RESEARCH ARTICLE

S-benzyl-cysteine-mediated Cell Cycle Arrest and Apoptosis Involving Activation of Mitochondrial-dependent Caspase Cascade through the p53 Pathway in Human Gastric Cancer SGC-7901 Cells

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Abstract

S-benzyl-cysteine (SBC) is a structural analog of S-allylcysteine (SAC), which is one of the major water-soluble compounds in aged garlic extract. In this study, anticancer activities and the underlying mechanisms of SBC action were investigated and compared these with those of SAC using human gastric cancer SGC-7901 cells. SBC significantly suppressed the survival rate of SGC-7901 cells in a concentration- and time-dependent manner, and the inhibitory activities of SBC were stronger than those of SAC. Flow cytometry revealed that SBC induced G2-phase arrest and apoptosis in SGC-7901 cells. Typical apoptotic morphological changes were observed by Hoechst 33258 dye assay. SBC-treatment dramatically induced the dissipation of mitochondrial membrane potential ($\Delta\Psi m$), and enhanced the enzymatic activities of caspase-9 and caspase-3 whilst hardly affecting caspase-8 activity. Furthermore, Western blotting indicated that SBC-induced apoptosis was accompanied by up-regulation of the expression of p53, Bax and the down-regulation of Bcl-2. Taken together, this study suggested that SBC exerts cytotoxic activity involving activation of mitochondrial-dependent apoptosis through p53 and Bax/Bcl-2 pathways in human gastric cancer SGC-7901 cells.

Keywords: Aged garlic extract - apoptosis-cell cycle - SGC-7901 cell - p53 signalling pathway

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Introduction

Garlic (Allium sativum) has been prescribed for the treatment of several human diseases for centuries. Recent interest has focused on the role of garlic and its chemical constituents in the prevention of cardiovascular disease and cancer (Mukherjee, 2003; Nagini et al., 2008). S-allylcysteine (SAC) is one of the major water-soluble compounds in aged garlic extract, which is derived from the degradation of S-alk(en)yl cysteine sulfoxides (ACSs) (Rose et al., 2005). A growing body of research suggests that SAC has anti-tumor (Hong et al., 2000; Chu et al., 2006), antioxidant (Imai et al., 1994) anti-bacterial (Shen et al., 1996), anti-fungal (Davis, 2005), anti-hepatotoxic (Zhang et al., 2007), and cardioprotective properties activities (Wang et al., 2009). S-benzyl-cysteine (SBC) is a structural analog of SAC with the same cysteine-containing structure. Therefore, we investigated the anticancer effects of SBC and compared these with those of SAC.

Gastric cancer is a serious public health problem although its incidence rate declined in the past several decades (Sun et al., 2009). According to the recent estimates of global cancer incidence, gastric cancer is still a leading cause of cancer-related death in the world, ranking the second in males and the forth in females (Danaei et al., 2005). Many investigations have demonstrated that apoptosis plays a pivotal role in the pathogenesis of tumors. Inducing apoptosis is an efficient approach to treat cancer (Penninger et al., 1996). Apoptosis can be triggered by either extrinsic or intrinsic signaling pathway. The extrinsic death-receptor pathway is activated by the engagement of the death receptor at the cell membrane with Fas-ligands, recruiting Fas-associated death domain to form a death-inducing signaling complex and ultimately leading to the activation of caspase-8 (Cryns et al., 1998). On the other hand, the intrinsic pathway is initiated by cellular response to stress stimuli, culminating in the activation of caspase-9. Caspase-8 and caspase-9 subsequently activate caspase-3 cascade that causes cell death by cleaving a variety of protein substrates (Sandhu et al., 2010). The p53 tumor suppressor gene can regulate cell cycle arrest, apoptosis, and DNA repair in a variety of cells (Hofseth et al., 2004). In addition, p53 participates

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directly in the intrinsic pathway of apoptosis by interacting with the proteins belonged with Bcl-2 family to induce the permeabilization of the outer membrane of mitochondria (Smith et al., 2010). Some Bcl-2 family members (such as Bax/Bcl-2) located on the mitochondrial membrane can alter the permeability of the mitochondrial membrane and trigger the activation of caspases, leading to apoptotic cell death (Chipuk et al., 2008; Yip et al., 2008). In this study, we found that SBC inhibited the proliferation of human gastric cancer SGC-7901 cells by inducing cell cycle arrest and apoptosis, and p53 and Bax/Bcl-2 signals played important roles in SBC-induced apoptosis.

Materials and Methods

Materials and reagents

SBC (CAS NO. 3054-01-1) and SAC (CAS NO. 21593-77-1) was provided by Hanhong Chemical Co., Ltd., Shanghai, China. Fetal bovine serum (FBS), RPMI 1640 medium, penicillin G, streptomycin and trypsin were obtained from the Invitrogen Corporation, USA. Hoechst staining kit, Cell cycle analysis kit, Apoptosis analysis kit, BCA protein analysis kit, JC-1 dye kit, Propidium iodide (PI) agents and Caspase Activity Assay Kit (Caspase-3, Caspase-8, Caspase-9) were purchased from Beyotime, China. The 3-(4,5)-dimethylthiahiazo(-z-y1)-3, 5-di-phenytetra-zoliumromide (MTT) was purchased from AMRESCO Inc., USA. Dimethyl sulfoxide (DMSO) and ribonuclease (RNase) were purchased from Sigma Chemical, USA. The PVDF membranes were purchased from Millipore Corporation, Massachusetts, USA. The polyclone antibodies to p53, Bax and Bcl-2 were purchased from Cell Signaling Technology XPTM, USA. Rabbit polyclone antibodies to GAPDH were purchased from Bioword Technology, MN, USA.

Cancer cell line and culture

Human gastric cancer SGC-7901 cell lines were obtained from Shanghai Institute for Biological Sciences, China. The cells were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin G and 100 mg/ml streptomycin and were kept at 37°C in a humidified 5% CO_2 incubator. The cells were passaged every 2-3 days and were always used at 80-90% of confluence at passages between 3 and 20 after de-freezing. The cells were seeded into different culture plates or dishes at corresponding density for respective assays.

Cell proliferation assay

The cell proliferation was measured by MTT method. Briefly, SGC-7901 cells were collected and seeded in 96-well plates at a density of 5×10^3 cells/well, and treated with phosphate buffered saline (PBS), various concentrations of SBC, SAC or positive control factor, 5-fluorouracil. After incubation for different time, the medium was removed and replaced with a fresh medium (180 μl/well). 20 μl of MTT solution (5 mg/ml) added to each well and the plates were incubated for additional 4 h at 37°C. Then, the medium was aspirated off, and 150 μl of DMSO were added to each well. The absorbance was read at 570 nm as test wavelengths using a microplate reader (TECAN Infinite 200). Cell viability was expressed as a percentage of the untreated cells.

Nuclear staining with Hoechst 33258

SGC-7901 cells were seeded in 24-well plates at a density of 5×10^3 cells/well, and incubated with SBC (5, 10 and 15 mM) for 24 h. Cellular monolayer in 24-well plates was fixed and stained with DNA fluorochrome Hoechst 33258 for 20 min. After washed with PBS, the morphological features of apoptosis (including cellular nucleus shrinkage, chromatin condensation, intense fluorescence and nuclear fragmentation) were monitored by fluorescence microscopy (Zeiss, German).

Measurement of apoptosis by Annexin V-FITC/PI staining

Flow cytometry was used to quantitatively detect the apoptotic rate. Cells (5×10^3/well) were seeded into 6-well plates and exposed to SBC at various concentrations (5, 10 and 15 mM) for 24 h, and then harvested and washed with phosphate buffered saline (PBS). Staining went along with 195 μl bonding buffer containing 5 μl Annexin V-FITC in the dark at room temperature for 10 min, and then added 10 μl PI in the dark 4°C for 10 min. The apoptotic cells were analyzed with FACScan flow cytometry (BD FACSCalibur).

Analysis of cell cycle by PI staining

Cells (3×10^5/well) were seeded into 6-well plates and exposed to SBC at various concentrations (5, 10 and 15 mM) for 24 h, and then harvested and washed with PBS, fixed in 70% ethanol at 4°C. Staining went along with PBS containing 40 μg/ml RNaseA and 10 μg/ml PI in the dark at room temperature for 30 min. The cell cycle was measured using FACScan Flow cytometry (BD FACSCalibur).

Fluorescent probe JC-1 binding assay for mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$m) was monitored using the fluorescent probe JC-1 dye. The cells with polarized mitochondria predominantly contained JC-1 in aggregate form, and fluoresced red or orange. Cells with depolarized mitochondria contained JC-1 predominantly in monomeric form and fluoresced green. After treated with SBC (5, 10 and 15 mM) for 48 hours, cells were directly incubated with 5 mM JC-1, or collected into 1.5 ml tubes and incubated with JC-1 for 20 minutes at 37°C. Then cells were assayed with inverted fluorescence microscopy (Zeiss, German) or FACScan Flow Cytometry (BD FACSCalibur). The green fluorescence recorded using an excitation wavelength of 490 nm and an emission filter of 525 nm. The red-orange fluorescence recorded using 560 nm and 590 nm. Flow cytometry was used to quantitatively detect the red and green fluorescence, and the ratio of red/green fluorescence used to demonstrate mitochondrial membrane potential. Data expressed as mean ± SD. of three different experiments.

Caspase activity assay

SGC-7901 cells were collected and homogenized in

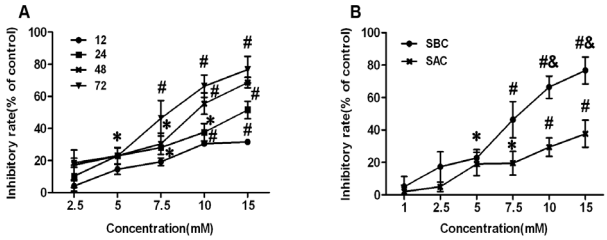

Figure 1. Effects of S-benzyl-cysteine (SBC) and S-allyl-cysteine (SAC) on Human Gastric Cancer SGC7901 Cell Survive Rate. (A) SGC7901 cells incubated with 2.5, 5, 7.5, 10 and 15 mM SBC for 12, 24, 48 and 72 hours, respectively. (B) SGC7901 cells incubated with 1, 2.5, 5, 7.5, 10 and 15 mM SBC or SAC for 72 hours, respectively. The survive rate was expressed as the optical density ratio of the treatment to control. Each value represents means ± SD in three independent experiments. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.01$ vs. control group, $^{\&}p < 0.05$ vs. SAC group

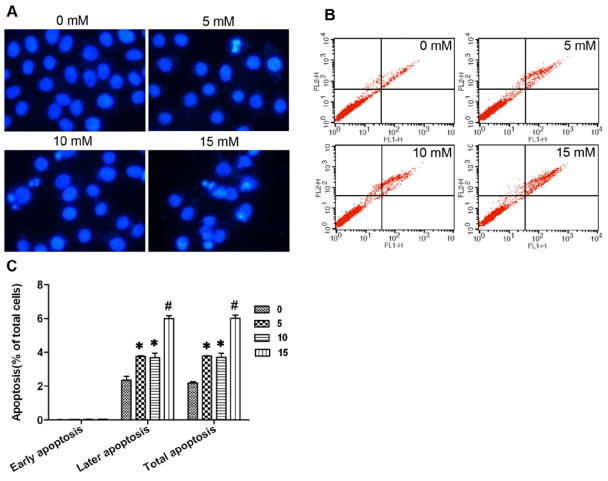

Figure 2. SBC Induced the Apoptosis of SGC7901 Cells. (A) Cells stained with Hoechst 33342 were imaged with fluorescence microscope (200×). The condensed and fragmented nuclei with bright stain were considered as apoptotic cells. (B) Apoptotic cells were analyzed by flow cytometry with Annexin V-FITC and propidium iodide (PI) staining. Early apoptotic cells (Annexin-V+) displayed in the lower right quadrant and late apoptotic cells (Annexin-V+ and PI+) were shown in the upper right quadrant. Necrosis cells were shown in the upper left quadrant (PI+). (C) The percentages of apoptotic cells were indicated by Annexin-V+ cells shown as the mean ± SD of 3 independent experiments. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.01$ vs. control group

cell lysis buffer. The protein concentration was measured using BCA protein assay kit. Total volume of 100 µl (100 µg protein extract, 50 µl reaction buffer supplemented with 10 mM DTT and substrates of pNA) was added to each tube. After incubating for 1.5 hr at 37°C, the formation of p-nitroanilide in the samples was measured with a microplate reader (TECAN Infinite 200) at 405 nm. Caspase-3, caspase-8, caspase-9 activities were assessed in parallel. The caspase activities were expressed by comparing with the results of the controls.

Western blotting assay

After the treatment with SBC (5, 10 and 15 mM) for 48 h, SGC-7901 cells were collected and homogenized in 200 μl RIPA lysis buffer. Cell lysate was centrifuged (12,000×g for 15 min at 4°C) and protein concentration was determined by the BCA protein assay kit. Equal amounts of protein were denatured and separated by 10% SDS-PAGE, then transferred onto PVDF membranes using a Bio-Rad miniprotein-III wet transfer unit. Nonspecific binding was blocked with 5% bovine serum albumin dissolved in TBST at room temperature for 1 h. Subsequently, the membranes were then washed three times and incubated with individual primary antibodies of p53, Bax and Bcl-2 at 4°C over night. The membranes were incubated with the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature followed by three washings. The signals were detected with a chemiluminescence ECL reagent and quantified by densitometry using a gel visualizer (Alpha Innotech, CA, USA). GAPDH served as the loading control, and the results were expressed as the percentage of control.

Statistical analysis

The data were expressed as means (standard errors of mean ± SD). The differences were analyzed by one-way analysis of variance (ANOVA) and Tukey's Studentized Range test. The differences were considered significant at $p<0.05$.

Results

SBC and SAC inhibited SGC-7901 cell proliferation

SGC-7901 cell survival rates were assayed by MTT method at 570 nm. As showing in Figure 1A, after incubate with 2.5, 5, 7.5, 10 and 15 mM SBC for 12, 24, 48 and 72 h, the inhibition rates of cells were notably increased in a concentration- and time-dependent manner ($p<0.01$). When treated respectively with 1, 2.5, 5, 7.5, 10 and 15 mM SBC or SAC for 72 h, the inhibition rates were elevated from 5.04% to 76.66% and 2.0% to 37.68%, respectively (Figure 1B). This result showed that the inhibitory activities of SBC were stronger than those of SAC $p<0.05$).

SBC induced apoptosis of SGC-7901 cells

SGC-7901 cells were treated with different concentrations of SBC and were analyzed by Hoechst 33258 staining. As showed in Figure 2A, normal cells exhibited regular and round shaped nuclei with a pallid blue, whereas apoptotic cells were characterized by the condensation and the fragmentation of nuclei with bright fluorescence. Flow cytometry analysis was used to quantify the apoptotic SGC-7901 cells after treatment with SBC (Figure 2B). The proportion of total apoptotic cells was increased from 2.04% to 6.17% (Figure 2C). The results revealed that SBC induced apoptosis in SGC-7901 cells in a dose-dependent manner ($p<0.01$).

SBC affected SGC-7901 cell cycle

After the administration with SBC, SGC-7901 cell

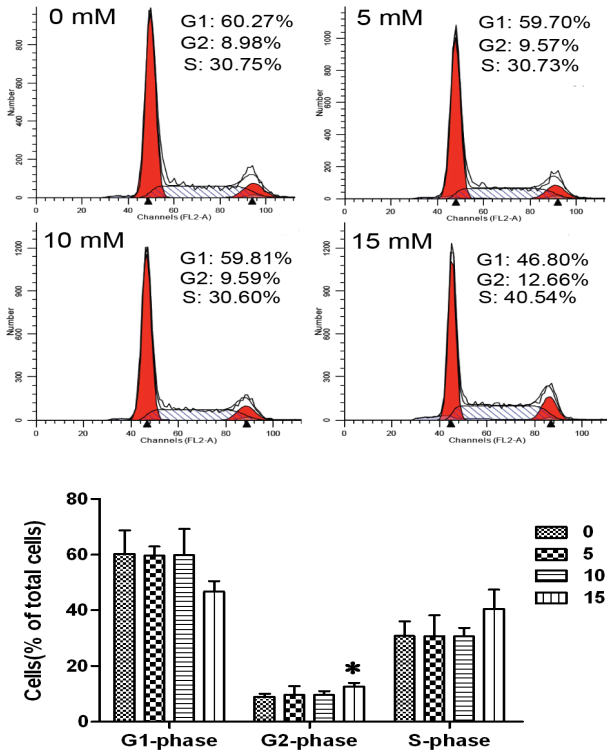

Figure 3. SBC Affected the Cell Cycle Progression of Human Gastric Cancer SGC7901 Cells. The cell cycle was analyzed by flow cytometry with PI staining. Data expressed as the mean ± SD of three different experiments. *$p < 0.05$ vs. control group

cycle was detected by flow cytometry. As compared to the control, G2-phase of SGC-7901 cells was elevated from 8.98% to 12.66%, whereas those at G1-phase and S-phase had almost no change (Figure 3). The above results indicated that SBC blocked the cell cycle at G2-phases in SGC-7901 cells ($p<0.05$).

Effect of SBC on mitochondrial membrane potential (ΔΨm)

Mitochondrial membrane potential (ΔΨm) was assayed by the fluorescent probe JC-1 dye. Figure 4 shows that the normal cells with red-orange fluorescence were reduced while the apoptotic cells with green fluorescence were augmented following SBC treatment. SBC-treatment dose-dependently decreased the amount of ΔΨm from 97.62% to 77.09% as compare with vehicle control ($p<0.05$).

Activation determination of caspase

To elucidate whether SBC activates the caspase-dependent cell death pathway, we measured the catalytic activity of caspase-3, caspase-8 and caspase-9 in SBC-treated SGC-7901 cells. After SGC-7901 cells were treated with 15mM SBC, caspase-3, caspase-8 and caspase-9 activities were increased to 201.52%, 128.34% and 322.73% in comparison with the internal control (Figure 5). The results showed that SBC significantly increased caspase-3 and caspase-9 activities and hardly affected the activity of cascase-8. Therefore, SBC induced apoptosis mainly through the mitochondrial-dependent caspase pathway.

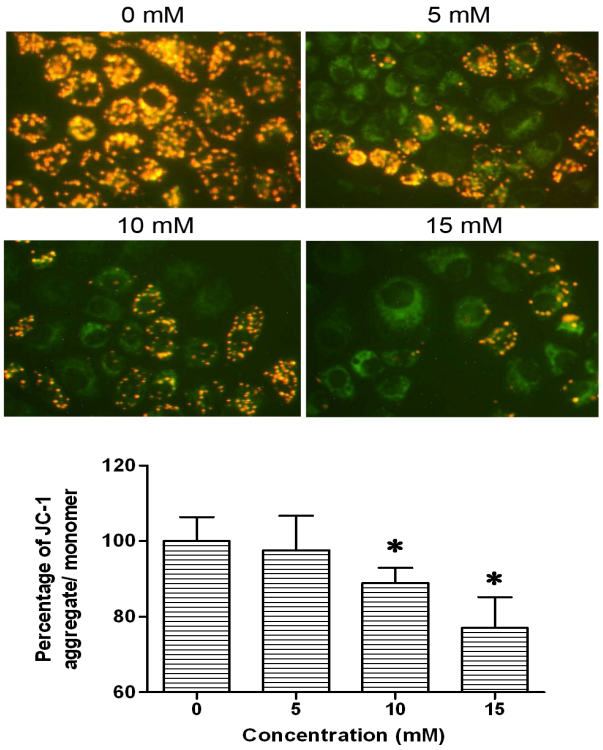

Figure 4. Effect of SBC on the Mitochondrial Membrane Potential (ΔΨm) in SGC7901 cells. After the application of SBC, cells were labeled with JC-1 over a 30 min period at 37°C, and fluorescence was monitored under the microscope (400×). Flow cytometry was used to quantitatively detect the red and green fluorescence, and the ratio of red/green fluorescence. Data expressed as mean ± SD. of three different experiments, *$p < 0.05$ vs. control group

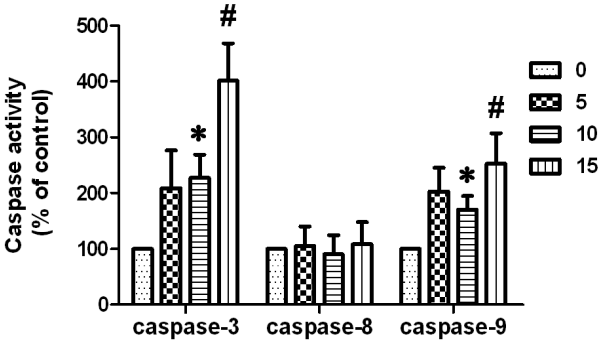

Figure 5. SBC Affected the Activities of Caspase-3, Caspase-8, and Caspase-9 in SGC7901 Cells. After the incubation with 5, 10 and 15 mM of SBC for 48 h, the enzymatic activities of caspase-3, caspase-8, and caspase-9 were tested by adding caspase-3 substrate DEVD-pNA, caspase-8 substrate IETD-pNA, or caspase-9 substrate LEHD-pNA. Caspase activities were determined in contrast to the control. The values are expressed as the mean ± SD of 3 independent experiments. *$p < 0.05$ vs. control group, #$p < 0.01$ vs. control group

Effect of SBC on expressions of p53, Bax/Bcl-2 proteins

To further elaborate on the possible mechanism underlying SBC-induced apoptosis, we tested the effect of SBC on the protein expressions of Bcl-2, Bax and p53. Western blotting analysis revealed that the Bcl-2 expression was obviously decreased to 39.48%, whereas

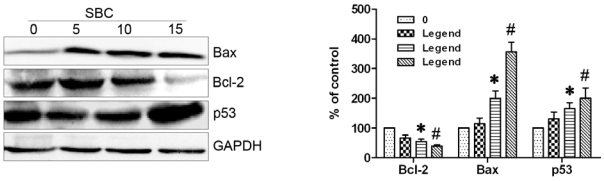

Figure 6. Western Blot Analysis of SBC on the Expression Levels of p53, Bcl-2 and Bax. Cells were treated with 5, 10 and 15 mg/ml of SBC for 48 h, after which whole cell extracts were prepared, and 50 μg proteins of these extracts were resolved by SDS-PAGE, and then probed with specific antibodies. Similar patterns of protein expression were obtained from three independent experiments with the similar results. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.01$ vs. control group

the Bax and p53 expression was increased to 356.03% and 200.21% as compared to GAPDH control (Figure 6). The results indicated that SBC up-regulation of the Bax/Bcl-2 ratio and p53 expression in a dose-dependent manner ($p<0.01$).

Discussion

Many researchers have demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which has become a principal mechanism for effective anticancer therapy (Kundu et al., 2005; Liu et al., 2012). In this study, we showed SBC could decrease the survival rate of human gastric cancer SGC-7901 cells in a concentration- and time-dependent manner, and the inhibitory activities of SBC were stronger than those of SAC (Figure 1A and Figure 1B). Apoptosis induction was a possible mechanism in the anti-proliferation activities of SBC, as it was confirmed by the cell morphological analyses of SBC-treated SGC-7901 cells, which showed the apoptotic characteristics, such as apoptotic bodies and nuclear condensation, in contrast to the control cells (Figure 2A). The flow cytometry data of Annexin V-PI dual staining indicated further that SBC could induce the apoptosis of SGC-7901 in a concentration-dependent manner (Figure 2B and 2C). Similar to apoptosis, cell cycle arrest is another index for cell growth inhibition. The cell cycle arrest can trigger proliferation inhibition and apoptosis in cancer cells (Pu, 2002; Chao, 2004). Our results showed that SBC induced SGC-7901 cells to arrest at the G2 phase. During cell cycle, the G2/M checkpoint is a potential target for cancer therapy. It prevents DNA-damaged cells from entering mitosis and allows for the repair of DNA damage (Wang, 2009).

A large number of genes involved in the regulation of apoptosis. Tumor suppressor gene p53 is a key element in the induction of cell cycle arrest and apoptosis following DNA damage or cellular stress through regulating the transcription and expression of proapoptotic protein Bax (Lane, 1992; Yamaguchi et al., 2003; Harris et al., 2005). The increased ratio of proapoptotic protein Bax/ antiapoptotic protein Bcl-2 results in the dispersal of mitochondrium transmembrane potential, which indicates the dysfunction of the mitochondria and triggers the cell death (Fujita et al., 1992; Schluep et al., 2009). Our results showed that SBC-treatment decreased the level of $\Delta\Psi m$ by using JC-1 stain and flow cytometry assay (Figure 4).

Caspase is an executer of apoptosis associated with the two signaling pathways. In the caspase family, either caspase-8 or caspase-9 and the subsequent effectors, caspase-3 cascades, are the key points in the apoptotic process. Regardless it is the mitochondrial pathway or the cell death receptor pathway, they ultimately activate caspase-3 that is essential for DNA fragmentation (Heimlich et al., 2004). Therefore, the activity of caspase-3 is considered as an appropriate measure of cytotoxic responsiveness (Hoffman et al., 2002). In this study, SBC could notably increase caspase-3 and caspase-9 activities and hardly affected caspase-8 activity in SGC-7901 cells. Therefore, we suggest that SBC may be a valuable target for the inhibition of the proliferation of gastric cancer based on the findings that SBC down-regulated the expression of Bcl-2 but up-regulated the expression of Bax and p53, which may subsequently activate the caspase cascade and mainly induce mitochondrial-dependent apoptosis.

In conclusion, this study combined morphology with biochemical techniques to demonstrate that SBC exerted its cytotoxic activity via p53 and Bax/Bcl-2 signal pathways in human gastric cancer cells.

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