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Research Article

Anti-Cancer Effects of Blazein on the Proliferation and Expression of Tumor Suppressor P53 and TNF-α in Human Lung Cancer Cells

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Abstract

Blazein was a new steroid that isolated from Agaricus blazei Murrill and identified by field desorption and electron ionization mass spectrometry. The potential cell growth inhibition by Blazein in cell proliferation was investigated by MTT assay. The effects of Blazein on the anti-proliferation and induce tumor suppression p53 and TNF-α on human lung A549 cell have been investigated. The inhibitory effect of Blazein on the proliferation of lungA549cells increased significantly at 24 hours and 48 hours at both in the 0.1 mM and 0.2 mM concentrations when compared with the control group in a concentration- and time-dependent manner. Western blot analysis revealed that the tumor suppressor p53 was up-regulated compared with control cells. P53 was increases in the Blazein groups suggesting that Blazein may enhance the anti-cancer effect in A549 cells significantly. The results of immunocytochemistry revealed that the expression levels of p53 and TNF-α were significantly elevated in the cytoplasm of tumor cells treated with Blazein. These results found that the new steroid, Blazein, has the inhabitation effects on the proliferation of cancer cell. Blazein induce tumor suppressor p53, also as TNF-α that suggests that Blazein has a specific role in strategy to prevention cancer, support further investigation on the anti-cancer effects of Blazein.

Keywords: Blazein; Agaricus Blazei Murrill; Himematsutake; Tumor Suppressor p53; Proliferation; TNF-α

Introduction

Lung cancer is one of the most common malignant tumors that exhibiting the highest mortality rate worldwide nowadays [1]. In Japan, lung cancer has the first highest morbidity rate among the malignant carcinoma. Secondhand smoke exposure had an increased risk of lung cancer mortality in Japanese wives [2]. Blazein was isolated from mushroom, used a folk remedies in Brazil, and shows pharmacologically tumoricidal activity in mouse tumor model [3-5]. The apoptotic bodied were clearly recognized in the colon cancer COLO201 cells that treated with 200 μM Blazein at 3 days. After 3 days, the culture colon cancer cells reduced due to apoptosis. Recent studies have proposed that Agaricus blazei has an antitumor effect on oncogenic K-ras^{G12V} driven lung adenocarcinoma genesis in mice [6].

P53 plays a key role in tumor suppression. Human p53 is defined as the guardian of the genome because its ability to control the expression of several genes and miRNAs affecting cellular processes, DNA repair, apoptosis, autophagy, proliferation, metabolism, and migration [7]. It was widely believed to mediate the main function of p53 in tumor suppression [8]. In the malignant change of pulmonary epithelial cells is accumulation of genetic and molecular alteration due the key mechanisms of proliferation and apoptosis. More crucially, tumor suppressor genes p53 inactivation at genetic, epigenetic lever eliminates important constraints on cell division at G1-check-point increasing cell proliferation. P53 inactivation may nullify both G1-arrest control and apoptosis, thus accelerating clonal expansion. TNF-α is also a powerful cytokine with critical roles in innate and adaptive immunity, and it is released pre-formed and/or induced in response to many pathogenic stimuli initially in the vanguard of the inflammatory cytokine factors [9,10]. In recent years, there has been interested in the parallels between chronic inflammation and cancer [11]. Therefore, it is no surprise that TNF-α has become a focus of this research.

To the best of our knowledge, the effects of Blazein on lung cancer cells have not yet been reported. In this study, the human non-small cell lung cancer A549 cell line was used to examine the effect of Blazein on the anti-proliferation and induce tumor suppression p53 and TNF-α on this cell. The underlying molecular mechanism was also elucidated to provide evidence of the potential clinical application of Blazein in tumor therapy.

Methods

Preparation and Purification of Blazein

Briefly, fresh fruiting bodies of Agaricus blazei Murrill (2.5 kg) were homogenized and extracted with acetone (5 L) as described in our previous study [5]. The extract was concentrated and partitioned into ethyl acetate and water layer. The ethyl acetate layer was concentrated and then washed with saturated $NaHCO_3$ solution and 1 M HCl, and dried with Na_2SO_4. The residue (7.6 g) obtained after removing the ethyl acetate was chromatographed on the column of silica gel (Wako Gel C200, 400 g) and separated into acidic, basic and neutral fractions. Repetitive chromatography of the neutral fractions

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using silica gel afforded Blazein (200 mg).

For the preparation of Blazein, high performance liquid chromatography (HPLC) was performed with a JASCO 8870-PV. 1H-nuclear magnetic resonance spectra were taken with JEOL, JNM-500 (Tokyo, Japan). Mass Spectrometry (MS) was performed using a Hitachi M-80 instrument (Tokyo, Japan). Blazein as described in our previous work [12,13] and used in this study (Figure 1).

Cell Culture and MTT Assay

The human non-small cell lung cancer A549cell line was cultured in a complete culture solution (RPMI-1640, $1x10^5$ cells/ml, RIKEN BioResource Center, Tsukuba, Japan) were supplemented with 10% FBS and stored at 37°C in a 5% CO_2 incubator. A549 cells at the logarithmic growth phase were randomly divided into 3 groups: Control, Blazein 0.1 mM, Blazein 0.2 mM. The anti-proliferative effect of Blazein on the A549 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazokium bromide (MTT) assay. Cells were plated at density of 2 x 10^3 cells/well into 96-well plate, treated with Blazein at various concentrations (0.1 mM, 0.2 mM), and incubated for 24 hours. Then, 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) was added to each wells, followed by incubation for 4 hours a 37°C. The medium was removed and replaced with 150 μl DMSO, and absorbance values were measured at 570 nm on a Bio-Rad model 680 microplate reader (Bio-Rad laboratories, Hercules, CA, USA). The inhibition rat was calculated as follows:

Inhibition rat (%) = (1-(absorbance of experimental group/ absorbance of control group)) x100

Western Blot Analysis for p53 Expression

After treatment with Blazein for 24 hours and 48 hours, cells were harvested and lysed using RIPA buffer (Cell Signaling Technology Inc., MA, USA) supplemented with phenylmethylsulfonyl fluoride (PMSF, Nacalai Tesque Inc.). Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (0.45 μm, Millipore). The membranes were blocked with Tris-buffered saline (TBST) containing 0.1% Tween-20 (Nacalai Tesque Inc.) and 5% bovine serum albumin (BSA, Sigma-Aldrich, Saint Louis, MO, USA), and incubated overnight at 4°C with anti-p53 rabbit polyclonal primary antibody (ab131442, abcam, USA). After washing with TBST, the membranes were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, Santa Cruz Biotechnology Inc., CA, USA) for 1 hour at room temperature, and finally developed with an electrochemiluminescence system (ECL, GE Healthcare, Little Chalfont, UK). Protein bands were detected using a LAS400 mini (Fujifilm, Tokyo, Japan), and band intensities of Western blots were quantitatively measured by calculating integrated grayscale densities in consistently sized windows incorporating each band using Image J software (Ver. 1.48). We used the ratio of the gray value of the target protein to the gray value of the β-actin as the final result, to produce a semi-quantitative analysis. All experiments were performed in triplicate.

Immunocytochemical analysis of p53 and TNF-α

Human lung cells were cultured overnight on culture slides (BD Falcon, Franklin Lakes, USA), with 2.5 x 104 cells/500 μl/well at 37°C in CO_2 incubator. After culture for 24 hours and 48 hours, the cells were fixed with 4% (v/v) formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and washed with PBS 3 times. The cells were treated with 1% (v/v) Triton X-100 for 20 min and then incubated with 5% (w/v) skim milk for 60 min at room temperature. Double immunofluorescence was performed to examine the co-localization of p53 and TNF-α on cells, by incubation with anti-p53 rabbit polyclonal primary antibody (1:400, ab131442, abcam, USA) and goat polyclonal anti-TNF-α antibodies (1:400, Santa Cruz Biotechnology) overnight at room temperature. Then the cells were incubated for 1 hours with the fluorescent secondary antibodies Alexa594-labeled donkey anti-rabbit IgG and Alexa488-labeled donkey anti-goat IgG antibodies (1: 400 each, Abcam Biotechnology, Cambridge, UK). The nuclei were stained with DAPI and the stained cells were examined under a florescent microscope (BX52, Olympus).

Results

Blazein inhibits cell proliferation in the human lung cancer A549 cell line

To examine the potential cell growth inhibition by Blazein in cell proliferation, we analyzed the effect of blazein on cell proliferation by MTT assay. A549 cells were treated with various Blazein concentrations. As shown in Figure 2a, 2b, the inhibitory effect of Blazein on the proliferation of A549 cells significantly increased at 24 hours and 48 hours at both in the 0.1 mM and 0.2 mM concentrations when compared with the control group. The results showed that Blazein significantly inhibited cell growth in lung cancer cell line in a concentration- and time-dependent manner (Figure 2). The findings suggest that Blazein exerts a significant influence on A549 cell line proliferation.

Blazein affects the expression of p53 protein in A549 cell line

To elucidate how Blazein affects the cell proliferation in A549 cells, tumor suppressor p53 protein was examined. After 24 hours or 48 hours treatment with Blazein, Western blot analysis revealed that the tumor suppressor p53 was up-regulated compared with control cells (Figure 3). P53 was significant increases in the Blazein groups. Those results suggest that Blazein may enhance the anti-cancer effect in A549 cells.

Blazein increase the expression of p53 and TNF-α in A549 cell line

A549 cells were treated with 0.1 mM and 0.2 mM of Agaricus blaze for 24 hours and 48 hours, and the double-stained with anti-p53

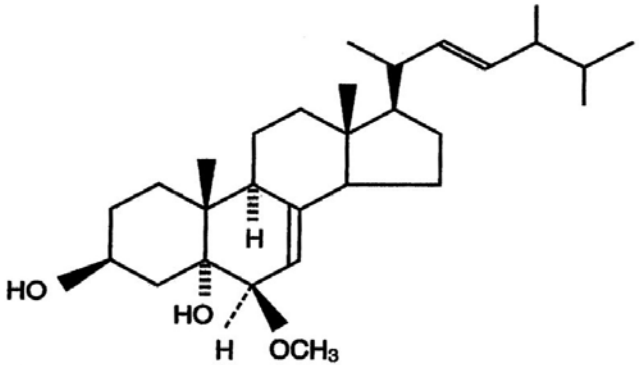

Figure 1: Chemical structure of Blazein.

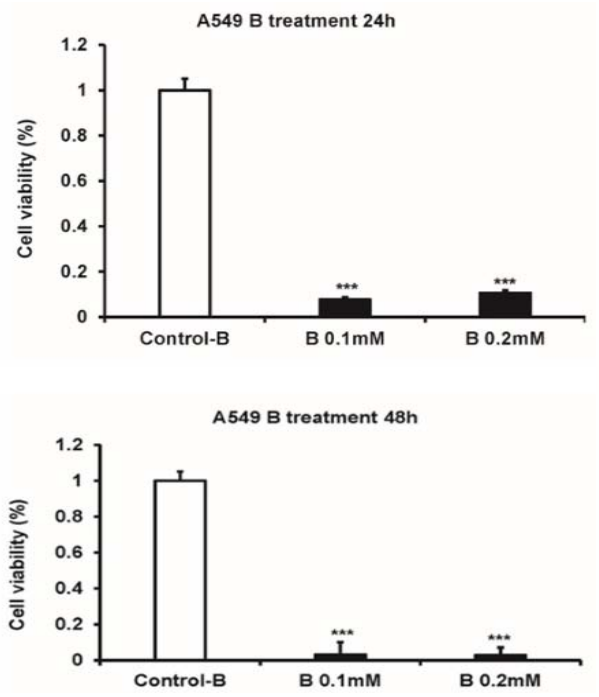

Figure 2: Effect of Blazein inhibited proliferation of human lung cancer A549 cell line on 24 hours and 48 hours. ***P<0.01 *vs.* control group.

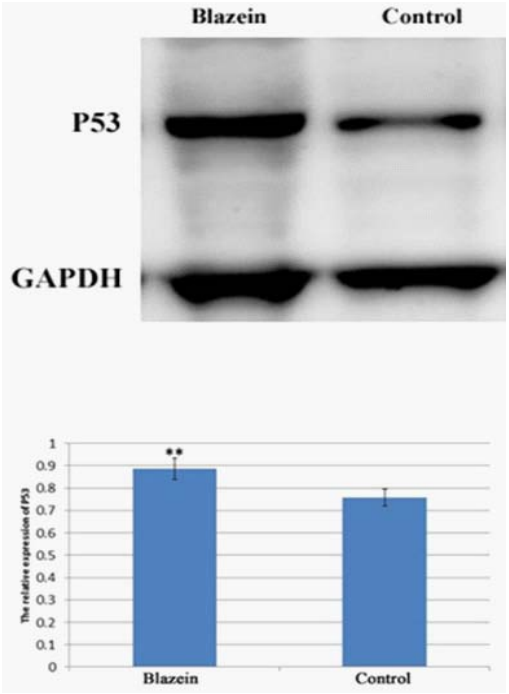

Figure 3: Effect of Blazein on the expression of p53 protein in human lung cancer A549 cell line. Values are expressed as the mean ± standard deviation; n=3. **P<0.01 *vs.* control group.

antibody and TNF-α antibody. The results of immunocytochemistry revealed that the expression levels of p53 and TNF-α were significantly elevated in the cytoplasm of tumor cells treated with Blazein, when

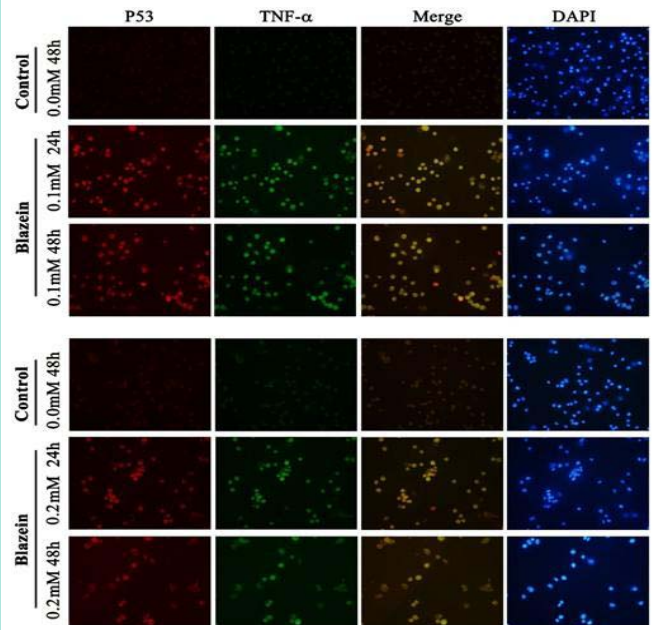

Figure 4: Co-localization of p53 and TNF-α in Blazein-treated human lung cancer A549 cell line. A549 cells were treated with 0.1 mM and 0.2 mM Blazein for 24 hours and 48 hours, and then double immunofluorescent analysis was performed to examine the co-localization of p53 and TNF-α as described in Methods. Stained cells were examined by a fluorescent microscope.

compared with the control group (Figure 4). In addition, DAPI fluorescence staining revealed that the numbers of p53 or TNF-α immuno-positive cells were increased with Blazein administration, and that the number of immuno-positive cells were significantly higher than in the control group (***P<0.01).

Discussion

In our previous studies, we demonstrated blazein induces cell death and morphological change indicative of apoptotic chromatin condensation in human lung cancer and stomach cancer cells [12]. The findings in our studies suggest that growth inhibition of tumor cells by Blazein results from the induction of apoptosis pathway. The present study demonstrates that Blazein, a new natural product prepared from Agaricus blazei Murrill, has a significant inhibit cell proliferation on human lung cancer cells and provides the new evident that Blazein has an inhibition effect on cancer cells.

P53 is one of the most widely studied tumor suppressor genes and play an important role in cell cycle regulation [7, 14]. Inactivation or a mutation in p53 can damage its DNA-binding properties and transcription factor function, thereby driving tumorigenesis. In the present study, treatment with Blazein for 24 hours and 48 hours, the result indicated that Blazein exerted a strong inhibitory effect on the proliferation of A549 cell in terms of suppression of tumor growth in a concentration- and time-dependent manner. The tumor suppressor p53 is an isoform of a protein encoded by homologous genes in various organisms. This homolog is crucial in multicellular organisms, where it prevents cancer formation, and thus functions as a tumor suppressor that bind to DNA and regulate gene expression to prevent mutations of genome. Our previously research reported that Blazein shows pharmacologically tumoricidal activity in mouse

tumor model [3-5]. The apoptotic bodied were clearly recognized in the colon cancer COLO201 cells that treated with 200μM Blazein at 3 days. Similar to this, this study indicated that Blazein treatment up-regulate the tumor suppressor p53 compared with control cells by Western blot analysis. P53 was significant increases in the Blazein groups. Those results suggest that Blazein may enhance the anti-cancer effect in A549 cells.

In conclusion, these results indicated that the new steroid, Blazein, has the inhabitation effects on the proliferation of cancer cell. Blazein induce tumor suppressor p53, also as TNF-α that suggests that Blazein has a specific role in strategy to prevention cancer, and thus, support further investigation on the anti-cancer effects of Blazein.

Availability of Data and Materials

The results used and analyzed during the current study are available from the corresponding author on reasonable request.

Author's Contributions

HIh: Preparation and analyzed Blazein and completed the draft. YA: Contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. MF: Contributed to the conception and supervision of the study, and the critical revision of the manuscript. EO: Contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data. HI: Contributed to the study conception and design, study supervision and critical revision of the manuscript. All authors read and approved the final manuscript.

References

1. Fang JY, Dong HL, Wu KS, Du PL, Xu ZX, Lin K. Characteristics and Prediction of Lung Cancer Mortality in China from 1991 to 2013. Asian Pac J Cancer Prev. 2015; 16: 5829-5834.
2. Hori M, Tanaka H, Wakai K, Sasazuki S, Katanoda K. Second hand smoke exposure and risk of lung cancer in Japan: a systematic review and meta-analysis of epidemiologic studies. Jpn J Clin Oncol. 2016; 47: 942-951.
3. 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: 277-284.
4. Itoh H, Ito H, Amano H, Noda H. Inhibitory action of a (1-->6)-beta-D-glucan-protein complex (F III-2-b) isolated from Agaricus blazei Murill ("himematsutake") on Meth A fibrosarcoma-bearing mice and its antitumor mechanism. Jpn J Pharmacol. 1994; 66: 265-271.
5. Kawagishi H, Inagaki R, Kanao T, Mizuno T, Shimura K, Ito H, et al. Fractionation and antitumor activity of the water-insoluble residue of Agaricus blazei fruiting bodies. Carbohydr Res. 1989; 186: 267-273.
6. Saito H, Hiroko Itoh, Fujishima M, Okumura E, Suzuki N, Ito H. Antitumor effect of glucomannan (ATOM) from the mycelium of liquid-cultured agaricus blazei murril on oncogenic K-ras driven lugh adenocarcinoma genesis in mice. Jpn Pharmacol Ther. 2018; 46: 1495-1501.
7. Reed SM, Quelle DE. p53 Acetylation: Regulation and Consequences. Cancers (Basel). 2014; 7: 30-69.
8. Liu J, Zhang C, Hu W, Feng Z. Tumor suppressor p53 and metabolism. J Mol Cell Biol. 2018; 11: 284-292.
9. Hehlgans T, Pfeffer K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor super family: players, rules and the games. Immunology. 2005; 115: 1-20.
10. Varfolomeev EE, Ashkenazi A. Tumor necrosis factor: an apoptosis JuNKie? Cell. 2004; 116: 491-497.
11. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor super families: integrating mammalian biology. Cell. 2001; 104: 487-501.
12. Itoh H, Ito H, Hibasami H. 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. Oncol Rep. 2008; 20: 1359-1361.
13. Itoh H, Fujishima M, Arakawa Y, Hibasami H, Nakada F, Ito H. Blazein induces apoptosis by a generation of active oxygen in human lymphoid leukemia molt 4B cells. 2014; 157: 597-602.
14. Brambilla E, Brambilla C. p53 and lung cancer. Pathol Biol (Paris). 1997; 45: 852-863.