JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Composition and Mechanism of Antitumor Effects of *Hericium erinaceus* Mushroom Extracts in Tumor-Bearing Mice

Sung Phil Kim,[†] Mi Young Kang,[§] Jae Ho Kim,[†] Seok Hyun Nam,[*,‡] and Mendel Friedman[*,∥]

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

[§]Department of Food Science and Nutrition, Kyoungpook National University, Daegu 702-701, Republic of Korea

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

ABSTRACT: We investigated antitumor effects of the following four extracts of freeze-dried *Hericium erinaceus* mushrooms in Balb/c mice intracutaneously transplanted on the backs with CT-26 colon cancer cells: HWE, hot water extraction by boiling in water for 3 h; MWE, microwaving in 50% ethanol/water at 60 W for 3 min; and ACE and AKE, boiling in 1% HCl or 3% NaOH for 2 h. HWE and MWE with a higher content of β-glucans, determined by an assay kit, than ACE and MKE were active in all bioassays. Gas chromatography/mass spectrometry analyses showed the presence of 40, 27, 16, and 13 compounds, respectively, in the four extracts. Daily intraperitoneal (ip) injections of HWE and MWE for 2 weeks significantly reduced tumor weights by 38 and 41%. Tumor regressions were associated with changes in the following cancer biomarkers as compared to phosphate buffer (PBS)-treated control mice: 2.7- and 2.4-fold increases in cytolytic activity of splenic natural killer (NK) cells; restored nitric oxide production and phagocytosis in peritoneal macrophages to 95−98% of normal levels; ∼2-fold increase in released pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1β, and interleukin-6 from macrophages; and ∼56 and ∼60% reductions in the number of blood vessels inside the tumor. The pro-angiogenic factors vascular endothelial growth factor (VEGF), cyclooxygenase 2 (COX-2), and 5-lipoxygenase (5-LOX) were also significantly reduced in mRNA and protein expression by tumor genes. Enzyme-linked immunosorbent assay of tumor cells confirmed reduced expression of COX-2 and 5-LOX (32 and 31%). Reduced COX-2 and 5-LOX expression down-regulated VEGF expression, resulting in inhibition of neo-angiogenesis inside the tumors. The results indicate that induction of NK activity, activation of macrophages, and inhibition of angiogenesis all contribute to the mechanism of reduction of tumor size.

KEYWORDS: *Hericium erinaceus* mushroom extracts, composition mice, antitumor effect, biomarkers, mechanism of tumor regression

INTRODUCTION

Mushrooms are a source of good quality proteins, containing up to 40% protein on dry weight basis (reviewed in ref 1). The essential amino acids of most species are found in the same proportion as in eggs. The vitamin content is also high, similar to that of yeast, except for thiamin. We found that for the four *Hericium erinaceus* extracts evaluated in the present stuydy, protein, carbohydrate, and mineral (ash) contents were all high, ranging (in % of dry wt) from 35.5 to 38.5, 33.8 to 39.5, and 14.6 to 19.0, respectively.[2] The moisture content was about 8%, and the fiber and fat contents were <1%. Low-fat mushrooms are a good source of essential nutrients and micronutrients. In addition, some mushroom varieties are reported to possess health-promoting antidiabetic, antioxidant, antitumor, cardiovascular, immunomodulating, antibacterial, and antiviral properties (reviewed in ref 3).

H. erinaceus, "Yambushitake" in Japan or "Houtou" in China, is an edible medicinal mushroom. In previous studies, we showed that two extracts of this mushroom upregulated nitric oxide (NO) production and phagocytosis[4] and induced apoptosis of U937 human monocytic leukemia cells.[2] To place the findings of the present study in perspective, we will first briefly mention selected reported observations on anticarcinogenic effects of mushrooms. Polysaccharides isolated from culture broth of *Hericium* spp. mushrooms have been shown to inhibit pulmonary metastatic effects in mice,[5] and a polysaccharide extract from the Maitake mushroom (*Grifola fondosa*) enhanced the antitumor activity of dendritic cell-based immunotherapy against murine colon cancer.[6] Another group has shown that oral administration of *Pleurotus ostreatus* mushrooms inhibits colitis-related colon carcinogenesis in mice.[7] In addition, a water-soluble polysaccharide isolated from *Lentinus edodes* mushrooms exhibited antitumor activity on Sarcoma 180 solid tumors implanted in BALB/c mice.[8] Oral ingestion of *L. edodes* mycelia inhibited melanoma growth in melanoma-bearing mice by mitigation of T-cell-mediated immunosuppression.[9] Additional examples are described below in the section Inhibition of Angiogenesis.

To demonstrate further the antitumor potential of *H. erinaceus* mushrooms, the major objective of this study was to determine the composition of four extracts by gas chromatography/mass spectrometry (GC/MS), the β-glucan content by a commercial

Received: May 16, 2011
Accepted: August 16, 2011
Revised: August 10, 2011
Published: August 16, 2011

assay kit, their abilities to reduce transplanted tumor size in mice, and the mechanism that governs tumor reduction.

MATERIALS AND METHODS

Materials. RPMI 1640 medium, Hanks' balances salt solution (HBSS), fetal bovine serum (FBS), and other cell culture reagents were purchased from Hyclone Laboratories (Logan, UT). Calcein-AM was purchased from Calbiochem (San Diego, CA). Hematoxyline, eosin Y, lipopolysaccharide (LPS), recombinant interferon-γ (rIFN-γ), and other reagents were from Sigma Chemicals (St. Louis, MO). The AMV reverse transcriptase and 2′-deooxyribonucleoside triphosphate (dNTP) mixture were obtained from Takara Bio (Kyoto, Japan). Polymerase chain reaction (PCR) primers were custom-synthesized and purified by Bioneer (Daejon, Korea).

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 the following solvents as previously described:[4] 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 Sox let extractor (Prolabo, Paris, France; MWE); and 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) with a cutoff molecular mass of 12 kDa against the same solvent. All extracts were lyophilized to powders. The amount of β-glucan in each extract was determined using a Mixed-Linkage-β-glucan Kit (Megazyme Intern Treland Ltd., Wicklow, Ireland).

Component Analysis by GC/MS. Lyophilized *H. erinaceus* extracts were derivatized in two steps to protect carbonyl function following the method of Kim et al.[10] Dried samples were dissolved in methoxyamine hydrochloride (100 μL; 20 mg/mL) in pyridine and reacted at 60 °C for 1 h. The acidic protons were exchanged against the trimethylsilyl group to increase the volatilities of the polar compounds using 100 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at 70 °C for 1 h. Each *H. erinaceus* extract was analyzed by GC/MS using a gas chromatograph, model 6890GC (Agilent Technologies, Inc., Santa Clara, CA), equipped with a mass spectrometer detector 5975 and DB-1 column (Agilent Technologies, stationary phase; polyethylene glycol, 30 m × 0.25 mm; i.d. 0.25 μm). The temperature was programmed at 70 °C (4 min) with an increase of 10 °C/min until 300 °C (6 min) was reached. Helium gas was used as the carrier with a flow rate of 1 mL/min. Both injector and detector temperatures were set at 250 °C. The injection was a split ratio of 25:1 in all cases. The injection volume was 1 μL. Mass spectra were recorded in electron ionization mode with ionization energy of 70 eV. Components were identified by retention times in the mass spectra and by comparing the mass spectra with those in a commercial library.[11]

Mammalian Cell Cultures. The CT-26 mouse colon carcinoma cell line and the Yac-1 splenic natural killer cells (NK)-sensitive mouse lymphoma cell line from the American Type Tissue Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS containing penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Mice. Pathogen-free female BALB/c mice (6 weeks old) 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 with pelletized commercial chow diet and sterile tap water ad libitum during the entire experimental period, as described in our previous studies with black rice bran[12] and liquid rice hull smoke.[10]

Table 1. β-Glucan Content of Four *H. erinaceus* Extracts[a]

sample	β-glucan (% of dry wt)
HWE	0.47 ± 0.02 a
MWE	0.51 ± 0.03 a
ACE	0.17 ± 0.01 b
AKE	0.13 ± 0.01 c

[a] Values expressed as means ± SDs (n = 3) in the column with the same letters are not significantly different at $p < 0.05$.

Tumor Transplantation and Treatment. BALB/c mice were intracutaneously transplanted with 1×10^6 cells of CT-26 mouse colon cancer cells in 200 μL of phosphate-buffered saline (PBS) into the lateral side of the back. 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 at the end of the treatments for the isolation of peritoneal macrophages and excision of tumor masses and spleens.

Tumor Growth. To evaluate the effects of each treatment, tumor masses were excised from the control and experimental groups of mice and weighed in an analytical balance.

NK Cell Cytolysis Assay. Spleen cells were isolated, and blood cells were removed as described by Trop et al.[13] Spleens were crushed through a stainless mesh (size 60) in complete medium (CM) consisting of RPMI 1640 medium and 10% FBS plus antibiotics. The NK activity was evaluated as follows. Briefly, splenic mononuclear cells were obtained by centrifuging the spleen cell suspension on 2 mL of histopaque-1077 (Sigma Diagnostics, St. Louis, MO) to recover the cells in the interface, which were then washed three times with CM. The cells were resuspended in CM at 1×10^6 cells/mL. Yac-1 cells, used as the target cell, were labeled with Calcein-AM ester according to the method of Roden et al.[14] Labeling of the cells (1×10^6 cells/mL) was in all cases performed at a final Calcein-AM concentration of 25 μM for 30 min. Purified mononuclear effector cells and labeled Yac-1 target cells were added to a 96-well plate and cocultured for 3 h at 37 °C (20:1 effector:target ratio). Following centrifugation at 400*g*, 100 μL of the supernatant from each well was harvested for measuring fluorescence released into medium using a spectrofluorometer (model RF-5301, Shimazu, Kyoto, Japan) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Spontaneous fluorescence release was determined by culturing the labeled target cells in CM without effector cells. Maximum fluorescence was obtained from wells where target cells were incubated with a lysis buffer (50 mM sodium borate, 0.1% triton X-100, pH 9.0). Specific lysis was calculated according to the following formula:

$$\%\ \text{lysis} = [1 - (\text{experimental fluorescence} - \text{background fluorescence})/(\text{maximum fluorescence} - \text{background fluorescence})] \times 100$$

NO Generation Assay. Isolation and purification of peritoneal macrophage cells from tumor-bearing mice treated with each *H. erinaceus* extract were performed according to the method of Narumi et al.[15] Cells exudated through lavaging with HBSS reagent were plated onto 60 mm tissue culture dishes ($1-5 \times 10^6$ cells/dish) to produce macrophage cells firmly adhered onto the dishes. NO was measured by determining the concentration of its stable oxidative

Table 2. Retention Times (t_R) of Identified Compounds in *H. erinaceus* Mushroom Extracts and Relative Proportions in % Peak Area

			peak area (%)			
peak no.	t_R (min)	compd (based on mass spectral data)	HWE	MWE	ACE	AKE
1	5.84	silanamine, *N*,*N*′-methanetetraylbis[1,1,1-trimethyl]	0.064	0.141		
2	5.93	2,6-dimethyl-4-nitrosophenol	0.099	0.169		
3	8.59	1-(3-methylbutyl)-2,3,4,6-tetramethylbenzene				2.22
4	8.69	L-alanine		0.099		
5	11.46	silanol, trimethyl-, phosphate	1.569	3.972	0.514	0.248
6	11.7	maleate	0.064			
7	11.7	ethyl malonate		0.122		
8	11.9	butanedioic acid	0.08		0.69	
9	12.44	fumaric acid	0.479	1.076	0.354	
10	12.51	2-(2-methylphenyl)-5*H*-6,7-dihydrocyclopenta[*d*]pyrimidine-4(3*H*)one	0.251			
11	13.39	4-aminoquinoline 1-oxide	0.094			
12	14.45	malic acid	4.097	7.088		
13	14.59	*N*,*O*-Bis-(trimethylsilyl)-2-pyrrolidone carboxylic acid	0.256	0.203		
14	14.84	L-aspartic acid	0.256	0.41		
15	14.97	erythritol			0.262	
16	14.97	butanal	0.145	0.602		
17	15.76	methanephrine	27.147			
18	15.99	glutamic acid		0.378		
19	16	6-hydroxy-2-aminohexanoic acid	0.154			
20	16.34	tartaric acid		0.094		
21	16.66	4,5-methanochrysene		0.174		
22	16.67	2-(2-methylphenyl)-5*H*-6,7-dihydrocyclopenta[*d*]pyrimidine-4(3*H*)one	0.669			
23	17.58	xylitol	34.398	26.205	12.127	16.919
24	18.3	citric acid	0.334	1.153		
25	18.38	L-lysine	0.616			
26	18.42	D-galactose	0.082			
27	18.43	α-D-galactopyranose	0.144	0.217	0.89	
28	18.92	pentanedioic acid				0.726
29	19.26	D-glucose		0.277		
30	19.39	2-phenyl-4-(propen-1-yl)-pyrimidine-5-carboxamide		0.516		
31	19.46	D-mannopyranose	0.196	0.158	0.16	
32	19.48	3,5-dimethoxymandelic acid	0.104			1.607
33	19.83	D-mannitol	2.601	2.34	1.182	
34	19.83	glucose oxime				0.214
35	20.12	9*H*-carbazole		0.555		
36	20.13	glucopyranose			1.177	
37	20.14	2,5-dimethoxymandelic acid				7.719
38	20.28	hexadecanoic acid			0.216	
39	20.49	1-(9-anthryl)-2-propyn-1-ol	0.203			
40	20.5	2-methylthio-3,4-dihydronaphtho[2,1-c]thiophene				1.246
41	20.51	*anti*-10-hydroxy-5,6,8,9-tetrahydro-5,9-methano-7*H*-benzocyclohepten-7-one			0.338	
42	21.3	inositol	0.297	0.137		
43	21.8	oleic acid			0.68	
44	21.8	*trans*-9-octadecenoic acid	0.046			0.087
45	22.04	octadecanoic acid	0.299		0.142	
46	23.77	silylated derivative of authentic 3-keto-2-methylvaleric acid	0.096			
47	23.78	uridine		0.176		
48	24.69	3,4-dimethoxymandelic acid	0.041			
49	24.77	5-nitro-3-phenyl-1*H*-indazole	0.035			
50	24.86	D-xylopyranose	0.185			
51	24.92	maltose	0.06			

dx.doi.org/10.1021/jf201944n |*J. Agric. Food Chem.* 2011, 59, 9861–9869

Table 2. Continued

peak no.	t_R (min)	compd (based on mass spectral data)	peak area (%) HWE	MWE	ACE	AKE
52	25.16	(2-methylcyclopent-1-enyl) (4,4-dimethyl-3-oxocyclopent-1-enyl)methane	0.051			
53	25.26	glucuronic lactone	0.081			
54	25.77	allose		0.192	0.274	
55	25.96	melibiose	0.278			
56	26.1	β-[(*S*)-citronellyl]-D-glucopyranoside	0.244			
57	26.27	4-pyrimidinecarboxyic acid, hexahydro-6-oxo-1,3-diphenyl-2-phenylimino)-, methyl ester			0.308	
58	26.28	condylon				0.081
59	26.28	β-D-ribofuranosyl		0.201		
60	26.34	1-dichloromethyl(dimethyl)silyloxybutane	0.236			
61	26.37	1-benzoimidazol-1-yl-3-(4-iodo-phenoxy)-propan-2-ol	0.342			
62	26.37	oleamide		1.14		
63	26.6	α-D-glucopyranoside, β-D-fructofuranosyl	0.535	0.958	0.633	
64	26.61	5-(*N*-*tert*-butoxycarbonyamino)-5-methyl-11-methylene-5,11-dihydro-6*H*-pyrido[4,3-*b*]carbazole				0.149
65	26.74	α-l-mannopyranoside				0.129
66	26.84	lactose				0.087
67	28.16	glucopyranose, β-D-glucopyranosyl	0.381			

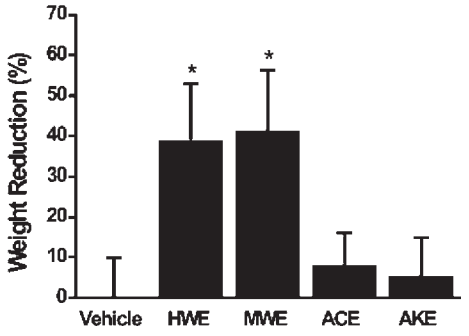

Figure 1. Effects of the four *H. erinaceus* extracts on tumor growth in vivo. BALB/c mice were intracutaneously transplanted with CT-26 mouse colon cancer cells (1×10^6 cells, 200 μL) and then subjected to daily ip injections of the extracts (10 mg/kg/mice). After 2 weeks, mice were sacrificed to measure tumor weight. Results are expressed as means $\pm$ SDs ($n = 10$). Bars with asterisks are significantly different between groups at $p < 0.05$ as compared with the vehicle control value.

metabolite nitrite, using the microplate method described by Xie et al.[16] with slight modification. Briefly, isolated peritoneal macrophages were cultured in a 96-well plate (1×10^5 cells/well) with rIFN-γ (10 U/mL) and LPS (100 ng/mL) for 48 h. To measure nitrite concentrations, culture medium (100 μL) was mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% *N*-(naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid] at room temperature for 15 min. The absorbance at 570 nm was determined with a microplate reader (model 550, Bio-Rad, Hercules, CA) using a standard calibration curve for sodium nitrite.

Phagocytotic Uptake Assay. The phagocytotic activity of peritoneal macrophage cells was measured following the method of Duperrier et al.[17] with some modification. Briefly, isolated peritoneal macrophages were cultured in a 60 mm culture dish (1×10^5 cells) with rIFN-γ (10 U/mL) and LPS (100 ng/mL) for 48 h. After stimulation, cells (1×10^4 cells) were resuspended in PBS (1 mL) containing 5% FBS and cultured at 37 °C for 15 min. They were then incubated with Dextran-FITC (1 mg/mL) at 37 °C for 1 h. The reaction was stopped with cold PBS containing 5% FBS and 1% sodium azide. The cells were then washed three times with cold PBS and analyzed on a FACSvantage instrument (Becton-Dickinson, Franklin Lakes, NJ).

Histology and Assessment of Tumor Vascularity. For histological analysis, the tumor tissue of the mice was fixed with 4% paraformaldehyde in 0.5 M phosphate buffer (pH 7.4). The tissues were rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sectioned into 4 μm and mounted onto glass slides. The sections were then dewaxed using xylene and ethanol and stained with hematoxylin and eosin Y (H&E). Blood vessels were counted in six blindly chosen random fields under the microscope at 200× magnification, and the microvessel density was recorded.

Reverse Transcription (RT) PCR of Cellular RNA. Total cellular RNA was prepared from tumor tissues following acid phenol guanidium thiocyanate−chloroform extraction.[18] For RT, total RNA (1 μg) was incubated with AMV reverse transcriptase (5 U) and oligo (dT18) as primer (100 ng). DNA amplification was then primed in a reaction mixture containing dNTP mix (400 μM), *Taq* polymerase (2.5 U), and primer sets (20 μM each) representing target genes. PCR was conducted using a thermocycler (model PTC-200, MJ Research Inc., Reno, NV) with one cycle for 5 min at 94 °C, followed by 30 cycles for 30 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C, and finally one cycle for 5 min at 72 °C. All amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with a UV illuminator. The intensity of the separated bands of DNA was quantified using a gel documentation system (mode LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan).

Western Blot Analysis of Cell Proteins. The tumor tissues were lysed and extracted in a homogenizer 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. The tissue extracts containing proteins (30 μg) were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membrane (Millipore, Billerica, MA).

The following primary antibodies were used for Western blot analysis: rabbit antimouse vascular endothelial growth factor (VEGF) polyclonal antibody (Santa Cruz, Delaware, CA), goat antimouse cyclooxygenase 2 (COX-2) polyclonal antibody (Santa Cruz), rabbit

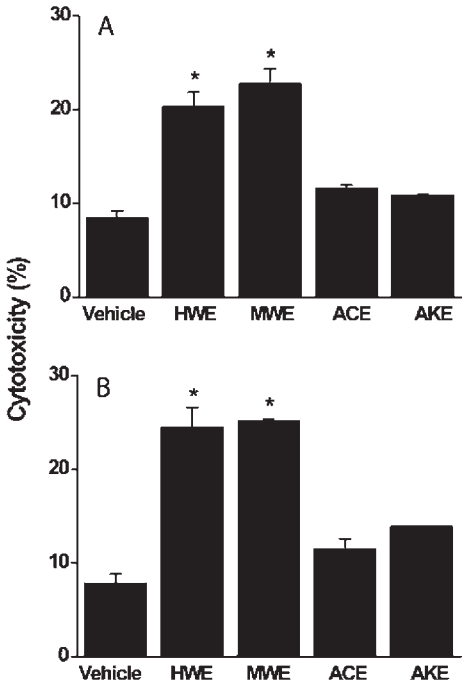

Figure 2. Effect of the *H. erinaceus* extracts on NK cytolytic activities. (A) NK cells from tumor-transplanted mice subjected to ip injection with each extract (10 mg/kg) were incubated with Yac-1 target cells labeled with Calcein-AM for 3 h (20:1 effector:target ratio). The NK cytolytic activity was evaluated by measuring Calcein-AM release from Yac-1 target cells using a fluorometer. (B) NK cells isolated from normal mice were treated with the mushroom extracts. They were then incubated with Calcein-AM to release from Yac-1 target cells. Results are expressed as means ± SDs (n = 10). Bars with asterisks are significantly different between groups at $p < 0.05$ as compared with the vehicle value.

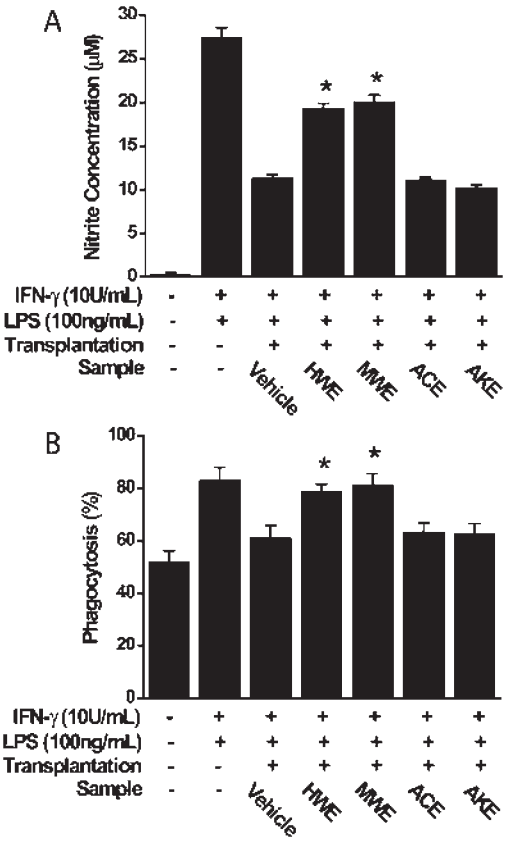

Figure 3. Up-regulation of NO production and phagocytosis activities of peritoneal macrophages from tumor-transplanted mice by the mushroom extracts. (A) Macrophage cells from tumor-transplanted mice subjected to ip injection with the extracts (10 mg/kg) were primed with rIFN-γ and then stimulated with LPS for 48 h. NO released into the culture medium was measured by the Griess method. (B) To induce phagocytosis, macrophage cells were stimulated with rIFN-γ and LPS for 48 h and then incubated with Dextran-FITC (1 mg/mL) for 1 h. The fluorescence intensity was then determined by flow cytometry. Results are expressed as means ± SDs (n = 10). Bars with asterisks are significantly different between groups at $p < 0.05$ as compared with vehicle value.

antimouse inducible nitric oxide synthase (iNOS) polyclonal antibody (Cell Signaling Tec., Danvers, MA), goat antimouse 5-lipoxygenase (5-LOX) polyclonal antibody (Abcam, Cambridge, MA), and antimouse β-actin monoclonal antibody (Millipore, Billerica, MA). After they were blocked with 5% skim milk, membranes were incubated with each primary antibody, followed by HRP-conjugated anti-IgG antibodies. Blots were developed using the ECL detection kit (Pierce, Rockford, IL). The intensity of the separated protein bands was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan). At least three separate replicates were determined for each experiment.

Enzyme-Linked Immunosorbent Assay (ELISA) of Cytokines and Eicosanoids. Extraction of eicosanoids prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) from tumor tissues was conducted by a described method.[19] Briefly, tumor tissues from tumor-bearing mice treated with various *H. erinaceus* extracts were homogenized in a phosphate buffer (pH 7.0) containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), and 10 mM ethylenediamine tetraacetic acid (EDTA). The homogenates were microcentrifuged at 14000g for 15 min at 4 °C to recover the supernatant. For quantitation of cytokines, peritoneal macrophages from each mice group were stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL) followed by recovery of the culture medium. Cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in the culture medium and eicosanoids LTB_4 and PGE_2 in the supernatants were determined by ELISA (Biosource International, Camarillo, CA) following the manufacturer's instructions. The absorbance of the final solution at 420 nm was measured in a microplate reader (model 550, Bio-Rad).

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

RESULTS AND DISSUSSION

Component Analysis of Extracts in Relation to Bioactivities. β-Gluco-oligosaccharides are structural components of biologically active polysaccharides. For example, polysaccharides from Shiitake (*L. edodes*) mushrooms called lentinan have been shown to possess strong antitumor effects.[20] Other examples are mentioned in the Introduction and under Inhibition of Angiogenesis.

Using a commercial assay kit, we measured the β-glucan content of the four *H. erinaceus* extracts. Table 1 shows that the β-glucan content of the HWE and MWE bioactive extracts was

Table 3. Mushroom Extracts Stimulate Release of Pro-inflammatory Cytokines in Peritoneal Macrophages from Tumor-Transplanted Mice[a]

	cytokine (pg/mL)		
sample	TNF-α	IL-1β	IL-6
control (−)[b]	56.457 ± 8.455 d	23.157 ± 3.023 d	18.318 ± 2.429 d
control (+)[c]	5712.51 ± 318.03 a	323.57 ± 14.235 a	410.219 ± 22.382 a
vehicle[d]	2113.91 ± 245.95 c	121.735 ± 11.354 c	155.767 ± 10.982 c
HWE	4200.87 ± 175.24 b	256.644 ± 17.32 b	327.289 ± 19.37 b
MWE	4317.34 ± 196.76 b	277.135 ± 20.304 b	354.512 ± 30.104 b
ACE	2412.65 ± 95.31 c	133.265 ± 10.309 c	162.023 ± 11.787 c
AKE	2331.97 ± 87.15 c	122.563 ± 12.092 c	147.375 ± 18.39 c

[a] Values expressed as means ± SDs ($n = 10$) in each column with the same letters are not significantly different at $p < 0.05$. [b] Normal mice macrophages not stimulated with rIFN-γ and LPS. [c] Normal mice macrophages stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL). [d] Tumor-transplanted mice macrophages subjected to ip injection with PBS only as vehicle and then stimulated with rIFN-γ and LPS.

about 3−4-fold higher than in the two inactive ACE and AKE fractions.

The analysis by GC/MS also revealed that the active extracts contained a larger number of characterized compounds than did the inactive extracts. Active HWE contained 40 compounds, and MWE contained 27; inactive ACE contained 16, and AKE contained 13 (Table 2). Evidently, a number of bioactive compounds were degraded during the acidic and basic extractions used to prepare ACE and AKE. It can be assumed that individual compounds in ACE and AKE are probably inactive.

Because the chemical nature of the identified compounds varied widely, we do not know which individual compound or mixtures of compounds may contribute to the observed regression of the implanted tumors. These observations suggest the need for further in vivo studies designed to ascertain the therapeutic potential of the mushroom components. Next, we discuss the results of the assays of the extracts.

Effect of *H. erinaceus* Extracts on Growth of Transplanted Tumors. The objective of the present study was to measure the inhibitory effect of *H. erinaceus* extracts on the growth of implanted tumors in mice and to determine several biomarkers that may be associated with the mechanism of growth inhibition. To accomplish this objective, CT-26 mouse colon cancer cells were employed for the intracutaneous transplantation of the tumors. The result (Figure 1) showed that ip injection of HWE and MWE for 2 weeks led to a reduction of tumor growth by 38 and 41%, respectively. By contrast, the extracts ACE and AKE used as internal controls induced only 7 and 5% reductions, respectively.

NK Activity. To find out whether NK cells were activated by the treatments, we determined changes in NK cytolysis activity. *H. erinaceus* extracts were intraperitoneally injected to the tumor-bearing mice for 2 weeks. The spleens from the sacrificed mice were then removed to separate NK cells (effector cells). To measure the level of calcein-AM released from the target cells caused by cytotoxic action of the effector cells, separated NK cells were incubated with a calcein-AM labeled YAC-1 cell line (target cells). The results show that, in response to the HWE and MWE treatments, NK cell activities were increased by 2.4- and 2.7-fold, respectively (Figure 2A).

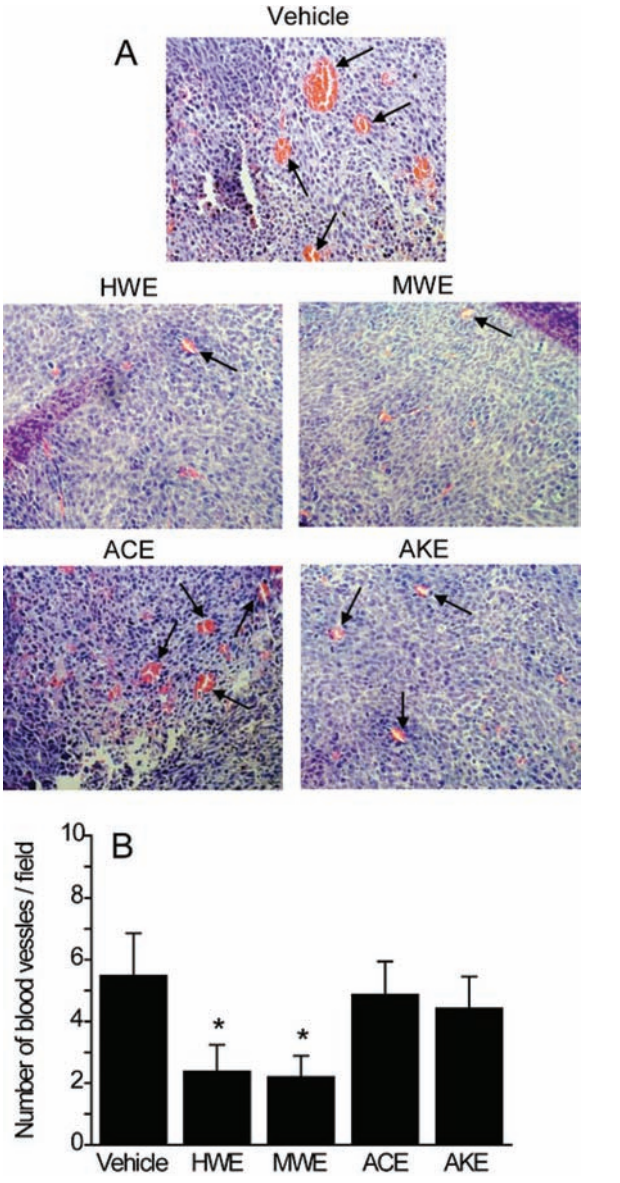

Figure 4. Antiangiogenic effect of mushroom extracts. (A) To observe blood vessel formation, paraformaldehyde-fixed and paraffin-embedded tumor sections were stained with hematoxylin and eosin Y (H&E). The images were photographed by microscope at 200× magnification. Arrows indicate blood vessels inside the tumor. (B) To record vessel density, blood microvessels were counted under a microscope in six randomly chosen fields. Results are expressed as means ± SDs ($n = 10$). Bars with asterisks are significantly different between groups at $p < 0.05$ as compared with the vehicle value.

We next examined whether HWE and MWE directly stimulated NK to exert a cytolytic action on tumor cells. For this experiment, NK cells were isolated from normal mice and then treated with various extracts ex vivo. The results depicted in Figure 2B show similar activation patterns of NK cytotoxicities to those observed with tumor-bearing mice; the treatment with HWE and MWE elevated NK cytotoxicity to tumors about 3-fold. ACE and AKE extracts were inactive. Evidently, both HWE and MWE can suppress tumor growth through activation of NK cells.

Macrophage Activity. Published studies indicate that physiological activities of macrophage cells are suppressed in tumor-bearing

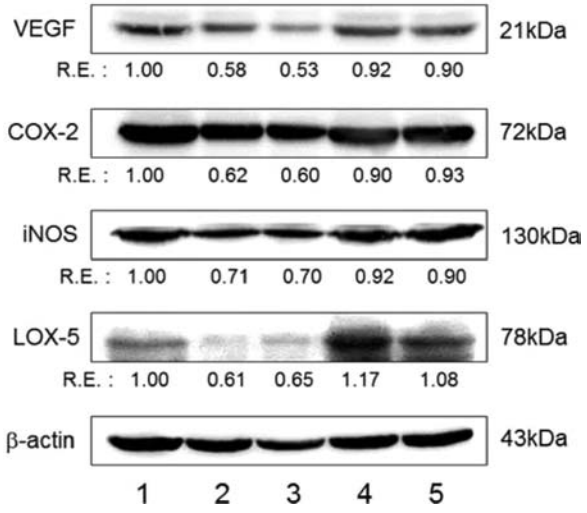

Figure 5. Western blot analysis of mushroom extract-modulated VEGF, COX-2, iNOS, and 5-LOX protein expression in transplanted tumors. The relative ratio is expressed as a relative expression (RE) value calculated from target gene/β-actin gene expression. β-Actin was used as a control for constitutively expressed protein. Intraperitoneally injected treatments: lane 1, control group, PBS; lane 2, HWE; lane 3, MWE; lane 4, ACE; and lane 5, AKE. Figures represent results from at least three individual experiments.

Table 4. Inhibitory Effect of Mushroom Extracts on Release of Eicosanoids in Transplanted Tumors[a]

	eicosanoids (pg/mL)	
sample	PGE_2	LTB_4
vehicle[b]	1065.43 ± 55.09 a	2629.04 ± 85.440 a
HWE	722.372 ± 13.371 b	1802.33 ± 37.859 b
MWE	713.052 ± 29.128 b	1902.33 ± 40.414 b
ACE	1064.92 ± 8.388 a	2779.75 ± 60.217 a
AKE	1048.85 ± 10.844 a	2705.67 ± 83.267 a

[a] Values are expressed as means ± SDs (n = 10) in each column with the same letters are not significantly different at $p < 0.05$. [b] Tumor-transplanted mice macrophages subjected to ip injection with PBS only as vehicle and then stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL).

animals.[21] It was therefore of interest to examine whether the mushroom extracts can restore suppressed macrophage activity to the level observed in normal mice. NO generation and phagocytosis were employed as parameters of macrophage activation. The results show that NO generation from rIFN-γ- and LPS-activated peritoneal macrophages from HWE- or MWE-treated tumor-bearing mice was increased by about 60% as compared to the vehicle-treated tumor-bearing mice (Figure 3A). Again, both ACE and AKE treatments failed to elevate NO production. Similar results were obtained for changes in phagocytosis activities in peritoneal macrophages from tumor-bearing mice. Figure 3B shows that treatment with HWE and MWE elevated phagocytotic activities of macrophages to 95 and 98% of normal levels. ACE or AKE extracts were inactive.

Pro-inflammatory Cytokine Secretion. As another parameter for macrophage activation, pro-inflammatory cytokine release (TNF-α, IL-1β, and IL-6) in response to priming with rIFN-γ and LPS was measured in peritoneal macrophages from

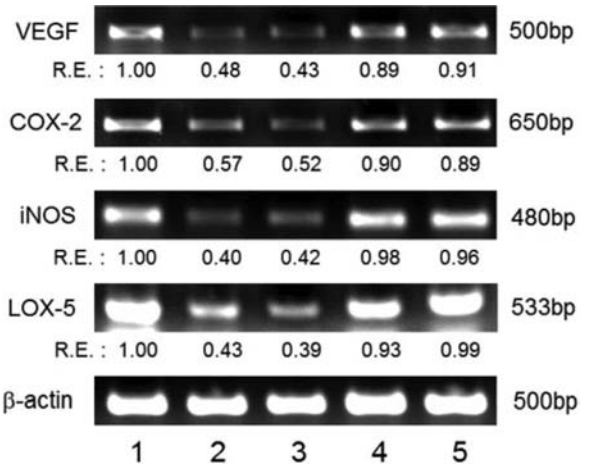

Figure 6. Semiquantitative analysis of mushroom extract-modulated gene expression for the synthesis of VEGF, eicosanoids, and NO in transplanted tumors. The relative ratio of each gene is expressed as a RE value calculated from target gene/β-actin gene expression. Intraperitoneally injected treatments: lane 1, control group, PBS; lane 2, HWE; lane 3, MWE; lane 4, ACE; and lane 5, AKE. Figures represent results from at least three individual experiments.

tumor-bearing mice (Table 3). ELISA analysis showed that production of the pro-inflammatory cytokines increased about 2-fold in cells from HWE- or MWE-treated tumor-bearing mice as compared to the vehicle-treated control. The suppressed macrophage activity was restored to about 85% of that in normal mice. These in vitro results suggest that restoration of macrophage activity by HWE and MWE is an important factor for tumoricidal or tumoristatic action of *H. erinaceus* extracts in vivo.

Inhibition of Angiogenesis. The antitumor activity of the extracts might in part be attributed to the inhibition of formation of new blood vessels inside the tumor, resulting in tumor cell death through hypoxia.[22] To demonstrate this possibility, we examined the effect of each extract on angiogenesis in the transplanted tumors by counting the number of blood vessels under the microscope in the control and experimental mice groups. As compared to the vehicle-treated control, blood vessel formation was significantly suppressed in the transplanted tumors treated with HWE and MWE by about 56 and 60%, respectively (Figure 4). It appears that HWE and MWE blocked new microblood vessel formation inside the tumor needed for tumor growth.

Regulation of Pro-angiogenic Factors. Two possible mechanisms may govern the suppression of new blood vessel formation in the transplanted tumors. The first is a direct effect of the mushroom extract. The second acts indirectly, involving inhibition of specific growth-related signals of vascular endothelial cell proliferation. The first possibility is ruled out because HWE and MWE did not inhibit angiogenesis in the chick embryo chorioallantoic membrane (CAM) assay (data not shown).[23]

We also investigated whether elevated angiogenesis-related gene expression levels in the transplanted tumors were down-regulated by the treatments. Total RNA of excised tumors was prepared, and transcription levels of VEGF, COX-2, 5-LOX, and iNOS genes were determined by RT-PCR. Figure 4 shows that both HWE and MWE, but not ACE and AKE, inhibited the known angiogenic factor VEGF by >50%.

Similar inhibition patterns were also observed by Western blot analysis of protein synthesis levels (Figure 5). PGE_2 and LTB_4,

Table 5. Primer Sets Representing Four Target Genes and the Internal Control β-Actin Gene

primer	sequence
VEFG sense	5′-TGACAAGCCAAGGCGGTGAG-3′
VEGF antisense	5′-CCTCCTCCCAACACAAGTCC-3′
COX-2 sense	5′-TCTCAACCTCTCCTACTAC-3′
COX-2 antisense	5′-GCACGTAGTCTTCGATCACT-3′
iNOS sense	5′-ATGTCCGAAGCAAACATCAC-3′
iNOS antisense	5′-TAATGTCCAGGAAGTAGGTG-3′
5-LOX sense	5′-ATGAGCTGTTTCTAGGCATGTACC-3′
5-LOX antisense	5′-GAATAAAGTACCCCTGACCCAGCC-3′
β-actin sense	5′-GTGGGGCGCCCCAGGCACCA-3′
β-actin antisense	5′-GTCCTTAATGTCACGCACGATTTC-3′

the respective arachidonic acid metabolites produced by COX-2 and 5-LOX, measured by ELISA (Table 4) confirmed the inhibitory actions of HWE and MWE on COX-2 and 5-LOX activities; HWE inhibited PGE_2 and LTB_4 by 32 and 31%, and MWE inhibited by 33 and 28%, respectively; ACE and AKE were inactive. The fact that the up-regulation of VEGF determined with target genes (Figure 6 and Table 5) was induced by PGE_2 supports our finding that down-regulation of COX-2 expression is associated with the blockade of blood vessel formation inside the tumors.[24,25]

The results indicate that reduction of tumor size is accompanied by modulation of gene expressions associated with tumor growth and the consequent effects on levels of the pro-inflammatory mediators and angiogenic factors. Although we do not know the mechanism of how HWE and MWE modulate expression of the COX-2 and 5-LOX, it is likely that down-regulation of COX-2 and 5-LOX might be the cause of repressed VEGF expression and subsequent tumor cell death due to a lack of diffusion of nutrients and oxygen to the tumor cells. Earlier studies also indicated that VEGF is the downstream mediator of 5-LOX in the induction of the angiogenic process and subsequent tumorigenesis.[26,27]

Related Studies on Inhibition of Angiogenesis. The present findings complement and extend reported observations on tumor-related angiogenesis induced by the following mushroom extracts and isolated pure compounds (described in chronological order). Ho et al. [28] showed that a polysaccharide isolated from the edible mushroom *Coriolus versicolor* inhibited angiogenesis and tumor growth in A180 tumor-bearing mice. Another study showed that the medicinal mushroom *Ganoderma lucidum* inhibited prostate cancer-dependent angiogenesis.[29] A water extract of the Maitake mushrooms (*G. fondosa*) inhibited angiogenesis in an in vivo chick CAM assay.[30] It has also been reported that oral administration of a β-1,3-D-glucan isolated from *Sparassis crispa* (Hanabiratake) edible mushrooms inhibited angiogenesis and reduced metastatic lung foci in female mice.[31] Chang et al.[32] showed that oral administration of an Enoki mushroom (*Flammulina velutipes*) protein inhibited angiogenesis and tumor size in hepatoma-bearing mice.[32] These observations and those of the present study indicate that inhibition of angiogenesis is associated with a reduction of tumor growth and metastasis induced by some mushroom extracts and isolated pure compounds.

The described findings indicate that daily ip administration for 2 weeks of *H. erinaceus* extracts to mice with transplanted tumors significantly reduced tumor size by about 40% as compared to mice treated with the vehicle. Compositional analysis showed that the nature, number, and concentrations of two bioactive extracts differed significantly from those present in two inactive extracts. Studies of the mechanism of the beneficial effects showed that the regression of the tumors was accompanied with changes in several in vitro and in vivo biomarkers (NK, NO, phagocytosis, tumor vascularity, histopathology, cellular RNA, and proteins; VEGF, COX-2, PGE_2, 5-LOX, LTB_4, TNF-α, IL1β, and IL-6) associated with normal and tumor cells. Of special interest is the finding that angiogenesis (restriction of blood flow to tumor cells) plays a role in the molecular mechanism of tumor regression induced by the mushroom extracts. Collectively, it appears that activation of macrophages, induction of NK cells, and inhibition of blood flow to tumor cells play a major role in the molecular mechanism of tumor regression. Stimulation of the immune system may also contribute to inhibition of tumor growth.[5,32,33] These considerations suggest that *H. erinaceus* mushrooms may have therapeutic potential against cancer.

AUTHOR INFORMATION

Corresponding Author

*Tel: 82-31-219-2619. Fax: 82-31-219-1615. E-mail: shnam@ajou.ac.kr or Mendel.Friedman@ars.usda.gov.

ACKNOWLEDGMENT

We thank Hye Min Lee of Gyeonggi Bio-Center (Suwon, Korea) for assisting with the GC/MS analysis and Carol E. Levin for assistance with the preparation of the manuscript.

ABBREVIATIONS USED

BSA, bovine serum albumin; CAM, chorioallantoic membrane; CM, complete medium; COX-2, cyclooxygenase 2; dNTP, 2′-deooxyribonucleoside triphosphate; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GS/MS, gas chromatography/mass spectrometry; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; ip, intraperitoneal; 5-LOX, 5-lipoxygenase; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; NK, splenic natural killer cells; NO, nitric oxide; PBS, phosphate buffer; PGE_2, prostaglandin E_2; RE, relative expression of genes and proteins; rIFN-γ, recombinant interferon-γ; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor

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