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Ethanolic Extract of *Agaricus blazei* Fermentation Product Inhibits the Growth and Invasion of Human Hepatoma HA22T/VGH and SK-Hep-1 Cells

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Abstract

Hepatoma is a leading cause of death in the world. SK-Hep-1 and HA22T/VGH cells are poorly differentiated human hepatocellular carcinoma cell lines with invasive and migratory abilities. *Agaricus blazei* (AB) is a mushroom with many biological effects and active ingredients, and the ethanolic extract of AB fermentation product (AB-pE) was demonstrated to inhibit the growth of hepatoma Hep3B and HepG2 cells in our previous study. In this study, we further investigated the anticancer and anti-invasive abctivities of the AB-pE. Results showed that the AB-pE inhibited the growth of SK-Hep1 and HA22T/VGH cells (with IC_{50} values of 26.8 and 28.7 μg/mL, respectively) and led cells toward apoptosis after 48 h of treatment. Activation of caspase-3 by AB-pE (12.5~200 μg/mL) in a dose-dependent manner was observed in both cell lines using fluorescence microscopy and flow cytometry. The apoptosis triggered by the AB-pE was regulated by the increased expression of Bax, the activation of caspase-3, caspase-9, and PARP, and the decreased expression of Bcl-2. Additionally, the AB-pE showed the potential ability to inhibit invasion of SK-Hep1 and HA22T/VGH cells according to the results of a Matrigel invasion assay. Our results suggested that the AB-pE may be a further developed for its potential against hepatoma due to its antiproliferative (via apoptosis) and anti-invasive activities in hepatoma cells.

Key words: *Agaricus blazei*, Anti-hepatoma activity, Antitumor invasion activity, SK-Hep-1 cells, HA22T/VGH cells

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies in both eastern and western countries, and approximately 90% of all cancer patients die from metastases (Wittekind and Neid, 2005; Moriwaki, 2002). SK-Hep-1 and HA22T/VGH cells are poorly differentiated human HCC cell lines which are difficult to treat and cause shorter survival rates compared to highly differentiated HCC, such as Hep 3B cells (Lerose et al., 2001; Wu et al., 2008). Recent work indicated that SK-Hep-1 and HA22T/VGH cells have migratory and invasive abilities (Wu et al., 2008), and many natural compounds from plants and foods were demonstrated to be inducers of apoptosis (Taraphdar et al., 2001). Therefore, developing health food products that can help reduce the incidence of hepatoma is an important approach for chemoprevention of cancers.

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The basidiomycete fungus *Agaricus blazei* (AB) Murill, also called himematsutake or agsrikusutake in Japanese, has traditionally been used as a health food source in Brazil and is prescribed as traditional medicine in Japan. Most studies reported that fruiting bodies of AB can confer immunomodulatory, hepatoprotective, antitumor, antiangiogenesis, antigenotoxic, antioxidative, and antidiabetes activities (Kim et al., 2005; Ahn et al., 2004; Izawa and Inoue, 2004; Barbisan et al., 2002; Martins de Oliveira et al., 2002; Takaku et al., 2001). The mycelia and fruiting bodies of AB have also been shown to induce apoptosis in different cancer cell lines, including leukemia, lymphoma, uterine cervical carcinoma, melanoma, and breast adenocarcinoma cells (Jin et al., 2007; Kawamura and Kasai, 2007). Because cultivating the AB fruiting bodies biotechnology requires several months and it is also difficult to control the quality of the products, many researchers are interested in using fermentation technology to produce large quantities of fungal products with consistent biological activities, including immunomodulatory, anticancer, and antioxidative activities (Su et al., 2008; Yu et al., 2009; Ker et al., 2005; Sorimachi et al., 2001). In our previous study, we found that the ethanolic extract of an AB fermentation product (AB-pE) cultured in medium containing black soybeans exhibited strong antihepatoma activity against Hep 3B and Hep G2 cells, and blazeispirols A and C were further demonstrated to be the major active compounds (Su et al., 2008).

The objective of this study was to evaluate the antihepatoma activity of the AB-pE against poorly differentiated hepatoma cells with high invasive activity. We studied here the antiproliferative activity and the expression of apoptosis-related proteins, such as Bax, Bcl-2, caspase-3, caspase-9 and poly(ADP ribose) polymerase (PARP) in SK-Hep-1 and HA22T/VGH cells, as a response to treatment with AB-pE. A Matrigel invasion assay was also used to investigate the anti-invasive effects of the AB-pE on both cell lines.Molecular and signaling mechanisms associated or involved in these bioactivities are revealed and discussed.

Materials and Methods

Materials and chemicals

The fermentation products of AB were obtained from Prof. Chin-Hang Shu of the Department of Chemical and Materials Engineering, National Central University (Taoyuan, Taiwan). Ethanol (95%) was purchased from Echo (Taipei, Taiwan). Dulbecco's modified Eagle medium (DMEM), antibiotic-antimycotic solution, penicillin-streptomycin solution, MEM non-essential amino acid solution, and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Grand Island, NY, USA). N,N-Dimethylfluoramide (DMF) was obtained from Lab-Scan (Dublin, Ireland). Trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sodium dodecylsulfate (SDS), ribonuclease (RNase) and propidium iodide (PI) were purchased from Sigma Chemical (St Louis, MO, USA).

Preparation of the ethanolic extracts of the fermentation product

The fermentation product of AB was lyophilized and ground into a powder. One gram of dry powder was extracted with 20 mL 95% ethanol at 30℃ for 24 h. The filtrate (AB-pE) was evaporated under a vacuum. The extract was dissolved in DMSO as the stock solution and stored at -20℃. For all experiments, the final concentrations of the tested compounds were prepared by diluting the stock solution with DMEM.

Analysis of blazeispirols A and C in the AB-pE

The AB-pE was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC), as previously described (Su et al., 2008). In brief, a Cosmosil 5C18-AR-II (5 μm, 250 mm × 4.6 mm i.d.) column (Nacalai Tesque, Kyoto, Japan) was used at a column temperature of 25℃. Gradient elution began with 80% methanol (in water and containing 0.5% acetic acid) and increased linearly to 84% in 15 min, to 86% in a further 15 min, and finally to 88% in 10 min. The flow rate was 0.7 mL/min, and the effluent was monitored at 243 nm. Quantification was performed by utilizing a standard curve of blazeispirols A or C. All analyses were carried out in triplicate.

Cell lines and culture

Human hepatoma SK-Hep-1 and HA22T/VGH cells were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). SK-Hep-1 and HA22T/VGH cells were cultured in complete DMEM (cDMEM containing DMEM, 10% FBS, 10 U/mL of penicillin, 10 μg/mL of streptomycin, and 100 μM non-essential amino acids; pH 7.0) at 37℃ in a 5% CO_2 atmosphere at 90% relative humidity.

Assessment of cell viability

SK-Hep-1 and HA22T/VGH cells were cultured in 96-well plates at a density of 5×10^3 cells/well with 100 µL cDMEM/well. After 24 h of incubation, the medium in the 96-well plate was replaced by 100 µL serum-free DMEM (containing DMEM, 10 U/mL of penicillin, 10 µg/mL of streptomycin, and 100 µM non-essential amino acids) containing different concentrations of samples (12.5~200 µg/mL) for 48 h. The sample was dissolved in DMSO, and the final concentration of DMSO in the medium was 0.1%. Then the medium was discarded, and 25 µL of an MTT solution (5 mg/mL PBS) and 100 µL serum-free DMEM were added to each well and reincubated for an additional 4 h. Aliquots of 100 µL of MTT lysis buffer (20 g SDS in 50 mL DMF and 50 mL water) were added to dissolve the formazan crystals, and then the plates were read at 570 nm in a microplate reader after 14~16 h (Mosmann, 1983). Cells not exposed to the sample served as the control with 100% survival. The concentration of the AB-pE required to inhibit cell growth by 50% (IC_{50}) was calculated by interpolation from a dose-response curve.

Flow cytometric analysis of the cell-cycle distribution and apoptosis

SK-Hep-1 and HA22T/VGH cells were cultured in 6-cm dishes at a density of 3.25×10^5 cells/dish with 6.5 mL cDMEM/dish. After 24 h of incubation, the medium was replaced with 6.5 mL serum-free DMEM containing different concentrations of samples (0~200 µg/mL). Cells were trypsinized by 0.5 mL of a trypsin-EDTA solution and collected by centrifugation (300 × g for 5 min). The pellet was further re-suspended with ice-cold PBS and centrifuged (300 ×g for 5 min), and then the supernatant was discarded and fixed with cold 70% ethanol. Cells were stored at -20 °C overnight. Fixed cells were centrifuged (300 ×g for 15 min) twice, and then the supernatant was discarded. Moreover, cells were mixed with 1× PBS containing 5% Triton X-100 and 2 mg/mL RNAase A, and then cells were stained with 1 mg/mL propidium iodide (PI). Data were collected from 10,000 cells by FACScan flow cytometry (BD Biosciences, San Jose, CA) within 30 min. The sub-G1 phase was analyzed with ModFitLT V3.0 software (Verity Software, Topsham, ME, USA).

Assay of caspase-3 activity

Caspase-3 activities in SK-Hep-1 and HA22T/VGH cells were evaluated as the protease activity of caspase-3 using PhiPhiLux G1D2 for the cytometric analysis and a G2D2 kit for the fluorescence microscopic assay (MBL,

Nagoya, Japan). A substrate of PhiPhiLux G1D2 and G2D2 which can penetrate the cell nucleus is converted to the fluorescent form as it is cleaved by the protease activity of caspase-3. The assessment was performed according to the manufacturer's recommendations (Becton Dickinson, San Jose, CA). In brief, treated SK-Hep-1 and HA22T/VGH cells were incubated with PhiPhiLux G1D2 or G2D2 for 45 min at 37℃ in a 5% CO_2 atmosphere, and then the caspase-3 activity was analyzed by fluorescence microscopy or flow cytometry.

Western blotting

SK-Hep-1 and HA22T/VGH cells were cultured in 10-cm dishes at a density of 2×10^6 cells/dish with 6.5 mL cDMEM/dish. After 24 h of incubation, the medium was replaced with serum-free DMEM containing different concentrations of sample (0~200 µg/mL) for 48 h. Proteins were extracted using RIPA lysis buffer [1% NP40, 0.5% sodium deoxycholate, 30 µL/mL aprotinin, 10 mg/mL phenylmethylsulfonylforid (PMSF), 100 mM sodium orthovanadat, 0.5% Triton-X 100], and cell lysates were collected in 1.5-mL microtubes. The cell lysate was set in liquid nitrogen for 5 min and then transferred to a 37℃ water bath for 5 min three times. To detect protein expression, 50 µg of protein of each cell lysate was mixed with six-fold sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 60% glycerol, and 0.6% bromophenol blue. The mixtures were boiled at 95℃ for 5 min and then subjected to 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 2~3 h. SDS-PAGE was carried out, and the proteins on the gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) at 150 V and 400 mA for 2 h. After washing three times with washing buffer (2.5 mM Tris, 150 mM NaCl, and 3% Tween 20; pH 7.4) for 5 min each, the membrane was incubated with blocking buffer (5% non-fat milk, 2.5 mM Tris, 150 mM NaCl, and 3% Tween 20; pH 7.4) for 1 h. After washing three times with washing buffer for 5 min each, the blots were incubated with the primary antibodies of anti-caspase-3, caspase-9, Bax, Bcl-2, PARP, and β-actin (1:1000) (Cell Signaling, Davers, MA, USA) overnight at 4℃. Blots were then incubated with anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP; 1:2000) (Cell Signaling) for 2 h at room temperature. Protein expression was detected by an enhanced chemiluminescence (ECL) Western blotting

detection system (General Electric, Fairfield, CT, USA), and the signals were exposed to x-ray film.

Invasion assay

A polycarbonate membrane with 8-μm pores that fit into a 24-well invasion chamber was obtained from Corning (Nagog Park, MA, USA). Matrigel (BD Biosciences, San Jose, CA, USA) (35 μL) was added to the upper compartment of the invasion chamber. SK-Hep-1 and HA22T/VGH cells (1×10^4 cells/100 μL) were added to the upper compartment of the invasion chamber. Various concentrations of AB-pE (25, 50, or 100 μg/mL) were loaded into the lower compartment of the invasion chamber. The Matrigel invasion chambers were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The Matrigel invasion chambers were washed with 1× PBS three times and then fixed with methanol. After staining with crystal violet, the filter inserts were removed from the wells, and the cells on the upper side of the filter were removed using a cotton swab. Cells which had invaded the lower surface of the filter were counted in five random fields by microscopy at a final magnification of 100x.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) and Duncan's multiple-comparison test (SAS Institute, Cary, NC, USA) to determine significant differences among the means ($p < 0.05$).

Results

AB-pE the principle of compounds that can confer major anti-hepatoma activity

It was demonstrated that blazeispirols A and C are the major components that contribute to the inhibition of growth of hepatoma Hep 3B and G2 cells (Su et al., 2008). Blazeispirols A and C in the AB-pE were detected by RP-HPLC as shown in Figure 1. The retention times (t_R) of blazeispirols A and C were 33 and 38 min, respectively, and their contents were 49.9 ± 8.9 and 14.2 ± 2.4 mg/g in the dry extract of the AB fermentation product, respectively (Figure 1).

Inhibitory ability of the AB-pE against HA22T/VGH and SK-Hep-1 cells

The inhibitory effects of AB-pE on the cell growth of HA22T/VGH and SK-Hep-1 cells were determined (Figure 2). When HA22T/VGH and SK-Hep-1 cells were treated with different concentrations of the AB-pE
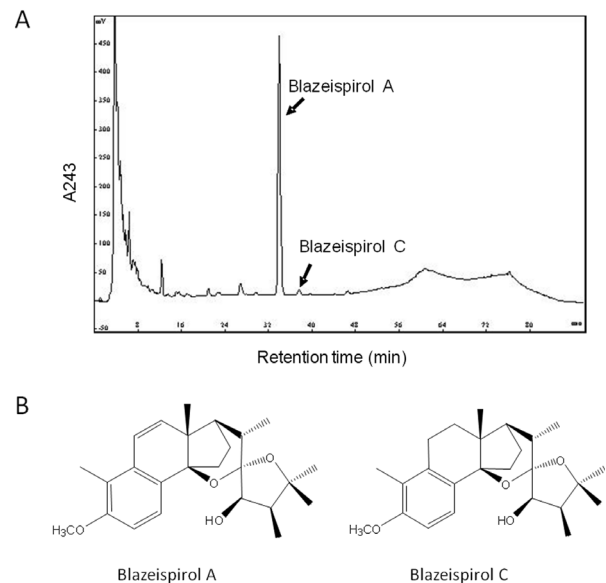

Figure 1. (A) HPLC chromatogram of the ethanolic extract of *Agaricus blazei* fermentation product (AB-pE). Conditions included a reversed-phase Cosmosil 5C18-AR-II column (5 μm, 250 mm ?4.6 mm i.d.); a mobile phase of solvent A (methanol/water/acetic acid, 80:20:0.5, v/v/v) and B (methanol/acetic acid, 100:0.5, v/v), and a linear gradient increase of methanol from 80% to 84% in 0~15 min, from 84% to 86% in 15~30 min, and finally from 86% to 88% in 30~40 min; a flow rate of 0.7 mL/min; and UV detection at 243 nm. (B) Structure of Blazeispirols A and C.
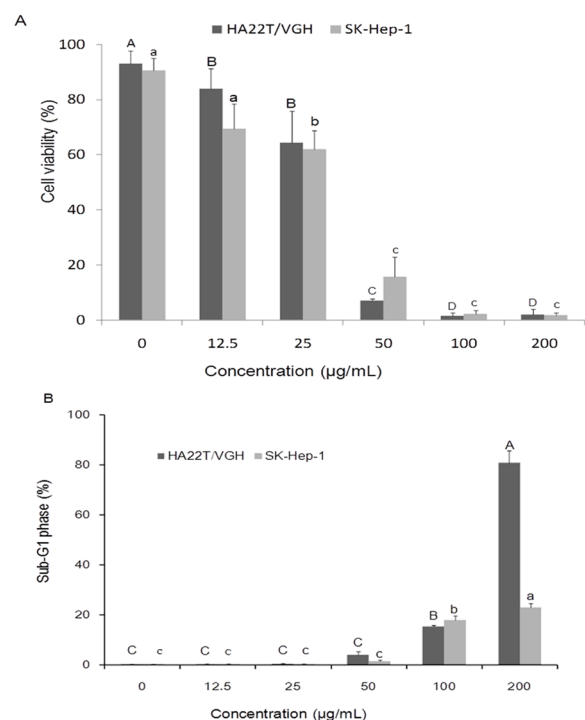

Figure 2. Effects of various concentrations of the ethanolic extract of Agaricus blazei fermentation product (AB-pE) on the cell viability and arrest at the sub-G1 phase in HA22T/VGH and SK-Hep-1 cells. Cells were seeded in complete DMEM for 24 h and then treated with the AB-pE (12.5, 25, 50, 100, and 200 μg/mL) for another 48 h. Cell viability (A) was determined using the MTT assay, and production of Ssub-G1 phase (B) was analyzed using flow cytometry. Each data represents the mean ± SD (*n* = 3). Numbers within each cell line with different letters significantly differ from the control at *p* < 0.05 using one-way ANOVA and Duncan's multiple-comparison test.

(12.5~200 μg/mL) for 48 h, cell viabilities decreased in a dose-dependent manner in both cell lines (Figure 2A). IC_{50} values against HA22T/VGH and SK-Hep-1cells were detected at 28.7 and 26.8 μg/mL, respectively. Treatment with AB-pE at 100 and 200 μg/mL resulted in altered cell morphologies, including cell floating (data not shown) and shrinkage (Figure 2B) in both HA22T/VGH and SK-Hep-1 cells. Additionally, to understand the inhibition mechanism of the AB-pE on growth of these cells, we analyzed the cell cycle activity, i.e., percentage of both cell lines in the sub-G1 phase, by flow cytometry (Figure 2B). The population of sub-G1 phase cells increased by 15.4% and 80.8% after 100 and 200 μg/mL AB-pE treatment, respectively, as compared to the control in HA22T/VGH cells. The AB-pE at 100 and 200 μg/mL significantly increased the percentages of SK-Hep-1 cells in the sub-G1 phase by 17.8% and 23%, respectively, compared to the control. These results suggest that the AB-pE induced apoptosis in both HA22T/VGH and SK-Hep-1 cells, and HA22T/VGH cells were more sensitive to the treatment than were SK-Hep-1 cells.

The AB-pE activated caspase-3 in HA22T/VGH and SK-Hep-1 cells

PhiPhiLux G1D2 and G2D2 were used to assay the activity of caspase-3 in HA22T/VGH and SK-Hep-1 cells treated with AB-pE to confirm the activity of apoptosis. Results from the fluorescence microscopic examination showed that more cells emitted green fluorescence in the AB-pE (50~200 μg/mL) treated in HA22T/VGH and SK-Hep-1 cells as compared to the untreated cells (Figure 3). Furthermore, the fluorescence intensity, e.g., the activity of caspase-3 altered by the AB-pE, was analyzed by flow cytometry. The average fluorescence intensity of 1×10^5 HA22T/VGH cells shifted to the right from 4.2 to 12.6, 18.2, 24.3, 39.5, and 87.7 mean fluorescence intensity (mfi), respectively, after treatment with 12.5~200 μg/mL AB-pE (Figure 4A). As shown in Figure 4B, for treatment with
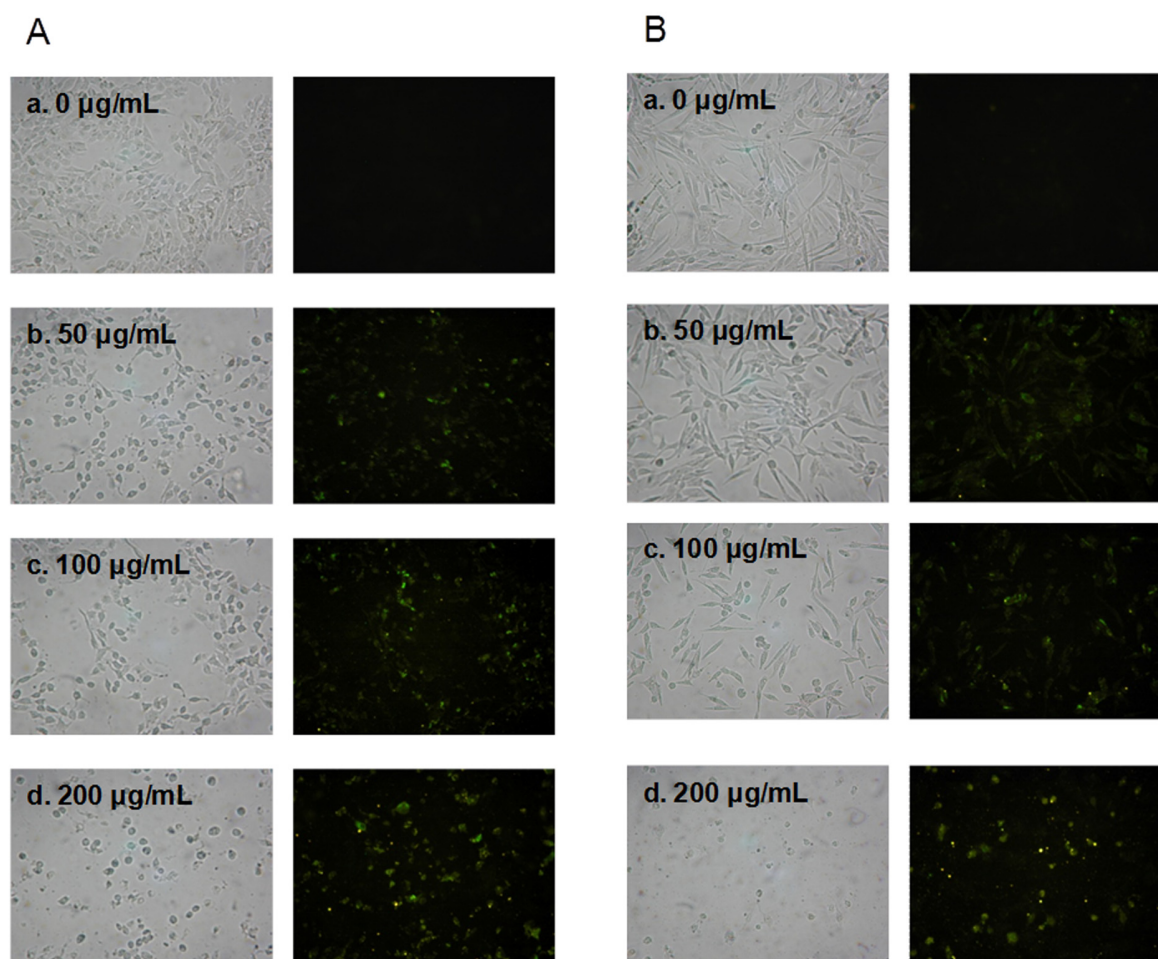

Figure 3. Effect of the ethanolic extract of Agaricus blazei fermentation product (AB-pE) on caspase 3 activity in HA22T/VGH (A) and SK-Hep-1 (B) cells (100x under an inverted-stage microscope equipped with phase contrast). Cells (3.25×10^5/dish) were incubated in serum-free DMEM and then treated with the AB-pE for another 48 h. Caspase-3 activity was determined using fluorescence microscopy.
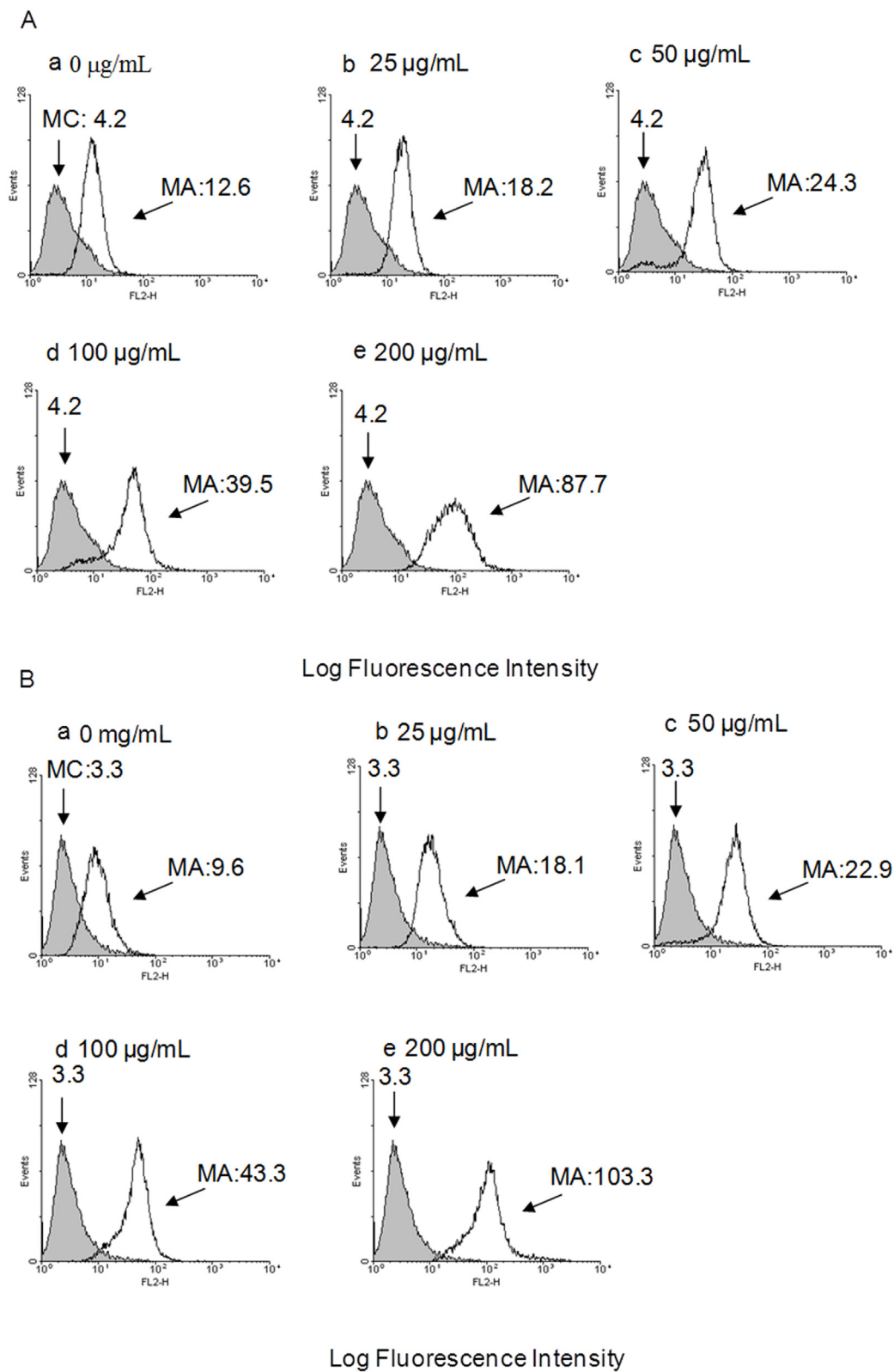
Figure 4. Effects of the ethanolic extract of Agaricus blazei fermentation product (AB-pE) on caspase-3 activity in HA22T/VGH (A) and SK-Hep-1 (B) cells. Cells (3.25×10^5/dish) were seeded in serum-free DMEM and then treated with the AB-pE for another 48 h. Caspase-3 activity was determined using flow cytometry. Data are shown as the log fluorescence, and the value is presented as the mean of the log fluorescence. MC, control cells; MA, treated cells.
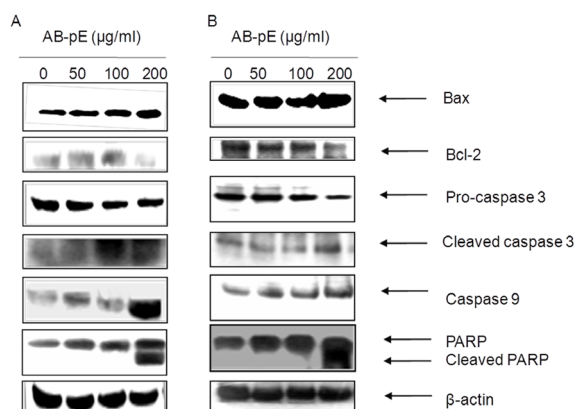
Figure 5. Effects of the ethanolic extract of Agaricus blazei fermentation product (AB-pE) on the protein expressions of Bax, Bcl-2, caspase-3, caspase-9, and PARP in HA22T/VGH (A) and SK-Hep-1(B) cells. After 48 h, cell lysates were collected and analyzed by Western blotting.

12.5~200 μg/mL AB-pE, the average fluorescence intensity of 1×10^5 cells shifted to the right from 3.3 to 9.6, 18.1, 22.9, 43.3 and 103.3 mfi, respectively. The data showed that 12.5~200 μg/mL of AB-pE increased the activities of caspase-3 in a dose-dependent manner in both HA22T/VGH and SK-Hep-1 cells when compared to the control. This result indicates that AB-pE stimulated the activities of caspase-3 in HA22T/VGH and SK-Hep-1 cells during the apoptotic process.

AB-pE induces apoptosis by regulating caspase-9, caspase-3, PARP, Bax and Bcl-2 activities

In order to further understand the apoptotic pathway of the AB-pE in HA22T/VGH and SK-Hep-1 cells, levels of five apoptosis-related proteins, including Bcl-2, Bax, caspase-9, caspase-3 and PARP, were assayed by Western blotting. AB-pE increased the expressions of the proapoptotic Bax and caspase-9 but decreased the expression of the antiapoptotic Bcl-2 (Figure 5). The AB-pE also activated caspase-3 and PARP. These results suggest that the AB-pE induced HA22T/VGH and SK-Hep-1 cells toward apoptosis by upregulating the expression of Bax, caspase-3, caspase-9 and PARP and downregulating Bcl-2.

AB-pE inhibits the invasive activity of HA22T/VGH and SK-Hep-1 cells

The effects of the AB-pE on invasive activity of HA22T/VGH and SK-Hep-1 cells were analyzed by a Matrigel invasion assay. The microscopy data show that 25, 50, and 100 μg/mL AB-pE inhibited the invasive ability of HA22T/VGH and SK-Hep-1 cells (Figure 6). The inhibitory rates of the invasive activity of AB-
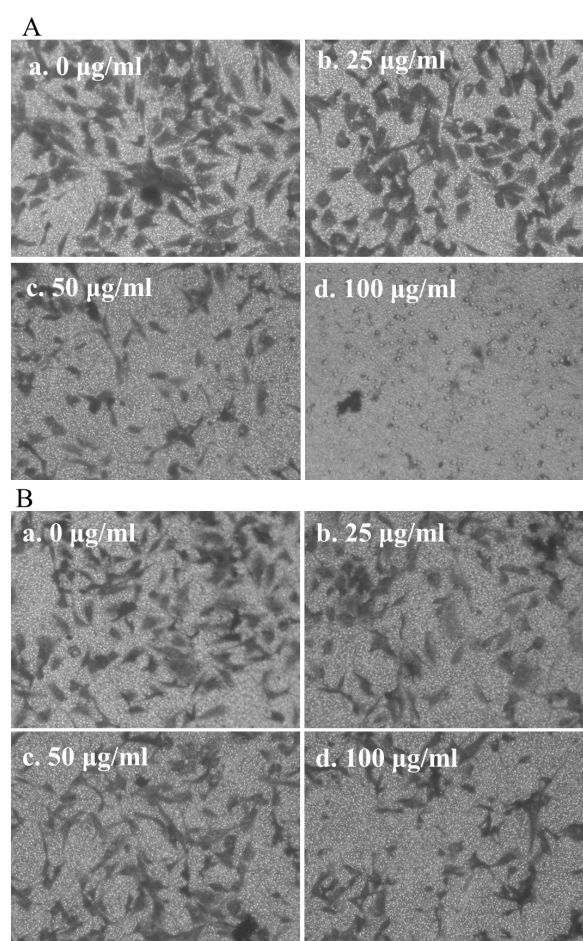

Figure 6. Effects of the ethanolic extract of Agaricus blazei fermentation product (AB-pE) on the invasive activity of HA22T/VGH (A) and SK-Hep-1 cells (B). Cells (1×10^4/dish) were incubated in the upper chamber with (25~100 μg/mL) or without the AB-pE for 24 h. Cells were photographed on the lower surface of chambers at 100x of an inverted-stage microscope equipped with phase contrast.

pE at 50 and 100 μg/mL were detected at 15.6% and 62.2%, repectively for SK-Hep-1 cells, and those of AB-pE at 25, 50, and 100 μg/mL were detected at 30.0%, 67.1%, and 95.7% for HA22T/VGH cells, respectively. These results showed that AB-pE conferred strong to suppressive activity against the invasive ability in both types of hepatoma cells.

Discussion

Various natural products have been shown to induce apoptosis in different cancer cell types and may have the potential to be further developed for cancer therapy and prevention (Taraphdar et al., 2001). An extracted compound from AB fruiting bodies is used as a complementary and alternative medicine for patients with a cancer history in Japan. Our previous study reported that the ethanolic extract from AB fermentation

products (AB-pE) cultured in medium containing black soybeans exhibited appreciable cytotoxicities against hepatoma Hep 3B and Hep G2 cells. Two major compounds isolated from the AB-pE, blazeispirols A and C, which contributed to the inhibition of growth of Hep 3B and Hep G2 cells, could be emp;oyed as biomarkers fro the fermentation process (Su et al., 2008). In the present study, we investigated the anticancer effect and mechanism of the AB-pE, containing 49.9 ± 8.9 and 14.2 ± 2.4 mg/g blazeispirols A and C, respectively, on the poorly differentiated HCC HA22T/VGH and SK-Hep-1 cell lines.

Apoptosis is a continuous physiologic process for programmed cell death accompanied by a non-inflammatory response and is one of the most active areas in of biomedical research (Schultz and Harrington, 2003). The stereotyped morphology of apoptosis includes condensation of nuclear heterochromatin, cell shrinkage, formation of apoptotic bodies, and DNA fragmentation (Taraphdar et. al. 2001; Samejima and Earnshaw, 2005). In this study, we found altered morphologies and decreased viabilities of HA22T/VGH and SK-Hep-1 cells when treated with AB-pE (Figure 2 and 3). Moreover, 100 and 200 μg/mL of the AB-pE increased the cell population in sub-G1 phase for both cell types tested, and HA22T/VGH cells were more sensitive to AB-pE than were SK-Hep-1 cells (Figure 2). According to these results, we suggest that AB-pE had a cytostatic effect against HA22T/VGH and SK-Hep-1 cells via apoptosis. Caspases are a group of cysteine proteases and key points in apoptosis. It was reported that $(1{\rightarrow}3)$-β-D-glucan extracted from AB fermentation products inhibited the growth of prostate cancer cells by inducing apoptosis via caspase-3 and -7 activation (Yu et al., 2009). In our study, the ethanolic extract from AB fermentation products which are enriched in blazeispirols A and C activated the caspase-3 activities in HA22T/VGH and SK-Hep-1 cells (Figure 3 and 4).

The Bcl-2 (B-cell lymphoma gene 2) protein family contains members with pro- and antiapoptotic activities (Dejean et al., 2006) The mitochondrion apoptosis-induced channel (MAC) plays a vital role in formation of the cytochrome C release channel early in apoptosis. Overexpression of Bax (Bcl-2-associated protein X), a proapoptotic protein, induces cell death via MAC formation and cytochrome C release (Dejean et al., 2006). Poly(ADP-ribose) polymerase (PARP), which play an important role in DNA repair, is a substrate cleaved by caspases during apoptosis (Decker and Muller, 2002). The hot-water extract of AB fruiting body preparations inhibited cell growth through apoptosis via the downregulation of Bcl-2, caspase-3 activation, and PARP cleavage in the human leukemic U937 cell (Jin et al., 2007). Hemicellulase-treated AB mycelia inhibited cell proliferation and induced apoptosis through activating p38 mitogen-activated protein kinase (MAPK) in different cell lines, such as leukemia, lymphoma, uterine cervical carcinoma, melanoma, and breast adenocarcinoma cells (Kawamura and Kasai 2007). In our study, we found that the AB-pE upregulated expression of proapoptotic Bax and caspase-9 proteins, downregulated expression antiapoptotic Bcl-2 protein, and activated expression of caspase-3 and PARP in a dose-dependent manner in HA22T/VGH and SK-Hep-1 cells (Figure 5). Blazeispirols A or C in the AB-pE may be the active constituents which induce apoptosis via a mitochondrial pathway in both cell types.

Metastasis is the major cause of death of cancer patients, and invasion is a critical event in cancer metastasis (Wittekind et al., 2005). In the poorly differentiated hepatocellular HA22T/VGH and SK-Hep-1 cells, protein kinase α (PKCα) is associated with regulation of cell migration and invasion. The lipid fraction containing ergosterol from AB fruiting bodies inhibited Matrigel-induced neovascularization in C57BL/6 mice (Takaku et al., 2001). In the present study, we reported that the AB-pE has the potential to inhibit the invasive ability of HA22T/VGH and SK-Hep-1 cells (Figure 6).

In conclusion, the AB-pE inhibited the growth of poorly differentiated hepatoma cells through induction of apoptosis. We believe this study provides initial evidence to show that the AB-pE can induce apoptosis via upregulating the expressions of proapoptotic Bax and caspase-9 proteins, downregulating antiapoptotic Bcl-2 protein, and activating caspase-3 and PARP. The AB-pE hence may have the potential to inhibit invasion of HA22T/VGH and SK-Hep-1 cells. Besides the apoptosis-related proteins Bax, Bcl-2, caspase-3, caspase-9, and PARP, additional future studies may help to demonstrate that the AB-pE induces apoptosis more through an intrinsic pathway or an extrinsic pathway by regulating Fas-associated protein with the death domain (FADD) and caspase-8. The mechanism of the AB-pE and its active compounds may warrant systematic evalution in future studies.

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