MMPs), tissue inhibitors of MMPs (TIMPs), and other proteins at the transcriptional level might be regulated by common factors and signal pathways.[17]

Published studies show that (a) an aqueous extract of the Cambodian mushroom *Phellinus linteus* inhibited experimental metastasis of B16BL6 melanoma cells in mice partly via regulation of the urokinase-type plasminogen activator system associated with tumor-cell-induced platelet aggregation;[19] (b) curcumin, a bioactive component in the spice turmeric, inhibited lung cancer cell invasion and metastasis by modulating E-cadherin expression,[20] induced a 54% inhibition

dx.doi.org/10.1021/jf400916c | J. Agric. Food Chem. 2013, 61, 4898–4904

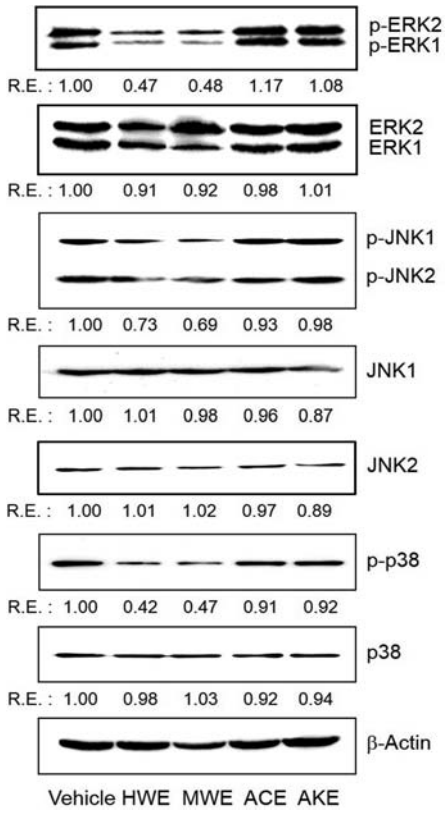

Figure 5. Effects of four *H. erinaceus* extracts on the activation and expression of three MAPKs in CT-26 cells. After incubation with each extract for 48 h, the cells were lysed, and total cellular proteins (30 μg) were resolved in SDS−polyacrylamide gels, after which they were transferred onto nitrocellulose membranes. The membranes were then probed with the indicated antibodies. Protein bands were visualized using the ECL detection system. β-Actin was used as a control for constitutively expressed cellular protein. Each protein was expressed as relative expression (RE) value calculated from the target protein/β-actin expression. The figure represents three independent experiments.

in nodule formation of CT-26 colon cancer cells in the lung,[21] inhibited the migratory and invasive ability of mouse hepatoma cells,[22] and helped regulate cell metastasis by inhibiting MP-2 and MMP-9 and up-regulating TIMPs gene expression in cancer cells;[23] (c) γ-tocotrienol, a component of natural vitamin E, inhibited the invasion and metastasis of human gastric carcinomal cells by a similar mechanisms;[24] and (d) the compounds decursin and decursinol, isolated from the roots of the nonfood plant *Angelica gigas*, inhibited lung metastasis of murine colon carcinoma.[9] The cited authors suggest that compounds that regulate MMP-2 and MMP-9 by targeting their upstream signaling molecules that mediate the expression of MMPs have the potential for treating cancer invasion and metastasis.

In the present study, we found that the hot water and microwaved 50% ethanol *H. erinaceus* mushroom extracts HWE and MWE effectively inhibited the proliferation and invasion of CT-26 colon carcinoma cells, as well as the metastasis and invasion of CT-26 cells to the lungs. The anti-invasive and antimetastasis activities of HWE and MWE might be ascribed to the suppression of ECM-degrading enzyme expression such as that of MMP-2, MMP-9, and u-PA via down-regulating the upstream MAPK signaling pathway. These results suggest that ERK and JNK activation through protein phosphorylation

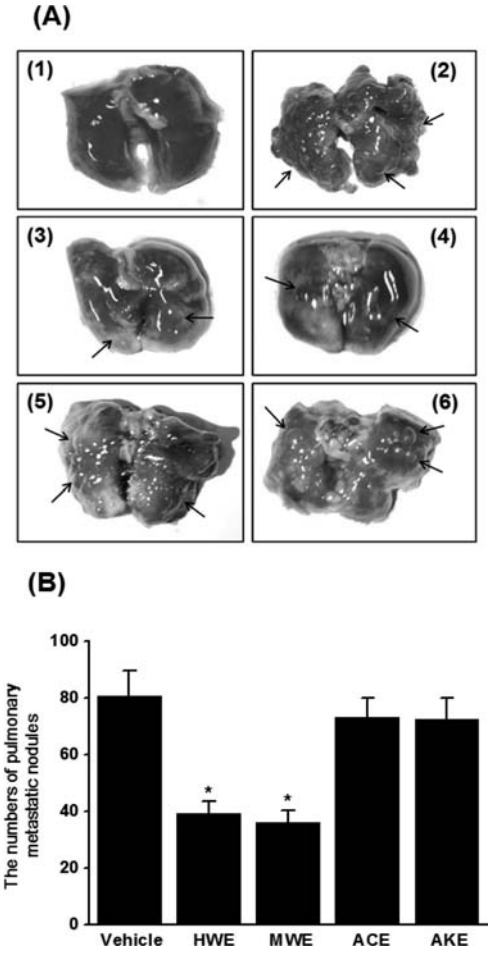

Figure 6. Effects of four *H. erinaceus* mushroom extracts on pulmonary tumor nodule formation through metastasis. BALB/c mice were intravenously transplanted with 1×10^5 cells of CT-26 cancer cells into the tail vein. Then the mice were divided into five groups ($n = 10$) and subjected to an intraperitoneal (ip) injection of each mushroom extract (10 mg/kg mouse) once a day for 2 weeks. Normal control group mice were administered the same volume of PBS only. (A) Lungs were excised to compare the pattern of pulmonary tumor nodule formation among the experimental groups. Panel 1, normal control; panel 2, CT-26 cell transplantation alone; panel 3, CT-26 cells transplantation plus HWE ip injection; panel 4, CT-26 cell transplantation plus MWE ip injection; panel 5, CT-26 cell transplantation plus ACE ip injection; panel 6, CT-26 cell transplantation plus AKE ip injection. Arrows indicate pulmonary tumor nodules. (B) The number of pulmonary tumor nodules is expressed as the mean ± SD. Bars with the same letters are not significantly different between groups at $p < 0.05$.

Table 1. Effects of Four *Hericium erinaceus* Extracts on Body and Lung Weights of CT-26 Cell-Transplanted Mice[a]

sample	body wt (g)	lung wt (g)
normal	25.175 ± 1.653a	0.172 ± 0.012b
CT-26 only	23.486 ± 1.598a	0.260 ± 0.020a
HWE treated	24.639 ± 2.004a	0.186 ± 0.015b
MWE treated	25.005 ± 1.836a	0.181 ± 0.013b
ACE treated	24.074 ± 1.711a	0.238 ± 0.021a
AKE treated	23.934 ± 1.690a	0.243 ± 0.017a

[a]Data are expressed as the mean ± SD ($n = 10$). Values in the same column not sharing a common letter are significantly different at $p < 0.05$.

dx.doi.org/10.1021/jf400916c | J. Agric. Food Chem. 2013, 61, 4898−4904

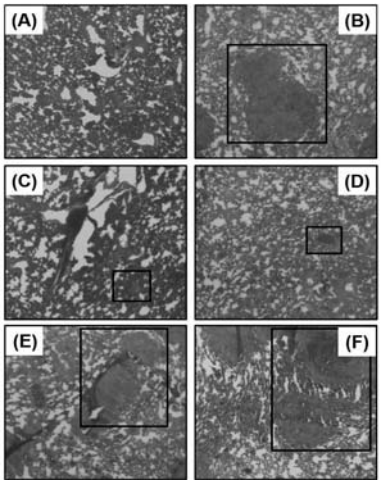

Figure 7. Changes in tumor nodule formation in lungs excised from four *H. erinaceus* mushroom extract-treated mice. To observe nodule formation, paraformaldehyde-fixed and paraffin-embedded lung tissue sections were stained with H&E. Panel A, normal control; panel B, CT-26 cell transplantation alone; panel C, CT-26 cells transplantation plus HWE ip injection; panel D, CT-26 cell transplantation plus MWE ip injection; panel E, CT-26 cell transplantation plus ACE ip injection; panel F, CT-26 cell transplantation plus AKE ip injection. Note the nodule size inside the box of each panel. The images were photographed by microscope at 100× magnification.

would be the most important modulator in this signaling pathway.

Most of the biomarkers that are associated with the inhibition of metastasis mentioned in the cited studies are similar to those observed in the present study. All of the reported studies seem to be consistent with the mechanistic aspects of the observed antimetastasis effect described in the present study for the mushroom extracts.

In conclusion, in this study we have attempted to elucidate the mechanism of the observed inhibition of metastasis of cancer cells from one organ to another in terms several factors (biomarkers) known to be associated with the prevention of metastasis. The findings of the previous and present studies suggest that the whole mushroom or the two bioactive mushroom extracts have multiple benefits. They can protect mice against lethal infection with *Salmonella* Typhimurium via stimulation the immune system, reduce inflammation, and inhibit carcinogenesis and metastasis of cancer cells in vivo. These results indicate the *H. erinaceus* edible mushrooms are a high quality functional food that can benefit human nutrition and may have the ability to help prevent and/or treat human cancers. Dietary evaluation of these beneficial antimicrobial and anticancer effects in humans, possibly using a reported 5% mushroom-containing bread formulation,[25] merits further study. It is worth noting that in addition to the cited health-promoting effects, *H. erinaceus* mushrooms are reported to exhibit other potential health benefits.[26−28] The present study complements our published studies on in vivo anticarcinogenic effects of the tomato glycoalkaloid α-tomatine,[29] the rice bran component γ-oryzanol,[30] and black rice bran.[31] Whether combinations of *H. erinaceus* mushrooms with these bioactive food ingredients would exhibit additive or synergistic beneficial effects also merits study.

AUTHOR INFORMATION

Corresponding Author

*(S.H.N.) Phone: 82-31-219-2619. Fax: 82-31-219-1615. E-mail: shnam@ajou.ac.kr. (M.F.) Phone: 01-510-559-5615. Fax: 01-510-559-6162. E-mail: Mendel.Friedman@ars.usda.gov.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Carol E. Levin for facilitating preparation of the manuscript.

ABBREVIATIONS USED

ACE, acid mushroom extract; ALK, alkaline mushroom extract; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HWE, hot water mushroom extract; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MTT, tetrazole 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MWE, microwaved 50% ethanol mushroom extract; NK, splenic natural killer cells; NO, nitric oxide; RE, relative expression of genes and proteins; TIMP, tissue inhibitor metalloproteinase; TNF-α, tumor necrosis factor-α; u-PA, urokinase-type plasminogen activator

REFERENCES

(1) Kim, S. P.; Kang, M. Y.; Choi, Y. H.; Kim, J. H.; Nam, S. H.; Friedman, M. Mechanism of *Hericium erinaceus* (Yamabushitake) mushroom-induced apoptosis of U937 human monocytic leukemia cells. *Food Funct.* **2011**, *2*, 348−356.

(2) Kim, S. P.; Kang, M. Y.; Kim, J. H.; Nam, S. H.; Friedman, M. Composition and mechanism of antitumor effects of *Hericium erinaceus* mushroom extracts in tumor-bearing mice. *J. Agric. Food Chem.* **2011**, *59*, 9861−9869.

(3) Kim, S. P.; Moon, E.; Nam, S. H.; Friedman, M. *Hericium erinaceus* mushroom extracts protect infected mice against *Salmonella* Typhimurium-induced liver damage and mortality by stimulation of innate immune cells. *J. Agric. Food Chem.* **2012**, *60*, 5590−5596.

(4) Lee, J. S.; Cho, J. Y.; Hong, E. K. Study on macrophage activation and structural characteristics of purified polysaccharides from the liquid culture broth of *Hericium erinaceus*. *Carbohydr. Polym.* **2009**, *78*, 162−168.

(5) Lee, J. S.; Hong, E. K. *Hericium erinaceus* enhances doxorubicin-induced apoptosis in human hepatocellular carcinoma cells. *Cancer Lett.* **2010**, *297*, 144−154.

(6) Jang, J.-H.; Noh, K.-H.; Choi, J.-N.; Jin, K.-S.; Shin, J.-H.; On, J.-H.; Cho, C.-W.; Jeong, W.-S.; Kim, M.-J.; Song, Y.-S. Effect of *Hericium erinaceus* mycelia supplementation on the oxidative stress and inflammation processes stimulated by LPS and their mechanisms in BALB/C mice. *J. Korean Soc. Food Sci. Nutr.* **2010**, *39*, 227−236.

(7) Kim, Y. O.; Lee, S. W.; Oh, C. H.; Rhee, Y. H. *Hericium erinaceus* suppresses LPS-induced pro-inflammation gene activation in RAW264.7 macrophages. *Immunopharmacol. Immunotoxicol.* **2012**, *34*, 504−512.

(8) Kim, S. P.; Choi, Y. H.; Kang, M. Y.; Nam, S. H. Effects of the extracts by extraction procedures from *Hericium erinaceus* on activation of macrophage. *J. Korean Soc. Appl. Biol. Chem.* **2005**, *48*, 285−291.

(9) Son, S. H.; Park, K.-K.; Park, S. K.; Kim, Y. C.; Kim, Y. S.; Lee, S.-K.; Chung, W.-Y. Decursin and decursinol from *Angelica gigas* inhibit the lung metastasis of murine colon carcinoma. *Phytother. Res.* **2011**, *25*, 959−964.

(10) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.

(11) Choi, D. S.; Kim, H.-J.; Yoon, J.-H.; Yoo, S.-C.; Jo, H.; Lee, S. Y.; Min, C. K.; Ryu, H.-S. Endometrial cancer invasion depends on

cancer-derived tumor necrosis factor-α and stromal derived hepatocyte growth factor. *Int. J. Cancer* **2009**, *124*, 2528−2538.
(12) Birkedal-Hansen, H.; Taylor, R. E. Detergent-activation of latent collagenase and resolution of its component molecules. *Biochem. Biophys. Res. Commun.* **1982**, *107*, 1173−1178.
(13) Bogenrieder, T.; Herlyn, M. Axis of evil: molecular mechanisms of cancer metastasis. *Oncogene* **2003**, *22*, 6524−6536.
(14) Blasi, F.; Carmeliet, P. uPAR: a versatile signalling orchestrator. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 932−943.
(15) Öste, R. E.; Friedman, M. Nutritional value and safety of heated amino acid-sodium ascorbate mixtures. *J. Agric. Food Chem.* **1990**, *38*, 1687−1690.
(16) Price, J. T.; Thompson, E. W. Mechanisms of tumour invasion and metastasis: emerging targets for therapy. *Expert Opin. Ther. Targets* **2002**, *6*, 217−233.
(17) Figueira, R. C. S.; Gomes, L. R.; Neto, J. S.; Silva, F. C.; Silva, I. D. C. G.; Sogayar, M. C. Correlation between MMPs and their inhibitors in breast cancer tumor tissue specimens and in cell lines with different metastatic potential. *BMC Cancer* **2009**, *9*, article 20, DOI: 10.1186/1471-2407-1189-1120.
(18) Tsubaki, M.; Satou, T.; Itoh, T.; Imano, M.; Ogaki, M.; Yanae, M.; Nishida, S. Reduction of metastasis, cell invasion, and adhesion in mouse osteosarcoma by YM529/ONO-5920-induced blockade of the Ras/MEK/ERK and Ras/PI3K/Akt pathway. *Toxicol. Appl. Pharmacol.* **2012**, *259*, 402−410.
(19) Lee, H.-J.; Lee, H.-J.; Lim, E.-S.; Ahn, K.-S.; Shim, B.-S.; Kim, H.-M.; Gong, S.-J.; Kim, D.-K.; Kim, S.-H. Cambodian *Phellinus linteus* inhibits experimental metastasis of melanoma cells in mice via regulation of urokinase type plasminogen activator. *Biol. Pharm. Bull.* **2005**, *28*, 27−31.
(20) Chen, H.-W.; Lee, J.-Y.; Huang, J.-Y.; Wang, C.-C.; Chen, W.-J.; Su, S.-F.; Huang, C.-W.; Ho, C.-C.; Chen, J. J. W.; Tsai, M.-F.; Yu, S.-L.; Yang, P.-C. Curcumin inhibits lung cancer cell invasion and metastasis through the tumor suppressor HLJ1. *Cancer Res.* **2008**, *68*, 7428−7438.
(21) Epstein, J.; Sanderson, I. R.; MacDonald, T. T. Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. *Br. J. Nutr.* **2010**, *103*, 1545−1557.
(22) Wang, S.; Yu, S.; Shi, W.; Ge, L.; Yu, X.; Fan, J.; Zhang, J. Curcumin inhibits the migration and invasion of mouse hepatoma Hca-F cells through down-regulating caveolin-1 expression and epidermal growth factor receptor signaling. *IUBMB Life* **2011**, *63*, 775−782.
(23) Hassan, Z. K.; Daghestani, M. H. Curcumin effect on MMPs and TIMPs genes in a breast cancer cell line. *Asian Pac. J. Cancer Prev.* **2012**, *13*, 3259−3264.
(24) Liu, H.-K.; Wang, Q.; Li, Y.; Sun, W.-G.; Liu, J.-R.; Yang, Y.-M.; Xu, W.-L.; Sun, X.-R.; Chen, B.-Q. Inhibitory effects of γ-tocotrienol on invasion and metastasis of human gastric adenocarcinoma SGC-7901 cells. *J. Nutr. Biochem.* **2010**, *21*, 206−213.
(25) Ulziijargal, E.; Yang, J.-H.; Lin, L.-Y.; Chen, C.-P.; Mau, J.-L. Quality of bread supplemented with mushroom mycelia. *Food Chem.* **2013**, *138*, 70−76.
(26) Nagano, M.; Shimizu, K.; Kondo, R.; Hayashi, C.; Sato, D.; Kitagawa, K.; Ohnuki, K. Reduction of depression and anxiety by 4 weeks *Hericium erinaceus* intake. *Biomed. Res.* **2010**, *31*, 231−237.
(27) Abdulla, M. A.; Fard, A. A.; Sabaratnam, V.; Wong, K.-H.; Kuppusamy, U. R.; Abdullah, N.; Ismail, S. Potential activity of aqueous extract of culinary- medicinal Lion's Mane mushroom, *Hericium erinaceus* (Bull.: Fr.) Pers. (Aphyllophoromycetideae) in accelerating wound healing in rats. *Int. J. Med. Mushrooms* **2011**, *13*, 33−39.
(28) Hiwatashi, K.; Kosaka, Y.; Suzuki, N.; Hata, K.; Mukaiyama, T.; Sakamoto, K.; Shirakawa, H.; Komai, M. Yamabushitake mushroom (*Hericium erinaceus*) improved lipid metabolism in mice fed a high-fat diet. *Biosci., Biotechnol., Biochem.* **2010**, *74*, 1447−1451.
(29) Friedman, M.; McQuistan, T.; Hendricks, J. D.; Pereira, C.; Bailey, G. S. Protective effect of dietary tomatine against dibenzo-[*a*,*l*]pyrene (DBP)-induced liver and stomach tumors in rainbow trout. *Mol. Nutr. Food Res.* **2007**, *51*, 1485−1491.
(30) Kim, S. P.; Kang, M. Y.; Nam, S. H.; Friedman, M. Dietary rice bran component γ-oryzanol inhibits tumor growth in tumor-bearing mice. *Mol. Nutr. Food Res.* **2012**, *56*, 935−944.
(31) Choi, S. P.; Kim, S. P.; Nam, S. H.; Friedman, M. Antitumor effects of dietary black and brown rice brans in tumor-bearing mice: relationship to composition. *Mol. Nutr. Food Res.* **2013**, *57*, 390−400.