# $\beta$ -Sitosterol Induces Anti-proliferation and Apoptosis in Human Leukemic U937 Cells through Activation of Caspase-3 and Induction of Bax/Bcl-2 Ratio

Cheol PARK,<sup>*a,b,#*</sup> Dong-Oh MOON,<sup>*c,#*</sup> Chung-Ho RHU,<sup>*d*</sup> Byung Tae CHOI,<sup>*e,f*</sup> Won Ho LEE,<sup>*b*</sup> Gi-Young KIM,\*,<sup>*c*</sup> and Yung Hyun CHOI\*,<sup>*a*</sup>

<sup>a</sup> Departments of Biochemistry, Dongeui University, College of Oriental Medicine; <sup>e</sup> Departments of Anatomy, Dongeui University College of Oriental Medicine; <sup>f</sup> Department of Biomaterial Control (BK21 Program), Dongeui University Graduate School; <sup>b</sup> Department of Biology (BK21 Program), Pusan National University; Busan 614–052, South Korea: <sup>c</sup> Faculty of Applied Marine Science, Cheju National University; Jeju-si, Jeju Special Self-Governing Province 690–756, South Korea: and <sup>d</sup> Division of Applied Life Science, Gyeongsang National University; Jinju 660–701, South Korea. Received December 14, 2006; accepted March 6, 2007

 $\beta$ -Sitosterol is the main dietary phytosterol found in plants and has been shown to inhibit proliferation and induce apoptosis in human solid tumors such as colon and breast cancers. However, the mechanism by which  $\beta$ sitosterol induces apoptosis is not completely understood in leukemic cells. This study investigated the mechanism of apoptosis induced by  $\beta$ -sitosterol in human leukemic U937 cells.  $\beta$ -Sitosterol induced cytotoxicity and apoptosis in U937 cells in a concentration dependent manner, as measured by hemocytometer counts, fluorescence microscopy, agarose gel electrophoresis, and flow cytometry analysis. The increase in apoptosis induced by  $\beta$ -sitosterol was associated with down-regulation of Bcl-2, degradation of poly-(ADP-ribose) polymerase (PARP) and phospholipase C (PLC)- $\gamma$ 1 protein, and activation of caspase-3.  $\beta$ -Sitosterol induced apoptosis was not associated with changes in the expression of Bcl-xL, Bax, or inhibitor of apoptosis proteins (IAPs). z-DEVD-fmk, a caspase-3 specific inhibitor, blocked caspase-3 activation and PARP degradation, and significantly attenuated  $\beta$ sitosterol-induced apoptosis. This suggests that caspase-3 activation is partially essential for  $\beta$ -sitosterol-induced apoptosis. Bcl-2 overexpression also significantly blocked caspase-3 activation and the decrease in PARP cleavage by  $\beta$ -sitosterol, and effectively attenuated the apoptotic response to  $\beta$ -sitosterol. These results show that  $\beta$ -sitosterol potently induces apoptosis in U937 cells and that  $\beta$ -sitosterol-induced apoptosis is related to the selective activation of caspase-3 and induction of Bax/Bcl-2 ratio.

Key words  $\beta$ -sitosterol; apoptosis; caspase-3; Bcl-2

Apoptosis is genetically programmed cell death that is essential for development, the maintenance of tissue homeostasis, and the elimination of unwanted or damaged cells from multicellular organisms.<sup>1)</sup> The aberrant regulation of apoptosis has been observed in many human diseases, such as neuronal conditions, autoimmune disease, and cancer.<sup>2)</sup> Therefore, understanding the mechanism of apoptosis is important for preventing and treating many diseases.<sup>3)</sup> Apoptosis is characterized by distinct morphological changes including plasma membrane blebbing, cell shrinkage, depolarization of the mitochondria, chromatin condensation, and DNA fragmentation. Several genes have been identified as either inducers or repressors of apoptosis. In particular, caspases are known to play key roles in the execution phase of cell death through various apoptotic stimuli.<sup>4,5)</sup> These cysteine-related proteases are present in cells as inactive proenzymes that are transformed into active tetramer following proteolytic removal of the prodomain and cleavage between the large and small subunits. Caspase activity is responsible, either directly or indirectly, for the cleavage of several intracellular proteins, which are characteristically proteolysed during apoptosis.<sup>6)</sup> The processing and activation of caspases can be regulated by molecules such as Bcl-2 and members of the inhibitory apoptosis protein (IAP) family. In most cases, apoptosis involves the release of cytochrome c from the mitochondria.<sup>7,8)</sup> In the cytosol, cytochrome c activates caspase-9, which in turn activates effector caspases such as caspase-3. Bcl-2 and its related proteins control the release of cytochrome c from the mitochondria and modulate the activity of caspases through a direct interaction with the active caspases.<sup>4,8)</sup> The induction of apoptosis in tumor cells has been shown to be the most common anti-cancer mechanism targeted by many cancer therapies. Therefore, there is a need to identify potential therapeutic anti-tumor drugs with potent and selective apoptotic effects.

Many epidemiological studies have shown that the regular consumption of fruits and vegetables has an inhibitory effect on the development of cancer.<sup>9,10</sup> This inhibition has been attributed to several different groups of compounds known as phytochemicals. Among them, sterols and sterolins, which are also known as phytosterols, are the plant counterparts of animal cholesterol.<sup>11)</sup> These molecules are not synthesized endogenously in humans but are derived solely from the diet through intestinal absorption.<sup>12-14</sup>) Previous studies have shown that  $\beta$ -sitosterol reduces carcinogen-induced cancer of the colon in rats,<sup>15)</sup> and exhibits anti-inflammatory,<sup>16,17)</sup> anti-angiogenic<sup>18)</sup> and immune-modulating properties.<sup>19,20)</sup> Several studies have indicated that  $\beta$ -sitosterol inhibits the growth of various cultured cancer cell lines that are associated with the activation of the sphingmyelin cycle,<sup>21,22)</sup> cell cycle arrest<sup>23,24)</sup> and the stimulation of apoptotic cell death.<sup>25,26)</sup> Although  $\beta$ -sitosterol is one of the major phytosterols detected in the blood, the underlying mechanisms of its action are not completely understood in leukemic cells.

This study investigated whether or not  $\beta$ -sitosterol induces apoptosis in human monocytic leukemia U937 cells and ex-

<sup>\*</sup> To whom correspondence should be addressed. e-mail: immunkim@cheju.ac.kr; choiyh@deu.ac.kr

amined the molecular events that mediate  $\beta$ -sitosterol-induced apoptosis in these cells. These results demonstrate that a treatment with  $\beta$ -sitosterol induces apoptosis in U937 cells, and suggests that the apoptosis induced by  $\beta$ -sitosterol appears to be related to the activation of caspase-3 and the induction of Bax/Bcl-2 ratio.

### MATERIALS AND METHODS

**Reagents**  $\beta$ -Sitosterol, 2-hydroxypropyl-cyclodextrin (CD), 4,6-diamidino-2-phenyllindile (DAPI), propidium iodide (PI) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2*H*tetrazolim bromide (MTT) were purchased from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was purchased from GIBCO-BRL (Gaithersburg, MD, U.S.A.) and caspase activity assay kit was obtained from R&D systems (Minneapolis, MN, U.S.A.). An enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, IL, U.S.A.). Caspase-3 inhibitor 1 (z-DEVD-fmk) was obtained from Calbiochem (San Diego, CA, U.S.A.). Any other chemicals not specifically cited above were purchased from Sigma.  $\beta$ -Sitosterol was complexed with 5 mM CD in order to solubilize it.

Cell Culture and Viability Assay The human leukemic U937 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Gibco BRL, Gaithersburg, MD, U.S.A.) at 37 °C and 5% CO<sub>2</sub>. The Bcl-2 overexpressing U937 cells were a generous gift from Dr. T. K. Kwon (Department of Immunology, Keimyung University School of Medicine, Taegu, Korea) and were maintained in a medium containing 0.7  $\mu$ g/ml geneticin (G418 sulfate). The experimental medium was made by supplementing the medium with  $\beta$ -sitosterol in the form of a CD complex.<sup>27,28)</sup> For the cell viability assay, the U937 cells were treated with  $\beta$ -sitosterol for 72 h and surviving cells were counted using trypan blue exclusion methods.

Agarose Gel Electrophoresis for DNA Fragmentation Assay After the  $\beta$ -sitosterol treatment, the cells were lysed in a buffer containing 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. The lysates were vortexed and cleared by centrifugation at 10000 **g** for 20 min. The DNA in the supernatant was extracted using a 25:24:1 (v/v/v) equal volume of neutral phenol: chloroform: isoamyl alcohol (Sigma) and analyzed electrophoretically on 1.2% agarose gels containing 0.1  $\mu$ g/ml ethidium bromide (EtBr, Sigma).

Flow Cytometry Analysis for Measurement of Sub-G1 Phase The cells were harvested and washed once with phosphate-buffered saline (PBS), fixed in ice-cold 70% ethanol and stored at 4 °C. Prior to analysis, the cells were washed once again with PBS, suspended in 1 ml of a cold PI solution containing 100  $\mu$ g/ml RNase A, 50  $\mu$ g/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 30 min in the dark. Flow cytometric analyses were carried out using a flow cytometer (FACS Caliber, Becton Dikinson, San Jose, CA, U.S.A.) and CellQuest software was used to determine the relative DNA content based on the presence of a red fluorescence.

Nuclear Staining with DAPI The cells were washed

with PBS and fixed with 3.7% formalin for 10 min at room temperature. The fixed cells were washed with PBS, and stained with DAPI solution for 10 min at room temperature. The cells were then washed twice with PBS and analyzed by fluorescence microscopy (Carl Zeiss, Germany).

Gel Electrophoresis and Western Blot Analysis The cells were harvested, lysed, and the protein concentrations were quantified using a Bio Rad protein assay (BioRad Lab., Hercules, CA, U.S.A.) according to manufacturer specifications. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.) by electroblotting. The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody and visualized by enhanced chemiluminescence (ECL) according to the recommended procedure. The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) and Calbiochem (Cambridge, MA, U.S.A.). The peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

Assay of Caspase-3 Activity The enzymatic activity of the caspases induced by  $\beta$ -sitosterol was assayed using a colorimetric assay kit according to the manufacturer's protocol. Briefly, the cells were lysed in a lysis buffer for 30 min on an ice bath. The lysed cells were centrifuged at 12000 g for 10 min, and 100  $\mu$ g of the protein was incubated with 50  $\mu$ l of a reaction buffer and 5  $\mu$ l of the colorimetric tetrapeptides at 37 °C for 2 h. Tetrapeptides included Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) for caspase-3. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

**Statistical Analysis** Unless otherwise indicated, each result is expressed as the mean $\pm$ S.D. of data obtained from triplicate experiments. A statistical analysis was performed by a paired Student *t*-test. Differences at *p*<0.05 are considered statistically significant.

## RESULTS

 $\beta$ -Sitosterol-Induced Anti-proliferation and Apoptosis in U937 Cells In order to investigate whether  $\beta$ -sitosterol induces anti-proliferation and apoptosis, U937 cells were stimulated with the indicated concentrations of  $\beta$ -sitosterol for 72 h and the number of viable cells was assessed. As shown in Fig. 1A, treatment with  $\beta$ -sitosterol for 72 h decreased the viability of U937 cells in a concentration manner. Compared with control cells  $[(1.9\pm0.2)\times10^5$  cells/ml], 15 and 20  $\mu$ M  $\beta$ -sitosterol significantly inhibited cell viability to  $(1.2\pm0.1)\times10^5$  and  $(0.8\pm0.2)\times10^5$  cells/ml, respectively. Further experiments were carried out to determine whether this inhibitory effect of  $\beta$ -sitosterol on cell viability is the result of apoptotic cell death. Morphological analysis following DAPI staining was performed to analyze the cells with nuclear chromatin condensation and apoptotic bodies. Very few apoptotic cells were observed in the control culture. The percentage of apoptotic cells cultured in the presence of  $\beta$ -sitosterol was increased in a concentration-dependent manner (Fig. 1B). We also investigated whether  $\beta$ -sitosterol induces DNA fragmentation in U937 cells. As shown in Fig. 1C, in-



Fig. 1. Induction of Anti-proliferation and Apoptosis by  $\beta$ -Sitosterol in Human Leukemic U937 Cells

Cells were plated at  $2 \times 10^4$  cells per 60-mm plate and incubated for 24 h. The cells were treated with different concentrations of  $\beta$ -sitosterol (0, 5, 10, 15 or 20  $\mu$ M) for 72 h. (A) The cell viability was measured using hemocytometer counts of trypan blue-excluding cells. (B) The cells were stained with DAPI. After 10 min incubation, the cells were washed with PBS and photographed with a fluorescence microscope using a blue filter. Magnification,  $\times 400$ . (C) After 72 h, the genomic DNA was extracted, and analyzed on a 1.2% agarose gel. M indicates a size marker of the DNA ladder. (D) The presence of cells with sub-G1 DNA content following treatment with  $\beta$ -sitosterol was evaluated using a flow cytometer in order to quantify the onset of apoptosis. The results are from one representative experiment of three performed that showed similar patterns. Each point represents the mean  $\pm$  S.D. of three independent experiments. The significance was determined by Student's *t*-test (\* p < 0.05 vs. untreated control).

creasing concentrations of  $\beta$ -sitosterol induced the progressive accumulation of fragmented DNA, which appeared as a typical ladder pattern of DNA fragmentation due to internucleosomal cleavage associated with apoptosis. We also analyzed the amount cells with sub-G<sub>1</sub> population using flow cytometry in order to quantify the effect of  $\beta$ -sitosterol on cell cycle distribution. As shown in Fig. 1D, the treatment with  $\beta$ sitosterol resulted in the dose-dependent accumulation of the sub-G<sub>1</sub> phase at 72 h. Treatment with 15  $\mu$ M and 20  $\mu$ MM  $\beta$ sitosterol resulted in a significant increase in the sub-G1 phase,  $18\pm3\%$  and  $28\pm4\%$ , respectively. Control cells treated with 5 mM CD did not experience changes in cell proliferation, viability, or morphology. This suggests that the U937 cells undergo apoptosis after exposure to  $\beta$ -sitosterol. Furthermore, there is a good correlation between the extent of apoptosis and the inhibition of growth.

Activation of Caspase-3 by  $\beta$ -Sitosterol The expression levels and activities of caspase-3 in U937 cells following 72 h exposure to various concentrations of  $\beta$ -sitosterol were measured in order to determine if  $\beta$ -sitosterol-induced apoptosis is associated with the activation of caspases. As shown in Fig. 2A, treatment with  $\beta$ -sitosterol significantly decreased the level of pro-caspase-3 and increased the active subunits of caspase-3 in a concentration-dependent manner. The activation of caspase often leads to the proteolytic cleavage of several target proteins such as poly-(ADP-ribose) polymerase (PARP) and phospholipase C (PLC)- $\gamma$ 1 proteins.<sup>29,30)</sup> The proteolytic activity of caspases was also determined using an *in vitro* assay with a colorimetric assay kit. As shown in Fig. 2B, treatment with  $\beta$ -sitosterol increased the caspase-3 activity (approximately 3-fold at 20  $\mu$ M  $\beta$ -sitosterol) in a concentration-dependent manner. This suggests that the activation of caspase-3 is a key step in the  $\beta$ -sitosterol-induced apoptotic pathway in U937 cells.

Inhibition of  $\beta$ -Sitosterol-Induced Apoptosis by Caspase-3 Inhibitor In order to confirm that the activation of caspase-3 is a key step in the  $\beta$ -sitosterol-induced apoptotic pathway, the U937 cells were pretreated with z-DEVD-fmk (50  $\mu$ M), a cell-permeable caspase-3 inhibitor, for 1 h, followed by a treatment with 20  $\mu$ M  $\beta$ -sitosterol for 72 h. Pretreament with z-DEVD-fmk significantly blocked caspase-3 cleavage and PARP degradation (Fig. 3A), and decreased caspase-3 activity to approximately 2-fold (Fig. 3B) in the  $\beta$ sitosterol-treated U937 cells. Furthermore, the blockade of the caspase-3 activity prevented the  $\beta$ -sitosterol-induced chromatin condensation (Fig. 3C), genomic DNA digestion (Fig. 3D) and a increase in the sub-G1 population (Fig. 3E). These results clearly show that  $\beta$ -sitosterol-induced apoptosis is associated with caspase-3 activation.

Effects of  $\beta$ -Sitosterol on Bax/Bcl-2 Ratio Levels of apoptotic and anti-apoptotic proteins following  $\beta$ -sitosterol treatment were examined by Western blotting in order to determine whether  $\beta$ -sitosterol induces U937 cell death by altering the ratio between Bax and Bcl-2. As shown in Fig. 4A, treatment of U937 cells with  $\beta$ -sitosterol did not cause significant changes in the expression of Bcl-xL, Bax, or cIAP-1.



Fig. 2. Activation of Caspase-3 and the Degradation of the PARP and PLC- $\gamma$ 1 Protein by  $\beta$ -Sitosterol in U937 Cells

(A) After 72 h incubation with  $\beta$ -sitosterol, the cells were lysed and the cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-caspase-3, anti-PARP and anti-PLC- $\gamma$ l antibodies. The proteins were visualized using an ECL detection system. Actin was used as the internal control. (B) The cell lysates from the cells treated with  $\beta$ -sitosterol for 72 h were assayed for *in vitro* caspase-3 activity using DEVD-pNA as substrates. Data indicates the amount of fluorescent cleavage products that were released. The results are from one representative experiment of three performed that showed similar patterns. Each point represents the mean  $\pm$  S.D. of three independent experiments. The significance was determined by Student's *t*-test (\* p < 0.05 vs. untreated control).





The cells were incubated with or without z-DEVD-fink for 1 h before being treated with  $\beta$ -sitosterol. (A) After 72 h incubation with  $\beta$ -sitosterol, the cells were lysed and the cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-caspase-3 and anti-PARP antibodies. The proteins were visualized using an ECL detection system. Actin was used as the internal control. (B) The cell lysates were made and used to measure the caspase-3 activity using DEVD-pNA, a caspase-3 substrate. The data is reported as the mean values from three independent experiments and the bars represent the standard deviations. (C) The cells were treated with z-DEVD-fink (50  $\mu$ M) for 1 h before a challenge with 20  $\mu$ M  $\beta$ -sitosterol for 72 h. The cells were stained with DAPI for 10 min and photographed with a fluorescence microscope using a blue filter. Magnification, ×400. (D) The genomic DNA was extracted from U937 cells grown under the same conditions as and run on 1.2% agarose gel in order to analyze DNA fragmentation. (E) The cells were pretreated for 1 h with or without z-DEVD-fink, and then with  $\beta$ -sitosterol for an additional 72 h. The cells were resents the mean ±S.D. of three independent experiments. The significance was determined by Student's *t*-test (\* p<0.05 vs. untreated control).

However,  $\beta$ -sitosterol significantly induced a concentrationdependent decrease in the expression levels of Bcl-2. cIAP-2 was slightly down-regulated. A densitometric analysis of the bands showed that  $\beta$ -sitosterol resulted in a dose-dependent increase in the Bax/Bcl-2 ratio that favors apoptosis (Fig. 4B). These results suggest that  $\beta$ -sitosterol specifically up-



Fig. 4. The Effects of  $\beta$ -Sitosterol on Bax/Bcl-2 Ratio in U937 Cells

(A) The cells were incubated with different  $\beta$ -sitosterol concentrations for 72 h. Cells were subsequently lysed and equal amounts of proteins were then separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies (anti-Bax, anti-Bcl-2, anti-Bcl-xL, cIAP-1 and cIAP-2) and detected by ECL. Actin was used as the internal control. (B) Densitometric analysis of the immunoblot data was used to quantify the levels of Bax and Bcl-2 in order to evaluate the effect of  $\beta$ -sitosterol on the Bax/Bcl-2 ratio. The results are from one representative experiment of three performed that showed similar patterns. Each point represents the mean±S.D. of three independent experiments. The significance was determined by Student's *t*-test (\*p<0.05 *vs.* untreated control).



Fig. 5. Inhibition of  $\beta$ -Sitosterol-Induced Caspase-3 Activation and PARP Cleavage by Bcl-2 Overexpression in U937 Cells

U937/vector or U937/Bcl-2 cells were treated without or with  $20 \,\mu\text{M} \beta$ -sitosterol for 72 h. (A) The cells were lysed and equal amounts of proteins were then separated by SDSpolyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies (anti-caspase-3 and anti-PARP) and detected by ECL. Actin was used as the internal control. (B) The cell lysates were made and then used to measure the caspase-3 activity using DEVD-pNA, a caspase-3 substrate. (C) The cells were stained with DAPI for 10 min and photographed with a fluorescence microscope using a blue filter. Magnification, ×400. (D) The genomic DNA was extracted and run on 1.2% agarose gels for DNA fragmentation. (E) The sub-G1 DNA content was evaluated using a flow cytometer. The graph represents the fraction of apoptotic cells. The data is reported as a mean±S.D. of three independent experiments.

## regulates Bax/Bcl-2 ratio in U937 cells.

Inhibition of  $\beta$ -Sitosterol-Induced Apoptosis by Overexpression of Bcl-2 We next investigated a potential role for Bcl-2 in  $\beta$ -sitosterol-induced apoptosis. As shown in Fig. 5A, treatment of U937 cells with  $\beta$ -sitosterol resulted in the induction of capase-3 cleavage following the cleavage of PARP, however overexpression of Bcl-2 significantly attenuated the cleavages of caspase-3 and PARP. We also evaluated

the *in vitro* activities of caspase-3 in U937 and U937/Bcl-2 cells treated with  $\beta$ -sitosterol. As shown in Fig. 5B,  $\beta$ -sitosterol treatment significantly increased caspase-3 activity in U937 cells, but not in U937/Bcl-2 cells. Furthermore, exposure to  $\beta$ -sitosterol induced apoptosis in U937/vector cells, as determined by the morphological changes, DNA fragmentation and measurements of the degree of sub-G1 accumulation (Figs. 5C, D). In contrast, Bcl-2 overexpression significantly blocked the formation of apoptotic bodies and DNA laddering. Bcl-2 significantly reduced the percentage of cells with sub-G1 DNA content (14±2%) following treatment with 20  $\mu$ M  $\beta$ -sitosterol (Fig. 5E). These results suggest that down-regulation Bcl-2 likely plays a major role in  $\beta$ -sitosterol-induced apoptosis through the cleavage of caspase-3 and PARP.

#### DISCUSSION

Recently, there has been considerable interest in using dietary prevention and chemoprevention to increase the lifespan. Dietary factors help prevent the development of cancer. The protective effects of a plant-based diet against cancer and other chronic diseases has been well documented.<sup>10,31</sup>  $\beta$ -Sitosterol is a plant compound known as a phytosterol, which is the counterpart of animal cholesterol.<sup>11</sup> Many biological effects of  $\beta$ -sitosterol have been described, including antiinflammatory, immune-modulating, and chemopreventive activities. These effects strongly suggest that  $\beta$ -sitosterol might be an effective natural component in cancer chemoprevention.

Awad *et al.*<sup>23,24)</sup> reported that  $\beta$ -sitosterol arrested human prostate and breast cancer cells in the G2/M phase of the cell cycle and induced apoptosis. These results suggest that the growth inhibitory and apoptosis inducing effects of  $\beta$ -sitosterol are the result of a block during this G2/M phase and that such cells do not enter the G1 phase. However, the precise mechanism by which  $\beta$ -sitosterol induces cell death is not completely understood. The aim of this study was to determine the capacity of  $\beta$ -sitosterol to induce apoptosis and identify the biochemical mechanisms of this induction in a human monocytic leukemic U937 cells.

Initially,  $\beta$ -situation was found to inhibit cell viability and promote the appearance of a sub-G1 cell population, which is considered to be a marker of cell death by apoptosis.<sup>32)</sup> Consistent with this observation  $\beta$ -sitosterol was shown to induce a dose-dependent increase in end-stage apoptotic events, including apoptotic body formation and DNA fragmentation. Exposing U937 cells to  $\beta$ -sitosterol resulted in the proteolytic activation of caspase-3, which is the main executioner of apoptosis. Activated caspases induce limited proteolysis of a number of cellular proteins, which are subsequently degraded and have been used as markers of chemotherapyinduced apoptosis. This study examined whether PARP and PLC- $\gamma$ 1, which are substrates of caspase-3,<sup>29,30)</sup> are cleaved in the cells treated with  $\beta$ -sitosterol. As expected, both proteins were clearly degraded in a  $\beta$ -sitosterol dose-dependent manner, which correlated with the activation of capase-3. Under the same conditions, z-DEVD-fmk prevented the  $\beta$ sitosterol-induced apoptosis by blocking not only caspase-3 activation but also PARP cleavage. This indicates that caspase-3 is the key molecule in responsible for mediating  $\beta$ - sitosterol-induced apoptosis in U937 cells.

Members of the Bcl-2 family, including Bcl-2, Bcl-xL and Bax, have been shown to regulate apoptosis. In particular, Bcl-2 has been reported to directly inhibit members of the caspase family, including caspases-3 and -9.8,33) In this study,  $\beta$ -sitosterol did not alter the expression levels of Bcl-xL and Bax in U937 cells but did selectively down-regulate the expression of Bcl-2. Although the mechanism by which  $\beta$ sitosterol decreases Bcl-2 content in U937 cells is unclear,  $\beta$ sitosterol has been shown to suppress down-stream caspases. Because Bcl-2 is a upstream molecule of caspase-3,<sup>33)</sup> it is possible that the observed decrease in the Bcl-2 content is a consequence of caspase-3-mediated processing after the  $\beta$ sitosterol treatment. Furthermore, Bcl-2 overexpression significantly attenuated  $\beta$ -sitosterol-induced apoptosis in U937 cells by inhibiting the caspase-3 activity and PARP cleavage. The transfection data shows that  $\beta$ -sitosterol-induced apoptosis and caspase-3 activation is related to the down-regulation of Bcl-2. Therefore, the data demonstrates that Bcl-2 downregulation contributes to the activation of caspase-3 in  $\beta$ sitosterol-induced apoptotic pathway.

In summary,  $\beta$ -sitosterol induces anti-proliferation and apoptosis in human leukemic U937 cells. The selective activation of caspase-3 and the down-regulation of Bcl-2 may mediate the  $\beta$ -sitosterol-induced apoptosis in U937 cells. Moreover, Bcl-2 overexpression attenuates  $\beta$ -sitosterol-induced apoptosis, and prevents the activation of caspase-3 and PARP. Therefore, Bcl-2 overexpression inhibits  $\beta$ -sitosterolinduced apoptosis through a mechanism that interferes with the down-regulation of caspase-3 in the execution of apoptosis. These results are expected to further contribute to the understanding of the anti-cancer activity of  $\beta$ -sitosterol.

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