Novel medicinal mushroom blend suppresses growth and invasiveness of human breast cancer cells

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Abstract. Mushrooms are an integral part of Traditional Chinese Medicine (TCM), and have been used for millennia to prevent or treat a variety of diseases. Currently mushrooms or their extracts are used globally in the form of dietary supplements. In the present study we have evaluated the anticancer effects of the dietary supplement, MycoPhyto® Complex (MC), a novel medicinal mushroom blend which consists of a blend of mushroom mycelia from the species *Agaricus blazei*, *Cordyceps sinensis*, *Coriolus versicolor*, *Ganoderma lucidum*, *Grifola frondosa* and *Polyporus umbellatus*, and ß-1,3-glucan isolated from the yeast, *Saccharomyces cerevisiae*. Here, we show that MC demonstrates cytostatic effects through the inhibition of cell proliferation and cell cycle arrest at the G2/M phase of highly invasive human breast cancer cells MDA-MB-231. DNA-microarray analysis revealed that MC inhibits expression of cell cycle regulatory genes (*ANAPC2, ANAPC2, BIRC5, Cyclin B1, Cyclin H, CDC20, CDK2, CKS1B, Cullin 1, E2F1, KPNA2, PKMYT1 and TFDP1*). Moreover, MC also suppresses the metastatic behavior of MDA-MB-231 by the inhibition of cell adhesion, cell migration and cell invasion. The potency of MC to inhibit invasiveness of breast cancer cells is linked to the suppression of secretion of the urokinase plasminogen activator (uPA) from MDA-MB-231 cells. In conclusion, the MC dietary supplement could have potential therapeutic value in the treatment of invasive human breast cancer.

Introduction

Edible and medicinal mushrooms can produce a variety of biologically active compounds and can be therefore described as a novel class of nutraceuticals which are widely used as dietary supplements (1). Recent epidemiological studies from Asia demonstrated that mushroom intake protects against cancer, specifically gastrointestinal (GI) cancer and breast cancer (2-4). The anticancer activities of mushrooms were mainly linked to the modulation of the immune system by branched polysaccharides (glucans), glycoproteins or peptide/protein-bound polysaccharides (5,6). Moreover, mushrooms contain minerals, vitamins (e.g., thiamin, riboflavin, ascorbic acid, and vitamin D), amino acids, and other organic compounds (7). Some of these natural mushroom compounds demonstrated specific activity against aberrantly activated signaling pathways in cancer cells and were able to modulate specific molecular targets in the cell function including cell proliferation, cell survival and angiogenesis (8).

MycoPhyto Complex (MC) is a dietary supplement consisting of a mixture of six varieties of mushroom mycelia, including *Agaricus blazei*, *Cordyceps sinensis*, *Coriolus versicolor*, *Ganoderma lucidum*, *Grifola frondosa* and *Polyporus umbellatus*, and additional ß-1,3-glucan isolated from the yeast, *Saccharomyces cerevisiae*. Interestingly, these specific mushrooms have been linked to different health promoting or disease preventing functions.

Agaricus blazei Murrill, popularly known as '*Cogumelo do Sol*' in Brazil, or '*Himematsutake*' in Japan, was originally discovered 50 years ago in Brazil (9,10). *A. blazei* is traditionally believed to treat many common diseases like atherosclerosis, hepatitis, hyperlipidemia, diabetes, dermatitis and cancer (10). The polysaccharide phytocomplex was suggested to be responsible for the immunostimulant and anti-tumor properties of *A. blazei*, probably through an opsonizing biochemical pathway (10). In addition, recent studies demonstrated anticancer activities of *A. blazei* through the induction of apoptosis by the activation of caspase-3 in prostate cancer cells (11) and inhibition of constitutively active NF-κB in leukemic cells (12). Moreover, *A. blazei* demonstrated anti-metastatic effect through the inhibition of MMP-9 in melanoma cells (13).

Cordyceps sinensis is the parasitic fungus that colonizes the larvae of moths (Lepidoptera), and is native to the high altitude (3500-5000 m) of Himalayas (14). *Cordyceps* has been a valued medicine in Traditional Chinese Medicine (TCM) for more than 2000 years, and has been used for the treatment of multiple disorders including respiratory, renal, hepatic, cardiovascular, immunologic, and nervous system, glucose metabolism, different inflammatory conditions and cancer

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(15). The anticancer activity of *C. sinensis*, was associated with the presence of cordycepin (3-deoxyadenosine) which demonstrated inhibition of cell proliferation (16,17), induction of apoptosis (18,19), and invasiveness (20). Mechanistically, *C. sinensis* extracts induced apoptosis by the activation of caspase-3 in leukemia cells and through the death receptor-mediated extrinsic and mitochondria-mediated intrinsic caspase pathways in lung cancer cells or by the inactivation of Akt kinase in breast cancer cells, respectively (21-23).

Coriolus versicolor is an obligate aerobe that is commonly found year-round on logs, stumps, tree trunks, and branches. The fungus occurs throughout the wooded temperate zones of Asia, Europe, and North America and may be the most common shelf fungus in the Northern Hemisphere (24). *C. versicolor* contains biologically active, structurally different protein-bound polysaccharide-K (PSK) and polysaccharo-peptide (PSP), which were approved in Asia for immuno-therapy or as biological response modifiers (BRMs) (24,25). In addition to the immunomodulatory activity, extracts of *C. versicolor* demonstrated direct effects on a variety of cancer cells. Therefore, *C. versicolor* induced apoptosis of breast cancer cells through p53 and Bcl-2 dependent and independent mechanisms (26) suppressed cell proliferation, and induced apoptosis of leukemia cells by mechanisms including inhibition of transcription factor NF-κB and down-regulation of expression of COX-2 (27). Moreover, direct cytotoxic effect of PSK, through the cell cycle arrest at G0/G1 phase and induction of apoptosis associated with the caspase-3 expression, was reported in various tumor cell lines derived from leukemia, melanomas, fibrosarcomas and cervix, lung, pancreas and gastric cancers (28).

Ganoderma lucidum (*Ling Zhi*, Reishi) is one of the important Asian fungi that have been recognized in China, Korea and Japan more than 4000 years ago (29). The biological activity of *G. lucidum* is usually associated with polysaccharides (mainly glucans and glycoproteins) and lanostane-type triterpenes (ganoderic acids, ganoderic alcohols and their derivatives) (30). The anticancer effects of *G. lucidum* have been attributed to the modulation of the immune system by polysaccharides (31,32), whereas triterpenes demonstrated the direct cytotoxic/killing effects on a variety of cancer cells including hepatoma, naso-pharynx carcinoma, lung carcinoma, sarcoma, breast cancer, and leukemia cells, respectively (33-37). *G. lucidum* extract (GLE), containing polysaccharides and triterpenes, suppressed proliferation and metastatic potential of breast cancer cells through the inhibition of Akt kinase and transcription factors AP-1 and NF-κB (38,39). Interestingly, GLE modulated estrogen receptor signaling and inhibited the oxidative stress-induced invasiveness of breast cancer cells (40,41). Moreover, GLE demonstrated anti-angiogenic activity by suppressing the secretion of vascular endothelial growth factor (VEGF) and transforming growth factor-ß1 (TGF-ß1) from prostate cancer cells (42).

Grifola frondosa (*Maitake*, which means 'dancing mushroom' in Japanese) is a popular culinary mushroom originally recognized in Japan and Korea. The anticancer activities of *G. frondosa* were originally described more than 30 years ago and are associated with the presence of 1,3-ß-glucan named grifolan LE (43,44). Suppression of tumor growth in mice by D-Fraction, 1,3-branched-1,6-ß-glucan isolated from *G. frondosa*, was associated with the stimulation of natural killer (NK) cells and activation of macrophages and differentiation of T-cells (45-47). Mechanistically, D-Fraction increased expression of TNF-α in NK cells and IL-12 release from macrophages and dendritic cells (45,47). Nevertheless, phase I/II trials with a polysaccharide extract from *G. frondosa* demonstrated depressed as well as enhanced immune functions in cancer patients (48). Interestingly, *G. frondosa* ameliorated colon inflammation and suppressed TNF-α expression in the colon tissue in an animal model of the inflammatory bowel disease (49). The direct anticancer effect was demonstrated on the gastric cancer cells by the induction of apoptosis via caspase-3-dependent and independent pathways (50).

Polyporus umbellatus (Zhu Ling in Chinese) has been used in TCM as a remedy for the urinary tract infection and as a diuretic, and for the treatment of hepatitis B (51-53). Anti-cancer activities of *P. umbellatus* have been demonstrated in animal studies (54-56) as well as in one clinical study with bladder cancer patients in China (57). Although the biological activity of *P. umbellatus* was associated with the presence of polysaccharides (58), the cytotoxic activity against leukemia cells was demonstrated with isolated triterpenes (polyporus-terones) isolated from *P. umbellatus* (59). In addition, other ergostane-type ecdysteroids named polyporoids demonstrated anti-inflammatory activity in a TPA-induced inflammation in mice (60). Nevertheless, the molecular mechanisms responsible for the anticancer activity of *P. umbellatus* were not addressed in these studies. Anticancer activity of yeast ß-glucans is associated with priming of neutrophils, macrophages and NK cells for cytotoxicity against tumors (61). Moreover, cancer immunotherapy was recently evaluated in the combination of yeast ß-glucans with anti-tumor antibodies (62).

In the present study, we evaluated anti-proliferative and anti-invasive properties of a dietary supplement MC on highly invasive human breast cancer cells.

Materials and methods

Cell culture and reagents. Human breast cancer cell line MDA-MB-231 was obtained from ATCC (Manassas, VA, USA) and was maintained in DMEM medium in the presence of penicillin (50 U/ml), streptomycin (50 U/ml), and 10% fetal bovine serum (FBS). Medium and supplements came from Gibco BRL (Grand Island, NY, USA). FBS was obtained from Hyclone (Logan, UT, USA). MycoPhyto Complex (MC), a mixture of *Agaricus blazei*, *Cordyceps sinensis*, *Coriolus versicolor*, *Ganoderma lucidum*, *Grifola frondosa* and *Polyporus umbellate* mycelia and ß-1,3-glucan purified from the yeast *Saccharomyces cerevisiae*, was supplied by EcoNugenics, Inc (Santa Rosa, CA, USA). MC stock solution was prepared by dissolving MC in dimethylsulphoxide (DMSO) at a concentration 25 mg/ml and stored at 4˚C.

Cell proliferation and cell viability. Cell proliferation was determined by the tetrazolium salt method, according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, MDA-MB-231 cells were cultured in a 96-well plate and treated at indicated times with MC (0-0.5 mg/ml). At the end of the incubation period, the cells were harvested and absorption was determined with an ELISA plate reader at

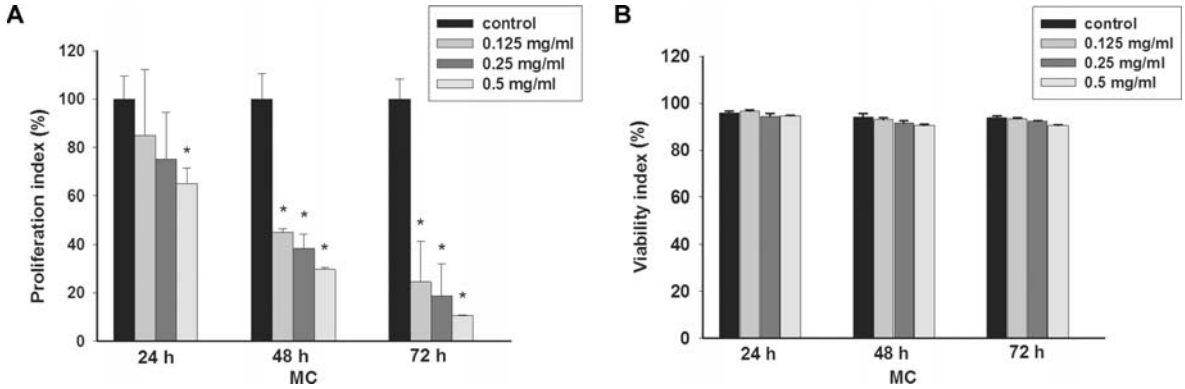

Figure 1. Effect of MC on the growth of breast cancer cells. MDA-MB-231 cells were treated with MC (0-0.5 mg/ml) for 24, 48 and 72 h. (A) Cell proliferation and (B) cell viability were determined as described in Materials and methods. Data are the means ± SD of triplicate determinations. Similar results were obtained in at least two additional experiments. *p<0.05.

570 nm, as described (38). Cell viability of MDA-MB-231 cells was determined after incubation with MC (0-0.5 mg/ml) for 24, 48 and 72 h by staining with trypan blue as described (63). Data points represent mean ± SD in the representative experiment of triplicate determinations. Similar results were obtained in two independent experiments.

Cell cycle analysis. MDA-MB-231 cells ($7.5x10^5$) were seeded in 100 mm dishes and cultured in DMEM media with 10% FBS for 24 h, followed by incubation with MC (0, 0.25 and 0.5 mg/ml) at 37˚C for 24 h. After incubation, the cells were harvested, washed with DPBS containing 1% FBS, and resuspended in propidium iodine (50 μg/ml). Samples were analyzed on a FACStar^{PLUS} flow cytometer (Becton-Dickinson, San Jose, CA, USA). The fractions of cells in different phase of the cell cycle (G0/G1, S, G2/M) are presented as a percentage of total cells analyzed.

DNA microarray analysis. MDA-MB-231 cells were treated with MC (0 and 0.5 mg/ml) for 24 h and total RNA isolated with RNAeasy (Qiagen, Valencia, CA, USA). This RNA was used for the evaluation of cell cycle regulatory genes with Cell Cycle Oligo GEArray according to the manufacturer's protocol (SABiosciences, Frederick, MD, USA), as previously described (64). The fold change of gene expression was determined by GEArray expression® analysis suite (SABiosciences).

Cell adhesion, migration, and invasion assays. Cell adhesion was performed with Cytomatrix Adhesion Strips coated with human vitronectin (Chemicon International, Temecula, CA, USA). Briefly, MDA-MB-231 cells were treated with MC (0-0.5 mg/ml) for 24 h, harvested, and counted. Cell adhesion was determined after 1.5 h of incubation at 37˚C (63). Cell migration of MDA-MB-231 cells treated with MC (0-0.5 mg/ml) was assessed in Transwell chambers in the DMEM medium containing 10% FBS (63). Invasion of MDA-MB-231 cells treated with MC (0-0.5 mg/ml) was assessed in Transwell chambers coated with 100 μl of Matrigel™ (BD Biosciences, Bedford, MA, USA) diluted 1:3 with DMEM, after 24 h of incubation (63).

uPA secretion. DMEM media from MDA-MB-231 cells treated with MC (0-0.5 mg/ml) for 24 h were collected and concentrated, and the secretion of uPA was detected by Western blot analysis with anti-uPA antibody (Oncogene Research Products, Cambridge, MA, USA), as described (63).

Western blot analysis. MDA-MB-231 cells were treated with MC (0-0.5 mg/ml) for 24 h. Whole cell extracts isolated from cells were prepared as described (40). Equal amounts of proteins were separated on 4-12% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated with the corresponding primary antibodies diluted 1:1000 in blocking solution, as follows: rabbit polyclonal antibodies against CXCR4 (Millipore, Temecula, CA, USA) and mouse monoclonal antibodies against Cox2, and ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight at 4˚C. Anti-mouse or anti-rabbit secondary antibodies were used to detect and visualize by the ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis. Data are presented as means ± SD. Statistical comparison between the control group (0 μg/ml of MC) and groups with different MC doses were carried out using two-sided Student's t-tests. $p<0.05$ was considered to be significant.

Results and Discussion

MC demonstrates cytostatic effect on the highly invasive breast cancer cells. Cancer metastasis, which consists of uncontrolled growth and invasive behavior of cancer cells, is one of the major medical problems in breast cancer patients (65). Although chemically synthesized chemotherapeutic agents demonstrated activity in the metastatic breast cancer setting (66), some of these chemotherapeutic drugs have undesirable toxic side effects. Therefore, the identification of natural anti-proliferative and anti-metastatic non-toxic agents is of particular interest and a unique blend of mushrooms as natural complexes demonstrated significant anticancer activities.

In the present study we evaluated if the mixture of mushrooms, MC, inhibits growth of highly invasive MDA-MB-231 breast cancer cells. As seen in Fig. 1A, increased concentration of MC (0-0.5 mg/ml) markedly suppressed proliferation of

Table I. Effect of MC on cell cycle distribution.

MC (mg/ml)	G0/G1	S	G2/M
0	72.8±0.8	24.9±1.0	3.1±0.5
0.25	73.5±2.2	22.6±1.3	3.8±0.7
0.50	71.4±2.3	16.4±2.7[a]	12.2±0.5[a]

Cell cycle analysis was performed as described in Materials and methods. Cell cycle distribution G0/G1, S and G2/M phase in %. The data are mean ± SD from three experiments. Statistical significance [a]$p<0.05$.

MDA-MB-231 in a dose- and time-dependent manner. To determine if the effect of MC on cancer cells is cytotoxic or cytostatic, we evaluated the cell viability after 24, 48 and 72 h of MC treatment. The viability of MDA-MB-231 cells was not affected by the MC treatment suggesting that the MC inhibits growth of breast cancer cells through its cytostatic effect (Fig. 1B). Although the previous studies with mushrooms or isolated mushroom extracts or their biologically active mushroom molecules demonstrated cytotoxic or pro-apoptotic effects, MC treatment significantly inhibited proliferation of breast cancer cells without any effect on viability of cells. For example, cytotoxic effect was demonstrated by PSK isolated from *C. versicolor* (28) or triterpenes isolated from *G. lucidum* or *P. umbelatus*, respectively (33-37,59). In addition, *A. blazei*, *C. sinensis*, *C. versicolor*, *G. frondosa*, and *G. lucidum* (11,18,19,26,50,67) induced apoptosis in a variety of cancer cells. Therefore, the mixture of medicinal mushrooms in MC can diminish the cytotoxic or pro-apoptotic effect of the individual mushroom. Alternatively, the cytostatic effect of MC can be the result of the synergistic or additive effects of low non-cytotoxic doses of these individual mushrooms.

MC induces cell cycle arrest at G2/M phase. In order to evaluate whether the cytostatic effect of MC is associated with the cell cycle arrest, MDA-MB-231 cells were treated with MC as described in Materials and methods. Cell cycle analysis revealed that MC induces significant cell cycle arrest at G2/M phase from 3.1% (control) to 12.2% (0.5 mg/ml MC) (Table I). As previously demonstrated cell cycle arrest at G2/M was induced by *A. blazei* in gastric epithethelial cells (68) or by cordyceptin isolated from *C. sinensis* in bladder cancer cells (69), whereas protein-bound polysaccharide (PSK) isolated from the *C. versicolor* induced cell cycle arrest at G0/G1 in a variety of cancer cells (28). Interestingly, different extract from *G. lucidum* demonstrate specific effects on cell cycle progression. Thus, extracts from *G. lucidum* induced cell cycle arrest at G0/G1 phase in breast cancer cells (38,40,70), whereas arrest at G2/M phase was induced in prostate, hepatoma and bladder cancer cells, respectively (67,71,72). On the other hand, isolated triterpenes from *G. lucidum* induced cell cycle arrest at G0/G1 and G2/M phase in macrophages (73), suggesting that cell cycle arrest depends on the specific biologically active compounds as well as particular cells.

In order to investigate whether MC-induced cell cycle arrest is associated with the expression of specific cell-cycle regulatory genes, we treated MDA-MB-231 cells with MC and performed DNA-microarray analysis as described in Materials and methods. As seen in Fig. 2 and Table II, MC down-regulated expression of *ANAPC2*, *ANAPC2*, *BIRC5*, *Cyclin B1*, *Cyclin H*, *CDC20*, *CDK2*, *CKS1B*, *Culin 1*, *E2F1*, *KPNA2*, *PKMYT1* and *TFDP1*. Some of the functional proteins (e.g., ANAPC2, ANAPC2 and CDC20) form the anaphase promoting complex/cyclosome (APC/C) inhibition of which can induce cell cycle arrest at G2/M phase (74). Inhibition of survivin (product of gene *BIRC5*) and CDC28 (product of gene *CKS1B*) was also associated with cell cycle arrest at G2/M phase (75,76). In addition, G2/M cell cycle arrest is controlled by Cyclin B1 and Cyclin H (CDK7), and inhibitors of CDK2 were developed as G0/G1 and G2/M inhibitors for cancer therapy (77,78). Down-regulation of transcription factors E2F1 and DP-1 (product of gene *TFDP1*) and Myt-1 protein (product of gene *PKMYT1*) was also linked to cell cycle arrest at G2/M phase in cancer cells (79,80). Therefore, the induction of cell cycle arrest of breast cancer cells by MC was associated with the down-regulation of expression of genes involved in G2/M phase.

Effect of MC on the invasive behavior of breast cancer cells. Invasive behavior of cancer cells is associated with their ability to adhere, to migrate and to invade the normal tissues. Breast cancer cells express integrin receptor $\alpha_v\beta_3$, and the interaction of $\alpha_v\beta_3$ with the extracellular matrix (ECM) protein vitronectin is involved in the adhesion of MDA-MB-231 cells to ECM (81). In addition, $\alpha_v\beta_3$ and vitronectin form a complex with urokinase plasminogen activator (uPA) and its receptor uPAR, and this whole complex activates the intracellular signaling

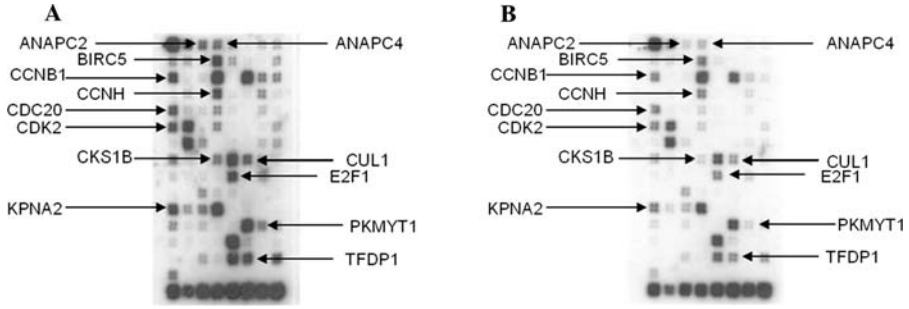

Figure 2. Effect of MC on the expression of cell cycle regulatory genes. MDA-MB-231 cells were treated with (A) control - 0 mg/ml MC or (B) 0.5 mg/ml MC for 24 h and RNA prepared and DNA-microarray analysis performed as described in Materials and methods. Similar results were obtained in one additional experiment.

Table II. Effect of MC on the expression of cell cycle regulatory genes.

Gene	Description	Fold change
ANAPC2	Anaphase promoting complex subunit 2	0.3
ANAPC4	Anaphase promoting complex subunit 4	0.5
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	0.7
CCNB1	Cyclin B1	0.6
CCNH	Cyclin H	0.7
CDC20	Cell division cycle 20 homolog	0.7
CDK2	Cyclin-dependent kinase 2	0.7
CKS1B6	CDC28 protein kinase regulatory subunit 1	0.3
CUL1	Cullin 1	0.6
E2F1	E2F transcription factor 1	0.7
KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0.7
PKMYT1	Protein kinase, membrane associated tyrosine/threonine 1	0.4
TFDP1	Transcription factor Dp-1	0.6

DNA-microarray analysis was performed with MDA-MB-231 cells treated with MC (0.5 mg/ml) for 24 h as described in Materials and methods. The data are representative from two independent experiments.

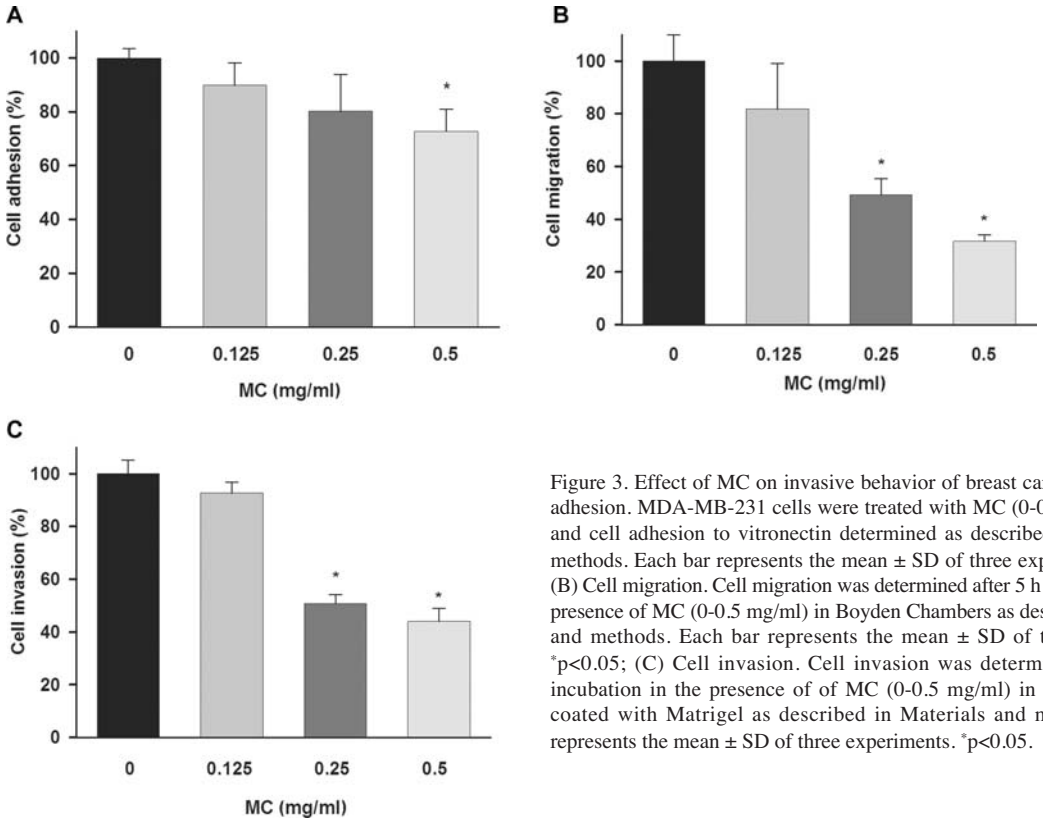

Figure 3. Effect of MC on invasive behavior of breast cancer cells. (A) Cell adhesion. MDA-MB-231 cells were treated with MC (0-0.5 mg/ml) for 24 h and cell adhesion to vitronectin determined as described in Materials and methods. Each bar represents the mean ± SD of three experiments. *p<0.05; (B) Cell migration. Cell migration was determined after 5 h of incubation in the presence of MC (0-0.5 mg/ml) in Boyden Chambers as described in Materials and methods. Each bar represents the mean ± SD of three experiments. *p<0.05; (C) Cell invasion. Cell invasion was determined after 24 h of incubation in the presence of of MC (0-0.5 mg/ml) in Boyden Chambers coated with Matrigel as described in Materials and methods. Each bar represents the mean ± SD of three experiments. *p<0.05.

responsible for cell adhesion and migration (82). To investigate if MC affects adhesion of invasive breast cancer cells, MDA-MB-231 cells were pretreated with MC (0-0.5 mg/ml) for 24 h and their adhesion to vitronectin was determined. As seen in Fig. 3A, adhesion of MDA-MB-231 cells to vitronectin was markedly suppressed by the MC treatment. The effect of MC on migratory potential of breast cancer cells was evaluated in MDA-MB-231 cells pretreated with MC (0-0.5 mg/ml) for 1 h

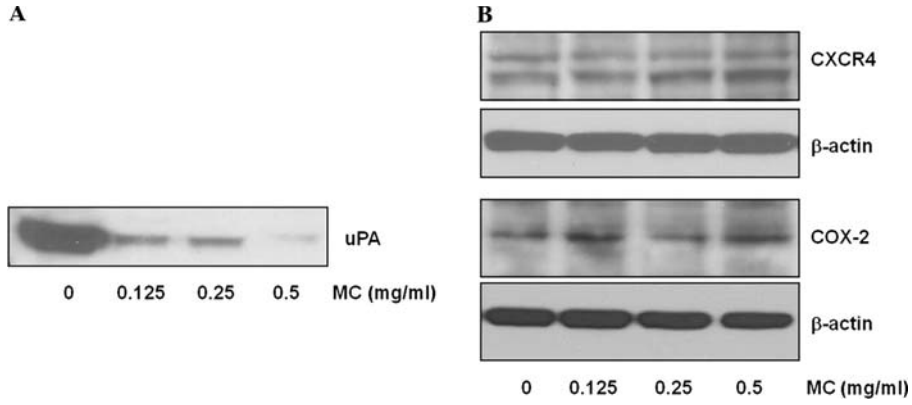

Figure 4. Effect of MC on the secretion of uPA and expression of CXCR4 and COX-2 in breast cancer cells. (A) MDA-MB-231 cells were treated with MC (0-0.5 mg/ml) for 24 h, and the secretion of uPA detected in conditioned media with anti-uPA antibody by Western blot analysis as described in Materials and methods. The results are representative of three separate experiments. (B) MDA-MB-231 cells were treated as described in (A), and the expression of CXCR4, COX2 and ß-actin determined as described in Materials and methods. The results are representative of three separate experiments.

and cell migration was determined after additional 4 h of incubation. As expected, MC also noticeably suppressed migration of breast cancer cells in a dose dependent-manner (Fig. 3B).

Cell invasion is associated with the enzymatic activity of uPA. uPA interacts with uPAR and converts plasminogen to plasmin which degrades ECM components and stimulates other proteolytic enzymes contributing to cell invasion (82). Therefore, we evaluated the effect of MC on cell invasiveness. MDA-MB-231 cells were plated on the Matrigel-coated filters in the presence of MC (0-0.5 mg/ml) and the amount of cells invaded through Matrigel counted after 24 h of incubation. As seen in Fig. 3C, MC markedly inhibited invasion of MDA-MB-231 cells in a dose-response manner.

In order to evaluate the effect of MC on the levels of uPA, conditioned media from MDA-MB-231 cells treated with MC (0-0.5 mg/ml) were collected and secretion of uPA was determined by Western blot analysis. As expected, MC markedly decreased secretion of uPA from MDA-MB-231 cells (Fig. 4A). These data are in concert with our previous paper demonstrating that sole mushroom *G. lucidum* inhibited invasive behavior of human breast and prostate cancer cells through the mechanisms including uPA/uPAR signaling (39). Moreover, ß-glucan isolated from *A. blazei* suppressed invasion of human ovarian cancer cells through the down-regulation of expression of uPA (83). On the other hand, apoptosis of human leukemia cells by proteins and peptide bound polysaccharides (PSP), extracted from *C. versicolor*, was associated with the increased expression of uPA (84). Alternatively, other mechanisms can be employed in the inhibition of cell invasiveness. In addition to uPA, chemokine receptor CXCR4 and cyclooxygenase-2 (COX-2) were also associated with invasiveness of breast cancer cells (85,86). In order to evaluate whether MC affects expression of these proteins, whole cell extracts from MDA-MB-231 cells used for the determination of uPA (Fig. 4A) were subjected to Western blot analysis with specific antibodies against CXCR4 and COX-2, respectively. However, MC treatment of MDA-MB-231 cells did not markedly affect expression of CXCR4 and COX-2 (Fig. 4B). Therefore, our data suggest that MC suppresses invasive behavior of breast cancer cells by the inhibition of secretion of uPA.

In conclusion, MC, which consists of mushrooms *Agaricus blazei*, *Cordyceps sinensis*, *Coriolus versicolor*, *Ganoderma lucidum*, *Grifola frondosa* and *Polyporus umbellate* and yeast ß-1,3-glucan, is a dietary supplement with the ability to suppress proliferation and invasive behavior of breast cancer cells. The biological effects of MC are probably mediated by the additive or synergistic effects of individual mushrooms. The mushroom based dietary supplement MC could have potential use in the treatment of invasive breast cancer.

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