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Betulinic Acid, a Naturally Occurring Triterpene found in the Bark of the White Birch Tree induces Apoptotic Cell Death in KB Cervical Cancer Cells through Specificity Protein 1 and its Downstream

Ji-Ae Shin[1], Eun-Sun Choi[1], Ji-Youn Jung[2], Nam-Pyo Cho[1], and Sung-Dae Cho[1]*

[1]*Department of Oral Pathology, School of Dentistry, Institute of Oral Bioscience, Brain Korea 21, Chonbuk National University, Jeonju 561-756, Korea*
[2]*Department of Companion and Laboratory Animal Science, Kongju National University, Yesan, Korea*
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ABSTRACT - Betulinic acid (BA), a naturally occurring triterpene found in the bark of the white birch tree, has been investigated to induce apoptosis in various cancer cells and animal models. However, there is no report of the chemopreventive effect of BA in cervical cancer cells. Using KB human cervical cancer cells as a model, we currently show that BA decreases cell viability and induces apoptotic cell death. The mechanism of the BA-induced anti-growth response in KB cells is due to the down-regulation of specificity protein 1 (Sp1) and its downstream targets, myeloid cell leukemia-1(Mcl-1) and survivin. Thus, BA acts as a novel chemopreventive agent through the regulation of Sp1 that is highly expressed in tumors.

Key words: Betulinic acid, Chemoprevention, Apoptosis, Cervical cancer

Cervical cancer is the second most popular malignancy in women in the world[1]. Although wide-spread implementation of diagnostic screening programs with increased percentages of patients having early lesions improves overall survivals of cervical cancer patients, the prognosis for advanced cancer still remains poor[2,3]. Thus, the development of new strategies through identifying potential targets is warranted.

Specificity protein 1 (Sp1) is an essential transcription factor for many genes necessary for the regulation of multiple aspects of tumor cell survival and growth and. The abnormal expression of Sp1 may contribute to cancer development and progression. It was also reported that Sp1 is strongly expressed in tumor tissues and the chemicals which attenuate the expression of Sp1 protein can inhibit tumor cell growth in pancreatic and esophageal cancer cells[4,5]. Recently, our group also reported that the down-regulation of Sp1 protein by tolfenamic acid inhibits the growth of human cervical cancer cells by the induction of apoptotic cell death. It suggests that Sp1 may be a good molecular target for the prevention of cervical cancer as well as other cancers and the development of the compounds targeting Sp1 as a novel class of chemopreventive agents could be meaningful for the treatment of cervical cancer.

Betulin is a lupine-derived triterpene that is in high concentration in birch bark and betulinic acid (BA); oxidation product of betulin has also been detected in bark extracts[6]. It is now known to be a component of various other plants that are widespread in tropical regions. BA was initially characterized as a highly selective inhibitor of human melanoma cell and tumor growth through the induction of apoptosis[7]. In addition, many previous studies over the past decade have reported that BA can induce apoptosis to inhibit cancer growth in various cancers including neuroblastomas, glioblastomas, gliomas and prostate cancer[8-11]. However, there is no report of the anticancer activity of BA in KB human cervical cancer cells. Therefore, we determine whether BA affects the proliferation of KB cells and evaluate unique mechanism of action of this compound.

Materials and Methods

Reagents

The antibodies for Poly(ADP-ribose) Polymerase (PARP) (Asp175), 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 pur-

*Correspondence to: Sung-Dae Cho, DVM PhD, Department of Oral Pathology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju 561-756, Korea
Tel: 82-63-270-4027, Fax: 82-63-270-4025
E-mail: efiwdsc@chonbuk.ac.kr

chased by Alexis (San Diego, CA, USA). 4'-6-Diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Cell Culture and Chemical Treatment

The KB human cervical cancer cells (American Tissue Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin (WelGENE Inc., Daegu, Korea) and 100 μg/mL streptomycin (WelGENE Inc., Daegu, Korea) in a humid atmosphere of 5% CO_2. Equal numbers of cells were seeded and allowed to attach overnight. The cells were treated with betulinic acid (BA; 2.5, 5 and 10 μM) diluted in DMEM with 5% FBS for 24, 48 and 72 hr.

MTS assay

The effects of BA on cell viability were estimated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cells were seeded in 96-well plates and incubated with various concentrations of BA. After the treatment with BA for 24.48 and 72hr, 30 μl of MTS (3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) solution was added to each well and the cells were incubated for 2hr at 37ºC. MTS solution was analyzed using a microplate reader (BioTeck Instruments Inc., Winooski, VT, USA) at 490 nm and 690 nm (background).

Western blot analysis

Whole-cell lysates were extracted with lysis buffer and quantified with *DC* Protein Assay (Bio-RAD). Equal amount of protein from each treatment group were separated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel and the separated proteins were transferred to Immun-Blot™ PVDF membrane (Bio-RAD). The membrane was blocked with 5% skim milk in TBS-T for 2hr at RT and maintained overnight at 4°C with the primary antibody, followed by maintained with horseradish peroxidase-conjugated secondary antibodies. The antibody-bound proteins were detected using an ECL Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.).

Detection of morphological apoptosis

Cell death was measured by DAPI staining (Sigma Chemical Co, MO). After an experimental treatment with 2.5, 5 and 10 μM of BA for 72 hr, KB cells were harvested by trypsinization and fixed in 4% paraformaldehyde at RT for 20 min. The cells were resuspended in PBS, deposited them on poly-L-lysin-coated slides, stained with a DAPI solution (2 μg/ml) and viewed under a fluorescence microscope.

Statistical Analysis

Statistical significance was assessed using a Student's t-test. A value of $p < 0.05$ compared to the solvent control was considered statistically significant.

Results and Discussion

The growth-inhibitory effect of betulinic acid (BA) in KB cells

BA was tested *in vitro* for its potential human cervical cancer cell growth inhibitory effect on KB cell lines using MTS assay which widely used to quantify cell viability. The results, summarized in Fig. 1, showed that BA exhibited an inhibitory effect on KB cells in a concentration-dependent manner. At higher concentration (10 μM) for 72 hr, the cell viability was 35.5%. Its IC_{50} value in KB cells was determined to be approximately about 7.9 μM. The results suggest that BA has a growth-inhibitory effect on KB human cervical cancer cells. These results are in agreement with other studies using BA in several different cancer cells[12,13].

BA induces apoptosis in oral cancer cell lines.

To examine whether BA induced apoptosis, DAPI staining was performed. The results showed the fragmentation and condensation of nucleus in the cells treated with BA (2.5, 5 and 10 μM) for 72 hr compared to the DMSO-treated cells (Fig. 2A). We next determined the level of poly (ADP)-ribose polymerase (PARP) cleavage by Western blot analysis (Fig. 2B). After BA treatment for 72 hr, cleaved PARP was detected in KB cells. Thus, we clearly showed that BA induced apoptosis in KB cells suggesting that the growth inhibition of KB cells by BA is due to apoptotic cell death.

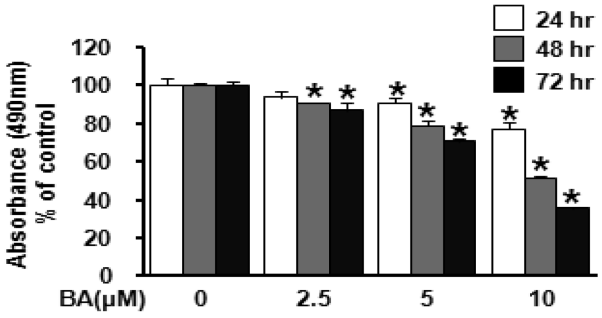

Fig. 1. The effect of BA on the viability of KB cells using MTS assay. KB cells treated with 2.5, 5 and 10 μM of BA for 24, 48 and 72 hr. Cell viability was determined using MTS assay. The graph was representative of three independent experiments and bar is mean ± SD. *$p < 0.05$ compared to DMSO-treated group.

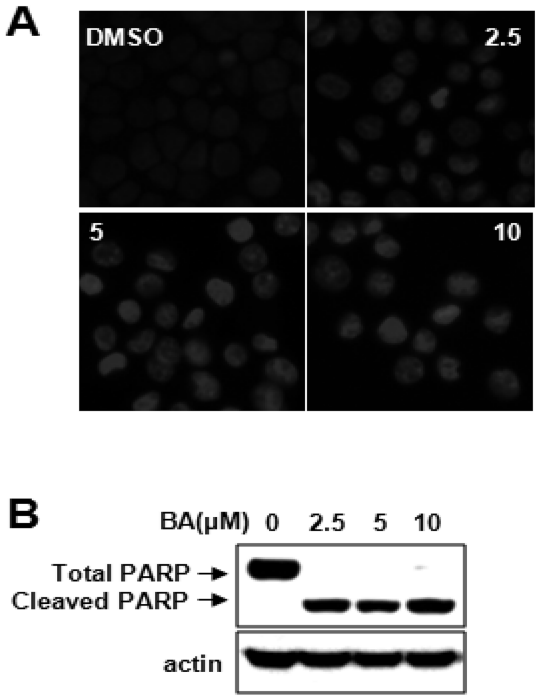

Fig. 2. The apoptotic activity of BA in KB cells after the treatment of BA for 72 hr. A, The effect of BA on the condensation and fragmentation of nucleus in KB cells. Fluorescence microscopy images of DAPI-stained KB cells showing the appearance of apoptotic morphology. B, The effect of BA on the cleavage of PARP in KB cells. The protein levels of cleaved PARP were determined by Western blot analysis. Actin was used to normalize the protein loading from each treatment.

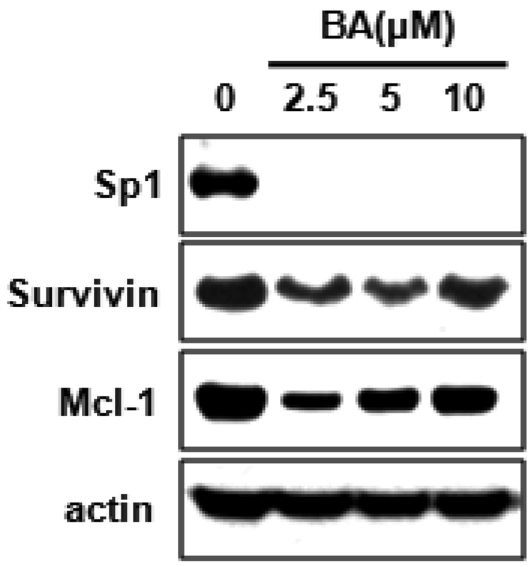

Fig. 3. The effect of BA on the regulation of Sp1, survivin and Mcl-1 protein expression. The protein levels were analyzed by Western blot analysis.

BA inhibits anti-apoptotic protein Sp1 and its downstream proteins in KB cells.

Specificity protein 1 (Sp1) is a member of the mammalian transcription factor family that binds to GC-rich sites containing GC-boxes. Recently, it was reported that Sp1 is highly overexpressed in many human tumors and cancer cell lines[14-18]. In the previous studies, it was also reported that targeting of Sp1 protein is a good treatment strategy for various cancer cells[4,5,12,19,20]. Therefore, we assessed whether BA affects Sp1 protein to induce apoptotic cell death in KB cells. As shown in Fig. 3, BA-treated KB cells exhibited decreased Sp1 expression. These results suggest that BA might inhibit Sp1 protein to exert its apoptotic activity in KB cells. Because BA modulates the expression of Sp1 protein, it is also important to determine the response on key candidates related to apoptosis in its downstream signaling pathway. Survivin and Mcl-1 are composed of highly GC-rich sequences in their promoters, so Sp1 can regulate these proteins. Therefore, the effect of BA on survivin and myeloid cell leukemia-1 (Mcl-1) in KB cells treated with BA for 72 hr was examined. The results showed that BA decreased survivin and Mcl-1 protein expression (Fig. 3). These *in vitro* results suggest that targeting Sp1 protein with BA to inhibit cervical cancer cells is quite effective through the inhibition of its downstream targets related to apoptotic cell death. Recently, our group reported that Sp1 protein can be a potential molecular target in cervical cancer prevention[21] and this study will support that a Sp1-decreasing compound such as BA may be a good candidate for the prevention of cervical cancer.

In summary, the results presented in this manuscript showed for the first time that BA induces apoptotic cell death by regulating the Sp1 protein in KB human cervical cancer cells. BA modulates the expression of several anti-apoptotic proteins, which are dependent on Sp1 proetien. Although we need to study anti-tumorigenic activity of BA in the *in vivo* animal model, all of these results indicate that BA may be a potential chemopreventive agent against cervical cancer.

요 약

흰자작나무의 껍질에서 발견된 자연적으로 발생한 triterpene인 betulinic acid (BA)가 다양한 종류의 암세포와 동물 모델에서 세포사멸을 유도하는 것으로 알려져 있다. 하지만 자궁경부암세포에서 BA의 화학적 암예방 효과는 연구되지 않은 상태이다. 따라서 이 연구에서는 사람 자궁경부암세포주인 KB세포를 이용하여, BA가 세포증식을 감소시키고 세포사멸을 유도하는 것을 확인하였다. KB 세포에서 BA에 의해 유도되는 세포증식의 억제는 specificity protein 1 (Sp1)과 Sp1의 표적단백질인 myeloid cell leukemia-1 (Mcl-1) 그리고 survivin의 감소 때문인 것으로 확인되었다. 따라서 BA는 자궁경부암에서 과다발현되는 Sp1을 조절하는 새로운

화학적 암예방 물질로서 작용할 수 있을 것으로 생각된다.

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