Betulinic Acid Induces Apoptosis in Human Mucoepidermoid Carcinoma Cells Through Regulating Specificity Protein 1 and Its Downstream Molecule, Survivin

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ABSTRACT - High-grade mucoepidermoid carcinomas (MECs) have difficulty in cure and 5-year survival rate is quiet low. Therefore, we need new therapeutic agents and molecular targets. Betulinic acid (BA) is one of the materials which is easily found in the world and shows tumor-suppress effects in various tumor types. In addition, many kinds of normal tissues have a resistance to BA treatment. In this study, we investigated the anti-proliferative activity of BA and its molecular targets in MC-3 human MEC cells using western blot analysis and DAPI staining. BA inhibited cell viability and induced apoptosis in MC-3 cells. It affected Specificity protein 1 (Sp1) and its downstream molecule, survivin whereas it did not affect myeloid cell leukemia-1 (Mcl-1). Therefore, we suggest that BA can be a potential anti-cancer drug candidate regulating Sp1 and survivin to exert apoptotic cell death.

Key words: Betulinic acid, Apoptosis, Mucoepidermoid carcinoma, Specificity protein 1, survivin

Introduction

Mucoepidermoid carcinoma (MEC) is the most frequently occurring primary malignancy of salivary glands in all age patients, representing 5% of whole salivary gland tumors and 35% of whole malignant tumors¹⁻³. Low-grade MECs rarely recur; after surgery, most patients are fully cured and the 5-year survival rate is 76-95%. Conversely, high-grade MEC is aggressive tumor that may have an invasive form of growth, recur and metastasize, and its 5-year survival rate is only 30-50%⁴. Therefore, it is important to find a new treatment agent and a potential molecular target for therapeutic strategies in MEC.

Natural materials have been used to treat human diseases for thousands of years⁵. Betulinic acid (BA) is a naturally occurring pentacyclic triterpenoid of the lupane class that belongs to the group of terpenes and is found abundantly in bushes and trees, forming the principal extractive (up to 30% of dry weight) of the bark of birch trees⁶⁻⁹. BA inhibits the growth of several tumors but normal tissues were shown to have the resistance to BA treatment in vitro⁶⁻¹⁰. Therefore, we regarded BA as a good potential anti-cancer drug candidate which has therapeutic effects with less side effects. In previous studies, the anticancer activity of BA was subsequently reported in several types of human malignancies, including head and neck tumors¹¹. However, there is no study for MEC with BA. Therefore we performed therapeutic effects of BA in MEC.

Materials and Methods

Reagents

The antibodies for Poly (ADP-ribose) Polymerase (PARP) (Asp175), cleaved caspase-3(Asp), Mcl-1 and survivin were obtained from BD Biosciences (San Jose, CA, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively. Antibodies for Sp1 and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Betulinic acid (BA) was purchased by Alexis (San Diego, CA, USA). 4′-6-Diamidino-2-phenylindole (DAPI) was acquired from Sigma-Aldrich Chemical Co. (St.Louis, MO, USA).

Cell Culture and Chemical Treatment

MC-3 human MEC cells were provided by Professor Wu Junzheng (Forth Military Medical University, Xi’an, China). MC-3 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/mL each of
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penicillin and streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. Equal numbers of cells were seeded and allowed to attach. When the cells reached 50-60% confluence, the cells were treated with DMSO or various concentrations of BA (7.5, 15 and 30 µM) diluted in DMEM with 5% FBS. BA was dissolved in 0.1% DMSO (vehicle control).

**DAPI staining**

Apoptotic cell death was determined morphologically using a fluorescent nuclear dye, DAPI. DAPI staining showed the number of apoptotic cells with chromatin condensation and nuclear fragmentation. MC-3 cells were incubated with DMSO or various concentrations of BA (7.5, 15, and 30 µM) for 24 h, then harvested by trypsinization, and fixed in 70% ethanol overnight at 4°C. The next day, the cells were stained with DAPI, deposited onto the slides, and finally viewed to detect apoptotic characteristics with a fluorescent microscope.

**Western blot analysis**

MC-3 cells were grown in 60 mm² dishes and treated with DMSO or BA. Whole-cell lysates were extracted and quantified using a protein assay kit. Total protein from each sample was mixed with 5 µl loading buffer and heated at > 90°C for 5 min. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto poly (vinylidene fluoride) (PVDF) membrane (Bio-Rad Laboratories). The membranes were blocked with 5% skim milk in TBST buffer at room temperature for 2 h, washed with TBST, and incubated overnight at 4°C with primary antibody. Washing was done with TBST, and incubation with horseradish peroxidase (HRP)-conjugated secondary antibody was done at room temperature for 2 h. After washing with TBST, detection was performed with ECL Western blotting reagents (Santa Cruz, CA). For loading control, the membranes were stripped and reprobed with HRP-conjugated anti-β-actin.

**Statistical Analysis**

The data were assessed for statistical significance using a Student’s t test. A value of p < 0.05 compared to the vehicle control was considered to be statistically significant.

**Results**

**BA Decreases the Cell Viability of MC-3 Cells**

To investigate the growth-inhibitory effect of BA in MEC, MC-3 cells were treated by DMSO or various concentrations of BA. The result showed that the number of cells decreased compared with the vehicle control in a concentration-dependent manner (Fig. 1A and B). We suggest that BA inhibits MC-3 cell growth.

**BA induces apoptosis in MC-3 cell line**

To examine whether BA-induced growth inhibition was occurred through apoptosis, we determined the level of PARP and caspase-3 cleavage, both of all present features of cells

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![Fig. 1. Cell growth inhibition caused by BA in human mucoepidermoid carcinoma (MC-3) cell line. Cells were treated with DMSO (vehicle control) or various concentrations of BA (7.5, 15, and 30 µM) for 24 h. A, Cell viability was determined by cell photo taken by optical microscope after 24 h (magnification ×200). B, Cells were counted using a hemocytometer (0.1-mm-deep). The results are expressed significance (*P < 0.05) compared to control group is indicated.](image)

![Fig. 2. Apoptotic effects are induced by BA in MC-3 cells. Cells were incubated with DMSO (vehicle control) or BA (7.5, 15 and 30 µM) for 24 h. Then, cells were harvested and prepared for Western blotting as described under Materials and Methods. Apoptotic marker protein, PARP and cleaved Caspase-3 in whole cell lysates, was detected by Western blotting, and actin was used to normalize the protein loading from each treatment.](image)
undergoing apoptosis, by Western blot analysis. After BA treatment for 24 hr, total PARP was decreased and cleaved Caspase-3 was detected in MC-3 cells (Fig. 2). Thus, these results indicated that BA induced apoptosis in MC-3 cells suggesting that the growth inhibition of MC-3 cells by BA is due to apoptotic cell death. To confirm whether BA induced apoptosis in MC-3 cell line, DAPI staining was performed. The results showed the fragmentation and condensation of nucleus in the cells treated with BA (7.5, 15 and 30 µM) for 24 h compared with the DMSO-treated cells (Fig. 3). Overall, these findings (Fig. 2 and 3) suggested that BA could induce apoptosis in MC-3 cells.

**BA inhibits anti-apoptotic protein Sp1 and its downstream protein, survivin, in MC-3 cells**

In previous studies, Specificity protein 1 (Sp1) might be a potential molecular target for various cancer treatment\(^{13}\). We performed whether BA affects Sp1 protein to induce apoptotic cell death in MC-3 cells. As shown in Figure 4, BA-treated MC-3 cells were exhibited the decrease of Sp1 expression in a concentration-dependent manner. This finding showed that Sp1 may be related with apoptosis by BA in MC-3 cells. Survivin and Myeloid cell leukemia-1 (Mcl-1) are downstream proteins of Sp1\(^{14,15}\). We investigated the effect of BA on survivin and Mcl-1 in MC-3 cells treated with BA for 24 hr. The results showed that BA decreased survivin but not Mcl-1 protein expression (Fig. 4). We can infer that Sp1 and survivin are related with apoptosis by BA in MC-3 cells.

**Discussion**

MEC is one of the most common malignant neoplasm of the salivary glands, exhibiting variable growth pattern and morphological diversity\(^{16,17}\). Therefore the finding of a new potent, less toxic anti-cancer agent is important. BA is contained in various plants throughout the plant kingdom and, hence, in the whole world and features potent antitumor activities\(^{18,19}\). Moreover, normal cells that have different origins reported to have resistance to BA than tumor cells\(^{20,21}\). Therefore, we regarded BA as an ideal agent which has therapeutic effects and less toxicity for cancer treatment. Objectives of this study are finding a therapeutic agent candidate, researching its effects in MC-3 cells and finding out molecular targets.

First of all, we examined the cell growth inhibition of BA in MC-3 cells. After treatment of various concentrations of BA in MC-3 cells, we can find the decrease of cell number and cell viability of MC-3 cells in a concentration-dependent manner. Then, we confirmed that the decrease of MC-3 cells by BA is due to apoptosis mechanism with apoptotic marker PARP and cleaved caspase-3. Also we found DNA fragmentation and condensation of MC-3 cells with BA treatment by DAPI staining. These results showed that BA induces apoptosis in MC-3 cells.

Specificity protein 1 (Sp1) is one of the members of transcription factor family that bind GC/GT-rich promoter elements\(^{22}\). Sp1 plays critical role in regulating cell differentiation, cell cycle and apoptosis-related genes influencing cell viability and growth\(^{23}\). In addition, Sp1 regulates thousands of genes, such as those encoding vascular endothelial growth factor (VEGF), p21CIP1/WAF1, 12(S)-lipoxygenase, phosphatase 2A (PP2A), and Sp1 itself\(^{23}\). Moreover, Sp1 levels

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**Fig. 3.** BA induced apoptotic cell death in MC-3 cells. Cells were incubated with DMSO (vehicle control) or BA (7.5, 15 and 30 µM) for 24 h. Then, cells were harvested and prepared for DAPI staining. DNA fragmentation and nuclear condensation were detected by fluorescence microscopy (magnification ×400). DNA fragmentation and nuclear condensation were quantified. \(*p < 0.05\) compared to the DMSO treatment group.

**Fig. 4.** The effect of BA on the regulation of Sp1, survivin and Mcl-1 protein expression. Cells were incubated with DMSO (vehicle control) or BA (7.5, 15 and 30 µM) for 24 h. Then, cells were harvested and prepared for Western blotting as described under Materials and Methods. Sp1, survivin and Mcl-1 protein in whole cell lysates, was detected by Western blotting, and actin was used to normalize the protein loading from each treatment.
 Werkclass, 브텍산드로조나드 효능과 MC-3 세포주에서의 분자 표적이 확인하고자 하였다. BA는 MC-3 세포주에서 세포생존을 저해하였고 세포사멸을 유도하였다. BA는 Sp1과 그의 하향 분자 표적인 survivin에 영향을 주었으나, 다른 하향 분자 표적인 Mcl-1에서는 유의한 변화를 일으키지 못하였다. 따라서, BA는 Sp1과 survivin을 조절하여 세포사멸을 일으키는 장애적인 항암제 후보가 될 수 있을 것이라 사료한다.

References


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