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Blazein of a new steroid isolated from *Agaricus blazei* Murrill (himematsutake) induces cell death and morphological change indicative of apoptotic chromatin condensation in human lung cancer LU99 and stomach cancer KATO III cells

HIROKO ITOH[1], HITOSHI ITO[3] and HIROSHIGE HIBASAMI[2]

Faculties of [1]Bioresources and [2]Medicine, Mie University, Tsu-city, Mie 514-0001;
[3]Research Institute of Mycology and Pharmacology, Tsu-city, Mie 514-0033, Japan

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Abstract. Blazein was isolated from mushroom (*Agaricus blazei* Murrill) and identified by Mass and ^{1}H-NMR as blazein. The effect of blazein on the DNA of human various cancer cells was investigated. DNA fragmentations by blazein to oligonucreosomal-sized fragments, a characteristic of apoptosis, were observed in the human lung LU99 and stomach KATO III cancer cells. The DNA fragmentations by blazein were observed from day 2 (KATO III cells) or day 3 (LU99 cells) after the addition of blazein to the culture cells. These findings suggest that growth inhibition by blazein results from the induction of apoptosis by the compound.

Introduction

It has been traditionally considered that extracts of the basidiomycete, *Agaricus blazei*, used as folk remedies in Brazil, are pharmacologically active. About three decades ago, following considerable effort, this strain was propagated in Japan and polysaccharides isolated from *Agaricus blazei* were shown to have tumoricidal activity in allogeneic (1,2) and syngeneic mouse tumor models (3). Although it has been postulated that the inhibitory action of these natural products results from the enhancement of host immunity against tumor growth, no direct evidence has yet been obtained.

Thus far we have purified several compounds extracted from the basidiomycete plant and evaluated the antitumor activity of those compounds (4-11). The efficiency of the antitumor compounds appears to be related to the propensity of tumor cells to respond to these compounds by apoptosis. Considerable attention has been focused on the sequence of events referred to as apoptosis, and the role of this process in mediating the lethal effects of antineoplastic agents in leukemia cells (12). Apoptosis is a highly regulated process that is characterized by cell shrinkage, membrane blebbing, chromatin condensation and the formation of a DNA ladder with multiple fragments of 180-200 bp caused by internucleosomal DNA cleavage (13).

In previous studies we demonstrated induction of apoptosis by phytol (9), sesamin (10), pheophorbide a (5), diol- and triol-types of phytol (6), 1,8-cineole (11), theaflavin (7), lupeol (4) and procyanidin (8) in cultured human lymphoid leukemia and stomach cancer cells.

In the present study, we isolated blazein which has antiproliferative properties from the mushroom (*Agaricus blazei* Murrill) and found induction of apoptotic morphological changes by blazein of the nucleus resulting from DNA fragmentation in human lung cancer LU99 and stomach cancer KATO III cells.

Materials and methods

Chemicals. RPMI-1640, Eagle's minimal essential and Dulbecco's modified Eagle's media, phytohemagglutinin-M and foetal calf serum were purchased from Gibco Laboratories (Grand Island, NY, USA). Lymphocyte separation medium was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). The other chemicals were of the highest grade and available from Nacalai Tesque Inc., Kyoto, Japan.

Apparatus. ^{1}H-NMR spectra were taken with JEOL, JNM-500 (Tokyo, Japan). Mass spectrometry (MS) was performed using a Hitachi M-80 instrument (Tokyo, Japan). For the preparation of blazein as described below, high performance liquid chromatography (HPLC) was performed with a JASCO 8870-PV.

Preparation of blazein and identification. Fresh fruiting bodies of *Agaricus blazei* Murrill were extracted with acetone as described (14). The extract was concentrated and partitioned between ethyl acetate and water. The ethyl acetate layer was concentrated and separated into acidic, basic and neutral

Correspondence to: Dr Hiroshige Hibasami, Faculty of Medicine, Mie University, Tsu-city, Mie 514-0001, Japan
E-mail: hibasami@nurse.medic.mie-u.ac.jp

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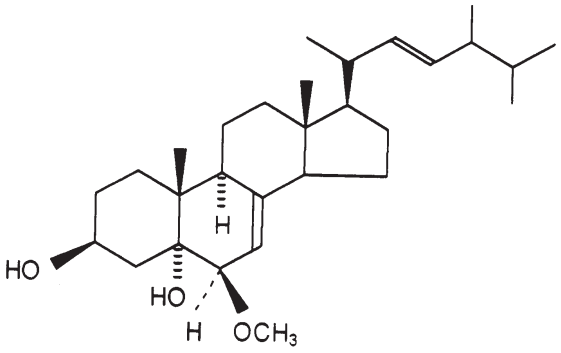

Figure 1. Chemical structure of blazein.

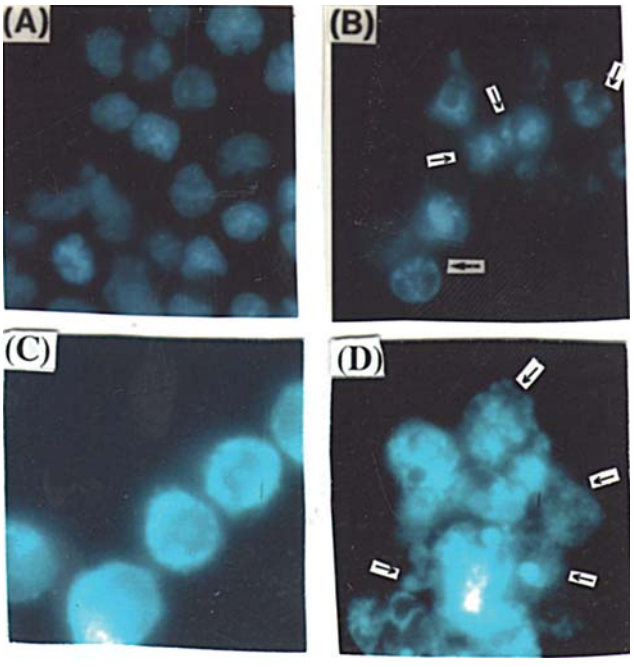

Figure 2. Morphological changes by blazein in the LU99 and KATO III cells. (A) Non-treated LU99 cells. (B) LU99 cells treated with 200 μM blazein. (C) Non-treated KATO III cells. (D) KATO III cells treated with 200 μM blazein. These cells were cultivated with blazein for 3 days, fixed with 1% glutaraldehyde, stained with Hoechst 33258 and observed under an epifluorescence microscope equipped with a cooled CCD camera (phometrics, P x L 1400) digital imaging system and Fuji pictorography 3000. Arrows indicate apoptotic cells.

fractions. Repeated chromatography of the neutral fractions using silica gel afforded Compound A. Compound A was identified as blazein using the apparatus described above. The chemical structure of blazein is shown in Fig. 1.

Cell culture. Human lung cancer LU99 cells were grown in RPMI-1640 medium containing 10% foetal calf serum, penicillin G (50 IU/ml) and streptomycin (50 μg/ml). Human stomach cancer KATO III cells were grown in 45% RPMI-1640 with 45% Eagle's minimal essential medium containing 10% foetal calf serum, penicillin G (50 IU/ml) and streptomycin (50 μg/ml). These cells were cultivated at 37˚C under humidified 95% air and 5% CO_2 atmosphere, and passaged every 4 days. Mycoplasma testing was routinely negative.

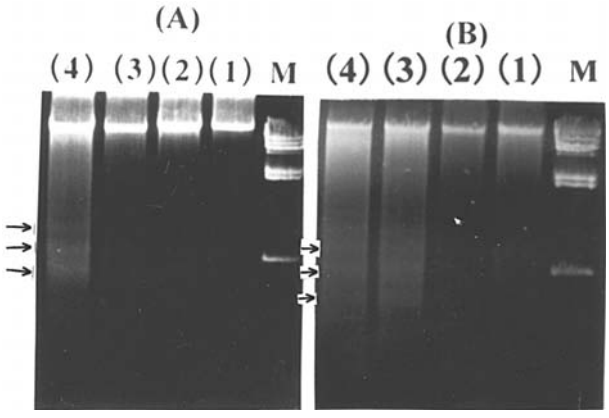

Figure 3. Dose-dependency of DNA fragmentation caused by blazein in the LU99 (A) and KATO III (B) cells. The cells were cultured in the presence of a vehicle (lane 1) or blazein at 50 (lane 2), 100 (lane 3) and 200 μM (lane 4) for 3 days. After the isolation of DNA from the blazein-treated cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid (pH 7.5) buffer containing 2 mM EDTA. M: λ DNA digested with *Hin*dIII.

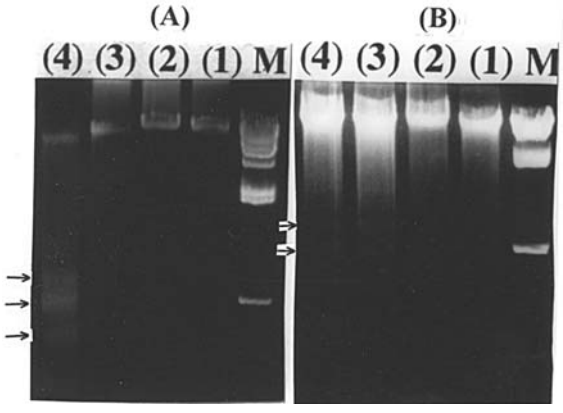

Figure 4. Time-course of DNA fragmentation caused by blazein in the LU99 (A) and KATO III (B) cells. The cells were cultivated in the presence of a vehicle (lane 1) or 200 μM blazein (lanes 2-4) for 1 day (lanes 2), 2 days (lanes 3) and 3 days (lanes 4). After the isolation of DNA from the blazein-treated cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid (pH 7.5) buffer containing 2 mM EDTA. M: λ DNA digested with *Hin*dIII.

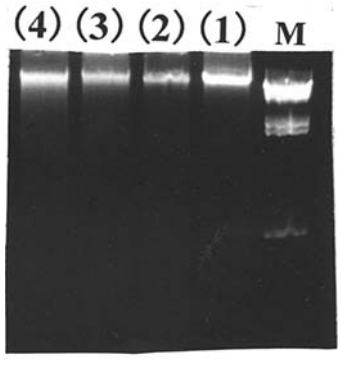

Figure 5. Effects of blazein on normal lymphocyte cells. The cells were cultured in the presence of a vehicle (lane 1) or blazein at 50 (lane 2), 100 (lane 3) and 200 μM (lane 4) for 3 days. After the isolation of DNA from the chalcone-treated cells, equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid (pH 7.5) buffer containing 2 mM EDTA. M: λ DNA digested with *Hin*dIII.

tion of human lymphocyte cells. Lymphocyte n medium (3 ml) was aseptically transferred to a centrifuge tube and the diluted blood (heparinized blood: physiological saline, 1:1) was layered over lymphocyte separation medium in the tube. The tube was centrifuged at 400 x g at room temperature for 20 min. The top layer of the clear plasma was removed, and the lymphocyte layer was transferred to a new centrifuge tube. An equal volume of PBS (-) was added to the lymphocyte layer in the tube and centrifuged for 10 min at room temperature at 260 x g. After centrifugation, the precipitated lymphocyte was washed again with PBS (-), and suspended in RPMI-1640 containing 10% FCS and 2% phytohemagglutinin-M (Gibco Laboratories). The obtained normal lymphocyte cells were cultivated in the presence of a vehicle or blazein.

Microscopic observations of the morphological change of LU99 and KATO III cells. Exponentially growing LU99 and KATO III cells were plated at the initial density of 3-4x10^5 cells/ml. After cultivating for 3 days in the presence of a vehicle or blazein, cells were fixed with 1% glutaraldehyde and stained with Hoechst 33258. The morphology of the stained cells was examined by an epifluorescence microscope with a cooled CCD camera digital imaging system and Fuji pictorography 3000 as described by Okumura *et al* (15).

Assay for DNA fragmentation. Various exponentially growing cancer cell lines were placed at 3-4x10^5 cells/ml. After cultivation in the presence of a vehicle or blazein for 1-3 days, cells were pelleted by slow centrifugation. DNA was isolated from the cell pellets as described previously (8). Equivalent amounts of DNA (2 μg) were loaded into wells of 2% agarose gel and electrophoresed in 40 mM Tris-acetic acid (pH 7.5) buffer containing 2 mM EDTA.

Results and Discussion

Morphological changes of LU99 and KATO III cells by blazein. Morphological changes showing apoptotic bodies were observed in the LU99 (Fig. 2B) and KATO III cells (Fig. 2D) after the addition of 200 μM blazein to the culture.

Induction of apoptosis by blazein in the cell lines. The significant growth inhibitory activity of blazein led us to investigate whether part of the effect was a result of the induction of apoptosis. The fragmentation of genomic DNA into oligonucleosomal-sized fragments (DNA ladder) is a characteristic of the occurrence of apoptosis.

As shown in Fig. 3, DNA ladders were observed in the LU99 cells (A), while those in the KATO III cells (B) were observed at the concentration of 100 and 200 μM blazein. As shown in Fig. 4, DNA ladders in the KATO III cells treated with 200 μM blazein were observed from day 2, while those in the LU99 cells were observed from day 3 after the addition of 200 μM blazein to the culture.

In contrast, we observed no induction of apoptosis by blazein in normal lymphocytes prepared from healthy volunteers (Fig. 5). Non-cancerous lymphocytes prepared from healthy volunteers were not affected by blazein, suggesting that the activities of these compounds are specific for cancer cells and would not be destructive to healthy tissues.

In conclusion, during apoptosis several characteristic morphological changes are induced in the cytoskeleton (leading to bleb formation) and in the nucleus (chromatin condensation and nuclear fragmentation). The search for better cancer chemotherapeutic agents is ongoing. A strategy to selectively induce apoptosis of stomach cancer cells without altering healthy cells is a significant objective for the development of new therapeutic techniques.

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