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β-Sitosterol Inhibits Cell Growth and Induces Apoptosis in SGC-7901 Human Stomach Cancer Cells

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β-Sitosterol is an important phytosterol found in plant food. It has been shown to have antiproliferative effects on cancers of the colon, breast, and prostate, but its effect on stomach cancer cells in vitro is unknown. Proliferation, cytotoxicity, and apoptosis in SGC-7901 human stomach cancer cells were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, clone formation, lactate dehydrogenase (LDH) leakage assay, acridine orange (AO)/ethidium bromide (EB) double staining, 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) staining, comet assay, and Western blotting. The results showed that β-sitosterol suppresses the proliferation and induces the cell cytotoxicity of SGC-7901 stomach cancer cells in a time- and dose-dependent manner. Cells treated with different concentrations of β-sitosterol also showed changes typical of apoptosis: morphological changes, DNA damage, increased expression of pro-caspase-3 and bax ($p < 0.05$), and activation of pro-caspase-3 and suppression of bcl-2 expression ($p < 0.05$). This study therefore revealed that β-sitosterol significantly inhibits the growth and induces the apoptosis of SGC-7901 human stomach cancer cells in vitro. The decrease of the bcl-2/bax ratio and DNA damage may be the critical mechanisms of apoptosis induced by β-sitosterol in SGC-7901 human stomach cancer cells.

KEYWORDS: β-Sitosterol; SGC-7901 stomach cancer cell; growth inhibition; apoptosis

INTRODUCTION

Phytosterols, or plant sterols, are essential phytochemicals that resemble cholesterol in structure and function but are enriched in plant foods such as nuts, peanuts, sesame seeds, soybean seeds, and unrefined plant oils and grains as well as their products such as cornflakes, wheat bran, and wheat germ (*1*). The most common plant sterols in foods are β-sitosterol, campesterol, and stigmasterol, representing about 50−65, 10−40, and 0−35% of the total phytosterols, respectively (*2*). Previous studies have demonstrated that phytosterols and their derivates may protect against many chronic aliments including arteriosclerosis, cancer, ulcer, and inflammation (*3*). Animal studies of the anticancer function have shown that a diet containing 2% mixed phytosterols inhibits cancer cell growth, reduces tumor size, and decreases the incidence of colon, breast, and prostate cancers induced by specific carcinogens (*4*−*10*). The results of in vitro studies have also shown that phytosterols, especially β-sitosterol, inhibit proliferation and induce apoptosis in colon, breast, prostate, liver, and murine fibrosarcoma cancer cells at very low concentrations of 2.4−32 μM (*6*, *7*, *9*, *11*−*14*). However, there are no reports on the effects of any phytosterols on stomach cancer except for a case control study from Uruguay, which showed that phytosterol intake has a strong inverse relationship with stomach cancer in humans (*15*).

Apoptosis, or programmed cell death, is a critical mechanism that allows multicellular organisms to maintain tissue integrity and function and to eliminate damaged or unwanted cells (*16*). Therefore, cancer cell apoptosis has been evaluated as a potential therapeutic target in recent years (*17*). Previous studies have shown that β-sitosterol induces apoptosis in specific cancer cells by affecting ceramide metabolism (*18*, *19*); activating caspase-3, ERK (*12*, *20*), and the Fas signaling pathway (*21*); increasing the expression of pro-apoptotic bax protein (*22*); restoring gap junctional intercellular communication (GJIC) (*14*); and affecting cell cycle kinetics (*23*) in specific cancer cells. However, details of the mechanisms by which β-sitosterol induces cell apoptosis and affects proliferation effect have not yet been fully elucidated. Also, there are no reports about the effect of β-sitosterol on apoptosis in stomach cancer cells ether in vitro or vivo.

Stomach cancer is one of the most common cancers and a major cause of death worldwide, especially in Asia. Although its rate of development and incidence have declined in recent years (*24*), it is still the second leading cause of cancer-related deaths, and to date there are no effective ways of preventing and treating it. Thus, it would be valuable to find potentially

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therapeutic antitumor dietary factors or drugs with potent and selective apoptotic effects on stomach cancer cells. The objectives of this study were to investigate the effect of β-sitosterol on proliferation and apoptosis in SGC-7901 stomach cancer cells in vitro and to study the possible mechanisms of action involved.

MATERIALS AND METHODS

Cell Culture. SGC-7901 human stomach cancer cells (Cancer Research Institute, Beijing, China) were maintained and subcultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS (Invitrogen), 2% L-glutamine (Sigma), and 1% antibiotic antimycotic solution (Invitrogen) in 75 cm^2 flasks at 37 °C under a constant humidified atmosphere of 5% carbon dioxide. The medium was changed every other day. For experiments, the concentration of FBS in the treatment medium was decreased to 5% when the β-sitosterol was added. β-Sitosterol (99.4%, TAMA Biochemical Co. Ltd.) stock solutions were prepared by dissolving β-sitosterol in 100% ethanol, and the final concentration of ethanol vehicle in the treatment medium was 0.5% (*25*). All of the control cells in this study were supplemented with medium containing the same concentration of ethanol vehicle (0.5%) as did the β-sitosterol-treated cultures. Trypan blue dye exclusion assay was used to count the viable cells used in this study.

Cell Proliferation and Clone Formation. The inhibitory effect of β-sitosterol on SGC-7901 cell proliferation was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay as described by Xu and others with some modifications (*26*). Briefly, the growing cells were harvested when they reached 80−90% confluence in the 75 cm^2 flasks and diluted to 2.0×10^4 cells/mL with culture medium. They were then seeded on 96-well plates at a density of 3×10^3 cells/well in 150 μL of medium. After 24 h for adhesion, the medium was removed and the cells were treated with 150 μL of medium containing 4, 8, 16, 32, and 64 μM β-sitosterol, respectively. Each treatment was conducted in six wells excluding the outside wells because they showed greater variance of absorption than those from the inner region. The medium was changed every other day. On days 1, 3, and 5, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS), then air-dried, and supplemented with 20 μL of 5 mg/mL MTT PBS solution for each well. The plate was shaken evenly and then incubated for 4 h under the same culture conditions. The MTT solution was removed carefully after incubation, and 150 μL/well of dimethyl sulfoxide (DMSO; Sigma) was added. The plate was shaken for 10 min, and the absorbance was measured at 570 nm in a microplate reader (Bio-Tech Instruments). The inhibition rate (IR) was the difference in absorbance values between the treated and control wells divided by the control absorbance.

For the clone formation experiment, 200 cells/well in 1.5 mL of medium was seeded in 6-well plates. After 24 h for adhesion, the medium was discarded, and 1.5 mL/well medium with 16 or 32 μM β-sitosterol was added. Control cells were treated with the same medium containing 0.5% ethanol vehicle. After treatment for 3 or 5 days, the media containing β-sitosterol were discard and cells were washed twice with prewarmed PBS; 1.5 mL/well medium without β-sitosterol and ethanol vehicle was added. Six-well plates with treated cells were incubated for a further 7 days under the same culture conditions, and the medium was changed every other day. After incubation, the cells were fixed with methanol and stained with Giemsa solution. Clones containing more than 50 cells were counted under an inverted phase microscope (Olympus).

Cytotoxicity Assay. Cytotoxicity induced by different concentrations of β-sitosterol was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Cells were seeded on 96-well plates at a density of 3×10^3 cells/well in 150 μL of medium. After 24 h of adhesion, the medium was removed and the cells were treated with 150 μL of medium containing 8, 16, 32, and 64 μM β-sitosterol, respectively. Each treatment was conducted in six wells. On days 1, 3, and 5, one plate was centrifuged at 4000 rpm at 4 °C for 5 min, respectively. Aliquots of supernatant medium and warm reagent of LDH assay kit (Sigma) were mixed in a new 96-well plate, and absorbance was recorded by a microplate reader at 340 nm. Cytotoxicity is presented as percentage of control values.

Changes in Cell Morphology. Cells were seeded in 75 cm^2 flasks at a density of 1×10^4 cells/cm^2. After 24 h of adhesion, treatment medium containing 8, 16, or 32 μM β-sitosterol was added to flasks, respectively. Control cells were treated with treatment medium supplemented with 0.5% ethanol vehicle. After 5 days of incubation, the cells in the flasks were observed under the inverted phase microscope (Olympus).

AO/EB Double Staining. Apoptotic morphology was investigated by double staining with acridine orange (AO; Sigma) and ethidium bromide (EB; Sigma) as described by Baskic and others (*27*) with some modifications. Briefly, cells treated for 5 days with 8, 16, or 32 μM β-sitosterol were harvested and washed twice with cold PBS. Cell pellets were resuspended and diluted with PBS to a concentration of 5×10^5 cells/mL. One microliter of mixed AO (100 μg/mL)/EB (100 μg/mL) water solution was mixed with 9 μL of cell suspension on a clean microscope slide, and the slide was immediately examined by fluorescence microscopy (IX70, SIF2 Olympus).

DAPI Staining. 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) staining was conducted according to the method described by Lock and others (*28*). Briefly, cells treated with 16 μM β-sitosterol for 5 days were harvested and washed twice with cold PBS, fixed with 100% ethanol for 20 min at room temperature, and then washed twice again with PBS. They were then incubated with 2 μg/mL DAPI solution for 30 min, and cell morphology was evaluated by fluorescence microscopy (IX70-SIF2 Olympus) after incubation. Cells with chromatin condensation, nuclear fragmentation, and nuclear condensation were recognized as apoptotic.

Comet Assay. The comet assay was performed as described by Tice et al. (*29*) and Lee et al. (*30*). Briefly, the harvested cells were diluted with PBS to 1×10^4 cells/mL. One hundred microliters of 0.8% normal melting point agarose was spread on frosted slides and covered with coverslips. After gelling for 5 min at 4 °C, the coverslips were gently removed and a mixture of 10 μL of cell suspension and 90 μL of low melting point (LMP, 0.6%) agarose was added rapidly and evenly to the frosted slides and allowed to solidify for 5 min at 4 °C in the dark. The slides were then placed in a tank with lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% SDS, 10% dimethyl sulfoxide, and 1% Triton X-100, pH 10.0) at 4 °C for 1 h in the dark, washed three times with PBS, and incubated in fresh alkaline buffer for 30 min at room temperature to allow the DNA to unwind. Electrophoresis was performed at room temperature in ice-cold alkaline electrophoresis buffer for 35 min at 25 V (1 V/cm, 300 mA). After electrophoresis, the slides were gently washed with PBS buffer and stained with 30 μL of EB solutions (20 μg/mL) and then rinsed carefully with PBS. They were then covered with coverslips and investigated with Comet 5.0, Kinetic imaging (Liverpool, U.K.).

Western Blotting. Cells harvested after culturing with medium containing 4, 8, 16, or 32 μM β-sitosterol for 5 days were washed with PBS and dissolved in lysis buffer [150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM dithiothreitol (DTT), 5 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL trypsin, 10 μg/mL aprotinin, 5 μg/mL leupeptin; pH 7.4] for 2 h at 4 °C, respectively, and the lysate was centrifuged at 12000*g* for 15 min at 4 °C. The protein in the supernatant was assayed using the Bradford method (*31*). Western blotting was performed as described by Liu and others (*32*) with some modifications. Briefly, 80 μg of protein from each sample was subjected to SDS electrophoresis and transferred to a PVDF membrane. The membrane was then blocked with PBS containing 5% nonfat milk at 4 °C for 1 h and incubated with primary antibodies at 4 °C for 12 h and corresponding secondary horseradish peroxidase-conjugated antibodies for 1 h at room temperature, respectively. After incubation, the protein bands were detected with 3, 3′-diaminobenzidine, and their densities were scanned and evaluated using a ChemiImager 4000 densitometer (Alpha Innotech). The experimental values were normalized to β-actin reactivity.

Statistical Analysis. All experiments were performed in triplicate, except the MTT and cytoxicity assay, for which there were six replicates, and the clone formation, for which there were five replicates. Values were expressed as means ± SD. All data were subjected to one-way analysis of variance (ANOVA) in the General Linear Models procedure using the Statistical Analysis System of Software Package (SAS Institute, 2006). Means were analyzed for significant differences using the Duncan multiple-range test. The significant difference level was set at $\alpha = 0.05$.

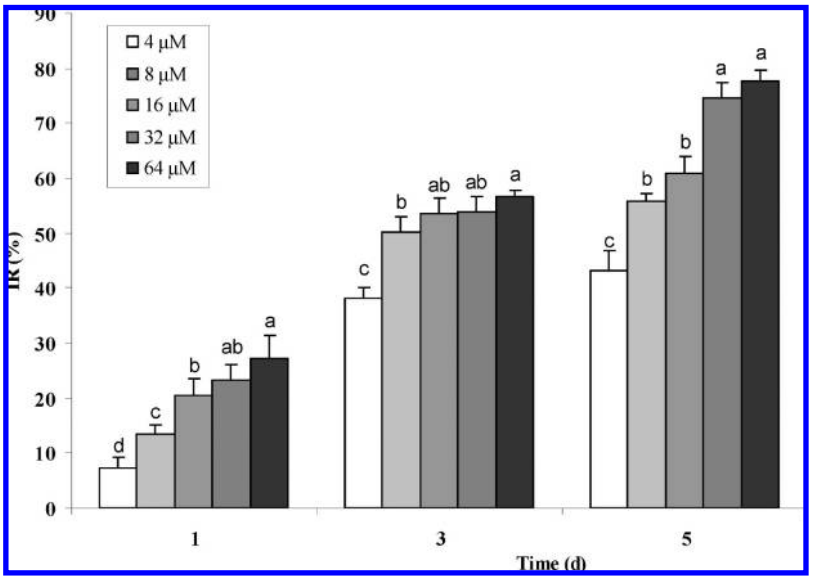

Figure 1. Effect of β-sitosterol on the proliferation in SGC-7901 human stomach cancer cells. Results are shown as mean ± SD ($n = 6$). Bars with different letters on the same day indicate significant differences ($p < 0.05$). IR, inhibition rate.

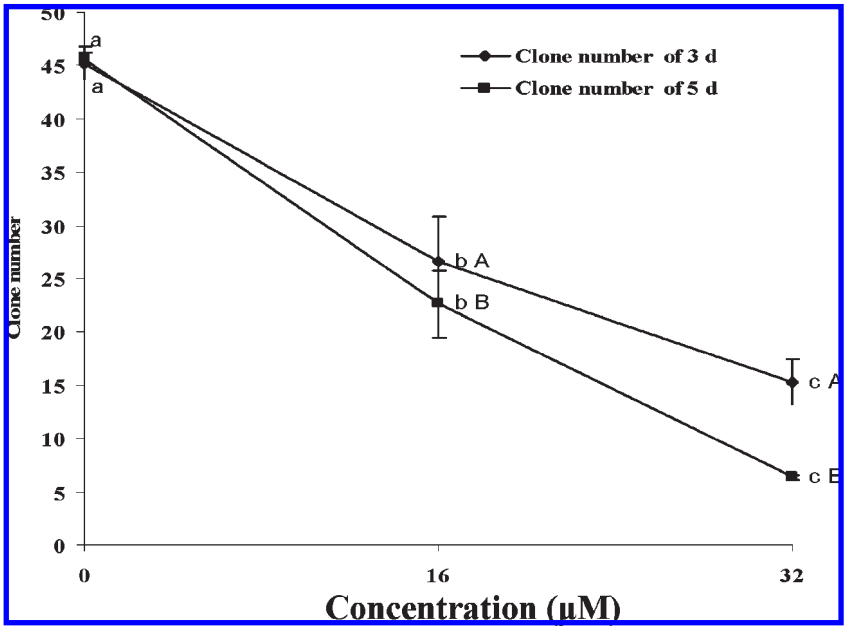

Figure 2. Clone formation as affected by different concentrations of β-sitosterol. Results are expressed as mean ± SD ($n = 5$), and the points with different lower case letters on the same day and with different capital letters in the same concentration were significantly different, respectively ($p < 0.05$).

RESULTS

Cell Proliferation and Clone Formation. MTT assay results showed that SGC-7901 stomach cancer cell proliferation was inhibited by β-sitosterol and that the inhibition rates were increased with increase of concentration and treatment time; however, no significant differences were observed among or between some concentrations on the indicated days (**Figure 1**). **Figure 2** shows the inhibition of clone formation by 16 and 32 μM β-sitosterol at 3 and 5 days. The results indicate that β-sitosterol significantly suppressed the formation of SGC-7901 cell clones in a time- and dose-dependent manner ($p < 0.05$). Thus, the MTT assay and clone formation experiments both suggested that β-sitosterol inhibits the proliferation of SGC-7901 human stomach cancer cells.

Cytotoxicity Induced by β-Sitosterol. As shown by **Figure 3**, LDH leakage induced by different concentrations of β-sitosterol was significantly different and dose-dependent on days 1, 3 and 5 ($p < 0.05$), except for those between 16 and 32 μM on days 3 and 5. These results showed that β-sitosterol induced cytotoxicity in SGC-7901 human stomach cancer cells and that the extent of cytotoxicity depended on β-sitosterol concentration and treatment time.

Morphological Changes Induced by β-Sitosterol. Figure 4 shows the inverted-phase microscopic images of the morphological changes in cells treated for 5 days by 8, 16, and 32 μM β-sitosterol. The cells became elongated, and they were fewer and more disorganized than the control cells treated only with 0.5% ethanol vehicle. The extent of changes in cell shape and density depended on β-sitosterol concentration. These results also demonstrate that β-sitosterol not only induced cell morphological changes but also inhibited the growth of SGC-7901 cells to some extent.

Apoptosis. Morphological changes in cells are one of the most important characteristics of apoptosis (*33*). The AO/EB double-staining results (**Figure 5**) demonstrated that cells supplemented

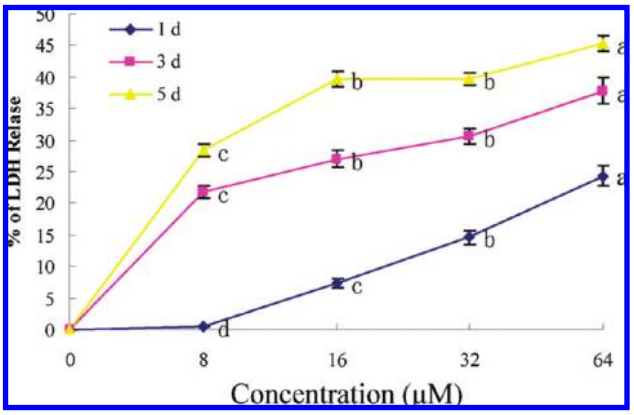

Figure 3. Effect of β-sitosterol on cytotoxicity in SGC-7901 cells. Percentage of LDH leakage is expressed as mean ± SD (n = 3), and concentrations on the same day with different letters are significantly different ($p < 0.05$).

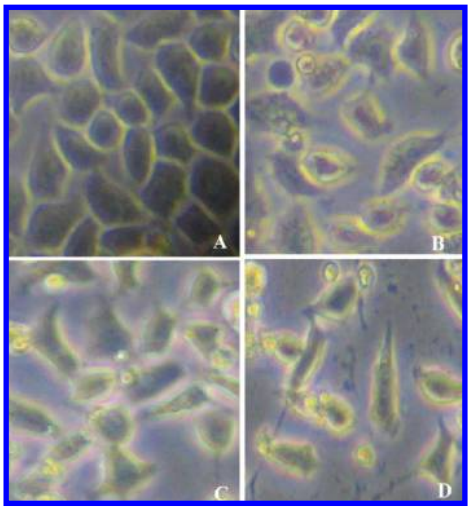

Figure 4. Effect of β-sitosterol on morphological changes of SGC-7901 human stomach cancer cells. Cells were treated with different concentrations of β-sitosterol in 75 cm^2 flasks for 5 days and observed under the inverted microscope (200×): **A**, 0 μM; **B**, 8 μM; **C**, 16 μM; **D**, 32 μM.

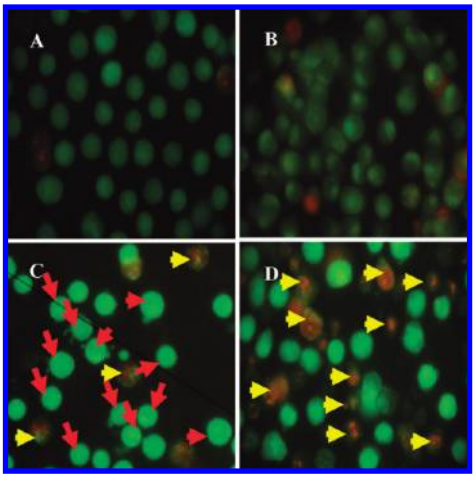

Figure 5. AO/EB staining results. Harvested cells treated with different concentrations of β-sitosterol were stained with AO/EB and observed under a fluorescent microscope (200×): **A**, control cells treated with medium containing 0.5% vehicle ethanol; **B**, 8 μM; **C**, 16 μM; **D**, 32 μM. Cells with red and yellow arrows were undergoing early apoptosis and late apoptosis, respectively.

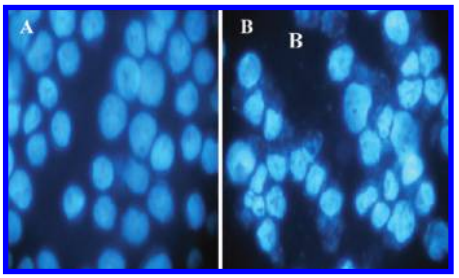

Figure 6. Effect of β-sitosterol on morphological changes in nuclear (200×): **A**, control cells treated with 0.5% vehicle ethanol; **B**, cells treated with 16 μM β-sitosterol. Cells were treated by 16 μM β-sitosterol for 5 days, fixed with methanol, and dyed with DAPI.

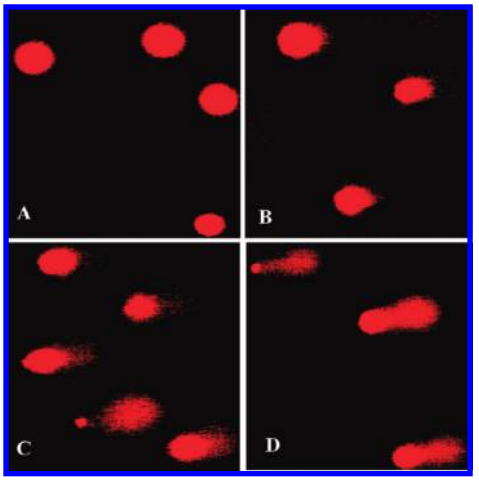

Figure 7. Comet images of cells treated for 5 days with different concentrations of β-sitosterol: **A**, control cells treated with 0.5% ethanoll; **B**, cells treated with 8 μM β-sitosterol; **C**, cells treated with 16 μM β-sitosterol; **D**, cells supplemented with 32 μM β-sitosterol.

with 8, 16, and 32 μM β-sitosterol for 5 days underwent both early apoptosis (cells with red arrows) and late apoptosis (cells with yellow arrows). The early apoptosis cells showed bright green areas with yellowish green fragmentation inside and membrane bubbles and apoptotic bodies outside (**Figure 5C**). The late apoptosis cells exhibited orange-yellow or red mixed with some green (**Figure 5D**). However, the control cells supplemented with 0.5% ethanol were round and stained uniformly green (**Figure 5A**), and very few necrosis cells were observed (uniform red) in this experiment. DAPI staining also showed that cells supplemented with 16 μM β-sitosterol for 5 days exhibited condensed and fragmented nuclei, indicative of apoptosis (**Figure 6**). Results from DAPI and AO/EB double staining are highly consistent in our study. Therefore, we conclude that β-sitosterol causes apoptosis in SGC-7901 cells.

Comet Assay. The comet patterns of the cells are shown in **Figure 7**. The comet tail length and amount of tail DNA in cells treated with the indicated concentrations of β-sitosterol for 5 days were increased with the concentration increase, but the comet image of control cells showed round and no comet tail. These results showed that β-sitosterol induced DNA damage in SGC-7901 stomach cancer cells and that the extent of damage depended on the β-sitosterol concentration.

Western Blotting. Figures 8−10 show changes in the expression of proteins closely related to cell apoptosis. Cells treated for 5 days with 4, 8, 16, and 32 μM β-sitosterol showed a dose-dependent increase in pro-caspase-3 expression, and pro-caspase-3 was cleaved into two fragments of 20 and 17 kDa (**Figure 8**),

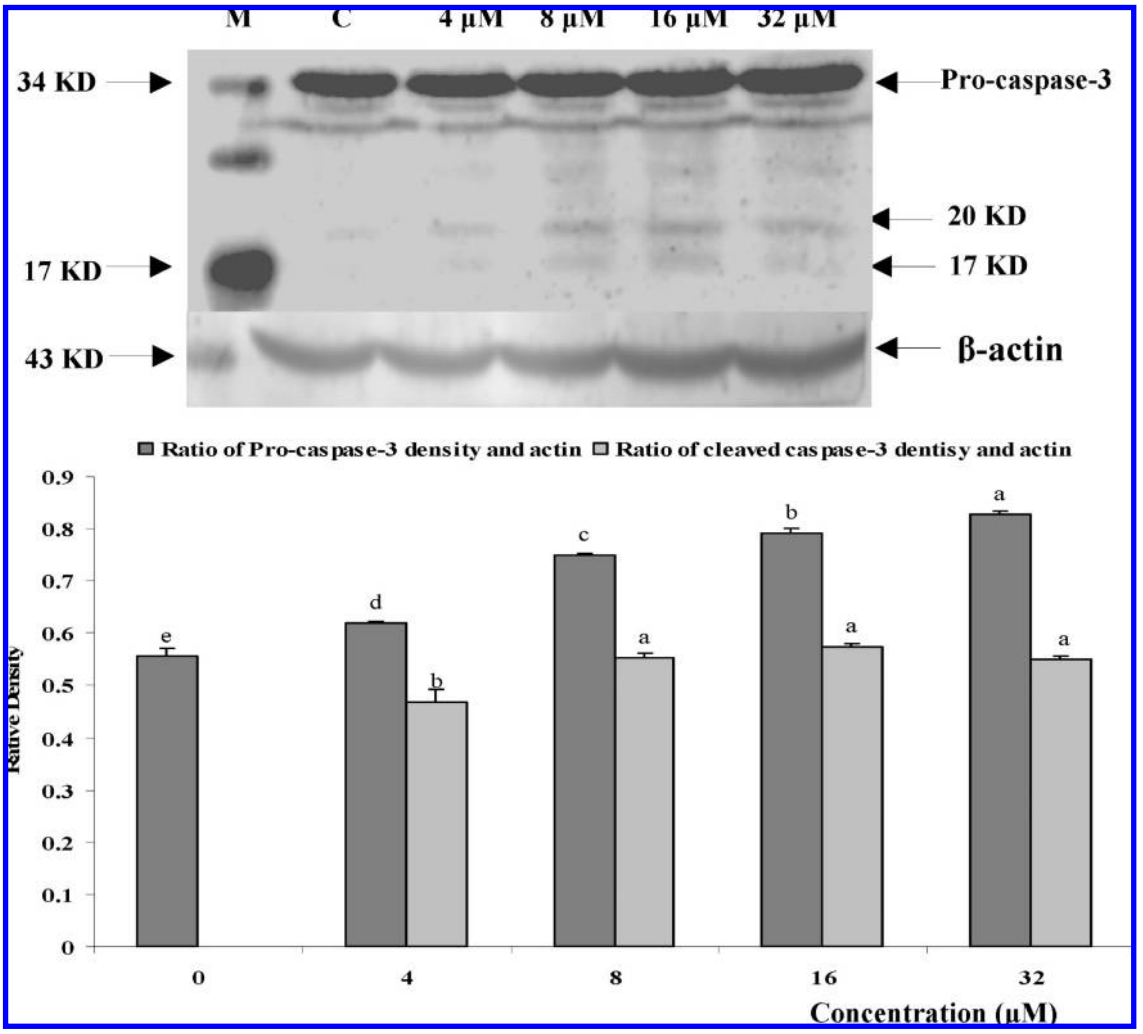

Figure 8. Effect of β-sitosterol on the expression of caspase-3. Expression of caspase-3 was determined by Western blotting, and density of pro-caspase-3 and cleaved caspase-3 was determined by Chemilmager 4000 densitometer. The relative density was the density of pro-caspase-3 or cleaved caspase-3 divided by the density of β-actin. Bars with different letters indicate significant differences ($p < 0.05$).

namely, it was activated to caspase-3, which is the critical executor of cell apoptosis. Bcl-2 expression was less than in the control cells, but no significant differences were observed among different concentrations of β-sitosterol except for the cells treated with 8 μM (**Figure 9**), whereas bax expression showed a dose-dependent increase (**Figure 10**). Scanning densitometry showed that pro-caspase-3 levels increased significantly from 11 to 48% above those of control cells as the β-sitosterol concentration increased from 4 to 32 μM β-sitosterol ($p < 0.05$); the relative density of the cleaved caspase-3 fragments (20 kDa) also increased from 4 to 16 μM β-sitosterol but decreased at 32 μM (**Figure 8**). The relative density of bcl-2 was decreased by 26−31% below the control values as the concentration of β-sitosterol increased from 8 to 32 μM, but no significant differences were observed in bcl-2 expression when the cells were treated with 16 and 32 μM β-sitosterol ($p > 0.05$) (**Figure 9**). Relative density analysis also showed that bax expression was increased significantly by 24.05−72.15% over control values as the β-sitosterol concentration increased from 8 to 32 μM ($p < 0.05$) (**Figure 10**).

DISCUSSION

Cell proliferation and viability are indispensable for the establishment of optimum cell culture conditions, assessment of cytotoxicity, and screening of anticancer drugs. In our study, 4−64 μM β-sitosterol suppressed the growth of SGC-7901 human stomach cancer cells. Sixteen micromolar was a common concentration used in most previous studies (*7*, *8*, *10*, *20*, *21*, *23*). The extents to which proliferation was inhibited by 16 μM β-sitosterol in our study on days 1, 3, and 5 were 20.53 ± 2.91, 53.58 ± 2.71, and 61.9 ± 3.09%, respectively. The day 3 result was higher than the 42% reported by Moon and others (*12*) in the murine fibrosarcoma cells, but much lower than the 80 and 81% reported by Awad and others in MDA-MB-231cells and HT-29 human colon cancer cells, respectively (*19*, *21*). We also observed that the inhibition of SGC-7901 cell line proliferation progressed slowly, in agreement with the reports on other cancer cell lines including MDA-MB-231, HT-29, and murine fibrosarcoma (*8*, *12*, *19*), but contrasting with Choi's study (*22*), in which it was found that HCT 116 colon cancer cells treated with 20 μM β-sitosterol for 2 days showed approximately 75% inhibition of proliferation. The differences may be attributable to the different varieties of cancer cell lines used in these studies. According to the cytotoxicity assay techniques, the MTT assay is also a most sensitive method to detect cytotoxicity (*34*). Our MTT results demonstrated that cytotoxicity induced by β-sitosterol was increased with increasing concentration and treatment time, but its results could not show the cell membrane integrity affected by β-sitosterol. Therefore, the LDH assay was performed, and its results demonstrated that β-sitosterol induced cytotoxicity in SGC-7901 stomach cancer cells. These results also implied that

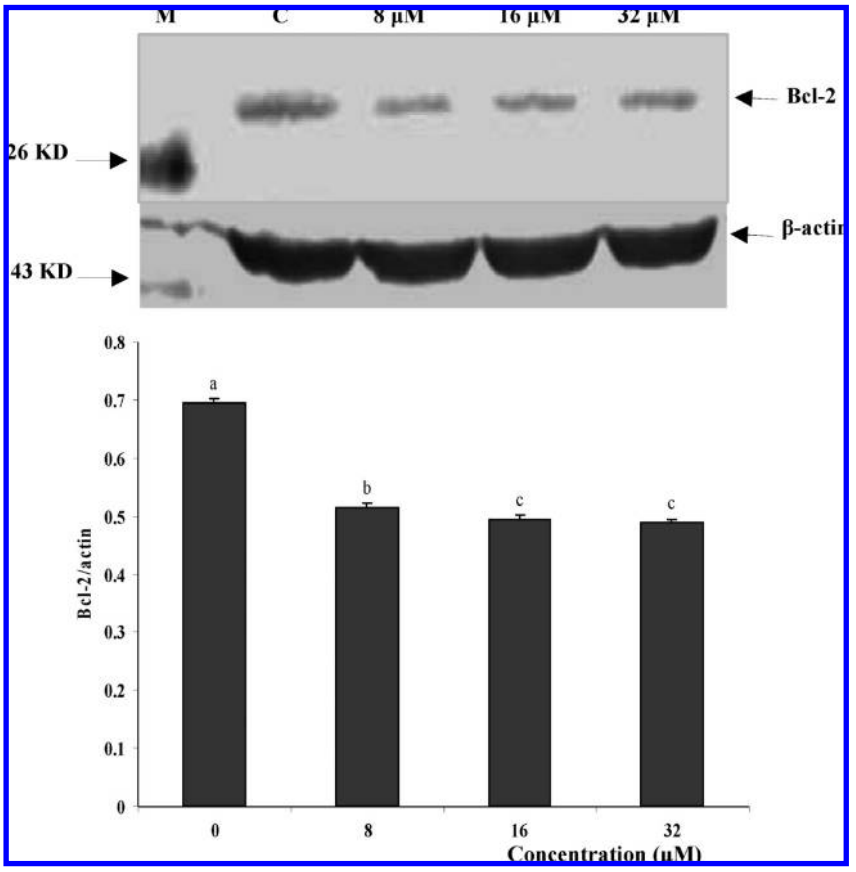

Figure 9. Effect of β-sitosterol on the expression of bcl-2. Cells were treated with different concentrations of β-sitosterol for 5 days, and bcl-2 was determined by Western blotting. Bcl-2 density was measured with a ChemiImager 4000 densitometer. The relative density was the density of bcl-2 divided by the density of β-actin. Bars with different letters indicate significant differences ($p < 0.05$).

cell membrane integrity was damaged or membrane permeability was changed by β-sitosterol. Because the structure and property of β-sitosterol are very similar to those of cholesterol, it can incorporate into cell membrane and affect the cell membrane lipid composition. Awad et al.'s study demonstrated that supplementation with 16 μM β-sitosterol for 9 days resulted in significant changes in membrane lipid composition (*35*). Moreover, activities of specific enzymes existing in membrane were also changed (*18*). Both the cell membrane composition and enzyme activity changes are probably reasons for membrane permeability increase or cell membrane damage. More details related to cytotoxicity in vitro still require research.

Apoptosis is a physiological mode of cell death and is characterized by morphological change and extensive DNA fragmentation, the frequency and time of appearance of which depend on the cell line and the apoptosis-inducing signal (*36*, *37*). Failure of apoptosis may promote survival and accumulation of cells to form tumors (*22*, *38*). Thus, apoptosis has become a target for eliminating cancer cells (*39*, *40*). To investigate the effect of β-sitosterol on apoptosis in SGC-7901 stomach cancer cells in vitro, AO/EB double staining, DAPI staining, and the comet assay were used to study the morphological changes and DNA damage, respectively. Our AO/EB and DAPI staining results indicate that β-sitosterol induced SGC-7901 human stomach cancer cell apoptosis, in good agreement with the DAPI staining results showing apoptosis in murine fibrosarcoma cells treated with β-sitosterol (*12*). Our DAPI staining results also showed that the nuclear morphology changed dramatically after β-sitosterol treatment, and DNA fragmentations were clearly observed (**Figure 6B**). This indicated that the DNA damages occurred in the cell apoptotic process. To further verify the DNA damages induced by β-sitosterol, the single-cell gel electrophoresis (SGCE) assay (comet assay), which has been shown by previous study to accurately reflect the measurement of DNA fragmentation and detect a cell undergoing apoptosis (*41*), was performed. The results showed that β-sitosterol induced DNA damages dose-dependently after cell treatment with β-sitosterol for 5 days. We can also conclude on the basis of the above results that DNA damage might be one of the mechanisms of SGC-7901 cell apoptosis induced by β-sitosterol.

Other results in the present study showed that exposure of SGC-7901 cells to different concentrations of β-sitosterol resulted in a dose-dependent pro-caspase-3 expression (4−32 μM) and proteolytic cleavage and activation of caspase-3, a critical enzyme in the execution phase of apoptosis and a hallmark of apoptosis (*42*). These results support the conclusion from the AO/EB double staining, DAPI staining, and comet assay experiments in our study and are similar to the conclusions from a study of the effect of 48 h β-sitosterol treatment on colon cancer cells (*33*), a much shorter time than the 5 days used in our study. Awad and others conducted an experiment on MDA-MB-231 breast cancer cells, and their results showed a significant increase in caspase-3 activity in MDA-MB-231 cells treated with 16 μM β-sitosterol for 3 days, but no change in caspase-3 protein expression (*20*). This latter result was similar to ours; we found that pro-caspase-3 expression did not differ significantly among treatments with different β-sitosterol concentrations for 3 days (data not shown). This may be because the treatment time was insufficient for an increase in protein expression but sufficient for enhancement of activity.

The bcl-2 family of proteins are the most important regulators of apoptosis and include both antagonists and agonists. Bcl-2 is an intracellular suppressor of apoptosis, which prolongs cells

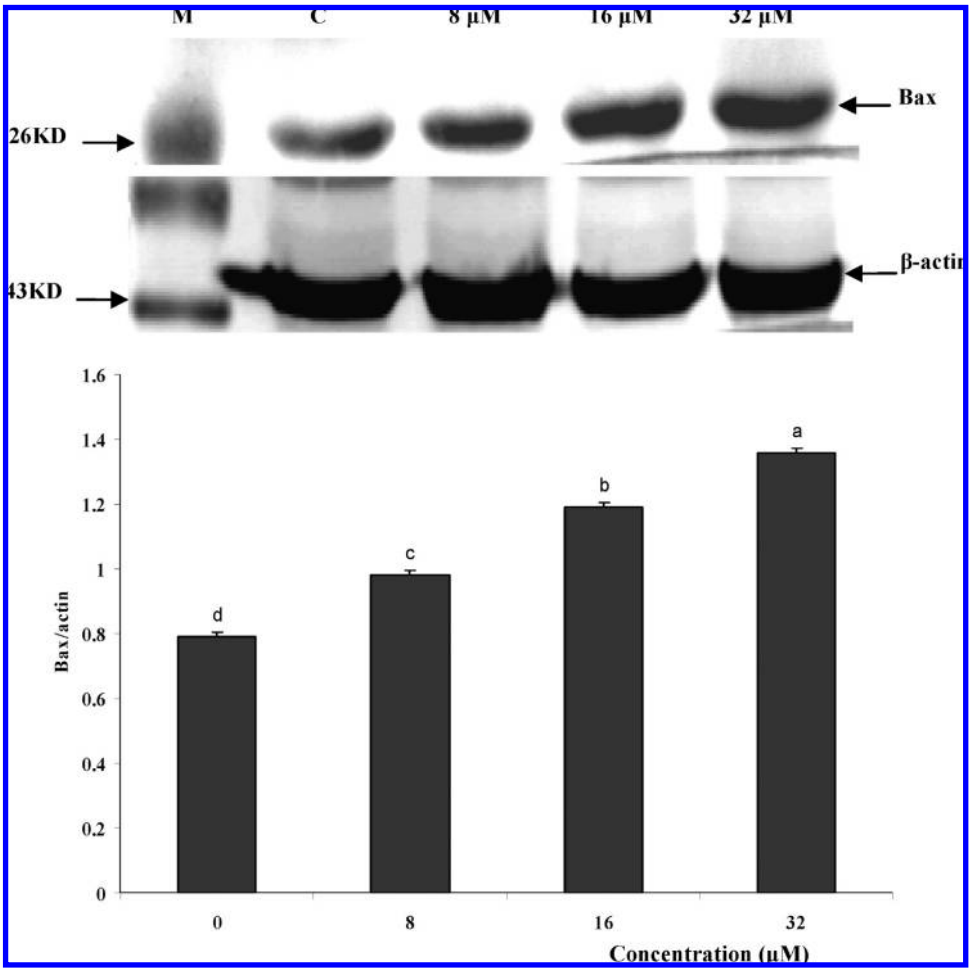

Figure 10. Effect of β-sitosterol on the expression of bax. Bax expression was determined by Western blotting. Expression density was measured with a ChemiImager 4000 densitometer. The relative density was the density of bax divided by the density of β-actin. Bars with different letters indicate significant differences ($p < 0.05$).

survival, whereas bax is an apoptotic agonist that promotes cell apoptosis. The bcl-2/bax ratio indicates whether and how a cell will respond to an apoptotic signal (*43*). Our results show that β-sitosterol decreased the bcl-2 expression significantly in comparison to control cells ($p < 0.05$) and caused a dose-dependent increase in bax expression. The bcl-2/bax ratio therefore decreased with increasing β-sitosterol concentration. This decrease may contribute to the activation of caspase-3 and induction of apoptosis via the mitochondrial apoptosis pathway.

β-Sitosterol is a major variety of phytosterols in plant food. Thus, people can ingest phytosterols from their everyday diet, and the daily intake amount is 160−400 mg/day (*44*). Therefore, the physiological concentration in human blood can be kept relatively constant by eating a diet enriched in phytosterols. Moreover, studies to date have demonstrated no obvious side effects of phytosterols, and they have already been used to lower human blood lipid levels and prevent cardiovascular disease (*2*, *3*). The effective dose of β-sitosterol used in anticancer studies is within the physiological human blood range (4−70 μM) (*45*). Thus, β-sitosterol may probably be used as a preventive agent against cancer in the future.

ABBREVIATIONS USED

SGC-7901, human gastric cancer cell line; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AO, acridine orange; EB, ethidium bromide; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; DTT, dithiothreitol; IR, inhibition rate; M, marker of standard protein; C, control; SGCE, single-cell gel electrophoresis.

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