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Barley grass extract causes apoptosis of cancer cells by increasing intracellular reactive oxygen species production

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Abstract. Cancer remains a leading cause of mortality worldwide, therefore food products are being investigated for potential prevention or treatment strategies. The ingredient, barley grass extract (*Hordeum vulgare* L.; Bex) is used to prevent or ameliorate various types of disease. In cancer, Bex has been revealed to inhibit tumor growth. However, its effect on cancer cells is yet to be clearly defined. In the present study, the effect of Bex on cancer cell growth was investigated. Bex inhibited the viabilities of breast and prostate cancer cells according to the results of MTT assays. Accordingly, Bex caused apoptosis, which was confirmed by Annexin V staining and western blot analysis for poly (ADP-ribose) polymerase and caspases. Furthermore, Bex increased the intracellular levels of reactive oxygen species (ROS), and *N*-acetyl-L-cystein blocked Bex-induced apoptosis. Therefore, the study demonstrated that Bex causes apoptosis of breast and prostate cancer cells by increasing intracellular ROS levels.

Introduction

Cancer is one of the leading causes of mortality worldwide (1,2). Despite numerous cancer studies and the development of various anti-cancer therapeutic agents, cancer remains dangerous. Anti-cancer therapeutic agents are chemically or biologically produced, and their effects are well defined (3-7). However, treatments continue to be associated with adverse effects and the majority of patients have an aversion to them (8).

Herbal products have long been used to prevent or treat diseases, including cancer (9-12). Furthermore, certain anti-cancer therapeutic agents that are chemically produced originate from herbal products and their chemical characteristics are modified (7,12-14). Typically, patients prefer to take herbal products (15-18); herbal products have historically been used as traditional medicines, such as traditional Chinese and Korean medicines, Kampo medicines and Ayurvedic medicine (13,14,19). Certain herbal products were demonstrated to treat cancer and/or reduce the side effects of cancer treatment (13,15-17,19-21). Therefore, herbal products are considered to be promising for cancer prevention and treatment.

Barley grass extract (*Hordeum vulgare* L.; Bex) has long been used as a food product. Its biological effects have also been addressed by various *in vitro* and *in vivo* studies, although evidence there is limited evidence of the efficacy of Bex against specific conditions (22). The effect of Bex on the immune system was revealed in *in vitro* and *in vivo* experimental sets (23-25). Accordingly, Bex inhibited atopic dermatitis in NC/Nga mice by altering the expression levels of cytokines (26). Similarly, Bex repressed lipopolysaccharide-induced inflammation (27). Furthermore, its effect in type 2 diabetes was revealed in a genetically engineered mouse model and patients (28,29). Therefore, the effects of Bex on particular diseases have been demonstrated at least in experimental systems. A previous study revealed that Bex caused apoptosis of leukemia and lymphoma cell lines (30); however, its effect in cancer remains unclear.

The present study examined the effect of Bex in different cancer cell lines, including breast cancer MDA-MB-231 cells and prostate cancer DU145 cells. Bex induced apoptotic cell death in MDA-MB-231 and DU145 cells. Furthermore, its effect resulted from an increased intracellular reactive oxygen species (ROS) level. Thus, the current study suggests that Bex may be useful for treating cancer, particularly breast and prostate cancer.

Materials and methods

Cell culture and herbal extract. MDA-MB-231 and DU-145 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Barley grass extract (Bex) was obtained from Chungbuk Agricultural Cooperation (Jecheon, South Korea). Bex was

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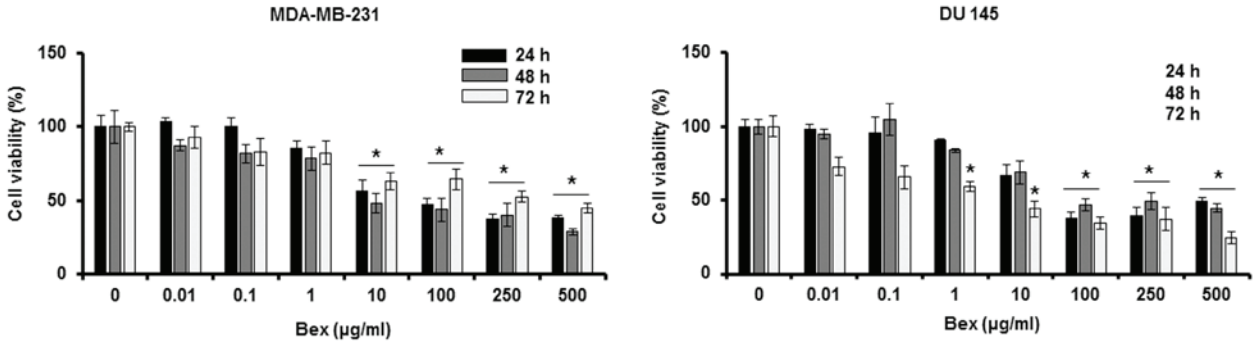

Figure 1. Bex reduces MDA-MB-231 and DU-145 cell viabilities. Cells were treated with different concentrations (0, 0.01, 0.1, 1, 10, 100, 250 and 500 μg/ml) of Bex for 72 h. Cell viabilities were measured at 24, 48 and 72 h. *P<0.05 vs. 0 μg/ml. Bex, barley grass extract.

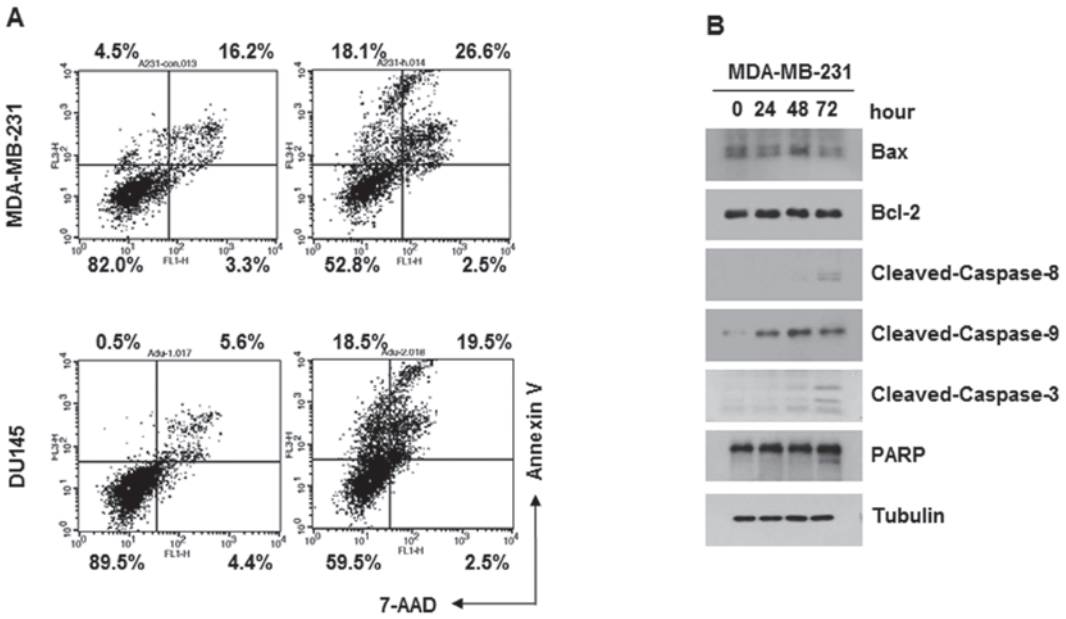

Figure 2. Bex induces apoptotic cell death. (A) Cells were treated with 100 μg/ml Bex for 24 h and subjected to Annexin V-fluorescein isothiocyanate and 7-AAD staining. (B) MDA-MB-231 cells were treated with 100 μg/ml Bex for 24 h and subjected to western blot analysis. Bex, barley grass extract; 7-AAD, 7-aminoactinomycin D; Bax, BCL2 associated X, apoptosis regulator; Bcl-2, B-cell lymphoma 2; PARP, poly (ADP-ribose) polymerase.

solubilized in water containing 0.01% dimethyl sulfoxide (DMSO). Therefore, all control groups in the experiment were treated with 0.01% DMSO.

Cell viability assay. Cell viability was examined using an EZ-CYTOX cell viability/cytotoxicity assay kit (cat. no. EZ-000, Daeil Lab Service, Seoul, South Korea) according to the manufacturer's instructions. Briefly, 100,000 cells per well were cultured in 96-well plates and treated with different doses (0, 0.01, 0.1, 1, 10, 100, 250 and 500 μg/ml) of Bex for 72 h. Cell viability at 24, 48 and 72 h was measured using a microplate reader at a wavelength of 450 nm. Experiments were performed in triplicate and repeated three times independently.

Apoptosis assay. Cells ($3x10^6$) were treated with different concentrations (0, 0.01, 0.1, 1, 10, 100, 250 and 500 μg/ml) of Bex for 24 h and stained with Annexin V-fluorescein isothio cyanate (FITC) and 7-aminoactinomycin D (7-AAD). Apoptotic cell death was determined using BD FACSCalibur flow cyto metry with BD MultiSET software (BD Biosciences, San Jose, CA, USA). For western blot analysis, $1x10^6$ cells were treated with 100 μg/ml Bex for 24 h and lysed with RIPA buffer. Protein (30 μg) was loaded onto SDS-PAGE and transferred to the membrane. After blocking with 5% milk, the membrane was incubated with an appropriate primary antibody for 1 h at room temperature. Anti-poly(ADP-ribose) polymerases (PARP) (cat. no. 9542), anti-cleaved caspase 8 (cat. no. 9496), anti-cleaved caspase 9 (cat. no. 7237), anti-cleaved caspase-3 (cat. no. 9664) and anti-β-tubulin (cat. no. 2146) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Intracellular ROS detection assay. ROS levels were measured using 10 μM 2',7'-dichlorofluorescin diacetate (H2DCF-DA; Molecular Probes; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells ($1x10^6$) were treated with 100 μg/ml Bex for 5 min and treated with H2DCF-DA for a further 24 h. The flow cytometry experiments were conducted in triplicate and repeated three times independently. Sigma-Aldrich *N*-acetyl-L-cystein (NAC; Merck KGaA, Darmstadt, Germany)

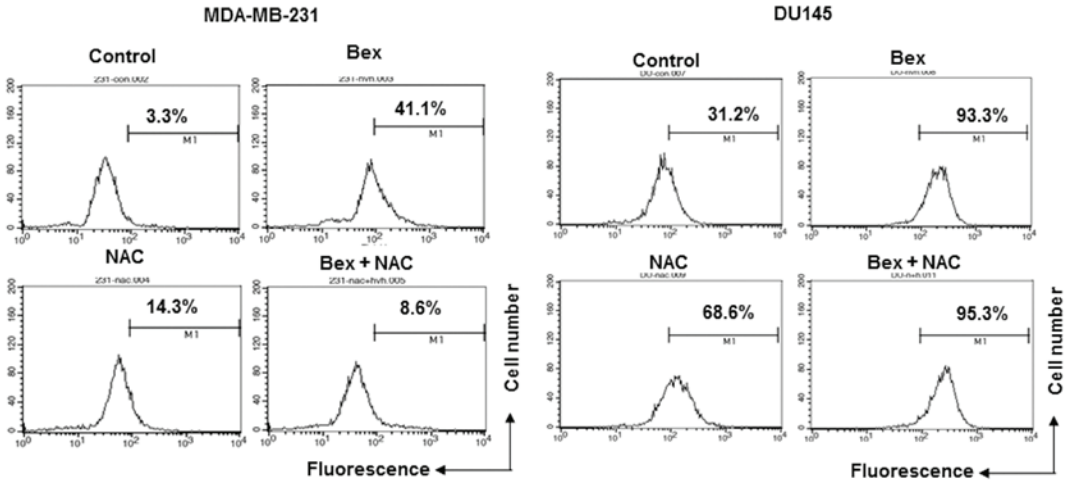

Figure 3. Bex increases intracellular reactive oxygen species level. MDA-MB-231 and DU-145 cells were pretreated with 10 mM NAC for 1 h before being treated with 100 μg/ml Bex for 24 h. Bex, barley grass extract; NAC, N-acetyl-L-cystein.

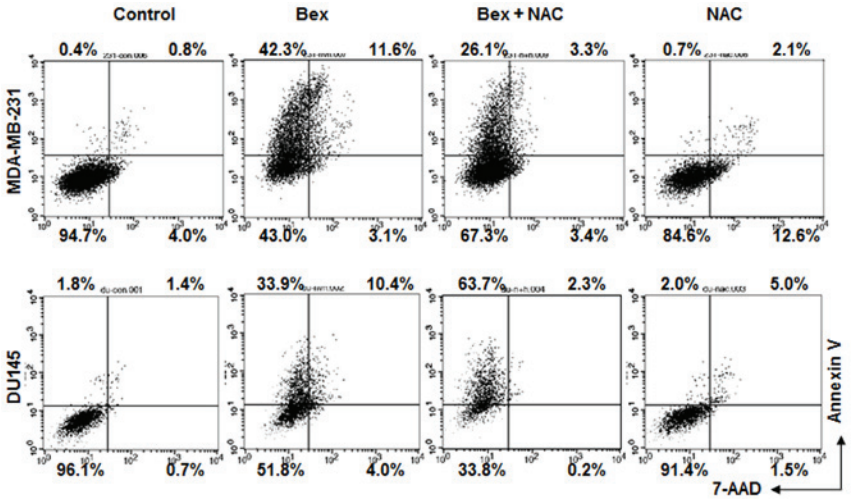

Figure 4. Bex-induced apoptosis requires intracellular reactive oxygen species production. Cells were treated with Bex for 24 h with or without NAC, and then subjected to Annexin V-fluorescein isothiocyanate and 7-AAD staining. Bex, barley grass extract; NAC, *N*-acetyl-L-cystein; 7-AAD, 7-aminoactinomycin D.

at 10 mM was used to inhibit ROS induction. Cells ($1x10^6$) were pretreated with 10 mM NAC for 1 h before being treated with 100 μg/ml Bex for 24 h.

Statistical analysis. All experiments were performed in triplicate and repeated three times independently. Statistical significance was evaluated using Student's t-test and analysis was conducted using SPSS version 24.0 software (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Bex inhibits cancer cell viability. To examine the effect of Bex on cancer cell viability, MDA-MB-231 breast cancer cells and DU-145 prostate cancer cells were treated with different concentrations (0, 0.01, 0.1, 1, 10, 100, 250 and 500 μg/ml) of Bex for 24, 48 and 72 h. Bex reduced the viability of those cancer cells in a dose-dependent manner (Fig. 1). Thus, the MTT assay data indicates that Bex inhibits cancer cell viability.

Bex causes apoptosis of cancer cells. Annexin V assays were performed to examine whether Bex induces apoptosis of cancer cells. MDA-MB-231 or DU-145 cells were treated with 100 μg/ml Bex for 24 h, followed by Annexin V-FITC and 7-AAD. Flow cytometry data indicated that Bex induced apoptosis of the two types of cancer cell (Fig. 2A). Consistently, Bex induced PARP cleavage and caspase activation in the MDA-MB-231 cells (Fig. 2B; data for DU-145 not shown). Thus, the current data indicates that Bex causes apoptosis of cancer cells.

Bex reduces the level of intracellular ROS. To examine whether Bex affects the production of intracellular ROS, MDA-MB-231 and DU-145 cells were treated with 100 μg/ml Bex for 1 h, and the intracellular ROS level was measured by detecting H2DCF-DA fluorescence using flow cytometry. Bex increased the intracellular ROS level in MDA-MB-231 breast and DU-145 prostate cancer cells (Fig. 3).

Bex-mediated increase of intracellular ROS level is critical for apoptosis. Whether Bex-induced apoptosis required

an increase of intracellular ROS level was subsequently examined. Bex-induced apoptosis was blocked in cells treated with NAC (Fig. 4). The data indicate that Bex-induced ROS accumulation is important for apoptosis.

Discussion

Bex has long been incorporated into diets for disease prevention. However, to the best of our knowledge, its effects in cancer are yet to be investigated. In the present study, Bex caused apoptosis of breast and prostate cancer cells by increasing the intracellular ROS level. The present data indicate that cancer could be, in part, treated using natural products in food. Bex is widely used in food. Therefore, the present study demonstrates that foods containing Bex may be useful for cancer treatment during therapeutic interventions.

A recent study demonstrated that Bex induced apoptosis of leukemia and lymphoma cell lines (30). While not shown in the present study, the data demonstrated no apoptotic effect of Bex in Jurkat T cells (data not shown). It is possible that the experimental conditions, such as the extraction method and concentration, may have influenced the controversial results. In the present study, Bex induced apoptotic cell death of highly metastatic MDA-MB-231 breast cancer cells and DU-145 prostate cancer cells. Thus, the anti-cancer effect of Bex is not limited to blood cancer. This is consistent with results obtained using Bex-treated B16 melanoma cells or HepG2 hepatoma cells (31,32). Bex is one of the ingredients in cereal and the anti-cancer effect of peptides from cereal has previously been demonstrated (33). Furthermore, meta-analyses indicated that cereal reduces cancer risk (34,35). Thus, the present study provides evidence that dietary components are beneficial for cancer prevention and treatment.

In conclusion, Bex induction of ROS was crucial for apoptotic cell death. While the chemical components to produce ROS and induce apoptotic cell death in those breast and prostate cancer cells require further investigation, this is the first study, to the best of our knowledge, that shows the role of Bex in cancer cell death.

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