

Original Article

Delayed Cell Cycle Progression and Apoptosis Induced by Hemicellulase-Treated *Agaricus blazei*

Masaki Kawamura¹ and Hirotake Kasai²¹Department of Alternative Medicine and Bioregulation, Faculty of Medicine and ²Department of Microbiology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan

We examined the effects of hemicellulase-treated *Agaricus blazei* (AB fraction H, ABH) on growth of several tumor cell lines. ABH inhibited the proliferation of some cell lines without cytotoxic effects. It markedly prolonged the S phase of the cell cycle. ABH also induced mitochondria-mediated apoptosis in different cell lines. However, it had no impact on the growth of other cell lines. ABH induced strong activation of p38 mitogen-activated protein kinase (MAPK) in the cells in which it evoked apoptosis. On the other hand, ABH showed only a weak p38 activation effect in those cell lines in which it delayed cell cycle progression with little induction of apoptosis. However, p38 MAPK-specific inhibitor inhibited both ABH-induced effects, and ABH also caused apoptosis in the latter cells under conditions of high p38 MAPK activity induced by combined treatment with TNF- α . These results indicate that the responsiveness of p38 MAPK to ABH, which differs between cell lines, determines subsequent cellular responses on cell growth.

Keywords: antiproliferation – cancer – cell lines – fungus – p38 MAPK

Introduction

Agaricus blazei Murill is a mushroom native to Brazil. This mushroom grows naturally near Piedade, a coastal Brazilian village, and it was eaten as a common food in this area. As the local people had lower than average frequencies of cancer and other age-related disorders, this basidiomycete has attracted attention due to the possibility that it may contain ingredients with beneficial health effects. There have been numerous studies regarding its activities both *in vitro* and in animal experiments. With regard to its anti-neoplastic activity, the features of *A. blazei* can be classified into three categories: immunomodulatory effects, such as activation of macrophages and NK cells (1–6); protective effects against genetic damage (7–12); and inhibitory effects on tumor cell growth, cell migration or tumor-induced neovascularization (3,13–15). Products derived from *A. blazei* have been consumed by many patients with cancer believing them to

have medicinal properties in Japan (16). However, little is known about its effects on humans. These products are used mostly under self rather than medical supervision. There is no clear medical justification for their use against cancer. Recently, their exaggerated effectiveness in humans, which is not supported by scientific evidence, has been widely advertised. In addition, cases of health hazards during their use have also been reported, although no causal relationships have yet been demonstrated between these products and deleterious effects on health. We doubt that these products live up to their touted roles as veritable panaceas against cancer. Furthermore, it is necessary to also determine the influences of their use on the effects of existing cancer treatments. Our previous study demonstrated bipartite characteristics of *A. blazei* extract in that it induced the maturation of murine bone marrow-derived dendritic cells and resulted in a Th1-biased response *in vitro*, but it also inhibited some responses induced by Toll-like receptor-mediated signaling, such as production of pro-inflammatory cytokines (17). *A. blazei* appears to have multiple biological activities.

In the present study, we examined the direct effects of *A. blazei* extract on growth of several tumor cell lines to

For reprints and all correspondence: Dr M. Kawamura, Department of Alternative Medicine and Bioregulation, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. Tel: +81-55-273-9539; Fax: +81-55-273-6728; E-mail: masakika@yamanashi.ac.jp

ascertain its efficacy against cancer and the limitations of its effectiveness. As it is difficult to abstract an essence from *A. blazei* due to its low aqueous solubility, *A. blazei* used here was digested with hemicellulases to achieve solubility in water. Hemicellulase-treated *A. blazei* (AB fraction H, ABH) showed a strong antiproliferative effect, and induced p38 mitogen-activated protein kinase (MAPK)-mediated cell growth delay and apoptosis in some cell lines. However, ABH did not have the same effect on growth of all cell lines. It had no effect on p38 MAPK activation in ABH-insensitive cells. These results suggested that cellular factors, which regulate p38 MAPK activation, hold the key to the susceptibility of cells to the effects of ABH on proliferation. Furthermore, as ABH induces a delay in cell cycle progression in S phase in sensitive cells, it may also have an influence on the therapeutic benefits of existing treatments against cancer.

Methods

Cell Lines

The cell lines used here were as follows: BALL-1 (B cell leukemia), CCRF-CEM (acute T lymphoblastic leukemia), Jurkat (T cell leukemia), THP-1 (monocytic leukemia), U937 (histiocytic lymphoma), HeLa (uterine cervical carcinoma), HMV-1 (melanoma), MCF7 (breast adenocarcinoma), supplied by the Cell Resource Center for Biochemical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Mycoplasma detection assay confirmed all cell lines to be mycoplasma-negative (MycoAlert™; Cambrex, Rockland, ME). The cells were maintained in RPMI 1640 medium (for non-adherent cells) or Dulbecco's modified Eagle's medium (for adherent cells) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT).

Materials

The following antibodies were used in this study: anti-cytochrome c mAb (Lab Vision Corporation, Fremont, CA), anti-phospho-p38 MAPK (Thr180/Tyr182) mAb (Cell Signaling Technology Inc., Danvers, MA), and fluorescein isothiocyanate-conjugated anti-human TNF cell surface receptor 1 (TNFR1) mAb (R&D Systems Inc., Minneapolis, MN). Recombinant human TNF- α was purchased from PeproTech Inc. (Rocky Hill, NJ). SB203580, SP600125 and PD98059 were obtained from EMD Biosciences, Inc. (Darmstadt, Germany). These are specific inhibitors of p38 MAPK, c-Jun N-terminal kinase (JNK) and MAPK extracellular signaling-regulated kinase (ERK) kinase (MEK), respectively.

Hemicellulase-Treated *A. blazei*

Hemicellulase-treated *A. blazei* (AB fraction H, ABH) was supplied by Japan Applied Microbiology Research Institute

Ltd (Yamanashi, Japan). Mycelia of *A. blazei* Murill were digested with 0.1% hemicellulases from *Trichoderma viride*, *Trichoderma harzianum*, *Aspergillus tamari* and *Aspergillus niger* for 1 h at 45°C. After inactivation of the enzymes at 70°C, the filtrate of the degradation product was lyophilized. ABH consists of 63.3% carbohydrates, 30.9% proteins, 0.3% lipids and minor components. The samples were examined for endotoxin by limulus amoebocyte lysate (LAL) assay (Seikagaku Corp., Tokyo, Japan). The endotoxin contents of samples were <0.02 EU per 1 mg of sample. The culture medium containing 0.1% ABH had a pH of 7.1–7.4 and iso-osmotic pressure (285 mOsm l⁻¹), determined by the freezing point depression method (Auto & Stat OM-6030; ARKRAY Inc., Kyoto, Japan). For comparison with ABH, mycelia of *A. blazei* Murill (Japan Applied Microbiology Research Institute Ltd) were water-extracted without hemicellulase. After heat treatment, the extract was lyophilized as described above.

Cell Growth and Viability

Cell growth was assessed by formazan formation induced by reduction of exogenous tetrazolium salt. Cells (1×10^4 per well for non-adherent cells or 4×10^5 per well for adherent cells) were cultured in flat-bottomed 96-well plates (BD Biosciences, San Jose, CA). After incubation for the indicated times, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt and 1-methoxy-5-methylphenazinium methylsulfate mixture (Tetra-Color ONE™; Seikagaku Corp., Tokyo, Japan) was added to each well. The optical density was measured at 450 and 630 nm (Molecular Devices Corp., Sunnyvale, CA) after 2 h. In another experiment, non-adherent cells (2 to 4×10^5 per well) were cultured in 24-well plates (BD Biosciences). The viable cell number in the cultures was counted by trypan blue dye exclusion method at the indicated times. The viability was determined as percentage of trypan blue-negative cells to total cells.

Apoptosis

Apoptosis was assessed by determining phosphatidylserine exposure on the cell membrane and DNA fragmentation. Cells were labeled with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI) in binding buffer (Biovision Inc., Mountain View, CA), and analyzed using FACSCalibur™ with CellQuest™ software (BD Biosciences). On the other hand, cells were incubated with $500 \mu\text{g ml}^{-1}$ proteinase K (Sigma-Aldrich, St Louis, MO), $500 \mu\text{g ml}^{-1}$ RNase (Sigma-Aldrich), and 1% SDS at 37°C for 30 min. Total DNA was extracted from these cultures using a DNA extraction kit (Wako Pure Chemical Industries Ltd, Osaka, Japan). DNA samples were electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining.

Cell Cycle

Cells were fixed with 70% ethanol (Wako) at 4°C for 2 h. After washing and centrifugation, the precipitates were

resuspended in $50 \mu\text{g ml}^{-1}$ PI solution including $250 \mu\text{g ml}^{-1}$ RNase, and incubated at 37°C for 30 min. DNA content in the cells was determined using FACSCalibur™ with CellQuest™ software. In cell cycle analyses, the signals of aggregating cells were removed on an FL-width/FL-area dot plot. In one experiment, CCRF-CEM cells were enriched at early S, mid-S or G_2/M and G_1 phases of the cell cycle with a high concentration of thymidine as described previously (18). Briefly, the cells were cultured in the presence of 2 mM thymidine (Sigma-Aldrich) for 24 h, and then cultured without thymidine for 12 h. The cells were further incubated with 2 mM thymidine for 14 h, followed by a 4, 10 or 16 h recovery period without thymidine for cell enrichment at early S, mid-S or G_2/M and G_1 phases, respectively.

Caspase Activity

Aliquots of cells (5×10^5) were stained with a carboxyfluorescein-labeled fluoromethyl ketone peptide (FAM-LEHD-FMK; Chemicon International Inc., Temecula, CA), which permeates the cell membrane and binds covalently to active caspase-9, in culture medium at 37°C for 1 h according to the manufacturer's instructions. After washing and PI staining, the caspase activity was analyzed by flow cytometry.

Western Blotting

The cytosolic protein fraction was prepared as described previously (19). Briefly, cells were suspended in extraction solution (20 mM HEPES, pH 7.5, 10 mM KCl, 1.9 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA) containing protease inhibitor cocktail (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and incubated on ice for 20 min. The homogenate was centrifuged at $1000 \times g$ for 5 min to pellet out nuclei and intact cells. The supernatant was further centrifuged at $14\,000 \times g$ for 30 min to collect the cytosolic (supernatant) fraction. Aliquots of $30 \mu\text{g}$ of the protein were separated by SDS-PAGE and transferred

onto PVDF membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline, and then incubated with $0.2 \mu\text{g ml}^{-1}$ anti-cytochrome c antibody. Blots were developed with horseradish peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence staining (Amersham Biosciences, Piscataway, NJ).

p38 MAPK Activation

Phosphorylated p38 MAPK in individual cells was detected by flow cytometric analysis for intracellular staining according to the manufacturer's protocol (Cell Signaling Technology Inc.; <http://www.cellsignal.com/support/protocols/>). Briefly, cells were suspended in PBS, and fixed with methanol-free formaldehyde (Polysciences Inc., Warrington, PA) at a final concentration of 2% for 10 min at 37°C . The cells were then permeabilized by adding ice-cold 100% methanol while gently vortexing so that final concentration was 90% methanol, followed by incubation on ice for 30 min. After washing, aliquots of the cells were incubated in PBS containing 0.5% BSA for 10 min and then rabbit anti-phospho-p38 MAPK mAb was added. After a further 30 min incubation and washing, the cells were reacted with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 30 min. After washing, the cells were analyzed by flow cytometry.

Results and Discussion

The Influence of ABH on Cell Proliferation Differed Between Cell Lines

Five human leukocytic cell lines were cultured with graduated concentrations of ABH, aqueous extract of *A. blazeyi* or aqueous extract of *A. blazeyi* containing 0.1% inactivated hemicellulases. After a 3 day incubation, cell proliferation was examined using tetrazolium salt and expressed relative to cell growth in culture medium only (Fig. 1). Although

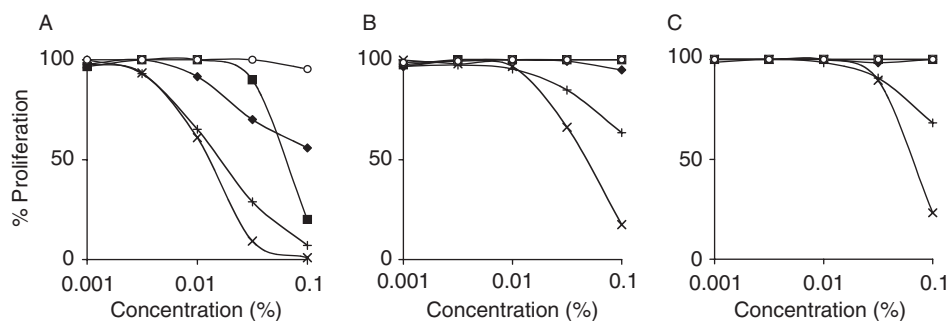


Figure 1. Hemicellulase-degradation enhanced the antiproliferative effect of *A. blazeyi* on some cell lines. Five leukocytic cell lines were cultured with the indicated concentrations of ABH (A), aqueous extract of *A. blazeyi* (B) or aqueous extract of *A. blazeyi* containing inactivated hemicellulases (C). Tetrazolium salt was added to the cultures after 3 days, and optical density was read. Percent proliferation indicates the proportion relative to cell growth in culture medium only. Data are representative of two separate experiments with similar results. Jurkat (plus), BALL-1 (cross), CCRF-CEM (closed diamond), U937 (closed square) and THP-1 (open circle).

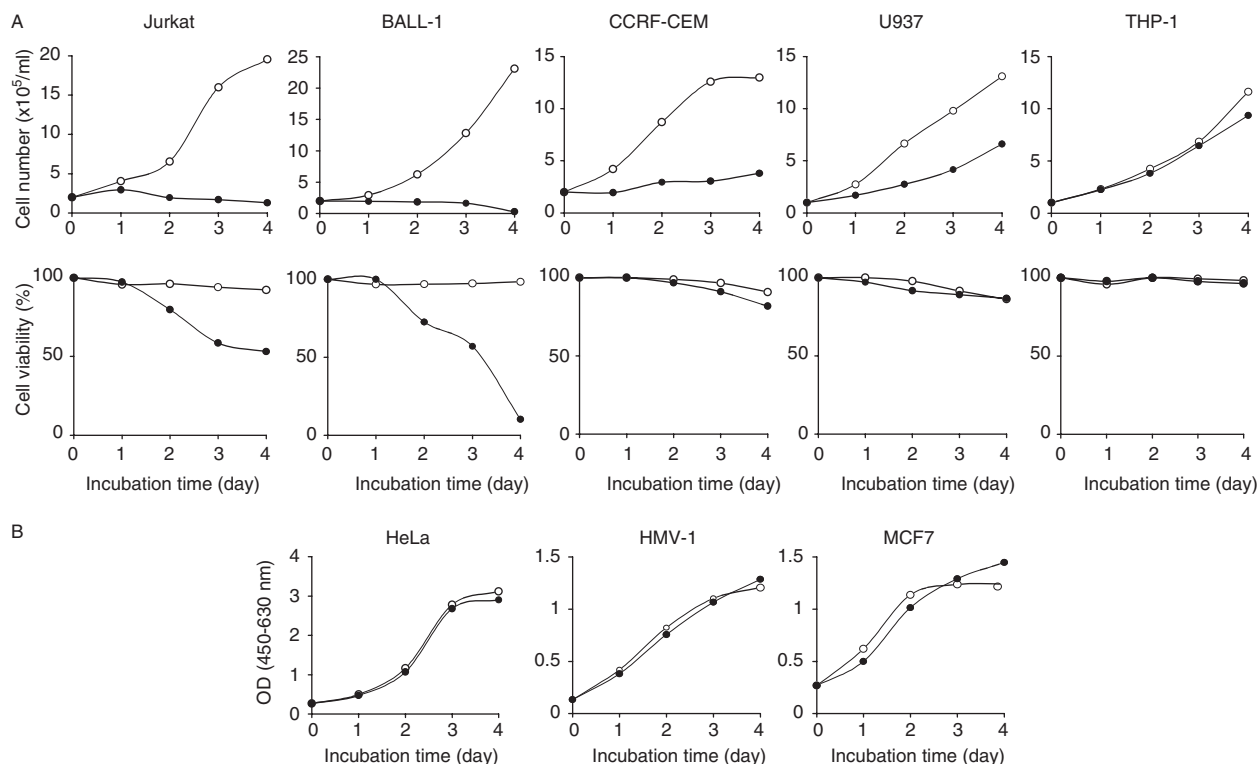


Figure 2. ABH had different effects on growth of several cell lines. The cells were cultured in the presence (closed circle) or absence (open circle) of 0.1% ABH for the indicated times (days). (A) The viable cell number (top) was assessed by the trypan blue dye exclusion method, and the viability (bottom) was determined as the percentage of trypan blue-negative cells to total cells. Data are representative of two separate experiments with similar results. (B) The proliferation of adherent cell lines was expressed as OD after addition of tetrazolium salt. Data are representative of two separate experiments with similar results.

aqueous extract of *A. blazei* showed an antiproliferative effect on Jurkat and BALL-1 cells, this substance had no effect on the cell growth of CCRF-CEM, U937 or THP-1 cells (Fig. 1B). However, hemicellulase-degradation of *A. blazei* not only enhanced its antiproliferative effect on Jurkat and BALL-1 cells, but also generated an effect on growth of the other cell lines (Fig. 1A). Jurkat and BALL-1 cells were more susceptible to ABH than the other cell lines examined; ABH inhibited their proliferation in a dose-dependent manner, and influenced their growth even at a relatively low concentration (0.003%). In addition, ABH strongly suppressed the growth of CCRF-CEM and U937 cells at a high concentration (0.1%). However, THP-1 cells responded poorly to ABH even at a high concentration (0.1%). On the other hand, addition of the same amount of inactivated hemicellulases to aqueous extract of *A. blazei* did not show such enhanced effects (Fig. 1C). Next, as the assay using tetrazolium salt does not discriminate between cell growth inhibition and cell injury, the viable cell number and viability of these cells, which were cultured with 0.1% ABH, were assessed every day by the trypan blue dye exclusion method (Fig. 2A). These cell lines showed different reactions to ABH. ABH produced severe injury to Jurkat and BALL-1 cells on Day 2 or later in culture, and resulted in a decrease in the number of viable cells over time. However, ABH showed

little cytotoxicity against CCRF-CEM or U937 cells, although it strongly inhibited their proliferation. In contrast, ABH had no effect on proliferation of THP-1 cells, as also shown in the assay using tetrazolium salt. In addition, no inhibition of proliferation was observed in three adherent cell lines (HeLa, HMV-1 and MCF7 cells), which were cultured in the presence of 0.1% ABH (Fig. 2B). ABH caused damage to some cell lines, inhibited proliferation of different cell lines without severe cytotoxicity or had no effect on growth of others. On the other hand, the same concentration of aqueous extract of *A. blazei* showed an antiproliferative effect but little cytotoxic effect on Jurkat and BALL-1 cells (data not shown). These results indicated that hemicellulase-degradation resulted in qualitative and/or quantitative changes in the nature of the effect of *A. blazei* on cell proliferation.

ABH Induced Apoptosis and Delay of Cell Cycle Progression in Responsive Cell Lines

To investigate the effects of ABH in more detail, we examined whether ABH caused apoptosis in these cells. Five leukocytic cell lines were cultured in the presence of 0.1% ABH for 3 days as described above. FACS analyses showed populations of annexin V-stained cells with different

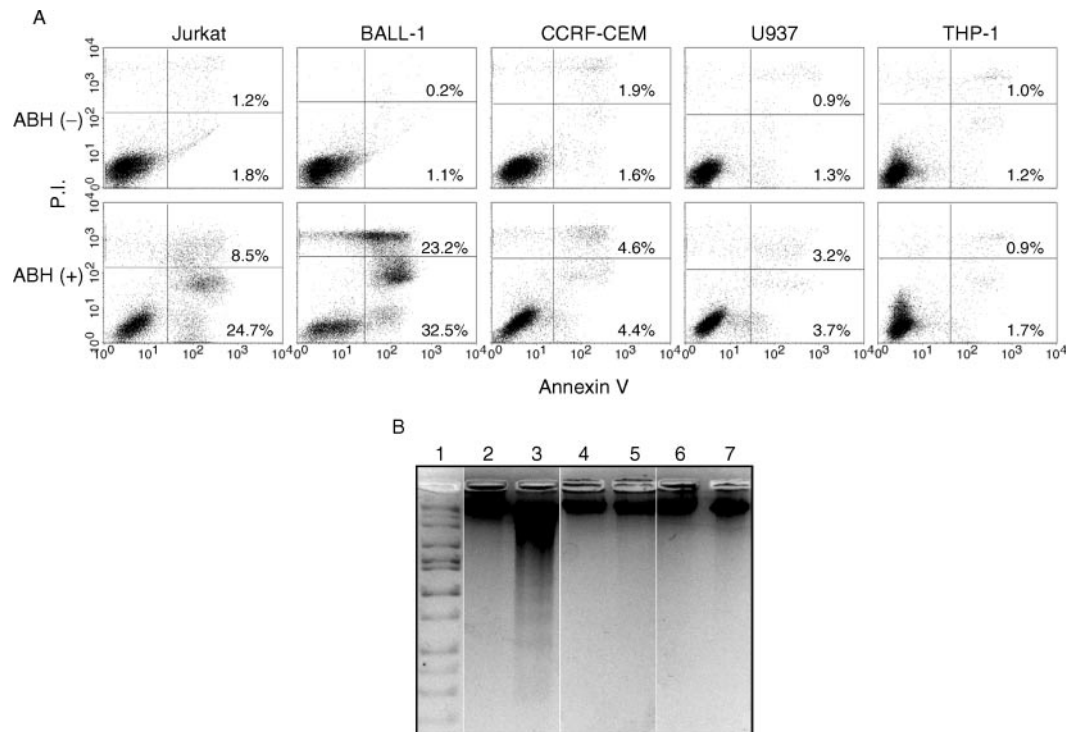


Figure 3. ABH induced apoptosis in some cell lines. **(A)** Apoptosis was assessed by phosphatidylserine exposure on the cell membrane. The cells were cultured in the presence or absence of 0.1% ABH for 3 days, and stained with fluorescein isothiocyanate-conjugated annexin V and PI. The numbers in dot plots indicate the percentage of the total cell number in each quadrant. Data are representative of three separate experiments with similar results. **(B)** Apoptosis was also assessed by determination of DNA fragmentation. BALL-1, CCRF-CEM and THP-1 cells were cultured in the presence (lanes 3, 5 and 7) or absence (lanes 2, 4 and 6) of 0.1% ABH. Total DNA was extracted from the cells, subjected to agarose gel electrophoresis and stained with ethidium bromide. Data are representative of two separate experiments with similar results.

staining properties of PI in ABH-treated Jurkat and BALL-1 cells (Fig. 3A). On the other hand, these populations were detectable but were markedly smaller in CCRF-CEM and U937 cells cultured with 0.1% ABH, which had a significant antiproliferative effect on these two cell lines. In addition, ABH did not induce apoptosis in THP-1 cells under the same conditions. Another characteristic feature of apoptosis, DNA fragmentation, which is detected as DNA ladder formation by electrophoretic separation on agarose gels, was also observed in ABH-treated BALL-1 cells (Fig. 3B, lane 3). However, fragments of apoptotic DNA were not detectable in ABH-treated CCRF-CEM cells (lane 5) in this assay, as was the case with ABH-treated THP-1 (lane 7) and all untreated cells (lanes 2, 4 and 6). These results indicated that ABH induced apoptosis in some cell lines, but that it also had other effects on cell growth, because it strongly suppressed the proliferation of CCRF-CEM and U937 cells without severe cytotoxic effects. Consequently, we postulated that ABH exerted an influence on cell cycle progression. To confirm this, five leukocytic cell lines, which were cultured with ABH as in the above assay, were fixed every 12 h and stained for DNA with PI. FACS analyses demonstrated the cycle phases of individual cells, allowing the measurement of the DNA contents of the cells (Fig. 4). ABH induced alterations in the distribution of cell cycle phases in four cell lines (Jurkat,

BALL-1, CCRF-CEM and U937) during incubation. Their dominant peaks on histograms shifted from G₁ to early S phase in the first 24 h. The majority or a portion of these cells moved synchronously in S phase during the next 12 h. On the other hand, the distribution pattern of cell cycle phases of ABH-treated THP-1 remained virtually constant throughout the experimental period, as was the case with all untreated cell lines (data not shown). Although the receptivity of these cell lines to ABH in alteration of cell cycle progression corresponded to that in cell proliferation, these results raised an additional question of whether this alteration resulted in inhibition of cell proliferation. Therefore, CCRF-CEM cells were pre-treated with a high concentration of thymidine, followed by different recovery periods without thymidine to enrich the cells in early S, mid-S or G₂/M and G₁ phases. These cells were then left untreated or were cultured with ABH as before (Fig. 5). The cells in early S phase at the onset (0 h) progressed synchronously in this phase during the first 6 h without ABH, and the bulk of the cells entered G₂/M phase within 12 h, and formed a dominant peak of G₁ phase, as seen on the histogram at 18 h (Fig. 5A, top). However, the cells in early S phase at the onset moved more slowly in this phase in culture with ABH, and almost all cells were still in S and G₂/M phases even after 30 h (Fig. 5A, bottom). Similarly, the cell cycle progression of cells in mid-S phase at the onset was

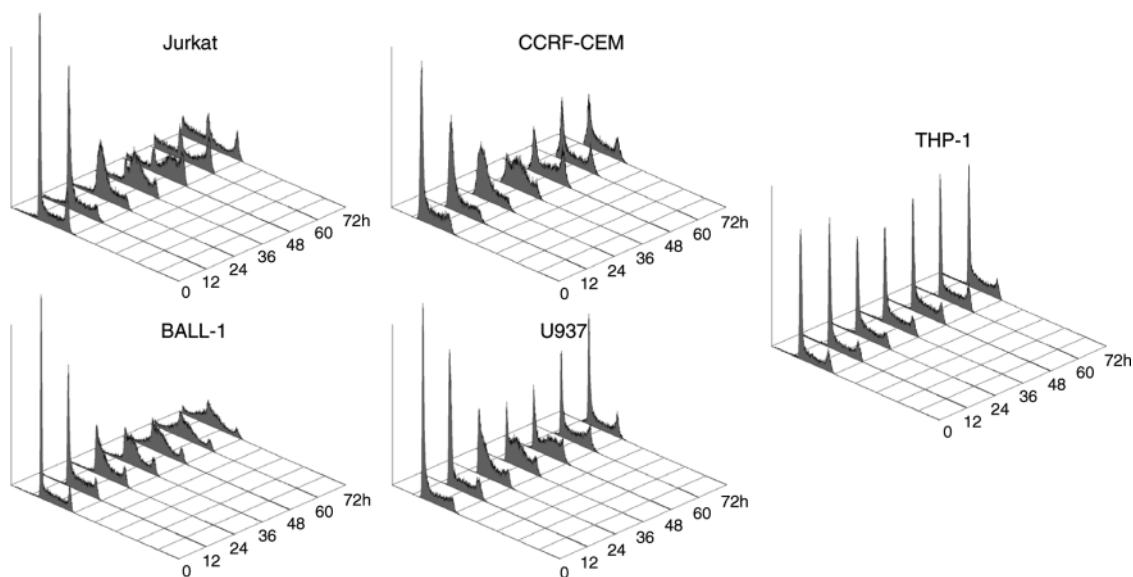


Figure 4. ABH induced S phase-synchronization of cell cycle progression in responsive cell lines. The cells were cultured with 0.1% ABH for the indicated times (hours), followed by fixation and staining for DNA. FACS analyses were used to determine the DNA contents of individual cells.

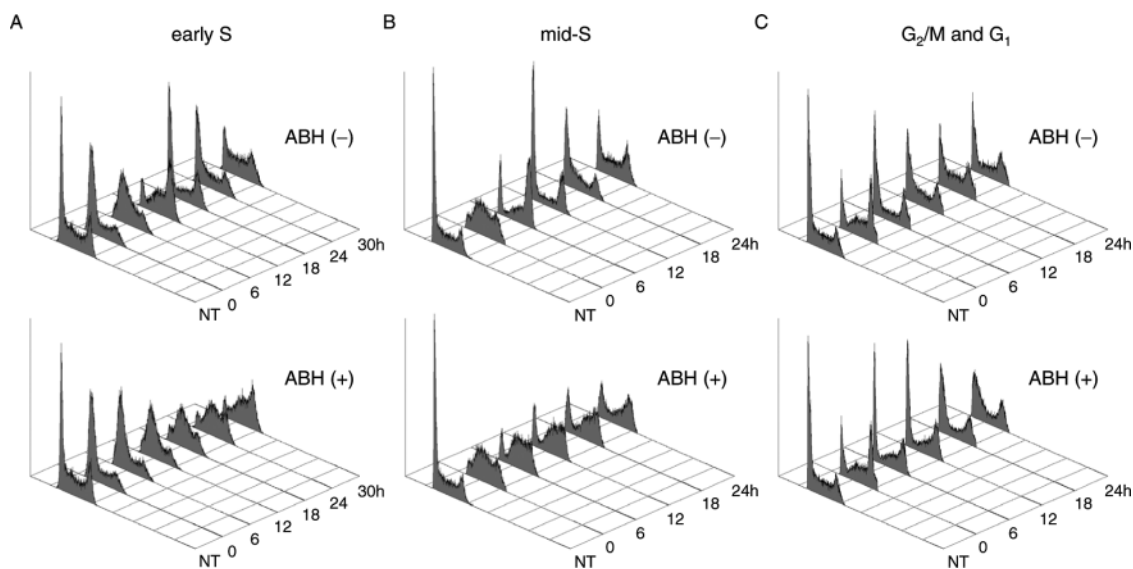


Figure 5. ABH induced a delay of cell cycle progression in S phase. CCRF-CEM cells were enriched in early S (A), mid-S (B) or G_2/M and G_1 (C) phases in advance, and then cultured in the presence or absence of 0.1% ABH for the indicated times (hours), followed by fixation and staining for DNA. FACS analyses were used to determine the DNA contents of individual cells. NT indicates that the cells were not treated with thymidine and ABH. Data are representative of two separate experiments with similar results.

markedly delayed in cultures to which ABH was added, and the cells did not form a dominant peak of G_1 phase even after 24 h unlike those cultured without ABH (Fig. 5B). In contrast, the cells in G_2/M phase at the onset made a transition to G_1 phase even in the presence of ABH to the same extent as those cultured without ABH, and then the G_1 peak formed by these cells shifted to early S phase (Fig. 5C). These results suggested that ABH delayed cell cycle progression in S phase resulting in concentration of the cells in this phase. The inhibition of cell proliferation was induced, at least in part, by exposure to ABH

in S phase and subsequent delay of cell cycle progression in responsive cells.

Mitochondria Played a Role in Apoptosis Induced by ABH

A recent study demonstrated that β -glucan extracted from *A. blazei* induced mitochondria-mediated apoptosis in a human ovarian HRA cell line (15). Therefore, we examined release of mitochondrial cytochrome c into the cytosol, which is one of

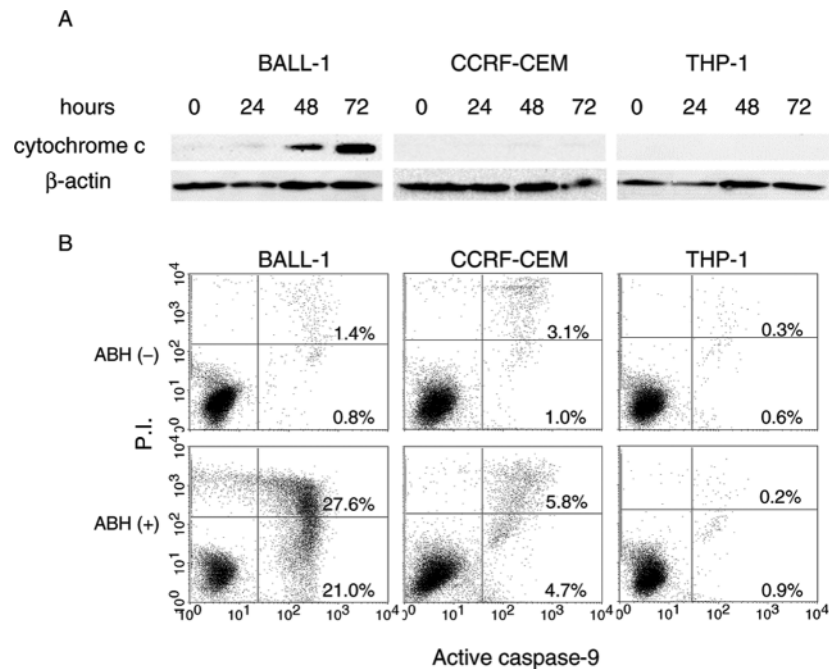


Figure 6. The mitochondria-mediated pathway participated in apoptosis induced by ABH. BALL-1, CCRF-CEM and THP-1 cells were cultured with 0.1% ABH. (A) The cytosolic fraction was prepared every 24 h, and subjected to western blotting analysis with anti-cytochrome c antibody. (B) These cells, which were cultured in the presence or absence of 0.1% ABH for 3 days, were stained with PI and a fluorescent-conjugated peptide specific for active caspase-9. The numbers in dot plots indicate the percentage of the total cell number in each quadrant.

early events in induction of mitochondria-mediated apoptosis (20), in cultures with ABH. BALL-1, CCRF-CEM and THP-1 cells, which were differentially sensitive to ABH, were cultured with 0.1% ABH, and the cytosolic fraction was prepared every 24 h. Western blotting analysis indicated that ABH treatment induced release of cytochrome c into the cytosol in a time-dependent manner in BALL-1 cells (Fig. 6A). The release of cytochrome c was slight in the first 24 h, but it increased to a large extent subsequently. On the other hand, ABH-treated CCRF-CEM cells showed the minimal release at a later stage in culture, and no release of cytochrome c was observed in ABH-treated THP-1 cells throughout the experimental period. Next, these cells were stained with PI and a fluorescent-conjugated peptide (FAM-LEHD-FMK), which binds active caspase-9, as the release of mitochondrial cytochrome c leads to activation of caspase-9, followed by activation of caspase-3, which initiates a set of events that culminate in apoptosis (21). FACS analyses detected larger populations of caspase-9-active BALL-1 cells in culture with ABH for 3 days (Fig. 6B) but not 1 day (data not shown). As expected, the degrees of caspase-9 activation corresponded to those of release of cytochrome c in ABH-treated CCRF-CEM and THP-1 cells.

p38 MAPK Activation was Responsible for ABH-Induced Effects

The study by Kobayashi *et al.* cited above also indicated that β -glucan extracted from *A. blazeyi* induced activation of p38

MAPK in HRA cells, and that p38 MAPK-specific inhibitor suppressed β -glucan-induced apoptosis (15). p38 MAPK has been shown to respond strongly to various stress stimuli, such as osmotic shock, lipopolysaccharide, inflammatory cytokines and irradiation (22–24), and studies using the inhibitor suggested that p38 activation is responsible for apoptosis induced by these stressors (25). Therefore, we also analyzed p38 activation after treatment with ABH in five leukocytic cell lines. These cells were incubated with 0.1% ABH for up to 64 h. The permeabilized cells were reacted with anti-phospho-p38 MAPK mAb, and the levels of phosphorylated p38 MAPK were measured by flow cytometry (Fig. 7). ABH induced strong p38 activation, which peaked within 15 min and returned to the basal levels in 60 min in both Jurkat and BALL-1 cells. On the other hand, ABH also activated p38 MAPK in CCRF-CEM and U937 cells, although the levels were very much lower than in Jurkat and BALL-1 cells. CCRF-CEM cells showed extremely weak and slow p38 activation in response to ABH, peaking at 16 h. However, these four cell lines showed p38 activation even after 64 h in culture. In contrast, ABH-treated THP-1 cells showed no p38 activation up to 64 h in culture. Next, we examined whether MEK and MAPK inhibitors attenuated the ABH-induced effects on cell growth. Four cell lines responsive to ABH were pre-treated with 10 μ M SB203580, a p38 MAPK-specific inhibitor, 4 μ M SP600125, a JNK-specific inhibitor or 10 μ M PD98059, a MEK-specific inhibitor, and then cultured with or without 0.1% ABH for 3 days. Their cell proliferation was examined using tetrazolium salt as described above, and

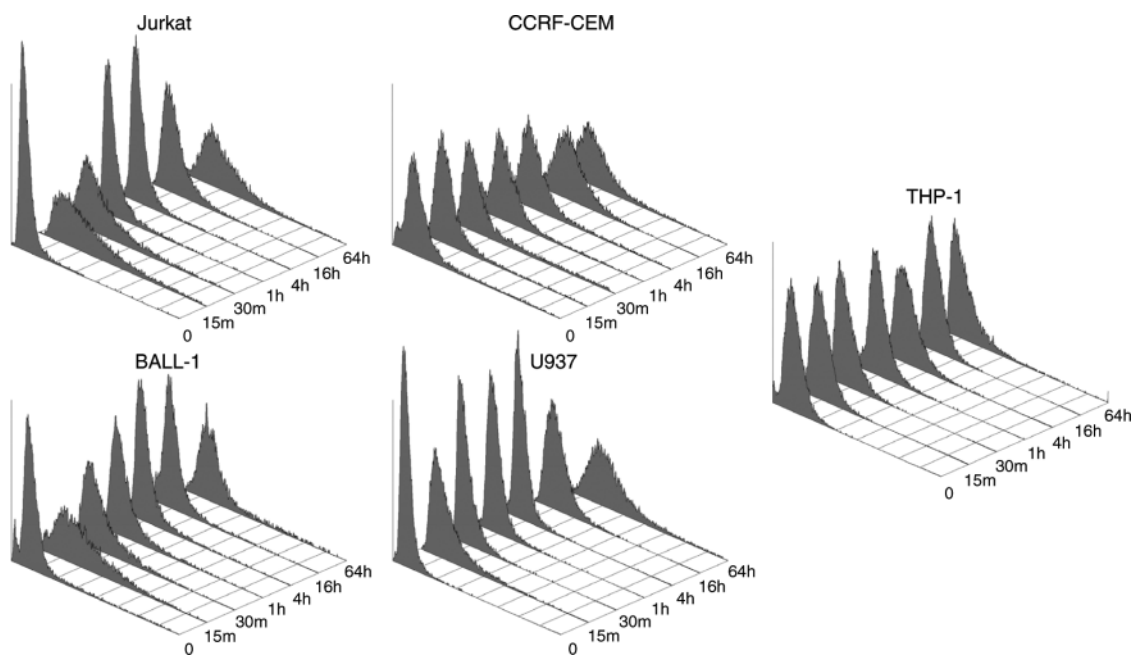


Figure 7. ABH induced different degrees of p38 activation. After the cells were fixed and permeabilized, the levels of phosphorylated p38 MAPK in the cells, which were exposed to 0.1% ABH for the indicated times, was measured by flow cytometry.

expressed relative to cell growth in cultures without ABH (Fig. 8A). These concentrations of inhibitors had no effect on growth of the four cell lines by themselves (data not shown). However, SB203580 weakened the antiproliferative effects of ABH on all of these cell lines, blocking the antiproliferative effects in CCRF-CEM and U937 cells almost completely. On the other hand, SP600125 and PD98059 had almost no influence on the ABH-induced effects, or rather increased the effect slightly, except in U937 cells. Increased concentrations of these two inhibitors (up to 50 μM) also did not improve the proliferation of ABH-treated cells (data not shown). FACS analyses confirmed that pre-treatment with SB203580 moderately or significantly inhibited ABH-induced apoptosis in Jurkat or BALL-1 cells, respectively (Fig. 8B). It was interesting to note that SB203580 also markedly improved the ABH-induced delay of cell cycle progression in S phase to almost the same degree as in cultures without ABH in CCRF-CEM cells, which had been synchronized in S phase at the onset of the experiment (Fig. 8C). These results indicated that ABH induced continual p38 activation, the levels of which differed between cell lines, and that this p38 activation was involved in not only apoptosis but also in the delay of cell cycle progression in S phase induced by ABH.

The Combination of ABH and TNF- α Induced Continual Strong Activation of p38 MAPK and Resulted in Apoptosis in CCRF-CEM and U937 Cells

ABH showed a weak cytotoxic effect in CCRF-CEM and U937 cells despite its strong antiproliferative effect (Fig. 2A).

It induced low levels of p38 activation in CCRF-CEM and U937 cells unlike BALL-1 and Jurkat cells, in which high levels of p38 activation resulted in both alteration of cell cycle progression and apoptosis. Therefore, we hypothesized that weak p38 activation by ABH did not result in apoptosis. However, we also suspected that these two cell lines were not susceptible to apoptosis regardless of the degree of p38 activation. Consequently, we attempted to increase the activity of p38 MAPK in these two cell lines. TNF- α has a wide variety of physiological activities. The ligation of TNF- α and its receptor on the cells has been shown to induce p38 activation (23). In addition, it has been shown that TNF- α triggers death receptor-mediated apoptosis (26). However, it also has anti-apoptotic effects, activating NF- κB (27). In fact, TNF- α (10 nM) had only a minor impact on growth of CCRF-CEM and U937 cells by itself (Fig. 9A). FACS analyses also showed that TNF- α induced little apoptosis in these two cell lines (Fig. 9B). However, the combination of 0.1% ABH and 10 nM TNF- α significantly augmented the cytotoxic effects on these cell lines. This combination decreased the cell viability of the cultures to far lower levels (Fig. 9A), and generated larger populations of annexin V-stained cells (Fig. 9B). Furthermore, the combination of ABH and TNF- α significantly augmented the activation of caspase-9 in CCRF-CEM cells (Fig. 9C), although TNF- α alone had little effect on the activation of caspase-9 in CCRF-CEM cells, and ABH also hardly activated caspase-9 in these cells (Fig. 6B). On the other hand, the growth of THP-1 cells was unaffected even by the same combination of ABH and TNF- α . Based on these results, the levels of p38 activation were measured in cultures with ABH and TNF- α (Fig. 10A). TNF- α induced sufficient levels

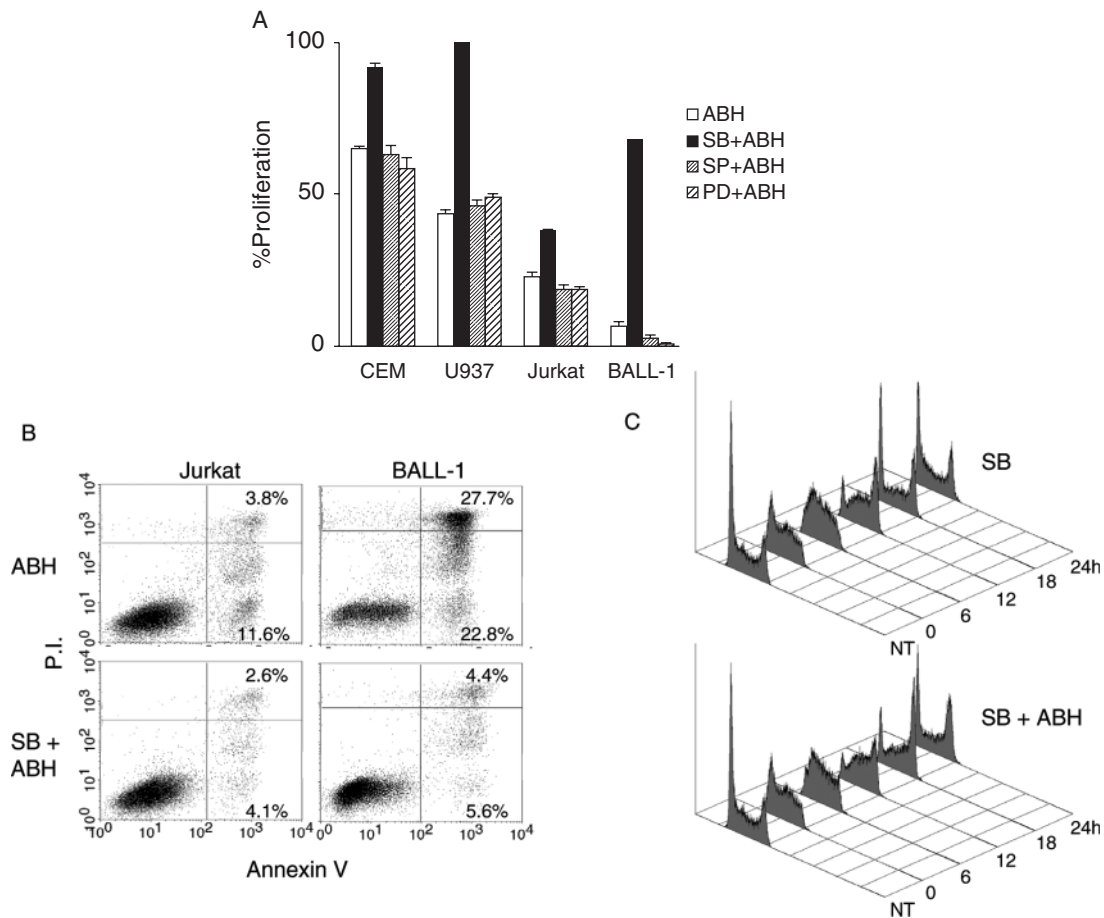


Figure 8. p38 activation was responsible for the effects of ABH. (A) The cells were pre-treated with 10 μ M SB203580 (SB), 4 μ M SP600125 (SP), 10 μ M PD98059 (PD) or 0.3% DMSO, which was used for dissolution of these MEK and MAPK inhibitors, for 30 min, and cultured in the presence or absence of 0.1% ABH. Tetrazolium salt was added to the cultures after 3 days. Percent proliferation indicates the proportion relative to cell growth without ABH. Data are shown as means \pm SD of duplicate cultures. Data are representative of two separate experiments with similar results. (B) Jurkat and BALL-1 cells were pre-treated with SB203580 (SB) or DMSO as described above, and cultured with 0.1% ABH for 3 days. The cells were stained with fluorescein isothiocyanate-conjugated annexin V and PI. The numbers in dot plots indicate the percentage of the total cell number in each quadrant. (C) CCRF-CEM cells were enriched in S phase in advance. The cells were pre-treated with SB203580 (SB) as described above, and cultured in the presence or absence of 0.1% ABH for the indicated times (hours), followed by fixation and staining for DNA. NT indicates that the cells were not treated with thymidine and ABH. Data are representative of two separate experiments with similar results.

of p38 MAPK activation immediately in these two cell lines, although it induced little apoptosis. On the other hand, p38 activation induced by combination of ABH and TNF- α was far stronger and longer lasting. These two cell lines showed a transient decrease in p38 MAPK activity after the rapid and strong activation, but the cells then showed restoration of the extremely strong activation at 16 and 64 h. Next, we examined whether this p38 activation was correlated with the enhanced cytotoxicity induced by combination of ABH and TNF- α . SB203580 significantly attenuated the enhanced cytotoxic effect on CCRF-CEM and U937 cells cultured with ABH and TNF- α (Fig. 10B). FACS analyses also confirmed that it blocked apoptosis induced by ABH and TNF- α (Fig. 10C). Although this enhancement of the cytotoxic effect was not due to upregulation of TNF type 1 receptor (TNFR1) on the cells by ABH (data not shown), the mechanism remains to be elucidated in detail. However,

these results suggested that the degrees of p38 activation induced by ABH, which were dependent on tumor cells and other stimuli, determined subsequent cellular responses on cell growth. We envisaged that some cell factors defined the responsiveness of p38 MAPK to ABH. Many molecules, such as TNF receptor family and Toll like receptors (28,29), protein kinase C (PKC) subtypes (30) and small G proteins (31), have been suggested to play a role in p38 activation. The quantitative or functional differences in these molecules between cell lines may result in differences in responsiveness to ABH. ABH is a crude extract from *A. blazei* mycelia and consists of multiple components. The components responsible for the ABH-induced effects demonstrated in the present study have not yet been identified. However, as hemicellulase-degradation of *A. blazei* enhanced the inhibitory effects on cell proliferation, some low molecular weight carbohydrate components may be involved in these effects, acting on the

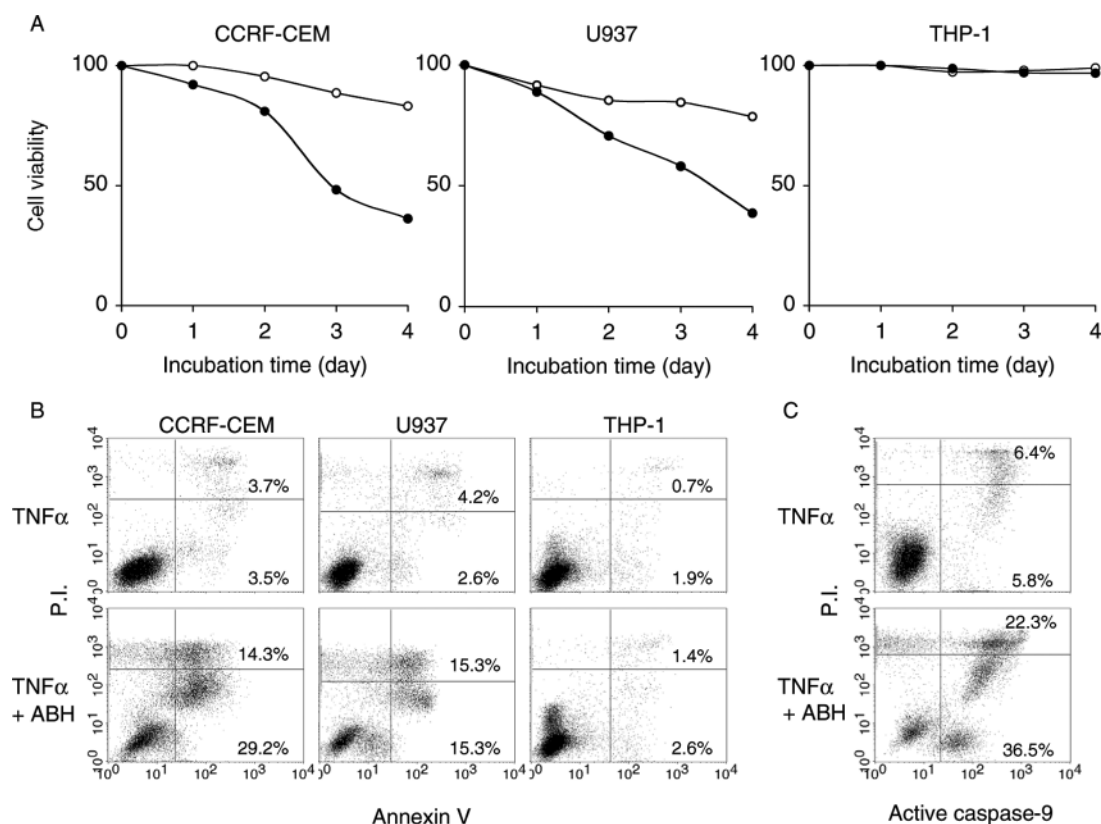


Figure 9. The combination of ABH and TNF- α increased susceptibility to apoptosis. CCRF-CEM, U937 and THP-1 cells were cultured with 10 ng ml^{-1} TNF- α in the presence or absence of 0.1% ABH. (A) Cell viability was assessed by the trypan blue dye exclusion method at the indicated times (open circle, without ABH; closed circle, with ABH). Data are representative of two separate experiments with similar results. (B) The cells were stained with fluorescein isothiocyanate-conjugated annexin V and PI on day 3. (C) CCRF-CEM cells were also stained with a fluorescent-conjugated peptide specific for active caspase-9 and PI on Day 3. The numbers in dot plots indicate the percentage of the total cell number in each quadrant.

receptors, signal proteins or transcriptional factors that have not yet been clarified.

Physiological Significance of ABH on the Antitumor Effects of Existing Treatments

Many patients with cancer in Japan have consumed *A. blazei* in the belief that it has medicinal properties. However, there is little evidence of its effectiveness against cancer in humans (32). It has been suggested that *A. blazei* improves immunological status, as demonstrated in animal experiments. Some recent studies indicated that *A. blazei* also has inhibitory effects on tumor cell growth, tumor-induced neovascularization, and cancer metastasis. In the present study, ABH induced inhibition of cell proliferation in some cell lines. However, our results showed that ABH does not have an antiproliferative effect on all tumor cells, and this suggested the limitation of its efficacy against cancer. Moreover, as ABH induced a delay in cell cycle progression in S phase in some tumor cells, it may also have an influence on the benefits of existing therapeutic regimens against cancer. For example, this feature of ABH may put patients at a disadvantage regarding the efficacy of radiation therapy for cancer. In general, it has been

demonstrated that tumor cells are more susceptible to irradiation in M phase and at the boundary of G₁/S of the cell cycle, whereas they can show resistance during the last half of S phase (33). Thus, the enrichment of tumor cells in S phase by ABH may cause attenuation of the effectiveness of radiotherapy. In contrast, tumor cells richer in S phase yield better effects of therapeutic heating and drugs that act specifically in S phase, such as antimetabolites. The synchronous delay of tumor cells in S phase by ABH may enable a number of cells to be exposed to the drugs in S phase for a long time. In addition, this may result in a decrease in time and frequency of administration of the drugs.

In conclusion, we examined the effects of ABH on proliferation of several tumor cell lines. ABH induced p38 MAPK-mediated delay of cell cycle progression and apoptosis in some cell lines. The growth of other cell lines showed poor or no responses to ABH. We hypothesized that the reactivity of cell growth to ABH was dependent on some cellular factor(s), which regulate p38 MAPK activation in response to ABH. These observations also indicate that the use of ABH as a complementary and alternative medicine is not necessarily appropriate for all tumors. The elucidation of these cellular factors in future studies will facilitate the appropriate use of ABH.

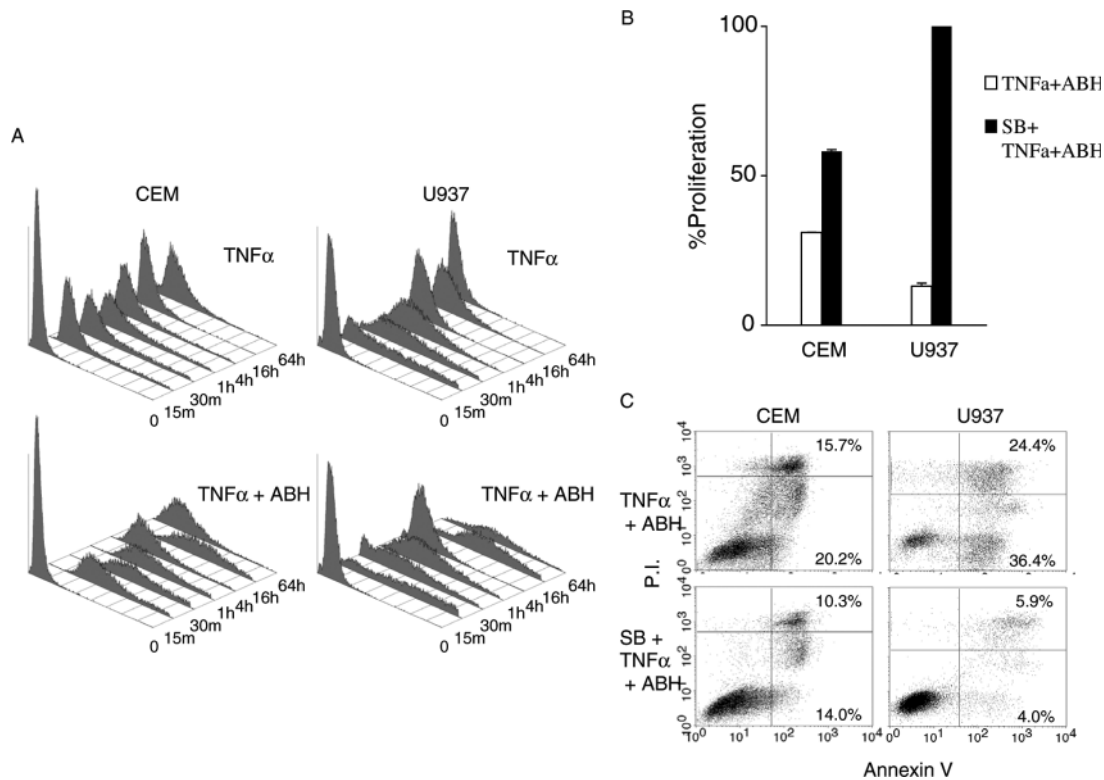


Figure 10. ABH and TNF- α resulted in continual strong p38 activation, which was responsible for enhanced cytotoxicity, in CCRF-CEM and U937 cells. (A) The levels of phosphorylated p38 MAPK in these two cell lines, which were exposed to 10 ng ml^{-1} TNF- α in the presence or absence of 0.1% ABH for the indicated times, were measured by flow cytometry. (B) These two cell lines were pre-treated with $10 \mu\text{M}$ SB203580 (SB) or 0.3% DMSO for 30 min, and cultured with 10 ng ml^{-1} TNF- α in the presence or absence of 0.1% ABH for 3 days. Tetrazolium salt was added to the cultures. Percent proliferation indicates the proportion relative to cell growth without ABH. Data are shown as means \pm SD of duplicate cultures. (C) The cells were also stained with fluorescein isothiocyanate-conjugated annexin V and PI. The numbers in dot plots indicate the percentage of the total cell number in each quadrant.

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References

- 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.
- Ebina T, Fujimiya Y. Antitumor effect of a peptide-glucan preparation extracted from *Agaricus blazei* in a double-grafted tumor system in mice. *Biotherapy* 1998;11:259-65.
- Fujimiya Y, Suzuki Y, Oshiman K, Kobori H, Moriguchi K, Nakashima H, et al. Selective tumoricidal effect of soluble proteoglycan extracted from the basidiomycete, *Agaricus blazei* Murill, mediated via natural killer cell activation and apoptosis. *Cancer Immunol Immunother* 1998;46:147-59.
- Sorimachi K, Akimoto K, Ikehara Y, Inafuku K, Okubo A, Yamazaki S. Secretion of TNF- α , IL-8 and nitric oxide by macrophages activated with *Agaricus blazei* Murill fractions *in vitro*. *Cell Struct Funct* 2001;26:103-8.
- Kaneno R, Fontanari LM, Santos SA, Di Stasi LC, Rodrigues Filho E, Eira AF. Effects of extracts from Brazilian sun-mushroom (*Agaricus blazei*) on the NK activity and lymphoproliferative responsiveness of Ehrlich tumor-bearing mice. *Food Chem Toxicol* 2004;42:909-16.
- Kasai H, He LM, Kawamura M, Yang PT, Deng XW, Munkanta M, et al. IL-12 production induced by *Agaricus blazei* fraction H (ABH) involves toll-like receptor (TLR). *Evid Based Complement Alternat Med* 2004;1:259-67.
- Delmanto RD, de Lima PL, Sugui MM, da Eira AF, Salvadori DM, Speit G, et al. Antimutagenic effect of *Agaricus blazei* Murrill mushroom on the genotoxicity induced by cyclophosphamide. *Mutat Res* 2001;496:15-21.
- Martins de Oliveira J, Jordao BQ, Ribeiro LR, Ferreira da Eira A, Mantovani MS. Anti-genotoxic effect of aqueous extracts of sun mushroom (*Agaricus blazei* Murill lineage 99/26) in mammalian cells *in vitro*. *Food Chem Toxicol* 2002;40:1775-80.
- Luiz RC, Jordao BQ, da Eira AF, Ribeiro LR, Mantovani MS. Mechanism of anticlastogenicity of *Agaricus blazei* Murill mushroom organic extracts in wild type CHO (K1) and repair deficient (xrs5) cells by chromosome aberration and sister chromatid exchange assays. *Mutat Res* 2003;528:75-9.
- Barbisan LF, Scolastici C, Miyamoto M, Salvadori DM, Ribeiro LR, da Eira AF, et al. Effects of crude extracts of *Agaricus blazei* on DNA damage and on rat liver carcinogenesis induced by diethylnitrosamine. *Genet Mol Res* 2003;2:295-308.
- Machado MP, Filho ER, Terezan AP, Ribeiro LR, Mantovani MS. Cytotoxicity, genotoxicity and antimutagenicity of hexane extracts of *Agaricus blazei* determined *in vitro* by the comet assay and CHO/HGPRT gene mutation assay. *Toxicol In Vitro* 2005;19:533-9.
- Bellini MF, Angeli JP, Matuo R, Terezan AP, Ribeiro LR, Mantovani MS. Antigenotoxicity of *Agaricus blazei* mushroom organic and aqueous extracts in chromosomal aberration and cytokinesis block micronucleus assays in CHO-k1 and HTC cells. *Toxicol In Vitro* 2006;20:355-60.
- 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.

14. Kimura 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.
15. 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.
16. 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.
17. Kawamura M, Kasai H, He LM, Deng X, Yamashita A, Terunuma H, et al. Antithetical effects of hemicellulase-treated *Agaricus blazei* on the maturation of murine bone-marrow-derived dendritic cells. *Immunology* 2005;114:397–409.
18. Knehr M, Poppe M, Enulescu M, Eickelbaum W, Stoehr M, Schroeter D, et al. A critical appraisal of synchronization methods applied to achieve maximal enrichment of HeLa cells in specific cell cycle phases. *Exp Cell Res* 1995;217:546–53.
19. Park MT, Choi JA, Kim MJ, Um HD, Bae S, Kang CM, et al. Suppression of extracellular signal-related kinase and activation of p38 MAPK are two critical events leading to caspase-8- and mitochondria-mediated cell death in phytosphingosine-treated human cancer cells. *J Biol Chem* 2003;278:50624–34.
20. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997;275:1132–6.
21. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997;91:479–89.
22. Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 1994;265:808–11.
23. Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, et al. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem* 1995;270:7420–6.
24. Bowen C, Birrer M, Gelmann EP. Retinoblastoma protein-mediated apoptosis after gamma-irradiation. *J Biol Chem* 2002;277:44969–79.
25. Benhar M, Dalyot I, Engelberg D, Levitzki A. Enhanced ROS production in oncogenically transformed cells potentiates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activation and sensitization to genotoxic stress. *Mol Cell Biol* 2001;21:6913–26.
26. Green DR. Apoptosis. Death deceiver. *Nature* 1998;396:629–30.
27. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science* 1996;274:782–4.
28. Sutherland CL, Heath AW, Pelech SL, Young PR, Gold MR. Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. *J Immunol* 1996;157:3381–90.
29. Schroder NW, Pfeil D, Opitz B, Michelsen KS, Amberger J, Zahringer U, et al. Activation of mitogen-activated protein kinases p42/44, p38, and stress-activated protein kinases in myelo-monocytic cells by *Treponema lipoteichoic acid*. *J Biol Chem* 2001;276:9713–9.
30. Berra E, Municio MM, Sanz L, Frutos S, Diaz-Meco MT, Moscat J. Positioning atypical protein kinase C isoforms in the UV-induced apoptotic signaling cascade. *Mol Cell Biol* 1997;17:4346–54.
31. Zhang S, Han J, Sells MA, Chernoff J, Knaus UG, Ulevitch RJ, et al. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J Biol Chem* 1995;270:23934–6.
32. 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.
33. Kiefer J. *Biological Radiation Effects*. Tokyo: Springer-Verlag Tokyo Inc., 1993.

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