FULL COMMUNICATION

Maitake (D Fraction) Mushroom Extract Induces Apoptosis in Breast Cancer Cells by *BAK-1* Gene Activation

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ABSTRACT   For many years mushrooms have been used empirically in traditional medicine to treat several diseases. Study of the maitake mushroom, with its immunomodulatory and antitumoral properties, has led to the isolation of several bioactive compounds. One of these, D fraction, is known to reduce tumor cell viability. This study examined the effect of isolated D fraction on viability and apoptosis of human breast cancer cells (MCF7). These cells were treated with maitake (D fraction) extract at 18 μg/mL, 36 μg/mL, 91 μg/mL, 183 μg/mL, or 367 μg/mL or were left untreated (control) for 24 hours. MCF7 incubation with the maitake extract resulted in decreased cell viability [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethox-yphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay] in a dose-dependent manner. Apoptosis was statistically significantly increased in a dose-dependent manner at every concentration tested (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay). Upon incubation with D fraction, a microarray assay revealed upregulation of BAK-1 and cytochrome c transcripts, 2 proteins directly involved in the apoptotic pathway. Reverse transcriptase polymerase chain reaction studies confirmed these findings; *BAK-1* was one of most overexpressed gene, as observed by microarray assay. These findings confirm the apoptotic effect of maitake D fraction in breast cancer cells and further highlight the involvement of cytochrome c release to the cytoplasm. Cytoplasmic release of cytochrome c, another player in the apoptotic pathway, was also increased after incubation with D fraction in a dose-dependent manner. This finding indicates that the effect of this compound involves mitochondrial dysfunction. The identification of the molecular mechanisms by which D fraction exerts its effects is crucial for the development of preventive and therapeutic strategies for cancer.

KEY WORDS: • *apoptosis* • **BAK-1** • *breast cancer* • *cytochrome c* • *maitake* • *mushroom*

INTRODUCTION

BREAST CANCER IS THE LEADING cause of death from cancer in women, the most common cancer among women worldwide, and the second most frequent cancer in the world. Its incidence is still increasing, with a total number of cases of 1,050,100 in 2002 compared with 572,100 in 1980. Breast cancer causes 370,000 deaths annually, representing 13.9% of cancer deaths in women, mainly in the industrialized countries.[1] While researchers search for the cause of the disease, an effective and rapid treatment is mandatory for these patients.

Maitake mushroom (*Grifola frondosa*) is a giant mushroom indigenous to northern Japan that has been used by Japanese herbalists for many years. Most research on maitake has focused on the use of maitake D fraction for treating various malignancies. The bioactive D fraction extracted from maitake is a protein-bound polysaccharide compound and is prepared by a standardized procedure developed by Maitake Products Inc. Among the various maitake fractions that have been prepared, D fraction was found to be the most potent for enhancing the immune system via oral administration or injection (both routes were effective), leading to the highest reduction in the rate of cancer proliferation.[2] Maitake D fraction has been reported to exert its antitumor effect in tumor-bearing mice by enhancing the immune system through activation of macrophages, T cells, and natural killer cells.[3] In a previous study, the combination of immunotherapy with the maitake D fraction and chemotherapy suggested that the D fraction might decrease the size of lung, liver, and breast tumors in patients with cancer.[2,4,5] In 1998, the Food and Drug Administration granted Maitake Products, Inc., an investigational new drug application to conduct a phase II pilot study using maitake D fraction among patients with advanced breast and prostate cancers. These ongoing studies are evaluating the immune stimulatory effect of D fraction on tumor size, immune assays, clinical symptoms, and patient quality of life.

The purpose of the current study was to investigate the direct effect of maitake D fraction on breast cancer MCF7 cells. We found that incubating MCF7 cells with maitake D fraction significantly decreased cell viability and induced cell death. Genomic analysis using complementary DNA (cDNA) microarrays revealed the upregulation of 22 pro-apoptotic and 7 anti-apoptotic genes compared with untreated MCF7 cells (control). *BAK-1* transcript at the messenger RNA level was found to be upregulated. After incubation with D fraction, cytoplasmic release of cytochrome c, another player in the apoptotic pathway, was also increased in a dose-dependent manner.

MATERIALS AND METHODS

Bioactive maitake D fraction

The bioactive D fraction was extracted from maitake mushroom, corresponding to the protein-bound polysaccharide compound, and was prepared by a standardized procedure developed by Maitake Products, Inc.

Cell cultures

The human breast cancer MCF7 cell line was obtained from the American Type Culture Collection. MCF7 cells were routinely cultured in Eagle minimal essential medium containing 10% inactivated fetal bovine serum and 1% penicillin/streptomycin. Cell culture media, fetal bovine serum, and penicillin/streptomycin were purchased from Invitrogen Life Technologies. Cells were grown at $37°C$ in a humidified 5% CO_2 atmosphere. In agreement with previous studies, incubations were performed for 24 hours in serum-free conditions.[6]

Labeling and cDNA human microarrays

We adopted the direct labeling of probes with amine-modified random primer, using $5\mu g$ of amplified RNA (aaRNA) as starting material. The $5\mu g$ of aaRNA ($8\mu L$) was combined with amine-modified random primer ($2\mu g/\mu L$, $1\mu L$) and ribonuclease inhibitor (5 units/μL, $1\mu L$). The mix was incubated at $70°C$ for 10 minutes and then chilled on ice for 10 minutes. Primer–RNA solution was added to the reverse transcriptase mix ($5\times$first-strand buffer, $6\mu L$; $50\times$ amplified deoxyuridine triphosphate [dUTP]/dinucleotide triphosphate [25 mM deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate; 15 mM deoxythymidine triphosphate; and 10 mM aminoallyl-dUTP], $0.6\mu L$; dithiothreitol, 0.1 M, $3\mu L$; Superscript II reverse transcriptase [Invitrogen/Life Technologies], $2\mu L$) and incubated at $42°C$ for 2 hours. The reaction was terminated by adding EDTA (0.5 M, $10\mu L$), and the RNA was hydrolyzed with NaOH (1 M, $10\mu L$) at $65°C$ for 30 minutes.

Probe purification

Probes were cleaned with a QIA quick polymerase chain reaction (PCR) purification kit (Qiagen); the Cy3- and Cy5-labeled products were combined and $30\mu L$ of water was ad-ded, followed by $500\mu L$ of Buffer PB. The samples were applied to QIA quick columns, which were centrifuged at 13,000 rpm for 1 minute, after which the flow through were discarded. To wash the columns, $750\mu L$ of Buffer PE was added, columns were spun again for 1 minute, and the flow-through was discarded. The washing step was repeated once more, and columns were spun again to remove residual ethanol. Fresh collection tubes were placed beneath each column, $30\mu L$ of Buffer EB was added, and tubes were incubated for 1 minute at room temperature. Columns were then centrifuged at 13,000 rpm for 1 minute, and the elution step was repeated once. Elutes were partially dried in a vacuum centrifuge and the volumes were adjusted to $23\mu L$ with water.

Hybridization and washing conditions

The following were added: $4.5\mu L$ of $20\times$saline–sodium citrate, $2\mu L$ of poly(A) (10 mg/mL), and $0.6\mu L$ of 10% (wt/vol) sodium dodecyl sulfate (SDS). The probes were then denatured at $98°C$ for 3 minutes. The products were pipetted onto arrays, coverslips were applied, and the slides were placed in a hybridization chamber (Corning). cDNA human microarrays containing 25,000 known genes were incubated in a $42°C$ water bath for 16 hours and were subsequently washed with $0.5\times$saline–sodium citrate, 0.01% (wt/vol) SDS, followed by $0.06\times$SSC, at room temperature for 10 minutes each. Slides were spun for 5 minutes at 800 rpm (130 g) at room temperature.

Array scanning and analysis

Arrays were read with an Affymetrix 428 fluorescent scanner (MWG Technologies) at 10-μm resolution and variable photomultiplier tube voltage settings to obtain the maximal signal intensities with less than 1% (wt/vol) probe saturation. The resulting images were analyzed by using ImaGene, version 4.2, and GeneSight software, version 4.0 (Biodiscovery, Inc.). After Lowess normalization, the data were analyzed by using GeneSigh 4.0 software (Biodiscovery).

Hierarchical clustering analysis

Hierarchical clustering was done by using the neighbor-joining method applied to the Pearson correlation distance matrix. In addition, other hierarchical clustering methods and other distance-generating functions (such as Euclidean) were used. Only marginal deviations between the methods and the distance matrices used were observed. We found the most stable results for the neighbor-joining method applied to the Pearson correlation distance matrix.

MTS toxicity assay

MCF7 cells (2×10^5 cells/mL) were allowed to grow until 70–90% confluence and were incubated with each treatment for 24 hours. Cells were washed twice with phosphate-buffered saline, and their viability was assessed by using Cell Titer 96 Aqueous ONE Solution Reagent (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay (Pro-

mega), according to manufacturer's instructions. Optical density was measured at 492 nm. Results are expressed as percentage of control, which was considered to be 100%.

TUNEL assays

Cells grown until 70% confluence onto glass coverslips were incubated with the different referred treatments for 24 hours. A TUNEL assay (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) was performed by using the In Situ Cell Death Detection kit (Roche Diagnostics), according to the manufacturer's instructions. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nucleus observed. Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2). The percentage of stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of nuclei stained with DAPI at×200 magnification field. One thousand nuclei were evaluated. Three independent experiments were performed.

Reverse transcriptase PCR

Reverse transcriptase (RT) PCR was performed to confirm the results obtained by cDNA array hybridization in MCF7 cell lysates. For each sample, 1.0 μg of RNA was reverse-transcribed in a reaction volume of 25 μL in the presence of 10 mM dNTP and 2 μL of RT-PCR enzyme mix. Gene-specific primers for *BAK-1* (forward: GAGAGCC TGCCCTGCCCTCT; reverse: CCACCCAGCCACCCCT CTGT), coamplified with a set of primers for the housekeeping gene *βActin,* were used in RT-PCR reaction. Quantifications were done in triplicate. The PCR products were separated on ethidium bromide–stained 2% agarose gel. The intensity of the fluorescence was automatically measured and integrated by using Genescan software (Image Master, Pharmacia).

Western blotting

Proteins were isolated from MCF7 total cell lysates by using Tripure (Roche Diagnostics). For cytoplasmic protein isolation, MCF7 cells cultures were subjected to the Abcam cell fraction protocol (Tufts-New England Medical Center and Molecular Cardiology Research Center). In brief, cells were lysed with 500 μL of cell-fractioning final solution and kept on ice for 20 minutes. Protein cytoplasmic fraction was then obtained upon centrifugation at 8000 rpm for 10 minutes. Pellets were then washed and proteins were quantified by using the bicinchoninic acid protein quantification assay kit (Pierce Biotechnology). Equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis with a 5% stacking gel. After electrophoresis, proteins were blotted into a Hybond nitrocellulose membrane (Amersham Biosciences) by using a mini-transblot electrophoretic transfer cell (Amersham Biosciences). Immunodetection for

cytochrome c and βactin (both at 1:2000 dilution) (Santa Cruz Biotechnology) was accomplished with enhanced chemiluminescence (ECL kit, Amersham Biosciences). Anti-goat secondary antibody conjugated with horseradish peroxidase (1:5000) was obtained from Santa Cruz Biotechnology. The relative intensity of each protein blotting analysis was measured by using a computerized software program (Biorad) and normalized with βactin bands to compare the expression of proteins in different treatment groups. Experiments were repeated 3 times.

Statistical analyses

All experiments were performed in triplicate. Quantifications are expressed as means ± standard deviations. Samples were evaluated by an analysis of variance test. Differences between experimental groups were analyzed by the Student *t*-test and were considered statistically significant whenever the *P* value was less than .05. Statistical analysis was performed using GraphPad.

RESULTS

Maitake induces the expression of apoptotic genes in breast cancer cells

Our objective was to determine whether D fraction from *G frondosa* can induce the apoptotic pathway in MCF7 cells. We performed a cDNA microarray using 25,000 known human genes. MCF7 cells were treated with increased concentrations of D fraction over 24 hours. Figure 1A shows a representative cDNA microarray image from each of the MCF7 cells treated with and without increased concentrations of maitake D fraction. In the right portion of Figure 1A, "a" corresponds to the square section indicated in the left microarray image. Three independent experiments were done. Figure 1B represents the box plot analysis after Lowess normalization under all conditions. Genes whose expression changed were considered statistically significant according to established algorithms, and the genes whose expression changed by at least 2-fold as a result of Maitake treatment were selected for further analysis. This combined analytic approach has previously been shown to identify differentially expressed genes with high sensitivity and specificity.

We identified 4321 gene sequences differentially expressed (*t*-test with false discovery rate, $P < .05$) after incubation with D fraction (at 91 μg/mL or 187μg/mL or 367 μg/mL during 24 hours) on MCF7 cells. Unsupervised hierarchical clustering performed by using the expression profiles of 4321 globally varying genes across the controls and Maitake-treated MCF-7 cells (data sets representing the 4 groups) revealed that samples clustered primarily according to D fraction treatment. Figure 1C represents the unsupervised hierarchical cluster 3 in all conditions. This finding suggested that the principal source of global variation in gene expression across these data sets was due to increased Maitake concentrations. In Figure 1C, black arrows indicate the specific variation in gene expression related to apoptosis due to Maitake treatment.
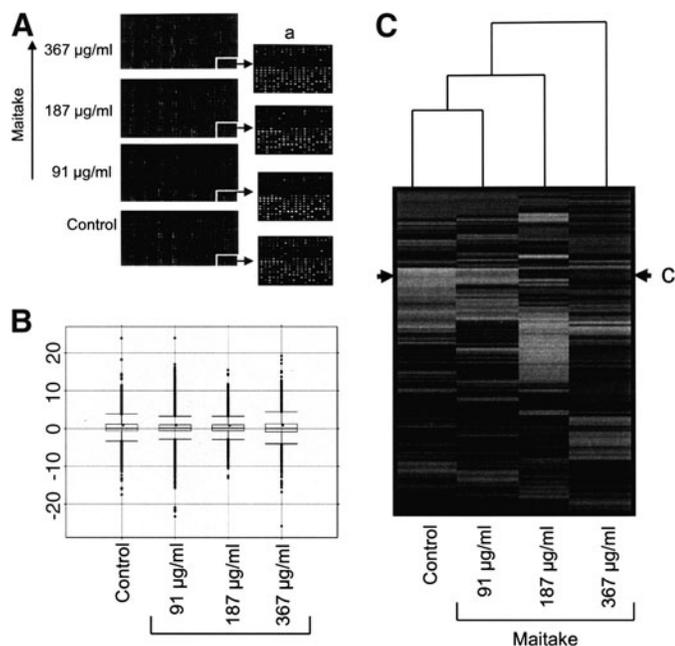
FIG. 1. Analysis of gene expression induced by maitake in breast cancer MCF7 cells. **(A)** Images of the cDNA microarrays employing 25,000 human known genes from MCF7 treated with and without (control) increased concentrations of maitake D fraction. The magnified right images (a) correspond to the indicated white square in each condition. The cDNA microarrays were performed against the human reference and used Cy3 fluorescent dye. In the microarrays, the green spots correspond with the Cy3 channel or the human reference; in the red channel (Cy5 dye) with red spots, we observed the genes overexpressed in relation to the control and the MCF7 treated cells. The yellow spots indicate the genes that were expressed in both channels (that is, were expressed equally for the human reference and for the sample). **(B)** The box plot analysis after Lowess data normalization, performed for each condition (see Materials and Methods section). **(C)** Two-dimensional unsupervised hierarchical clustering of the data matrix, consisting of 25,000 genes by 4 samples and representing the control and maitake-treated MCF-7 cells. Rows represent genes and columns represent sample conditions. The cDNA microarray experiments were performed in triplicate. Black arrows (c) indicate the 29 apoptosis related-genes that were significantly overexpressed after maitake treatment (Table 1).

Only 29 apoptotic genes were statistically significantly upregulated after maitake treatment (Table 1). *BAK-1* was one of the pro-apoptotic genes with the most increased expression after incubation with D fraction. Accordingly, expression of *BAK-1* was upregulated by D fraction treatment in a dose-dependent manner; expression was increased almost 25-fold upon incubation with $367 \mu g/mL$ of D fraction when compared with controls (Table 1). Table 1 lists these 29 genes and the average ratio value with respect to control. Four of 29 genes were related to induction of apoptosis (pro-apoptotic genes): *BAK-1* (BCL2-antagonist/killer 1), *BCL2L13* (BCL2-like 13 [apoptosis facilitator]), *BID* (BH3 interacting domain death agonist), and *NUPR1* (nuclear protein 1). One gene, cytochrome c1 (*CYC1*), was related to electron transport from mitochondria; 17 were directly involved in apoptosis; and 7, including *MPO* (myeloperoxidase), *TNFAIP8*

(tumor necrosis factor α–induced protein 8), *VDAC-2* (voltage-dependent anion channel 2), and *BCL2L2* (BCL2-like 2), were related to anti-apoptosis (anti-apoptotic genes). We decided to begin studying the apoptotic pathway with one of the genes most activated by maitake in the MCF7 cells.

Maitake directly induces cells death in breast cancer cells

To determine whether maitake D fraction can directly induce cell death in MCF7 cells, we treated cells with increased concentrations of maitake and evaluated cell viability and cell death by using a Time Lapse Microscope; pictures were taken every 10 minutes for 24 hours. Figure 2A shows a representative image (from 3 independent experiments) of MCF7 cell culturing treated with and without maitake at different concentrations. These correspond to the images taken 1 hour, 5 hours, 10 hours, and 24 hours after treatment. Microscope images were set up at×20 amplification. After we analyzed the video data and counted the relative percentage of cell deaths under each condition, we found a significant increase in cell death with dose-dependent maitake treatment; apoptosis was highest at the maximum concentration of $367 \mu g/mL$, reaching almost 98% cell death (Fig. 2B). We observed a high percentage of cell death under all conditions because of the stress (incubation at room temperature and not-humidified atmosphere controlled) that cells suffer with the Time Lapse equipment. The atmosphere was not controlled for humidity.

D fraction decreased MCF7 cell viability and increased apoptosis

The evidence that maitake D fraction exerted anticancer effects and directly induced cell death prompted us to measure the effect of this compound on viability of MCF7 cells and to examine whether the trigger mechanisms for cell death are related to apoptosis. Cell viability was first examined with MTS assay in MCF7 cell cultures incubated with 5 different concentrations of D fraction. The number of viable cells decreased gradually with increasing concentrations of D fraction (Fig. 3). In fact, the highest concentration of D fraction resulted in a significant decrease in viability compared with control values (Fig. 3).

To evaluate whether this decrease in cell viability was due to increased apoptosis, a TUNEL assay was performed. MCF7 (4×10^4) cells were incubated with the same 5 concentrations of maitake D fraction for 24 hours, and the percentage of apoptotic cells was quantified. Treatment with this fraction, at any concentration, led to a statistically significant increase in the number of apoptotic cells in a dose-dependent manner (Fig. 3B). Of note, nearly 95% of the cells became apoptotic when treated with the highest concentration of maitake fraction ($367 \mu g/mL$) (Fig. 3B). Figure 3C shows a representative image of these latter findings. As observed, treatment with this fraction increased the number of apoptotic cells (green) compared with the untreated culture.

TABLE 1. APOPTOTIC GENE EXPRESSION PROFILE IN MCF7 ABNORMALLY EXPRESSED
AFTER INCUBATION WITH D FRACTION MAITAKE RELATIVE TO UNTREATED CELLS

Gene Number	Gene ID	Gene Symbol	Gene Name	Biological Function	Ratio per D Fraction Concentration			
					367 μg/mL	183 μg/mL	92 μg/mL	37 μg/mL
1	H52673	BAK1	BCL2-antagonist/killer 1	Induction of apoptosis	25.022	4.526	1.234	0.417
2	W74646	BCL2L13	BCL2-like 13 (apoptosis facilitator)	Induction of apoptosis	22.828	10.526	4.615	0.586
3	AA457705	IER3	Immediate early response 3	Apoptosis	20.131	19.520	13.957	1.163
4	AA700443	SEMA6A	Sema domain, (semaphorin) 6A	Apoptosis	19.494	16.485	1.862	0.585
5	R55303	NGFR	Nerve growth factor receptor	Apoptosis	16.425	8.194	6.934	0.407
6	AA927951	FXR1	Fragile X mental retardation	Apoptosis	12.056	6.495	3.523	0.691
7	T64469	NUPR1	Nuclear protein 1	Induction of apoptosis	8.750	7.620	2.030	0.693
8	AA872011	AXUD1	AXIN1 up-regulated 1	Apoptosis	7.659	7.363	1.368	1.330
9	R96507	ADAMTSL4	ADAMTS-like 4	Apoptosis	6.842	16.334	6.139	1.201
10	AA018124	HD	Huntingtin (Huntington disease)	Apoptosis	6.161	4.981	1.620	0.418
11	AA625671	DAP3	Death associated protein 3	Apoptosis	5.949	3.590	1.159	0.421
12	AA919063	BID	BH3 interacting domain death agonist	Induction of apoptosis	5.941	8.429	1.125	0.362
13	AA634466	PPP1R13B	Protein phosphatase 1	Apoptosis	5.790	4.597	2.524	0.972
14	AA447774	CYC1	Cytochrome c-1	Electron transport	5.752	8.168	3.116	0.286
15	AA777555	TNFRSF19	Tumor necrosis factor receptor	Apoptosis	5.589	7.404	1.272	0.730
16	N70382	CCAR1	Cell division cycle and apoptosis regulator 1	Apoptosis	5.537	13.580	8.613	0.471
17	AA878235	UNC5D	Unc-5 homolog D (C. elegans)	Apoptosis	4.998	3.721	0.899	0.636
18	AA236957	ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor	Apoptosis	4.731	28.364	8.283	0.881
19	AA181808	APIP	APAF1 interacting protein	Apoptosis	4.644	12.486	2.357	0.749
20	AA458464	CTNNBL1	Catenin, beta-like 1	Apoptosis	3.526	9.187	4.688	1.406
21	AA704483	ITGB2	Integrin, beta-2	Apoptosis	2.956	5.006	2.412	0.570
22	AA044059	VDAC1	Voltage-dependent anion channel 1	Apoptosis	2.685	3.684	3.137	1.059
23	AA703058	MPO	Myeloperoxidase	Anti-apoptosis	24.131	7.729	7.636	0.343
24	AA454588	BCL2L2	BCL2-like 2	Anti-apoptosis	13.216	16.176	8.567	0.353
25	AA002126	BIRC3	Baculoviral IAP repeat-containing 3	Anti-apoptosis	10.558	10.239	1.838	1.152
26	H57855	TNFAIP8	Tumor necrosis factor, a-induced protein 8	Anti-apoptosis	9.454	6.262	8.567	0.449
27	AA857093	VDAC2	Voltage-dependent anion channel 2	Anti-apoptosis	8.228	12.687	5.652	0.687
28	N32514	SFRP1	Secreted frizzled-related protein 1	Anti-apoptosis	7.613	7.805	2.386	0.743
29	AA703531	IL24	Interleukin 24	Anti-apoptosis	7.323	3.138	2.645	0.404

Maitake increased BAK-1 *gene expression in MCF7 cells*

Expression of the *BAK-1* gene was greatly increased after incubation with D fraction. Accordingly, expression of *BAK-1* was upregulated by D fraction treatment in a dose-dependent manner. *BAK-1* transcript was examined by using RT-PCR assay. As illustrated in Figure 4, the messenger RNA level of *BAK-1* increased upon treatment with maitake D fraction at increasing concentrations, confirming the previous findings obtained by microarray assay. In these assays a housekeeping gene, *βactin*, was used as a control for gene expression. Figure 4 shows the ratio of *BAK-1* gene expression with respect to *βactin* (top panel) and the RT-PCR–specific bands at increased maitake concentrations (bottom panel).

Maitake increased cytochrome C cytoplasmic level in MCF7 cells

To further investigate the possible mechanisms by which maitake D fraction exerts its apoptotic effects, the expression of cytochrome c was addressed. Cytoplasmic lysates were obtained from cultures previously incubated with the 5 different concentrations of maitake D fraction or were left
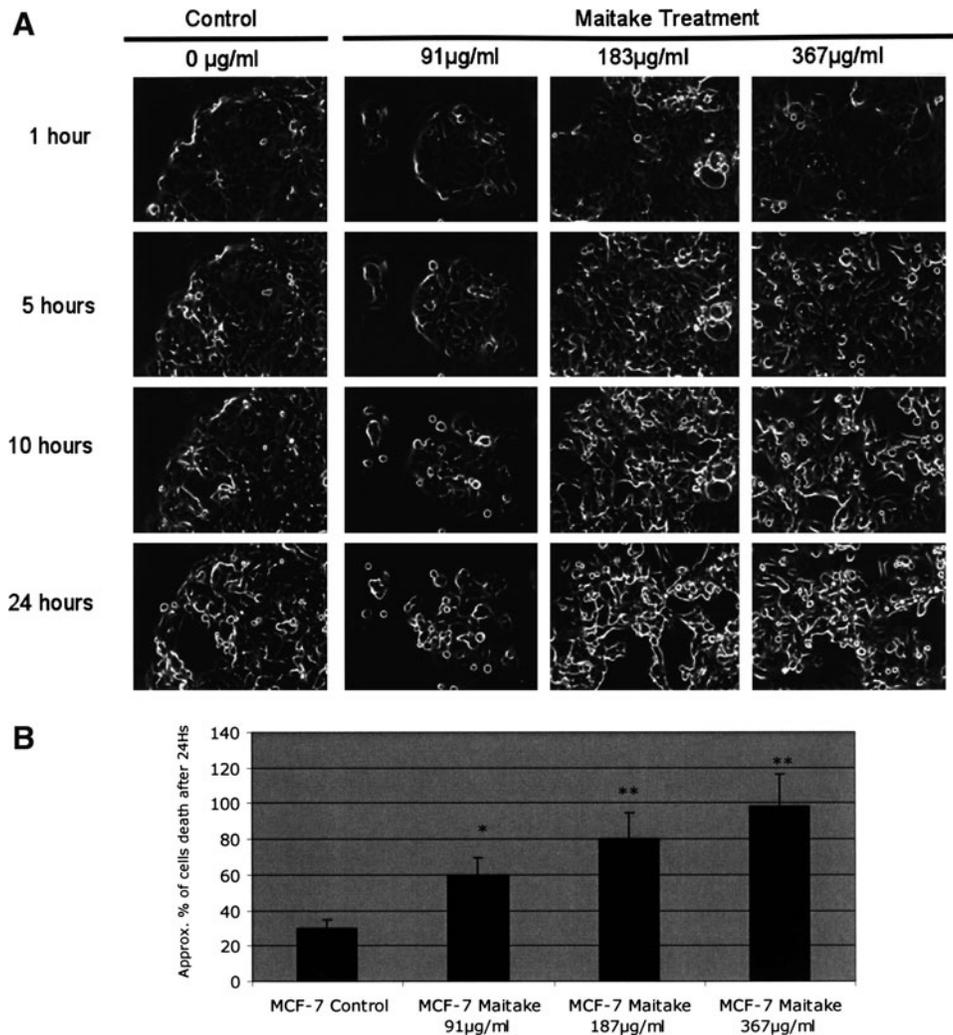
FIG. 2. Analysis of cell death induced by maitake in MCF7 cells using the Time Lapse microscope. MCF7 cells at 70% confluence were treated with and without (control) increased concentrations of maitake D fraction The experiments were performed in triplicate. Cells were placed in the Time Lapse microscope under a CO_2 atmosphere at room temperature for 24 hours. The camera was set up to take pictures every 10 minutes, using the $\times 20$ objective. Images and video were analyzed by using specific software. **(A)** The representative image corresponding to the cell culture at 1 hour, 5 hours, 10 hours, and 24 hours after maitake treatment in the conditions indicated in the figure. **(B)** The approximate percentage of dead cells observed in each image after 24 hours of maitake incubation.

untreated for 24 hours; cytochrome c expression was then examined by Western blotting. Although no significant findings were observed, a dose-dependent increase in the cytoplasmic cytochrome c occurred upon treatment with the compound. This finding indicates that D fraction promoted cytochrome c release from the mitochondria (Fig. 5).

DISCUSSION

Breast cancer mortality has remained almost unchanged for the past 5 decades. The condition is second to lung cancer as a cancer-related cause of death.[7,8] The failure to eradicate this disease is largely due to the inability to identify a specific etiologic agent, determine the precise time of initiation, and resolve the molecular mechanisms responsible for cancer initiation and progression. Until the

exact cause of breast cancer is found, researchers are investigating antitumoral agents and their potential clinical applications. An effective therapy is becoming increasingly important in fighting this disease. Current breast cancer treatments involve surgery, followed by radiation and hormonal therapy or chemotherapy. Some of these treatments have toxic side effects. The drugs used in chemotherapy affect normal cells as well as cancer cells. Researchers are trying to develop targeted drugs that attack only cancer cells and do not harm normal cells. One approach is to look for compounds that can block genes involved in transforming a normal cell into a cancer cell.

Recently, several in vitro studies have shown that pentamer (from the family of flavonoids derived from chocolate) inhibits growth of breast cancer cells.[9,10] In addition, for thousands of years, humans have used medicinal
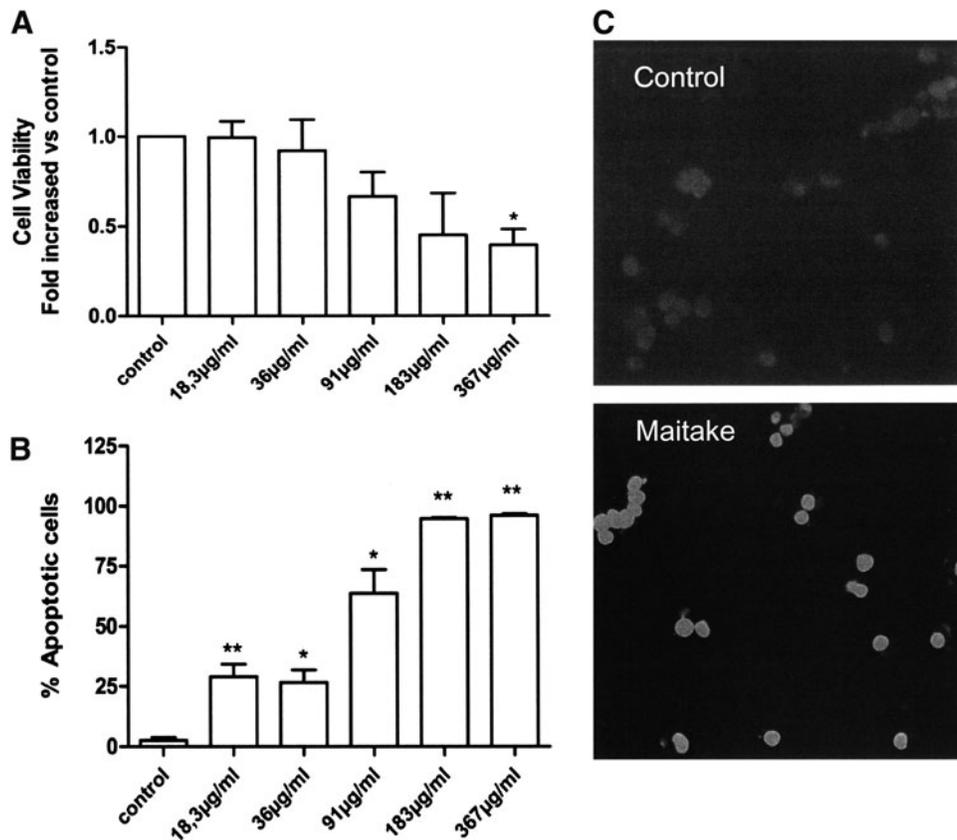
FIG. 3. MCF7 cell viability was evaluated after incubation with 5 concentrations of maitake D fraction. **(A)** Cell viability was assessed by MTS assay. Results are expressed in absorbance values at 540 nm and are the fold-increase relative to control cell cultures. Three independent experiments were performed in triplicate, with identical results. *$P = .05$ vs. control ($n = 9$). **(B)** Apoptosis was increased at every incubation as evaluated by TUNEL assay, reaching statistical significance for every concentration of D fraction. *$P < .05$ vs. controls; **$P < .01$ vs. control. Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. Experiments were performed in triplicate, with identical results ($n = 9$). **(C)** Immunofluorescence for apoptoticc cells (green). Note the increased number of apoptotic cells in cells treated with 367 μg of maitake fraction D (*bottom panel*) in comparison to untreated cells (*top panel*). Representative images are shown. Magnification×200.

mushrooms because of their healing potential. A rich folkloric tradition has passed the knowledge of mushrooms down through the ages, particularly in Japan and China, where mushrooms are supported by decades of scientific research and are imbedded in the cultural consciousness. U.S. researchers have been exploring these fascinating organisms for their medicinal properties. The future for mushrooms as a nutritional supplement looks bright as long as clinical studies continue to support the mushroom's properties as a powerful healing agent with the ability to improve human health.

In this study we demonstrated that D fraction of the maitake mushroom induced apoptosis in MCF7 breast cancer cells. These findings were corroborated by a reduction in cell viability with 5 different concentrations of this fraction. Although several genes promoting apoptosis were upregulated, as assessed by cDNA microarray analyses, the current study examined *BAK-1* and cytochrome c within these cells. Our RT-PCR *BAK-1* results confirmed the increased expression of *BAK-1*. The BAK-1

protein localizes to mitochondria and induces apoptosis by accelerating the opening of the mitochondrial voltage-dependent anion channel (VDAC), which leads to a loss in membrane potential and the release of cytochrome c. Pro-apoptotic proteins of the Bcl-2 family initiate apoptosis by blocking the anti-apoptotic activity of Bcl-2 and Bcl-xL by binding to their mitochondrial binding sites or by triggering the activation of pro-apoptotic Bax/Bak.[11] Cytochrome c is an intermediate in apoptosis, a controlled form of cell death used to kill cells in the process of development or in response to infection or DNA damage. Cytochrome c is released by the mitochondria in response to pro-apoptotic stimuli.[12] Finding the exact molecular mechanism of apoptosis activation induced by maitake in breast cancer cells is our objective.

Evidence demonstrated that *BAK-1* promotes cell death and counteracts the protection from apoptosis provided by Bcl-2.[13,14] Chittenden et al.[13] found that enforced expression of *BAK-1* induced rapid and extensive apoptosis of serum-deprived fibroblasts, implying that *BAK-1* may be
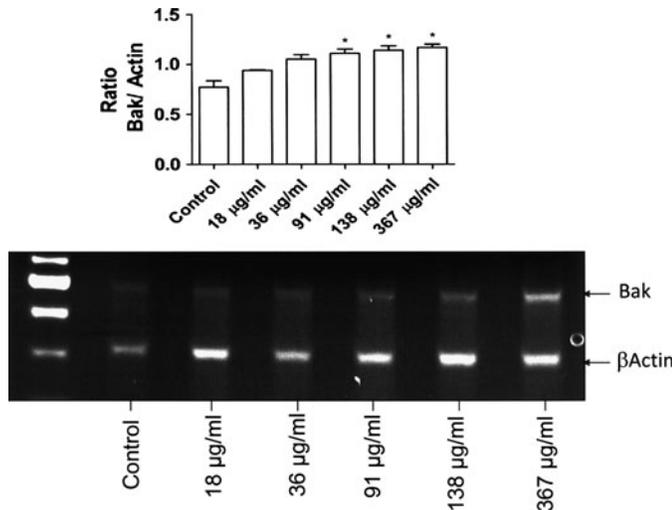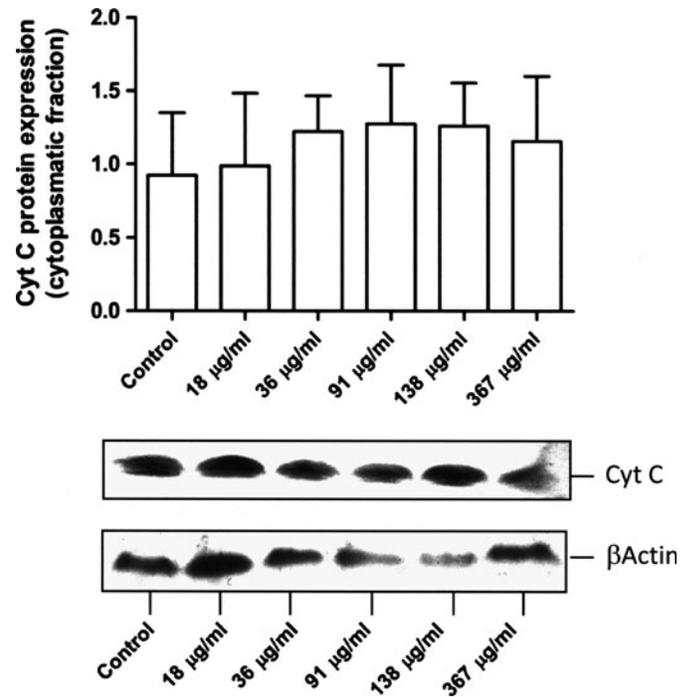
FIG. 4. Expression of *BAK-1* in MCF7 whole cell lysates after incubation with different concentrations of maitake D fraction over 24 hours. Reverse-transcriptase polymerase chain reaction analysis for *BAK-1* and *βActin* was carried out by using RNA derived from vehicle-treated (control) and 5 concentrations of maitake D fraction MCF7 cell cultures. *BAK-1* was upregulated by the compound in a dose-dependent manner. Bars correspond to intensity ratios of *BAK-1* gene expression after normalization to *βActin*. Results are representative of 3 independent experiments. Statistically significant differences were found for incubation with the 3 highest D fraction concentrations. $*P < .05$ vs. controls.

FIG. 5. Immunoblotting for cytochrome c after incubation with maitake D fraction. Increased expression of cytochrome c was observed after incubation with increasing doses of D fraction, although this finding was not statistically significant. Equivalent protein loading was confirmed by probing stripped blots for *βActin,* as shown. The bottom image shows a representative Western blot sample from 3 independent experiments ($n = 6$). Cyt c, cytochrome C.

directly involved in activating cell death machinery. Accordingly, Kiefer et al. reported that the *BAK-1* gene product primarily enhances apoptotic cell death after an appropriate stimulus.[14] In fact, during transduction of an apoptotic signal into the cell, an alteration in the permeability of the mitochondria membranes causes the translocation of the pro-apoptotic protein cytochrome c into the cytoplasm; this, in turn, activates death-driving proteolytic proteins known as caspases. Bcl-2 family members, which may play both anti-apoptotic or pro-apoptotic roles, regulate cell death by controlling this mitochondrial membrane permeability during apoptosis.

Our cDNA microarrays results showed that D fraction from maitake can induce the expression of 29 apoptosis-related genes; 22 of these are related to apoptosis induction and 7 are related to anti-apoptosis or apoptosis regulation. Positive and negative genetic and environmental regulators controls whether the pro-apoptotic or anti-apoptotic pathway is chosen. Pro-apoptotic gene activation will lead to cell death, while deactivation of the gene will block apoptotic pathways. Our results show that genetic regulation can also be modified by maitake treatment in MCF7 breast cancer cells.

Our study demonstrates that *BAK-1* appears to be an essential gateway to the mitochondrial dysfunction that is required for cell death in response to maitake in MCF7 cells. However, more research must be done to determine the exact apoptosis activation and its regulation. We found that the pro-apoptotic messenger RNA VDAC-1 is significantly

upregulated in the MCF7 cells treated with maitake in a dose-dependent manner; the increased reached 2.6-fold at $367\,\mu g$ of maitake per mL after 24 hours of treatment. We also found that maitake treatment upregulated *Bcl-2-like 2* (13.21-fold increase with respect to control), a gene involve in the anti-apoptotic pathway. In 1999, Shimizu et al.[15] showed that the recombinant pro-apoptotic proteins Bax (Bcl2-associated X protein) and BAK-1 accelerate the opening of VDAC, whereas the anti-apoptotic protein BCLXL closes VDAC by binding to it directly. Bax and BAK-1 allow cytochrome c to pass through VDAC out of liposomes, but passage is prevented by BCLXL. In agreement with this, VDAC-1–deficient mitochondria from mutant yeast did not exhibit a Bax/BAK-1–induced loss in membrane potential and cytochrome c release, both of which were inhibited by BCLXL. Shimizu et al.[15] concluded that the Bcl-2 family of proteins binds to the VDAC in order to regulate the mitochondrial membrane potential and the release of cytochrome c during apoptosis. On the basis of this finding, we can postulate that maitake D fraction stimulates *BAK-1* gene expression, thereby allowing cytochrome c to pass through VDAC-1 out of liposomes in MCF7 cells.

In this study, we found that VDAC-2 is upregulated by Maitake-treatment in MCF7 cells. Cheng et al. reported that *BAK-1* aggregates with the mitochondrial outer membrane protein VDAC-2, a VDAC isoform present in low abun-

dance that interacts specifically with the inactive conformer of *BAK-1*.[16] Cells that are deficient in VDAC-2 but express the highly abundant VDAC-1 exhibited enhanced *BAK-1* expression.[16] Conversely, overexpression of VDAC-2 selectively prevented *BAK-1* activation and inhibited the mitochondrial apoptotic pathway. From our results, we can probably assume that VDAC1 enhanced the expression of *BAK-1* in the MCF7 cells after maitake treatment; however, the upregulation of VDAC-2 could partially block BAK-1 protein and prevent cell death by apoptosis in less that 40% of cells at 367 μg of fraction D per mL, as was observed in the TUNEL assay results. Of note, this result shows that 60% of MCF7 cells treated with maitake will die as a result of apoptosis at the maximum concentration of D fraction after 24 hours of treatment.

BH3 interacting domain death agonist (BID) messenger RNA was found to be upregulated by maitake treatment in MCF7 cells (5.9-fold increase at 367 μg/mL). Another pro-apoptotic activity occurs through the cytoplasmic protein, Bid. This molecule is found in the cytoplasm in an inactive form. When cleaved by activated caspase-8 from the extrinsic pathway, Bid causes a structural change to Bax, making it similar to the structure of the anti-apoptotic molecule, Bcl-2, and thereby allowing Bax to translocate to the mitochondria.[17–20] Death signals activate "BH3-only" molecules, such as tBID, BIM, or BAD, which displace VDAC-2 from *BAK-1*, enabling homo-oligomerization of *BAK-1* and apoptosis.[16] Thus, we can conclude that VDAC-2 regulates the activity of *BAK-1* and provides a connection between mitochondrial physiology and the core apoptotic pathway.

Many pro-apoptotic signals engage the apoptotic machinery by releasing cytochrome c from mitochondria.[21–24] cDNA microarray data showed upregulation of apoptotic peptidase activating factor 1 interacting protein (APAF1) in breast cancer cells after maitake treatment in a dose-dependent manner. Cytoplasmic cytochrome c induces APAF-1 oligomerization, resulting in recruitment and activation of caspase 9 within the apoptosome.[25–27] Caspase 9, in turn, is known to activate downstream effectors caspase 3 and 7.[28] Finally, our cDNA microarray results demonstrate that caspase 7 and caspase 1 apoptosis-related genes in MCF7 cells were upregulated after 24 hours of maitake treatment (24.5- and 10-fold increases, respectively, at 183 μg/mL; $P < .001$) (data not shown). It is well known that caspase proteins are the target key regulatory and structural proteins for proteolysis leading to cell death.[28]

β-glucans are not expressed on mammalian cells and are recognized as pathogen-associated molecular patterns by pattern recognition receptors, primarily the C-type lectin receptor dectin-1; they also interact via the complement receptor 3 (CR3).[29–32] Dectin 1 is a small type II transmembrane receptor with a lectin-like carbohydrate recognition domain that recognizes β1,3-linked and β1,6-linked glucans and intact yeast, while CR3 is a widely expressed β2 integrin containing a lectin domain, which mediates carbohydrate recognition.[29–32] Maitake D fraction is rich in β1,3-linked glucans and probably interacts with a pattern recognition receptor in the cell membrane. A specific receptor for maitake in the mammalian cells has not yet been found.

On the basis of these results, Figure 6 summarizes the putative molecular mechanism involved in the apoptosis activation induced by *G frondosa* D fraction in breast cancer cells. Maitake triggers a cell stress signal pathway after interacting with the pattern recognition receptor in the cell membrane; it then induces the activation of the pro-apoptotic protein BAK-1 through activation of BID by caspase 1 or 7 and through activation of other BH3 initiator proteins. Activated BAK-1 proteins oligomerize at the mitochondrial membrane and cause the release of several mitochondrial factors, such as cytochrome c, which, in combination with APAF-1 and procaspase 7, form an apoptosome. Activated caspase 7 probably then activates caspase 1, allowing apoptosis to proceed. Bcl-2L2 blocks the activation of *BAK-1* (Fig. 6).

In conclusion, the present study showed that the D fraction from maitake mushroom has strong anti-cancer properties in breast cancer cells, namely by exerting pro-apoptotic effects and reducing tumor cell viability. This action was accompanied by an increase in pro-apoptotic genes, which was further corroborated by an increase in *BAK-1* gene expression, cytochrome c release to the cytoplasm, and activation of caspase 7 and caspase 1. Given the established epidemiologic and experimental findings regarding natural molecules, in particular from mushroom compounds, on cancer development and delay in progression, understanding the molecular mechanisms inherent in these effects is of utmost importance. This knowledge may lead to the development of therapeutic or preventive strategies for highly incident neoplasias, such as breast cancer.
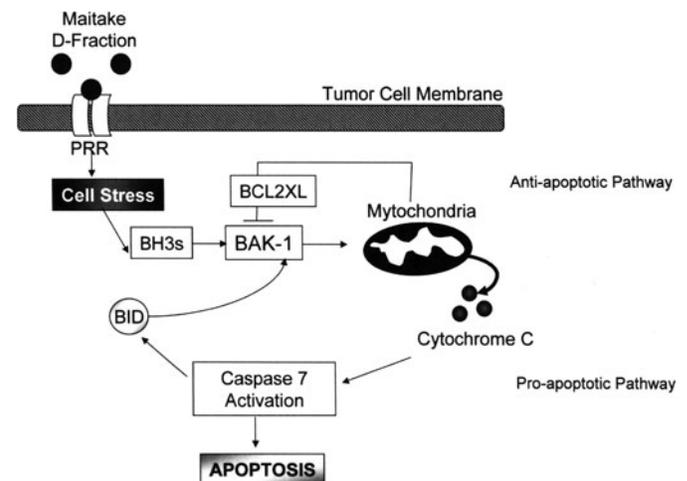
FIG. 6. Putative molecular mechanism of apoptosis activation induced by maitake in MCF7 cells. This image summarizes the results obtained in this work. The gene expression involved in the apoptotic and anti-apoptotic pathway induced by D fraction in MCF7 cells is explained in the Discussion section. PRR, pattern recognition receptors.

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