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RESEARCH ARTICLES

Inhibitory mechanisms of *Agaricus blazei* Murill on the growth of prostate cancer in vitro and in vivo

Ching-Han Yu[a], Shu-Fen Kan[a], Chin-Hang Shu[b], Ting-Jang Lu[c], Lucy Sun-Hwang[c], Paulus S. Wang[a,d,*]

[a]*Department of Physiology, School of Medicine, National Yang-Ming University, Shih-Pai, Taipei 11221, Taiwan, Republic of China*
[b]*Department of Chemical and Materials Engineering, National Central University, Jhong-Li 32001, Taiwan, Republic of China*
[c]*Institute of Food Science and Technology, National Taiwan University, Taipei 10617, Taiwan, Republic of China*
[d]*Department of Medical Research and Education, Taipei City Hospital, Taipei 10341, Taiwan, Republic of China*

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Abstract

Agaricus blazei Murill (*A. blazei*) has been conventionally used as a health food for the prevention of cancer. However, little is known about the direct effects and action mechanisms of *A. blazei* on human prostate cancer. In the present study, the effects of *A. blazei* on the growth of human prostate cancer were examined in vitro and in vivo. *A. blazei*, especially the broth fraction, inhibited cell proliferation in both androgen-dependent and androgen-independent prostate cancer cell lines. The broth of *A. blazei* induced lactate dehydrogenase leakage in three cancer cell lines, whereas the activities of caspase 3 and the DNA fragmentation were enhanced the most in androgen-independent PC3 cells. The protein expressions of apoptosis-related molecules were elevated by the broth of *A. blazei* in PC3 cells. Oral supplementation with the broth of *A. blazei* (with the higher ratio of β-glucan) significantly suppressed tumor growth without inducing adverse effects in severe combined immunodeficient mice with PC3 tumor xenograft. Tumor xenografts from *A. blazei*-fed mice showed decreased proliferating cell nuclear antigen-positive cells and reduced tumor microvessel density. Based on these results, we found that the broth of *A. blazei* may directly inhibit the growth of prostate cancer cell via an apoptotic pathway and suppress prostate tumor growth via antiproliferative and antiangiogenic mechanisms. We therefore suggest that *A. blazei* might have potential therapeutic use in the prevention and treatment of human prostate cancer.

Keywords: *Agaricus blazei*; Human prostate cancer; Apoptosis; Antitumor activity

1. Introduction

The edible mushroom *Agaricus blazei* Murill (*A. blazei*), a species of Basidiomycetes native to Brazil, is often consumed as food or as a drink around the world. *A. blazei* has been popularly consumed as a complementary treatment for various diseases, including diabetes, hyperlipidemia, arteriosclerosis, chronic hepatitis, and cancer [1–4]. In Asia, particularly in Japan, *A. blazei* is the mostly used adjuvant for the prevention and treatment of cancer after the removal of the major tumor [4,5]. The well-known component of *A. blazei*, β-glucan, inhibits the growth of sarcoma 180 in mice and improves quality of life through elevating the immune activity in cancer patients [6,7]. In addition, the polysaccharide–protein complex also shows antitumor activities due to immunological host-mediated mechanisms [8–10]. In addition, recent studies have found that the β-glucan from *A. blazei* inhibits tumor growth not only by accelerating the immune activity but also through its direct antitumor capability [11]. Some other substances of *A. blazei*,

Abbreviations: *A. blazei*, *Agaricus blazei* Murill; PARP, poly(ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yle)-2,5-diphenyltetrazolium bromide; OD, optical density; LDH, lactate dehydrogenase; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-UTP end labeling; SCID, severe combined immunodeficient; PCNA, proliferating cell nuclear antigen.

* Corresponding author. Department of Physiology, School of Medicine, National Yang-Ming University, Shih-Pai, Taipei 11221, Taiwan, Republic of China. Tel.: +886 2 28267082; fax: +886 2 28264049.
E-mail address: pswang@ym.edu.tw (P.S. Wang).

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ergosterol and blazeispirols, have antitumor effects with their antiangiogenic activities [5,12–14]. Nevertheless, no epidemiological evidence and few studies stand for the beneficial effects of the crude aqueous fermentative products of this mushroom, which is the most common type of product in the market. Only the soluble protein-bound polysaccharide acid-treated fraction has shown some direct antitumor activities as seen by selective cell cycle arrest and apoptosis induction in Meth A tumor cells in vitro [2]. Therefore, the direct antitumor effects of fermentative-cultured *A. blazei* on prostate cancer were investigated in the present study.

In 2007, there were about 218,890 males detected with prostate cancer in the United States [15]. In addition, the incidence and mortality of prostate cancer have also been increasing in Asian countries in the past decade. Traditionally, radiation therapy and surgery were the curative treatments for prostate cancer in situ. Now, androgen ablation is the mainstream treatment for metastatic prostate cancer; however, there is still no effective treatment for hormone-refractory prostate cancer. Therefore, it is necessary to develop some novel and effectual treatments for hormone-insensitive prostate cancer.

Induction of cell apoptosis has been the target mechanism for cancer treatments. Caspase cascade is a well-known key pathway in the apoptotic signal transduction. Caspases are divided into two types of subfamilies: upstream initiator caspases (caspases 8 and 9), which are involved in regulatory events, and downstream effector caspases (caspases 3, 6, and 7), which are direct responses to the change in cell morphological events and to the cleavage of nuclear protein poly(ADP-ribose) polymerase (PARP) [16]. The intrinsic pathway (the mitochondria) is one of the major apoptotic pathways in the upstream of caspase cascade [17]. Bcl-2 family members translocate to mitochondria and mediate the membrane potential to induce cytochrome *c* release. The cytosolic cytochrome *c* is further involved in the signaling of caspase activation and finally causes cell apoptosis.

The major objectives of the present study were to explore antiproliferative and antitumor effects occasioned by *A. blazei* with the measurement of cell proliferation, cytotoxicity, and protein expressions of apoptosis-related molecules in vitro on both androgen-dependent and androgen-independent prostate cancer cell lines and on the detection of tumor growth in the in vivo study on androgen-independent PC3 cells. Such investigations have, in part, demonstrated the antitumor effects of *A. blazei* upon prostate cancer and assisted in providing a complementary treatment for cancer patients.

2. Materials and methods

2.1. Isolation of polysaccharides and analysis of (1→3)-β-D-glucan from A. blazei

The fermentative products were shredded by a grinder for polysaccharide extraction. The crude polysaccharides were extracted three times with boiling water for 2 h each from the shredded products. The extracts were concentrated using a rotor evaporator (RE111, Buchi, Flawil, Switzerland). Four volumes of 95% (v/v) ethanol were added to precipitate the crude polysaccharides. The crude polysaccharides were further treated with cetylpyridinium chloride, amyloglucosidase (A-7255, Sigma, St. Louis, MO, USA), and protease (P-7026, Sigma) to remove the small amounts of proteins, uronic acids, starch-like α-D-glucans, and hexosamines.

Aniline blue was used to detect the existence of (1→3)-β-D-glucan in the crude polysaccharide. The method was similar to that described previously [18,19]. The concentrations of polysaccharide and (1→3)-β-D-glucan are shown in Table 2.

2.2. Cells and culture conditions

The androgen-dependent human prostate carcinoma cell line LNCaP and androgen-independent prostate cancer cell lines DU145 and PC3 were purchased from the Culture Collection and Research Center of the Food Industry Research and Development Institute (Taiwan, ROC). Cell lines were maintained in RPMI 1640 (Gibco Laboratories, Buffalo, Grand Island, NY, USA) (for LNCaP) or in Dulbecco's modified Eagle's medium (Gibco Laboratories) (for DU145 and PC3) with 10% fetal calf serum (PAA, Pasching, Austria) in an atmosphere of 5% CO_2 at 37°C.

2.3. Cell proliferation assessment

Cell proliferation was determined with the use of the modified colorimetric 3-(4,5-dimethylthiazol-2-yle)-2,5-diphenyltetrazolium bromide (MTT) assay. After cells were attached, they were challenged with different concentrations of fermentative products from *A. blazei* or with curdlan (Sigma) or β-D-glucan (Sigma). The MTT (Sigma) assay was performed on Days 1, 2, 3, and 4 as described previously [20]. The proliferation index of each day referred to the optical density (OD) of that day divided by the OD of Day 0. Each experimental condition was performed in three preparations and repeated four times.

2.4. Cytotoxicity assessment by lactate dehydrogenase assay

Cytotoxicity was measured using a cytotoxicity detection kit (Boehringer–Mannheim, Mannheim, Germany). The detailed procedure was described previously by Kan et al. [21]. Each experiment was performed in four preparations. Results were expressed as the percentage of lactate dehydrogenase (LDH) leakage. The activity of LDH in mouse serum was examined with the use of an LDH kit (BioAssay Systems, Hayward, CA, USA) following the instruction of the manufacturer.

2.5. Apoptosis detection

An apoptosis detection kit (Promega, Madison, WI, USA) was used to measure fragmented DNA by terminal deoxynucleotidyl transferase-mediated deoxy-UTP end labeling (TUNEL). After treatment with *A. blazei*, cells

were harvested and then fixed by the formaldehyde. TUNEL assay was performed as recommended by the manufacturer and analyzed with the use of a flow cytometer (Beckman Coulter, San Diego, CA, USA).

2.6. Measurement of caspase 3 activity

The cells treated with different fermentative products of *A. blazei* for 72 h were harvested and washed twice by phosphate-buffered saline. Cell pellets were obtained, and the enzyme activity of caspase 3 was detected with the use of a Caspase Colorimetric Assay System (R&D Systems, Minneapolis, MN, USA) as recommend by the manufacturer. Results were expressed as the percentage of the control group.

2.7. Immunoblotting analysis

The method of immunoblot was performed as described by Kan et al. [20]. After culture under the treatment of different fermentative products of *A. blazei* for 72 h, cells were harvested and lysed in RIPA buffer. The immunoblot was performed with the antibodies of caspase 3 (Imgenex, San Diego, CA, USA), caspase 7 (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Sigma). After being washed three times by TBST, the blot was incubated with horseradish peroxidase-conjugated goat antimouse or anti-rabbit secondary antibody (Promega), and proteins were visualized using enhanced chemiluminescence detection (Western blotting reagents, Amersham International, Bucks, UK).

2.8. Isolation of cytosolic fractions

Release of cytochrome *c* from mitochondria to cytosol was measured by immunoblot as described previously [22]. Cytosolic fraction was then concentrated to 50–100 μl using a centrifugal concentration device for 10-kDa molecular mass (microcon YM-10, Millipore, Bedford, MA, USA) following the manufacturer's instruction. The fraction was separated on 15% SDS–PAGE and detected by mouse monoclonal antibody at a dilution of 1:400 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Each experimental condition was repeated three times.

2.9. Isolation of nuclear fraction

The expression of PARP in nucleus was examined by Western blot. After *A. blazei* treatment, cells were incubated in hypotonic buffer A (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, 1 μl/ml of PMSF, and 1 μl/ml of aprotinin, pH 7.9) for 10 min on ice. Buffer B (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, and 2.5% NP-40, pH 7.9) was further added in cell-suspended buffer A. After centrifugation at 1000*g* for 10 min, the supernatant was removed and the nuclear pellet was homogenized in buffer C (20 mM Hepes, 0.45 M NaCl, and 1 mM EDTA, pH 7.9) for 20 min on ice. The homogenate was centrifuged at 10,000*g* for 15 min. Equal amounts of nuclear extracts were subjected to 7% SDS and detected by the antibody of PARP (Cell Signaling Technology). Each experimental condition was repeated three times.

2.10. Animals

Male severe combined immunodeficient (SCID) mice (6 weeks old) were obtained from the Tzu-Chi University Laboratory Animal Center and housed under specific pathogen-free conditions at the National Yang-Ming University Laboratory Animal Center. Animal care was done in accordance with the National Yang-Ming University's guidelines for animal care.

2.11. In vivo tumor xenograft model

Six-week-old male SCID mice were injected subcutaneously with 2×10^6 PC3 cells in the right flank to establish PC3 tumor xenografts. On the next day, the mice were randomly divided into three groups: group I (n=7), normal drinking water (control); group II (n=7), 0.1% A-b (w/v) (broth of Sample A) in drinking water; and group III (n=7), 0.2% A-b (w/v) in drinking water for 8 weeks. The amount of A-b feeding was designated according to the dose for humans as suggested by the regulations of health food of the Department of Health, Executive Yuan, ROC. Body weight, diet, and water consumption were recorded two times per week throughout the study. Once xenografts started growing, their sizes were measured twice weekly. The tumor volume was calculated by the formula $0.5236\times L1\times(L2)^2$, where L1 is the long axis of the tumor and L2 is the short axis of the tumor. At the end of the experiment, tumors were excised, weighed, and stored in 4% paraformaldehyde/phosphate-buffered saline until additional analysis.

2.12. Immunohistochemical detection of tumor xenografts

Paraffin-embedded tumor xenograft sections (thickness=5 μm) were deparaffinized and hydrated to visualize CD31 and proliferating cell nuclear antigen (PCNA) expressions. Antigen retrieval was done by incubating the sections in boiling 10 mM citrate buffer, pH 6.0, for 20 min. Endogenous peroxidase activity was inhibited by 3% (v/v) H_2O_2 in methanol. Sections were then incubated with rabbit polyclonal anti-CD31 antibody (Abcam, Cambridge, UK) or mouse monoclonal anti-PCNA antibody (Cell Signaling Technology) at dilutions of 1:50 and 1:100, respectively. Finally, the sections were stained with the use of a polymer-horseradish peroxidase IHC detection system (BioGenex, San Ramon, CA, USA) following the manufacturer's instruction. Proliferating cells were quantified by counting the PCNA-positive cells (brown) and the total number of cells at 10 randomly selected fields at ×400 magnification. The proliferation index (per ×400 microscope field) was determined as the number of PCNA-positive cells multiplied by 100 and then divided by the total number of cells. CD31-stained (brown) microvessels

Table 1
Fermentation conditions of the fermentation products produced by using soybean or black bean as part of the liquid fermentation medium of *A. blazei*

Sample[a]	Fermentation conditions[b]					
	Fermentation system (l)	Fermentation mode	Temperature (°C)	Medium[c]	DO (dissolved oxygen)	Fermentation time (days)
A	500	One stage	28	A	≧50	7
B	500	Two stages[d]	28	A for the first stage and A′ for the second stage	≧50 for the first stage and 10 for the second stage	13
C	500	Two stages	28	A for the first stage and B for the second stage	≧50 for the first stage and 10 for the second stage	17
D	500	Two stages	28	A for the first stage and C for the second stage	≧50 for the first stage and 10 for the second stage	17

[a] The samples were obtained from Dr. Chin-Hang Shu (Department of Chemical and Materials Engineering, National Central University).
[b] Initiated fermentation conditions: working volume=350 l, inoculum ratio=10%, agitation=50 rpm, air flow rate=100 l/min, and pH 5.
[c] Medium A: 5 g/l of fructose, 15 g/l of sucrose, 20 g/l of soybean, 3 g/l of KH_2PO_4, and 1 g/l of $MgSO_4 \cdot 7H_2O$. Medium A′ contains 2.5 g/l of fructose, 7.5 g/l of sucrose, 10 g/l of soybean, 1.5 g/l of KH_2PO_4, and 0.5 g/l of $MgSO_4 \cdot 7H_2O$. Medium B contains 5 g/l of glucose, 1.5 g/l of malt extract, 10 g/l of black bean, 1.5 g/l of KH_2PO_4, 0.5 g/l of $MgSO_4 \cdot 7H_2O$, and 0.05 g/l of vitamin B_1. Medium C contains 5 g/l of glucose, 1.5 g/l of malt extract, 10 g/l of soybean, 1.5 g/l of KH_2PO_4, 0.5 g/l of $MgSO_4 \cdot 7H_2O$, and 0.05 g/l of vitamin B_1.
[d] The first stage is from Day 1 to Day 5, and the second stage is from Day 6 to Day 13 or 17.

were quantified in 10 random microscopic (×400) fields per tumor.

2.13. Statistics

All values are given as the mean±S.E.M. In some cases, Student's *t* test was used. In others, means were tested for homogeneity by one-way analysis of variance, and the difference between specific means was tested by Duncan's multiple-range test [23]. The difference between two means was considered statistically significant when $P<.05$.

3. Results

3.1. The level of polysaccharide in the fermentative product of A. blazei

Table 1 shows the fermentative conditions for each of the products. The fermentative system and temperature, 500 l and 28°C, respectively, were identical in the four products. The fermentative period in Sample A was one stage, but in Samples B–D, there were two stages. In Stage 1, *A. blazei* was incubated in Medium A, which contained 20 g/l of soybean as the nitrogen source and then fermented with Medium A′ for Sample B, with Medium B for Sample C, and with Medium C for Sample D in Stage 2. After the fermentation, Samples A–D were further examined for contents of carbohydrate, polysaccharide, and (1,3)-β-D-glucan, shown in Table 2. The concentrations of the total carbohydrate were similar between the four samples. However, the levels of polysaccharide and (1,3)-β-D-glucan were higher in Samples A and D. The ratios of (1,3)-β-D-glucan in polysaccharide were similar in Samples A (1.26%), C (1.00%), and D (1.14%), but the ratio was low in Sample B (0.52%).

3.2. Antiproliferative effects of A. blazei and β-glucan on human prostate cancer cell

Fig. 1 shows the effects of different fermentative products of *A. blazei* and β-glucan on the proliferation of prostate cancer cell lines measured by MTT assay. The fermentative conditions and relative products are shown in Table 1. Among these four fermentative products of *A. blazei*, Sample A was most effective on antiproliferation in the three human prostate cancer cell lines. Within Sample A, the proliferation of the three cell lines was mostly inhibited by the broth of Sample A (A-b) (data on mycelium and whole product not shown). In LNCaP cells, cell growth was significantly ($P<.01$) suppressed by A-b at 400 and 800 μg/ml after 3–4 days of treatment (Fig. 1A). Whereas A-b at 400 and 800 μg/ml inhibited the proliferation in DU145 and PC3 cells after 1–4 days of treatment. Antiproliferative effects of A-b seemed to be more prominent in the hormone-independent cell lines. Three prostate cancer cell lines were treated with two types of β-glucan to verify whether β-

Table 2
The carbohydrate content of fermentation products produced from *A. blazei*

Sample	Total carbohydrate (mg/dl)	Polysaccharides (mg/dl)	(1,3)-β-D-glucan (mg/dl)	(1,3)-β-D-glucan ratio in polysaccharides (%)
A	947.3	561.0	7.1	1.26
B	938.0	412.9	2.2	0.52
C	975.4	367.9	3.7	1.00
D	954.3	628.1	7.2	1.14

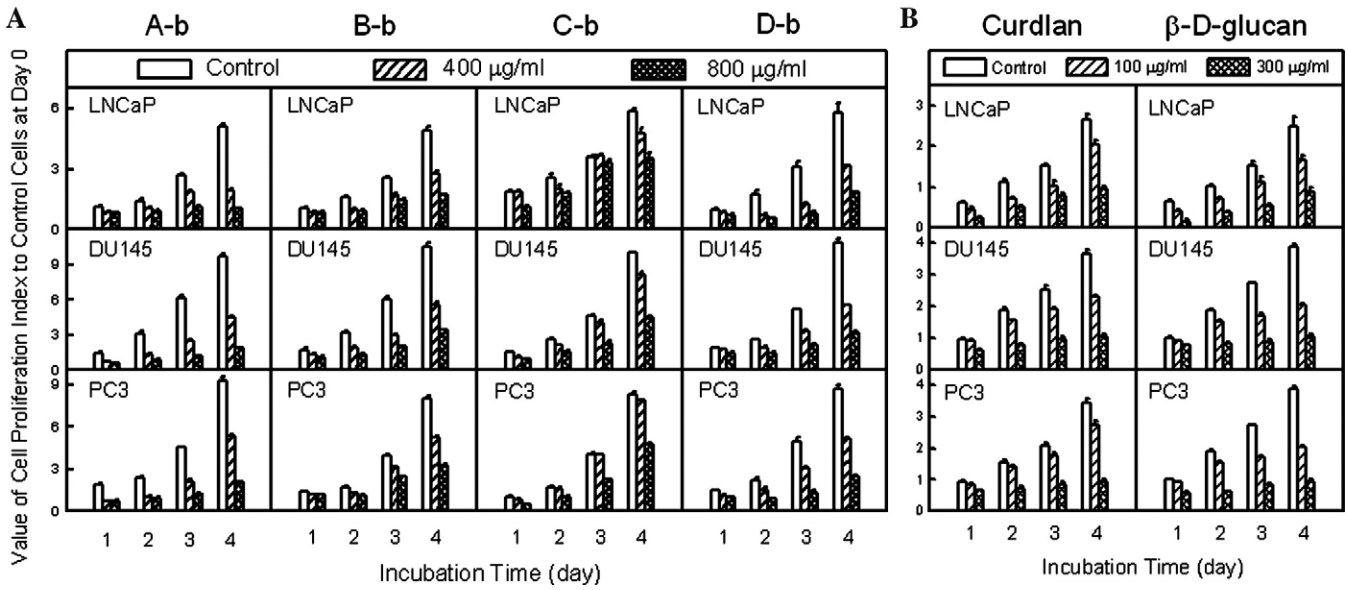

Fig. 1. Inhibitory effects of fermentative products of *A. blazei* and β-glucan on the proliferation of prostate cancer cells. Cell proliferation was measured by MTT assay after treatment at the concentrations of (A) 0, 400, and 800 μg/ml for *A. blazei* or (B) 0, 100, and 300 μg/ml for β-glucan following an incubation period of 1–4 days. The proliferation index was calculated by the OD values of each day divided by the OD values of Day 0. Each value represents the mean±S.E.M.

glucan would be one of the main bioactive compounds from *A. blazei* inhibiting the proliferation of cancer cell lines. Curdlan is the (1→3)-β-glucan from *Alcaligenes faecalis*, and β-D-glucan is from barley. Both types of β-glucan showed the inhibitory effects on the growth of prostate cancer at concentrations of 100 and 300 μg/ml from Day 2 to Day 4 (Fig. 1B).

3.3. Cytotoxicity of *A. blazei* on human prostate cancer cell lines

Since the broth was more effective on antiproliferation, as shown in Fig. 1, the broths of four types of fermentative products were used to measure the cytotoxicity on the three human prostate cancer cell lines. The effects of cytotoxicity were measured by the LDH assay at the concentration of 800 μg/ml after a 72-h treatment (Fig. 2). In comparison with the cytotoxic effects of different fermentative broths in the three cell lines, the broths of Samples A and D (A-b and D-b, respectively) were more effective than the broths of Samples B and C (B-b and C-b, respectively). The percentages of cytotoxicity of A-b and D-b in LNCaP, DU145, and PC3 cells are about 10%, 20%, and 15%, respectively. Parallel with the results of MTT assay, the broth of *A. blazei* was more cytotoxic to the androgen-independent prostate cancer cell lines.

3.4. Induction of apoptotic signals after *A. blazei* treatment

Apoptotic mechanism was involved in the death of *A. blazei*-treated cancer cells [11,24]. To examine whether *A. blazei* stimulated apoptosis in prostate cancer cells, we measured the DNA fragmentations and the activities of the effector caspase caspase 3 in the three prostate cancer cell

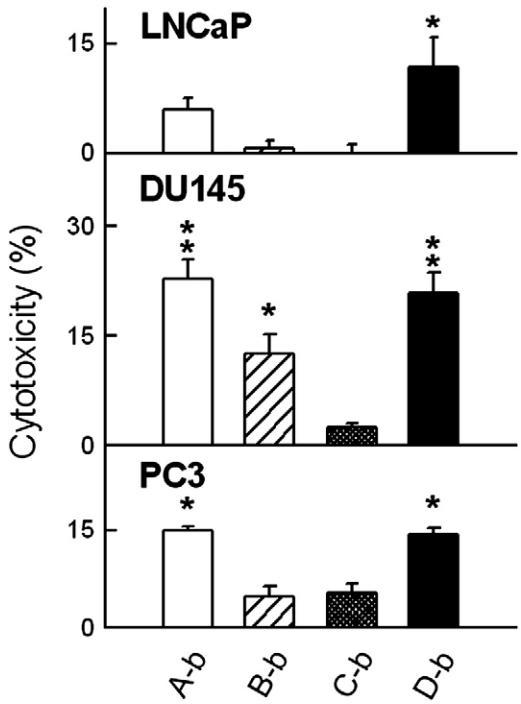

Fig. 2. Cytotoxic effects of different *A. blazei* broths on three prostate cancer cell lines. Cytotoxicity was detected by LDH assay and induced by *A. blazei* broths at the concentration of 800 μg/ml after a 72-h treatment. Cytotoxicity levels were calculated by the OD values using the following formula: (*X*-Low Control)/(High Control−Low Control)×100%, where *X* is the OD value of samples. Each value represents the mean±S.E.M. **P*<.05 and ***P*<.01, as compared with the cytotoxicity induced by *A. blazei* broths at the concentration of 100 μg/ml.

lines after treatment at the concentration of 800 μg/ml for 72 h (Fig. 3A and B). In Fig. 3A, the broth of *A. blazei* caused DNA fragmentation in the three prostate cancer cell lines. In LNCaP cells, the percentage of cells under the bar increased from 63% to 77% in average after the broth treatment. It increased from 57% to approximately 76% and from 52% to about 84% in DU145 and PC3 cells, respectively. Cell number under the bar increased by 22%, 33%, and 62% in LNCaP, DU145, and PC3 cells, respectively. Therefore, the major effect of *A. blazei* on the

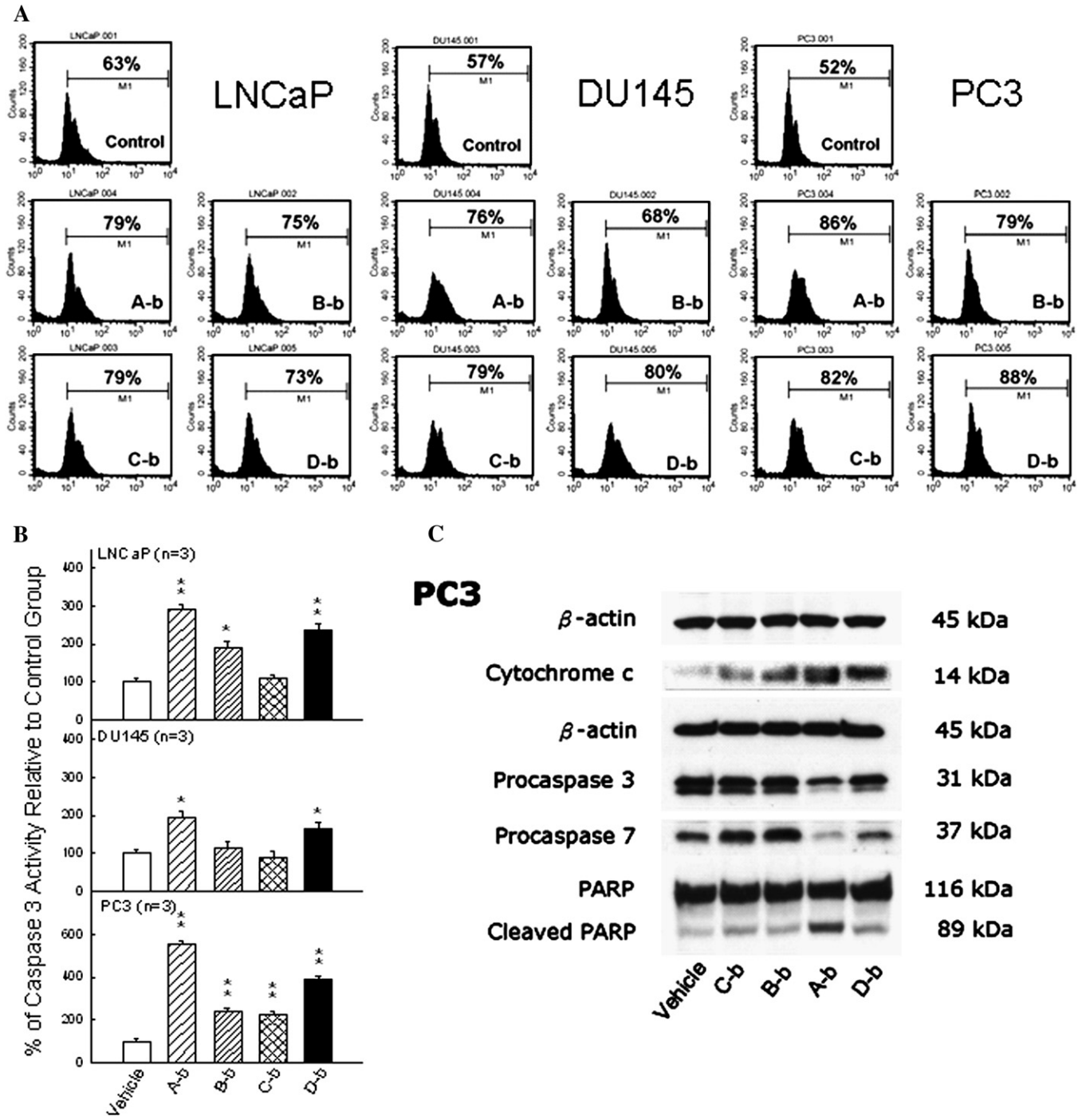

Fig. 3. Effects of *A. blazei* broths on inducing cell apoptosis in prostate cancer cells. (A) Cells were treated with the broths of *A. blazei* at the concentration of 800 μg/ml for 72 h. DNA fragmentation was detected by a TUNEL kit and assessed on a flow cytometer. (B) Caspase activities were assayed by a colorimetric kit after treatment at the concentration of 800 μg/ml for 72 h. Activities were calculated as the percentage to the control group. Each value represents the mean±S.E.M. **P*<.05 and ***P*<.01, as compared with the value for the vehicle group. (C) Cells were incubated with the broth of *A. blazei* for 72 h. Whole cell lysates, nuclear fractions, and cytosolic fractions were subjected to 10%, 7%, and 15% SDS-PAGE, respectively. Each lane was loaded with 100 μg of protein. Similar results were obtained in three other experiments.

induction of DNA fragmentation could be found in PC3 cells. The broths of Samples A and D (A-b and D-b, respectively) elevated the activities of caspase 3 more significantly ($P<.01$ in LNCaP and PC3 cells; $P<.05$ in DU145 cells) than the broths of Samples B and C (B-b and C-b, respectively) (Fig. 3B). In LNCaP cells, A-b and D-b increased the activities of caspase 3 to approximately 2.5 to 3 times. However, this elevation is about 1.5 to 2 times in DU145 cells. In PC3 cells, A-b and D-b up-regulated caspase 3 activity to about 4 to 6 times. The elevation of caspase 3 activity by the broth of *A. blazei* was more significant in hormone-independent PC3 cells, consistent with the results of DNA fragmentation.

3.5. Elevations of apoptosis-related protein expressions by *A. blazei*

According to the previous results, caspase 3 activity was elevated by the broth of *A. blazei* more significantly

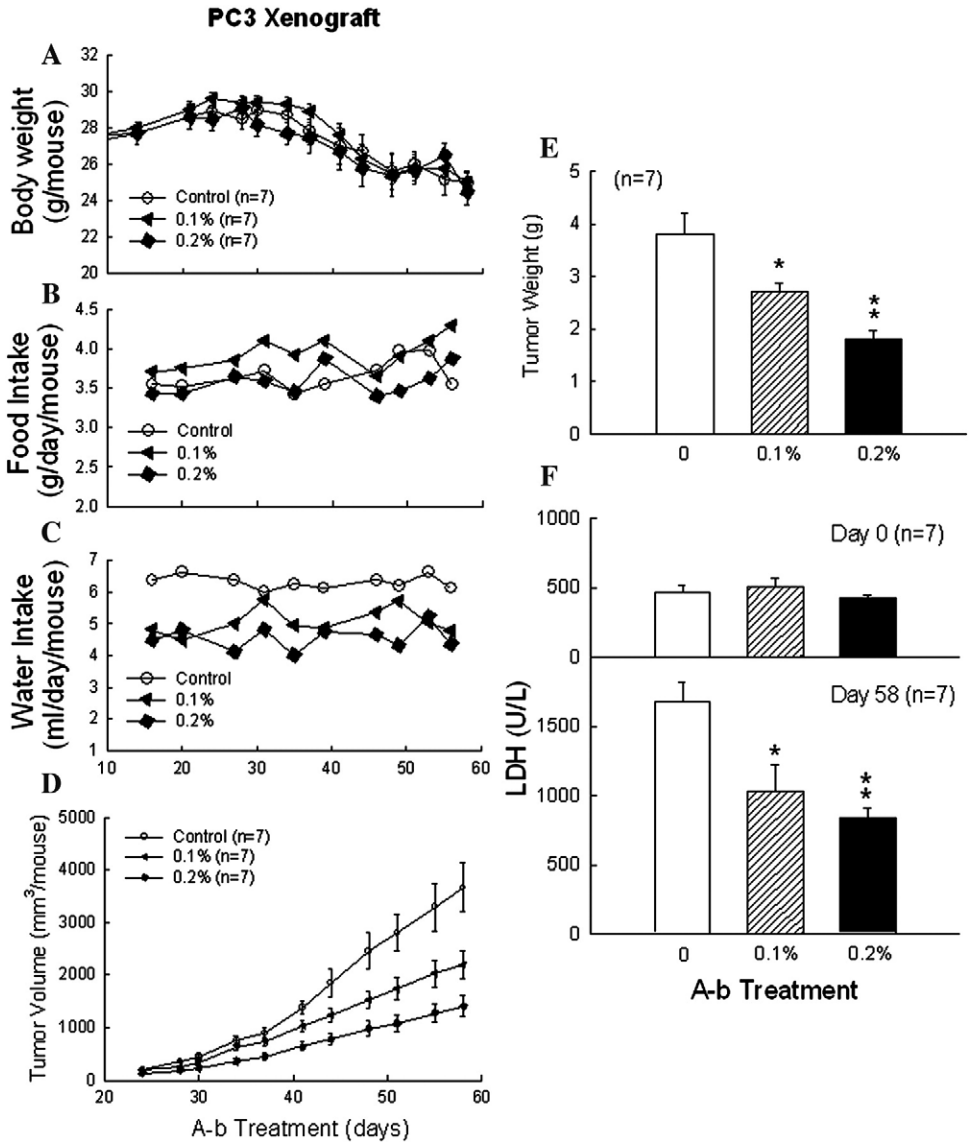

Fig. 4. Effect of *A. blazei* (A-b) feeding in SCID mice during PC3 tumor xenograft study. (A) Body weight of each mouse in different groups was recorded twice a week from Day 21 to Day 58 and was represented as the mean±S.E.M. of seven mice in each group. Daily diet (B) and water intake (C) were also recorded in each group. (D and E) Approximately 1 million PC3 cells were subcutaneously injected in the right flank of each mouse. At the same time, A-b feeding (0%, 0.1%, and 0.2%) was started. Tumor sizes were measured twice weekly in two dimensions throughout the study. The tumor volume was calculated by the formula $0.5236\times L1\times(L2)^2$, where L1 is the long diameter and L2 is the short diameter. Tumor volume (in cubic millimeters per mouse) is represented as the mean±S.E.M. of seven mice in each group. At the end of this study, tumors were excised and weighed. Weight of tumor (in grams per mouse) is represented as the mean±S.E.M. of seven tumors from individual mice. (F) The amount of LDH activity was detected in the mouse serum of Days 0 and 58 under the instruction of the manufacturer. *$P<.05$ and **$P<.01$, as compared with the value for the vehicle group.

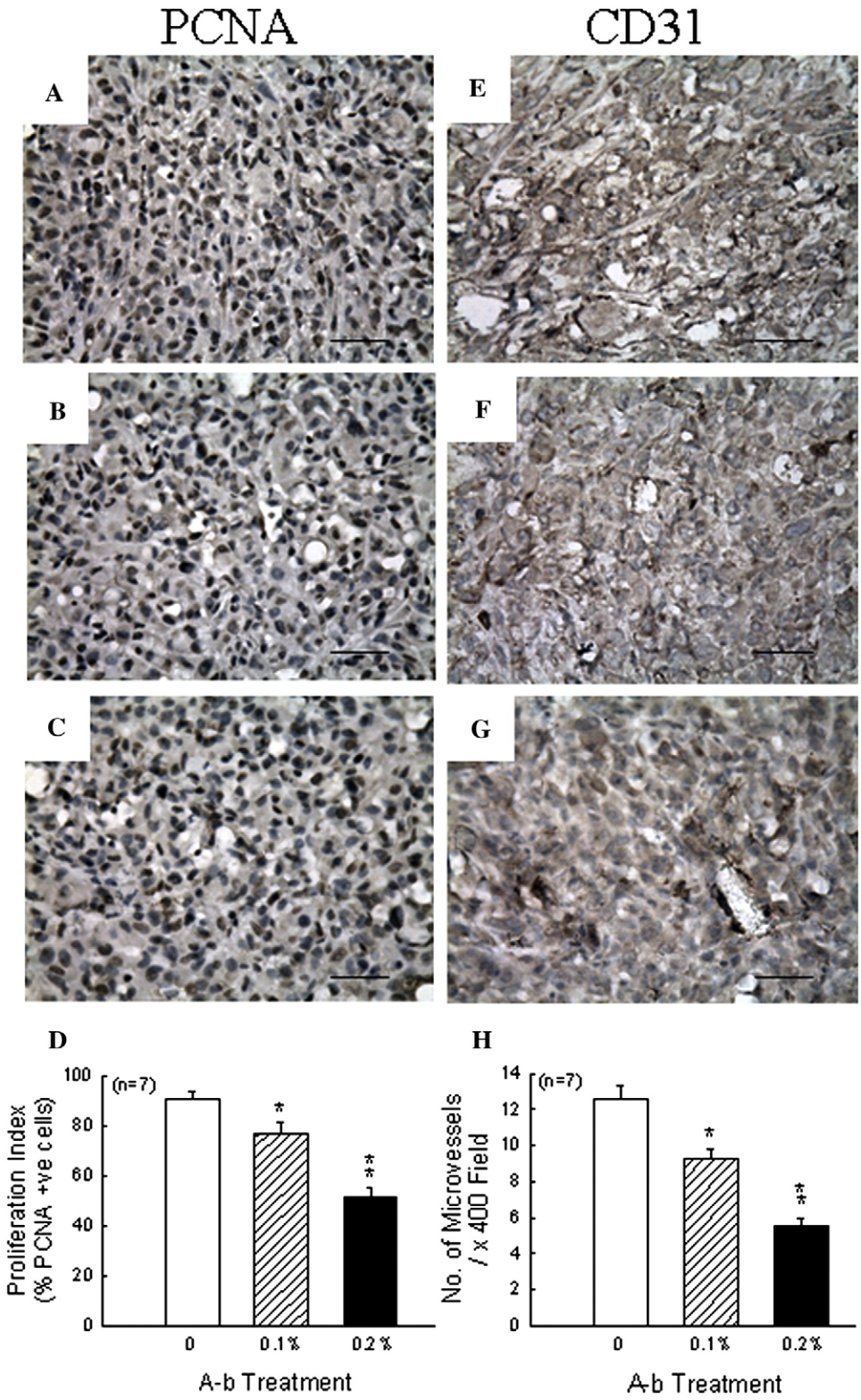

Fig. 5. In vivo antiproliferative and antiangiogenic effects of *A. blazei* (A-b) feeding in PC3 tumor xenograft in SCID mice. At the end of the study, tumors were excised and tumor sections were stained by immunohistochemistry for PCNA (A–D) and for endothelial cell-specific antigen CD31 (E–H). Bars represent 255 μm. (D) PCNA-positive cells were quantified for proliferative index, which was calculated by the number of positive cells multiplied by 100 and then divided by the total number of cells counted under ×400 magnification in 10 randomly selected areas in each tumor sample. (H) Microvessels (CD31 positive) were used for the assessment of intratumoral microvasculature in tumor samples. The CD31-positive areas (brown) were recorded under ×400 magnification in 10 randomly selected areas in each tumor sample. The data are the mean±S.E.M. of seven tumor samples from individual mice. *$P<05$ and **$P<01$, as compared with the value for the vehicle group.

in PC3 cells. Therefore, the protein expressions of apoptosis-related molecules were measured by immunoblot analysis in PC3 cells after the treatment of *A. blazei* broth for 72 h (Fig. 3C). The whole cell lysates, the nuclear fractions, and the cytosolic fractions were used to detect the caspases, PARP, and cytochrome *c*, respectively. The protein levels of procaspases 3 and 7 were down-regulated after A-b and D-b treatment. In addition, the proteolytic forms of PARP were detectable in nucleus after A-b treatment. The cytosolic cytochrome *c* was also elevated after treatment, especially in the A-b group.

3.6. Effects of A. blazei feeding on PC3 tumor xenografted SCID mice

In vivo tumor xenograft growth in mouse is a preferred animal model to study the anticancer effects of any compound in a preclinical model. In the present study, we used subcutaneous hormone-refractory human PC3 prostate tumor xenograft growth in SCID mouse as a model to assess the in vivo anticancer efficacy of *A. blazei* against prostate carcinoma growth. A-b was given in drinking water at 0.1% and 0.2% (w/v) doses for 8 weeks. The primary parameters used in the assessment of gross toxicity of a test compound in cancer chemoprevention/therapy studies include body weight, diet, and water consumption [25,26]. Accordingly, we assessed whether A-b feeding to mice during the 8-week study period caused any adverse health effect in terms of changes in animal body weight, diet, and water consumption. Fig. 4A shows that the body weight increased during the first 4 weeks but slightly decreased during the last 4 weeks in the three groups. In A-b-fed mice, body weight and diet were similar to those of the control group and did not show any significant alteration (Fig. 4A and B). However, the water consumption profiles of the A-b-fed group were lower than those of the control group (Fig. 4C). Overall, A-b did not show any adverse effect on PC3 tumor xenografted mice.

3.7. Antitumor effects of A. blazei feeding on hormone-refractory PC3 tumor xenografts

On the basis of the data showing inhibition of PC3 prostate cancer growth by A-b in vitro, we then assessed whether this effect of A-b might be revealed in vivo. As shown in Fig. 4D, the control group (normal drinking water) of mice showed a progressive tumor xenograft growth during the entire study. However, the A-b feeding resulted in a clear separation in tumor growth curves, showing a decreased rate of tumor growth as compared with that in the control group. The effect of A-b oral feeding was more prominent, starting from the fifth week to the sixth week of the study. This observation suggested that a consistent level of A-b might be necessary for its observable in vivo antitumor effects in prostate carcinoma. The inhibitory effect of A-b on PC3 tumor xenograft growth was more evident and significant at the end of the study. After 8 weeks, the tumor volume of control mice was 3661±466 mm^3 of tumor volume/mouse. A-b feeding at 0.1% and 0.2% dose levels resulted in 2200/±272 mm^3 of tumor volume/mouse ($P<.05$) and 1407±200 mm^3 of tumor volume/mouse ($P<.01$), accounting for 45% and 62% inhibition in tumor growth, respectively (Fig. 4D). Consistent with the tumor volume results, the wet tumor weight measurements at the end of the study also showed a significant reduction in A-b-fed groups (Fig. 4E). In this case, compared with the control group of mice with 3.79±0.40 g/mouse tumor weight, A-b feeding at 0.1% and 0.2% dose levels showed 2.72±0.15 and 1.80±0.16 g/mouse tumor weight, accounting for 28% ($P<.05$) and 53% ($P<.01$) inhibition, respectively (Fig. 4E). The activity of LDH in mouse serum is shown in Fig. 4F. Before the xenograft (Day 0), the LDH levels in the three groups were about 400–500 U/l. After the inoculation for 58 days, the value of LDH elevated to 1679.5 U/l in the control group. In the A-b feeding groups, the activities of LDH were 1035.5 and 836.5 U/l. At 0.1% and 0.2% dose levels, the LDH levels were 62% ($P<.05$) and 50% ($P<.01$) in comparison with the control group, respectively.

3.8. Antiproliferative and antiangiogenesis effects of A. blazei feeding in prostate tumor xenografts

According to the antitumor effects of A-b in vivo, we further examined whether this effect is associated with an in vivo alteration in cell proliferation and/or angiogenesis. In the results of proliferative analysis (Fig. 5A–D), the number of PCNA-positive cells in A-b feeding groups was less as compared with that of the control group. Quantification of these results showed that A-b decreased the proliferation index (percentage of PCNA-positive cells) from 91.2±2.8 (control) to 77.3±4.2 (0.1% A-b, $P<.05$) and 51.2±4.3 (0.2% A-b, $P<.01$), which accounted for 15% and 44% inhibition, as compared with those of the control group, respectively (Fig. 5D). In the data of angiogenesis detection (Fig. 5E–H), microscopic analysis of the tumor sections showed that A-b inhibited microvessel density in PC3 tumor xenografts. Quantification of microvessels showed 9.3±0.6 (0.1% A-b, $P<.05$) and 5.5±0.5 (0.2% A-b, $P<.01$) microvessels/×400 field in the tumor sections as compared with control group, showing 12.5±0.8 microvessels/×400 field, which accounted for 26% and 56% decreases in microvessel density in the tumors from 0.1% and 0.2% in the A-b feeding groups, respectively (Fig. 5H). Taken together, these results suggest the possible involvement of both antiproliferative as well as antiangiogenic effects of *A. blazei* in the in vivo inhibition of prostate tumor growth.

4. Discussion

In the present study, we demonstrated that (a) the fermentative products of *A. blazei* and β-glucan showed inhibitory effects on the proliferation of prostate cancer cells, (b) cytotoxic effects were induced by the broth of *A. blazei* in prostate cancer cells, (c) the broth of Sample A (A-b) caused

apoptotic effects in androgen-independent PC3 cells by increasing the DNA fragmentation and activating the apoptosis-related proteins, and (d) the oral administration of A-b inhibited the PC3 xenograft tumor growth in SCID mice via antiproliferative and antiangiogenic mechanisms without affecting the body weight and food intake of mice.

A. blazei is a widely used health food with expectation of certain pharmacological activities. Bioactive compounds of *A. blazei* have been reported for decades, including polysaccharides, cytotoxic steroids, lectin, and antimutagens [27,28]. Linoleic acid and ergosterol extracted from *A. blazei* display antimutagenic and antineovascularization characteristics, respectively [4]. Previous studies suggested that polysaccharide from *A. blazei* might be important both as a prophylaxis against cancer and as functional food [7]. In addition, various polysaccharides and protein-bound polysaccharides have been identified from *A. blazei* and have demonstrated the antitumor activity in vitro and in vivo via direct and indirect effects, such as (1,3)-β-D-glucan, (1,6)-β-D-glucan, and their protein-bound complexes [2,8,9,11,28–31]. The way to elevate the content of the active substance, polysaccharides, in the broth of *A. blazei* is to add the soybean or black bean, which also contains polysaccharides in the fermentation medium of *A. blazei.* In this study, four types of fermentation media were used to produce four fermentative products, Samples A–D (Table 1), and the levels of polysaccharides in the four samples are shown in Table 2. In comparison with Samples C (367.9 mg/dl) and D (628.1 mg/dl), soybean was more able to enhance the polysaccharide production in *A. blazei*. Furthermore, in the samples using soybean as fermentative medium, the condition of Medium A for one-stage fermentation (Sample A) and Medium C for two-stage fermentation (Sample D) resulted in higher levels of polysaccharides and (1,3)-β-D-glucan production.

Results examined by MTT assay showed that the broth of Sample A (A-b), which contained higher levels of polysaccharides and (1,3)-β-D-glucan than other samples, had greater inhibitory effects on cell proliferation than other fermentative products (Fig. 1). It suggested that the product generated with soybean (20 g/l) in the medium for the fermentation period of 7 days might be a proper condition to produce the antitumor substance by *A. blazei*, and the substance might be secreted to broth. In Fig. 1B, β-glucan shows the directly inhibitory effects on prostate cancer cells. Table 2 shows that a higher concentration of β-glucan was detected in Samples A and D compared with Samples B and C. These data indicate that β-glucan might be one of the bioactive compounds in *A. blazei* suppressing the growth of prostate cancer. Results of cytotoxicity on prostate cancer cell lines caused by the broth of *A. blazei* were found to be the greatest in the A-b and D-b groups correlated with the MTT assay (Fig. 2). In addition, the cytotoxicity that resulted from the broth of *A. blazei* treatment was more significant on androgen-independent cell lines than on the androgen-dependent LNCaP cell line, which is more relative to the primary prostate cells. These results speculated that the broth of *A. blazei* might target on the malignantly rapid proliferative cells. A portion of the antiproliferative effects of the broth of *A. blazei* examined by the MTT assay resulted from the cytotoxic character of *A. blazei*.

Based on previous studies, the direct antiproliferative effects induced by *A. blazei* on cancer cells are via antimetastatic and apoptotic pathways in addition to cytotoxic signal. Treatment of β-D-glucan extracted from *A. blazei* directly stimulates apoptotic signaling in HRA ovarian cancer cells [11]. Extracts of *A. blazei* induce cell cycle arrest and apoptosis in gastric cancer cells and leukemia U937 cells [24,32]. Consistent with the cytotoxic effects, A-b and D-b were more effective in caspase 3 activation than the other two fermentative broths (Fig. 3B). The *A. blazei* induced the DNA fragmentation and elevated the caspase 3 activity more in PC3 cells than in the other two cell lines (Fig. 3A and B). It was suggested that *A. blazei* was more capable of inducing apoptosis in androgen-independent prostate cancer cells, PC3, than in androgen-dependent prostate cancer cells, LNCaP. It might result from the characteristics of *A. blazei* targeting on rapid-growth cancer cells. In Fig. 3C, the increases of protein expression of apoptosis-related proteins were caused by the broth of *A. blazei* on PC3 cells. These data indicate that the broth of *A. blazei* directly induced cell apoptosis in hormone-independent prostate cancer cells via cytochrome *c* release, caspase activation, and PARP cleavage.

Since the broth of *A. blazei* showed direct antitumor effects on prostate cancer cells in vitro, it would be important to know if *A. blazei* would still be effective in the in vivo study without causing any adverse effect. In Fig. 4A, there is no significant difference in body weight among the three groups. The decrease in body weight in the three groups in the last 4 weeks might have resulted from the growth of xenograft tumor in the mice. Because the smell and taste of the broth of *A. blazei* were different from those of the broth in the control group, the water consumptions of A-b groups were slightly less than those of the control group (Fig. 4C). Although the water intakes were different among groups, the significant antitumor effects were still observed in A-b-fed groups (Fig. 4D and E). The LDH level in serum provides reliable data about the tumor progression in the mice [33]. In Fig. 4F, the LDH activity in A-b feeding groups showed 38% and 50% inhibition, respectively, which are parallel with the results of tumor volume. Although the broth of *A. blazei* did induce a cytotoxic effect in the in vitro study (about 10–20%), A-b feeding did not induce further cytotoxic effects in the mice in comparison with the control group. These results suggest that the broth of *A. blazei* provided specific antitumor effects in PC3 prostate cancer cell xenograft tumor.

Inhibiting cell proliferation and decreasing angiogenesis are two major mechanisms interfering with tumor growth. Based on the results of PCNA stain (Fig. 5A–D), *A. blazei* inhibited prostate xenograft tumor growth via an antiproliferative pathway. Tumor angiogenesis is a reliable biomarker and a therapeutic target for prevention and therapy of

various cancers, including prostate cancer [34–36]. Angiogenesis is required at every stage of tumor progression after a tumor goes beyond a certain size [35–37]. Several phytochemicals from the diet modulate the balance between proangiogenic and antiangiogenic growth factors and influence the course of pathological angiogenesis [38]. The results of CD31 immunohistochemical staining showed the decrease in tumor angiogenesis by A-b feeding (Fig. 5E–H). These observations might result from the antiangiogenic efficacy of the broth of *A. blazei* in prostate tumors [4,39]. In summary, the above results suggest that the broth of *A. blazei* might have in vivo antitumor effects targeting cell proliferation and angiogenesis, causing the inhibition of prostate tumor growth.

Taken together, our data indicate that the broth of *A. blazei* shows antiproliferative and antitumor growth effects in prostate cancer in vitro and in vivo through an apoptotic pathway without causing significant side effects in mice. To our knowledge, this is the first investigation about the direct antitumor effects of *A. blazei* on an animal model without effects on the immune system. Consumption of the broth of *A. blazei* might be an adjuvant or complementary treatment for patients with prostate cancer.

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References

[1] Delmanto RD, de Lima PLA, Sugui MM, da Eira AF, Salvadori DMF, Speit G, et al. Antimutagenic effects of *Agaricus blazei* mushroom on the genotoxicity induced by cyclophosphamide. Mutat Res 2001;496:15–21.

[2] Fujimiya Y, Suzuki Y, Takakura R, Ebina T. Tumor-specific cytocidal and immunopotentiating effects of relatively low molecular weight products derived from the basidiomycete, *Agaricus blazei* Murill. Anticancer Res 1999;19:113–8.

[3] Menoli RC, Mantovani MS, Ribeiro RL, Speit G, Jordao BQ. Antimutagenic effects of the mushroom *Agaricus blazei* Murill extracts on V79 cells. Mutat Res 2001;496:5–13.

[4] Takaku T, Kimura Y, Okuda H. Isolation of an antitumor compound from *Agaricus blazei* Murill and its mechanism of action. J Nutr 2001;131:1409–13.

[5] Yoshimura K, Ueda N, Ichioka K, Matsui Y, Terai A, Arai Y. Use of complementary and alternative medicine by patients with urologic cancer: a prospective study at a single Japanese institution. Support Care Cancer 2005;13:685–90.

[6] Ahn WS, Kim DJ, Chae GT, Lee JM, Bae SM, Sin JI, et al. Natural killer cell activity and quality of life were improved by consumption of a mushroom extract, *Agaricus blazei* Murill Kyowa, in gynecological cancer patients undergoing chemotherapy. Int J Gynecol Cancer 2004;14:589–94.

[7] Mizuno M, Morimoto M, Minato K, Tsuchida H. Polysaccharides from *Agaricus blazei* stimulate lymphocyte T-cell subsets in mice. Biosci Biotechnol Biochem 1998;62:434–7.

[8] Itoh H, Ito H, Amano H, Noda H. Inhibitory action of (1→6)β-D-glucan–protein complex (FIII-2-b) isolated from *Agaricus blazei* ("Himematsutake") on Meth A fibrosarcoma-bearing mice and its antitumor mechanism. Jpn J Pharmacol 1994;66:265–71.

[9] Ito H, Shimura K, Itoh H, Kawade M. Antitumor effects of a new polysaccharide–protein complex (ATOM) prepared from *Agaricus blazei* (Iwade strain 101) "Himematsutake" and its mechanisms in tumor-bearing mice. Anticancer Res 1997;17:277–84.

[10] Kawagishi H, Inagaki R, Kanao T, Mizuno T, Shimura K, Ito H, et al. Fractionation and antitumor activity of the water-soluble residue of *Agaricus blazei* fruiting bodies. Carbohydr Res 1989;186:267–73.

[11] Kobayashi H, Yoshida R, Kanada Y, Fukuda Y, Yagyu T, Inagaki K, et al. Suppressing effects of daily oral supplementation of beta-glucan extracted from *Agaricus-blazei* Murill on spontaneous and peritoneal disseminated metastasis in mouse model. J Cancer Res Clin Oncol 2005;131:527–38.

[12] Hirotani M, Sai K, Hirotani S, Yashikawa T. Blazeispirols B, C, E, and F, des-A-ergostane-type compounds, from the cultured mycelia of the fungus *Agaricus blazei*. Phytochemistry 2002;59:571–7.

[13] Hirotani M, Sai K, Nagai R, Hirotani S, Takayanagi H, Yoshikawa T. Blazeispirane and protoblazeispirane derivatives from the cultured mycelia of the fungus *Agaricus blazei*. Phytochemistry 2002;61:589–95.

[14] van den Bemd GJ, Pols HA, van Leeuwen JP. Anti-tumor effects of 1,25-dihydroxyvitamin D_3 and vitamin D analogs. Curr Pharm Des 2000;6:717–32.

[15] Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.

[16] Ranger AM, Malynn BA, Korsmeyer SJ. Mouse models of cell death. Nat Genet 2001;28:113–8.

[17] Shi Y. A structural view of mitochondria-mediated apoptosis. Nat Struct Biol 2001;8:394–401.

[18] Wood PJ, Fulcher RG. Specific interaction of aniline blue with (1→3)-β-D-glucan. Carbohydr Polym 1984;4:49–72.

[19] Young SH, Jacobs RR. Sodium hydroxide-induced conformational change in schizophyllan detected by the fluorescence dye, aniline blue. Carbohydr Res 1998;310:91–9.

[20] Kan SF, Huang WJ, Lin LC, Wang PS. Inhibitory effects of evodiamine on the growth of human prostate cancer cell line LNCaP. Int J Cancer 2004;110:641–51.

[21] Kan SF, Yu CH, Pu HF, Hsu JM, Chen MJ, Wang PS. Anti-proliferative effects of evodiamine on the growth of human prostate cancer cell lines DU145 and PC3. J Cell Biochem 2007;101:44–56.

[22] Gottlieb RA, Granville DJ. Analyzing mitochondrial changes during apoptosis. Methods 2002;26:341–7.

[23] Steel RGD, Torrie JH. Principles and procedures of statistics. New York: McGraw-Hill; 1960.

[24] Jin CY, Choi YH, Moon DO, Park C, Park YM, Jeong SC, et al. Introduction of G2/M arrest and apoptosis in human gastric epithelial AGS cells by aqueous extract of *Agaricus blazei*. Oncol Rep 2006;16:1349–55.

[25] Singh RP, Tyagi AK, Zhao J, Agarwal R. Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein kinases and induction of apoptosis. Carcinogenesis 2002;23:499–510.

[26] Sporn MB, Suh N. Chemoprevention: an essential approach to controlling cancer. Nat Rev Cancer 2002;2:537–43.

[27] Kawagishi H, Katsumi R, Sazawa T, Mizuno T, Hagiwara T, Nakamura T. Cytotoxic steroids from the mushroom *Agaricus blazei*. Phytochemistry 1988;27:2777–9.

[28] Kawagishi H, Nomura A, Yumen T, Mizuno T, Hagiwara T, Nakamura T. Isolation and properties of a lectin from the fruiting bodies of *Agaricus blazei*. Carbohydr Res 1988;183:150–4.

[29] Ohno N, Furukawa M, Miura NN, Adachi Y, Motoi M, Yadomae T. Antitumor β-glucan from the cultured fruit body of *Agaricus blazei*. Biol Pharm Bull 2001;24:820–8.
[30] Lee YL, Kim HJ, Lee MS, Kim JM, Han JS, Hong EK, et al. Oral administration of *Agaricus blazei* (H1 strain) inhibited tumor growth in a sarcoma 180 inoculation model. Exp Anim 2003;52:371–5.
[31] Oshiman K, Fujimiya Y, Ebina T, Suzuki I, Noji M. Orally administered beta-1,6-D-polyglucose extracted from *Agaricus blazei* results in tumor regression in tumor-bearing mice. Planta Med 2002; 68:610–4.
[32] Jin CY, Moon DO, Choi YH, Lee JD, Kim GY. Bcl-2 and caspase-3 are major regulators in *Agaricus blazei*-induced human leukemic U937 cell apoptosis through dephosphorylation of Akt. Biol Pharm Bull 2007;30:1432–7.
[33] Arguello F, Sterry JA, Zhao YZ, Alexander MRA, Shoemaker RH, Cohen HJ. Two serologic markers to monitor the engraftment, growth, and treatment response of human leukemias in severe combined immunodeficient mice. Blood 1996;87:4325–32.
[34] Ali IU, Senger DR, Smith LEH. Angiogenesis as a potential biomarker in prostate cancer chemoprevention trials. Urology 2001;57:143–7.
[35] Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nat Med 2000;407:249–57.
[36] Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2002;2:727–39.
[37] Gimbrone Jr MA, Leapman S, Cotran R, Folkman J. Tumor dormancy in vivo by prevention of neovascularization. J Exp Med 1972;136: 261–76.
[38] Singh RP, Agarwal R. Tumor angiogenesis: a potential target in cancer control by phytochemicals. Curr Cancer Drug Targets 2003;3:205–17.
[39] Kumura Y, Kido T, Takaku T, Sumiyoshi M, Baba K. Isolation of an anti-angiogenic substance from *Agaricus blazei* Murill: its antitumor and antimetastatic actions. Cancer Sci 2004;95:758–64.