RESEARCH COMMUNICATION

Molecular Mechanisms of Celery Seed Extract Induced Apoptosis via S Phase Cell Cycle Arrest in the BGC-823 Human Stomach Cancer Cell Line

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Abstract

Background: Mechanisms of apoptosis in tumor cells is an important field of tumor therapy and cancer molecular biology. Loss of cell cycle control, leading to uncontrolled proliferation, is common in cancer. Therefore, the identification of potent and selective cyclin dependent kinase inhibitors is a priority for anti-cancer drug discovery. There are at least two major apoptotic pathways, initiated by caspase-8 and caspase-9, respectively, which can activate caspase cascades. Apoptosis triggered by activation of the mitochondrial-dependent caspase pathway represents the main programmed cell death mechanism. This is activated by various intracellular stresses that induce permeabilization of the mitochondrial membrane. Anti-tumor effects of celery seed extract (CSE) and related mechanisms regarding apoptosis were here investigated in human gastric cancer BGC-823 cells. Methods: CSE was produced by supercritical fluid extraction. Cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and apoptosis by flow cytometry using Annexin/PI staining and DAPI staining and a laser scanning confocal microscope (LSCM). Cell cycling was evaluated using PI staining with flow cytometry and expression of cell cycle and apoptosis-related proteins cyclin A, CDK2, bcl-2 and bax was assessed by immunohistochemical staining. Results: CSE had an anti-proliferation effect on human gastric cancer BGC-823 cells in a dose- and time-dependent manner. After treatment, the apoptotic rate significantly increased, with morphological changes typical of apoptosis observed with LSCM by DAPI staining. Cell cycle and apoptosis related proteins, such as cyclin A, CDK2 and bcl-2 were all down-regulated, whereas bax was up-regulated. Conclusions: The molecular determinants of inhibition of cell proliferation as well as apoptosis of CSE may be associated with cycle arrest in the S phase.

Keywords: Celery seed extract - gastric cancer cells - apoptosis - cell cycle

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Introduction

Gastric cancer is one of the most frequent digestive system cancers and the leading cause of death among malignancies in most of country in the world (Jemal et al., 2010). Recent evidence suggests that apoptosis of cells is closely related to the occurrence, progress and metastasis of tumors (Hung et al., 2008). Study of the mechanisms of apoptosis in tumor cells is an important field of tumor therapy and molecular cancer biology (Ye et al., 2007; Yang et al., 2009). Apoptosis is regarded as the preferred mechanism for managing cancer cells (Fesik 2005) .

The search for putative bioactive compounds from natural plant sources is currently a highly active area of scientific research. Celery *Apium graveolens* L (Fam. Umbelliferae), is widely grown in the temperate zone as a garden crop, and its leaf stalks are used as a popular vegetable in most of Asian countries and extract of celery has been recommended in Chinese herbal medicine as a remedy for hypertension and the "cleansing" of blood. In addition, celery seeds are used as a homeopathic treatment for rheumatism, arthritis, and gout (Woods et al., 2001). Phytochemical investigations of celery seeds are reported to possess anti-inflammatory and antioxidant property and are use to elevate liver and spleen indurations (Lewis et al., 1985). Data were confirmed by Zheng et al. (Zheng et al., 1993) who showed sedanolide-induced GST activity in mice. Study found sedanolide to inhibit benzópyrene induced forestomach tumour formation in mice. Surprisingly, no further work on CSE and apoptotic modulation on human stomach cancer cell lines BGC-823 via cell cycle arrest has been reported. its mechanisms in human gastric cancer cells remain unknown. We investigated the effects of CSE on cell growth and apoptosis in gastric cancer cells, particularly focusing on inhibition of cell proliferation, cell cycle distribution, and the signaling pathways involved in CSE-induced apoptosis.

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Several studies have shown that the apoptosis might be induced due to cell cycle arrest (Hartwell and Kastan 1994; Vermeulen et al., 2003). Therefore, inhibition of the cell cycle has been appreciated as a target for the treatment of cancer (McDonald and El-Deiry 2000; Owa et al., 2001). Inhibition of tumorigenesis often involves modulation of signal transduction pathways, leading to cell cycle arrest and, consequently, apoptosis.

Materials and Methods

Chemicals and reagents

Celery seed was purchased from Jiachen biotechnology company (Shenzhen, China). RPMI-1640 medium, HEPES (4-hydroxyethyl piperazine ethanesulfonic acid), fetal calf serum and trypsogen were purchased from GIBCO (Canada). 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, and trypsin were purchased from Amresco Chemical Co. Ltd. (USA). Propidium iodide (PI) was purchased from Sigma (USA). The Annexin V/PI-FITC apoptosis detection kit was purchased from BD Biosciences (USA). The primary antibodies for Bcl-2, Bax, CDK2, cyclinA , β-actin and the secondary antibody were acquired from Santa Cruz Biotechnology (USA), and all chemicals were of analytical grade and were obtained from Tianjin Chemical Reagents Co. Ltd. (Tianjin, China).

Preparation of celery seed extract (CSE)

Supercritical fluid extraction (SFE), was scaled-up 100-fold using a preparative HA221-50-06 SFE system (SITEC Co. Ltd., Switzerland). A 350 g amount of sample was placed in the extraction vessel with a 1,000 mL capacity, and extracted statically for 1.5 h and then dynamic extraction for 2.5 h by flowing liquid CO_2 at a rate of 40 L/h. The pressure and temperature in the extraction vessel was 25 MPa and 45°C respectively, and which changed to 6 MPa and 35°C in the separate vessel. The extract in supercritical fluid was depressed directly into separate vessels. The crude extract obtained was brown grease and some semisolid (98 g). This extracts were named CSE and dissolved in RPMI 1640 medium in the required amount stored in a refrigerator at 4°C until used.

Cell culture

BGC-823 cells were obtained from the Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China), cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100μg/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO_2; the medium was changed every other day. When the cultures were 80 to 90% confluent, the BGC-823 cells were washed with phosphate-buffered saline (PBS, pH 7.4), detached with 0.25% trypsin, centrifuged and re-plated onto 96- or 24-well plates at an appropriate density according to each experimental scale.

Cell viability and cytotoxicity

The cultured cells at the exponential growth phase were harvested from the culture flasks by trypsin and then resuspended in fresh medium. The cell suspensions were dispensed into a 96-well microplate at 100 μl/well and incubated in an incubator with 5% CO_2 at 37°C. After 24 h, 200 μl of various concentrations (0 to 500 μg/mL) of CSE were added and incubated for 24, 48, and 72 h to evaluate their anti-proliferation effects on BGC-823. The cell proliferation in the microplate was determined using the MTT (3-(4,5-dimethylthiazol-2-yle)2,5-diphenyl-tetraloziumbromide) assay after incubation (Chang et al., 2008). Twenty microliters of PBS solution containing 5 mg/ml MTT was added to each well. After incubation for 4 h, the cells from each well were solubilized with 100 μl DMSO for optical density determination at 570 nm. Cell proliferation activity was expressed as the percentage of MTT counts of treated cells relative to those of the control (% of control). The percentage of cell growth inhibition was calculated as follows:

Inhibition (%)=[A570(control)–A570(drug)]/A570 (control)×100%

Observation of morphological changes

The BGC-823 cells were seeded in six-well plates (2.0×10^5 cells/well) and incubated in RPMI1640 at 37°C in an atmosphere of 5% CO_2 for 24 h. The cells were treated with several concentrations of CSE. After incubation for 12 h, cellular morphology was observed using a phase contrast microscope (Nikon, Japan). The photographs were taken at a magnification of 40×.

The treated cells were fixed with 95% ethanol for 30 min at 4°C, washed in PBS, and stained with 1 mmol/L DAPI for 30 min at room temperature. The morphological changes in the nuclear chromatin were observed a laser-scanning confocal microscope (LSCM, Bio-Rad Radiance 2100, U.S.A) with 488-nm excitation and 525-nm emission wavelengths.

Flow cytometry analysis of cell apoptosis

BGC-823 cells were cultivated in RPMI1640 with 10% fetal bovine serum. Before the cell density was modulated to 1×10^5 cells, cell synchronization was conducted to force the cells to the G0 phase via a serum-free culture for 24 h, and the cells were washed twice with PBS before being suspended in a binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 140 mM NaOH, and 2.5 mM $CaCl_2$). Five microliters of fluorescein isothiocyanate (FITC)-labeled Annexin V was mixed with 100 μl of cell suspensions containing 1×105 cells, and the cells were incubated at room temperature for 5 min. Thereafter, 50 μl of propidium iodide (PI) solution (10 μg/ml) was added to the cells, followed by an additional 5-min incubation period. The scatter parameters of the cells (20,000 cells per experiment) were analyzed using a FAC Scan flow cytometer and Cell Quest analysis software (Becton–Dickinson, CA, USA). Four cell populations were identified according to the following interpretations: viable population in the lower-left quadrant (low-PI and FITC signals), early apoptotic population in the lower-right quadrant (low-PI and high-FITC signals), necrotic population in the upper-left quadrant (high-PI and low-FITC signals), and late apoptotic or necrotic population in

the upper-right quadrant (high-PI and high-FITC signals).

Analysis of cell cycle phase distribution by flow cytometry

PI is a highly water soluble, fluorescent compound that cannot pass through intact membranes and is generally excluded from viable cells. It binds to DNA by intercalating between the bases in double stranded nucleic acids of exposed nuclei (Lima et al., 2002). After fixed and permeabilized cells were stained with PI, the relative fluorescence intensity of PI was measured by flow cytometry as an indirect measure of the cellular DNA content.

After 12 h of culture with medium containing 50, 100, 300mg/L CSE, BCG-823 cells were collected by trypsinization, washed with PBS and fixed in ice-cold 70% ethanol for at least 24 h. The cells were washed twice with PBS by centrifuging at 1000 rpm for 10min. The cells were resuspended in 3ml of PBS for 5min. Then, the cells were stained with 1ml PI (0.1mg/mL with 0.1% TritonX-100) and incubated in the dark for 30min. The samples were analyzed by flow cytometry (FACSCalibur, BD, USA). Cells with a lower DNA content than that of the G1 phase of the cell cycle were considered as hypodiploid cells (sub-G1 phase). The percentage of cells in a particular phase was analyzed using the CellQuest and Modfit software (Beckman, USA). A flow cytometer equipped with a 15mW argon laser and an excitation line of 488 nm was used. Fluorescence intensity of PI was detected at an emission wavelength 630 nm.

Western blot analysis

The 20 μg of protein in each 20-μl sample was electrophoresed through 10% SDS-PAGE gels as previously described (Rasmussen et al., 2008). Separated proteins were incubated with primary antibodies overnight at 4°C, transferred to nitrocellulose membranes, and blocked with a 5% skim milk solution. They were incubated with secondary antibodies for 1 h at 37°C. Each antigen-antibody complex was visualized by enhanced chemiluminescense (ECL) western blotting detection kits (Amersham Pharmacia Biotech, Piscataway, NJ), and band densities were determined using Chemi Doc Software (BioRad); β-Actin was used as a loading for normalization.

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are given as the mean ± SE. Statistical analysis was performed using the statistical software package SPSS 13.0 (SPSS). A p-value of 0.05 (two-sided) was considered statistically significant.

Results

Cytotoxic activity of CSE on BGC-823 cells

As shown in Figure 1A, untreated BGC-823 cells grew well with clear skeletons, whereas cells treated with CSE exhibited cytoplasmic shrinkage and either detached from each other, floated in the medium, or became distorted and blurry under a phase contrast microscope. The number of sloughed cells increased

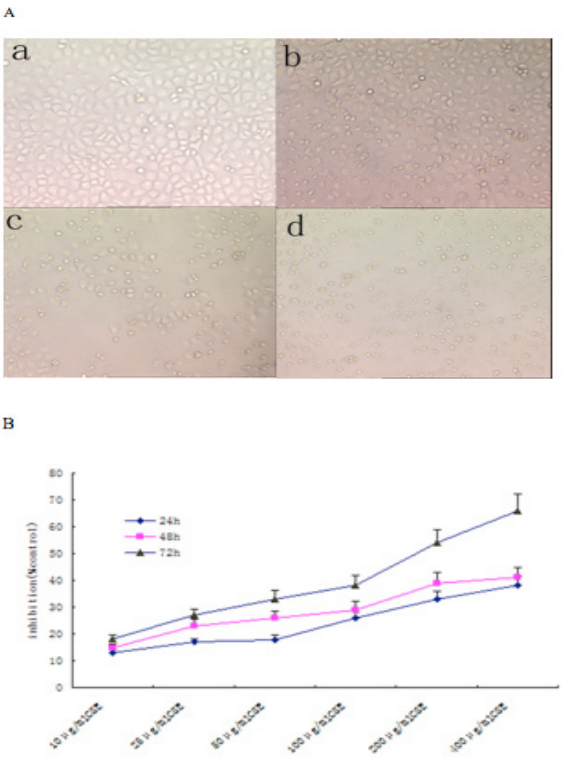

Figure 1. CSE Inhibition of the Viability of BGC-823 Cells. (A) Morphological changes of BGC-823 cells exposed to CSE for 24 h imaged with a phase contrast microscope at 40×. a: vehicle treated; b: 50 μg/ml; c: 100 μg/ml; d: 300 μg/ml. (B) Effect of CSE on BGC-823 cells viability. BGC-823 cells were treated with CSE at the indicated concentrations for 0-72 h. Cell viability was then determined by MTT assay and expressed as the mean±SD, n = 3. The OD value at 570 nm is proportional to the number of cells with CSE

with increasing drug concentrations. The MTT assay showed that CSE significantly inhibited the viability of BGC-823 cells (Figure 1B). The cells were incubated in the absence or presence of various concentrations of CSE for specified time periods. The MTT assay showed that CSE decreased the viability of BGC-823 cells in a concentration-dependent and time-dependent manner ($P<0.05$ and $P<0.01$, respectively).

Effect of CSE on apoptosis in BGC-823 cells

To identify whether CSE induces apoptosis, the treated cells were also stained with Annexin V-FITC/PI, and the population of apoptotic cells was analyzed by flow cytometry. As seen in Figure 2A, the drug treatment significantly increased the proportion of apoptotic cells. In the vehicle treated samples, 10.81±1.16% of cells stained positive for Annexin V-FITC and PI, while CSE treatment showed increases of 15.73±2.12%, 31.36±3.19%, and 41.86±4.42% in early and late stage apoptosis ($P<0.01$). These results demonstrated the ability of CSE to induce apoptosis mainly early stage apoptosis in BGC-823 cells. The morphologic changes of cells treated in the manner described above were also observed with LSCM by DAPI staining. As shown in Figure 2B, typical morphological changes, such as the formation of apoptotic bodies, appeared after the cells were treated for 24 h with 300 μg/ml CSE, whereas the vehicle treated cells did not show the evident apoptotic morphological changes.

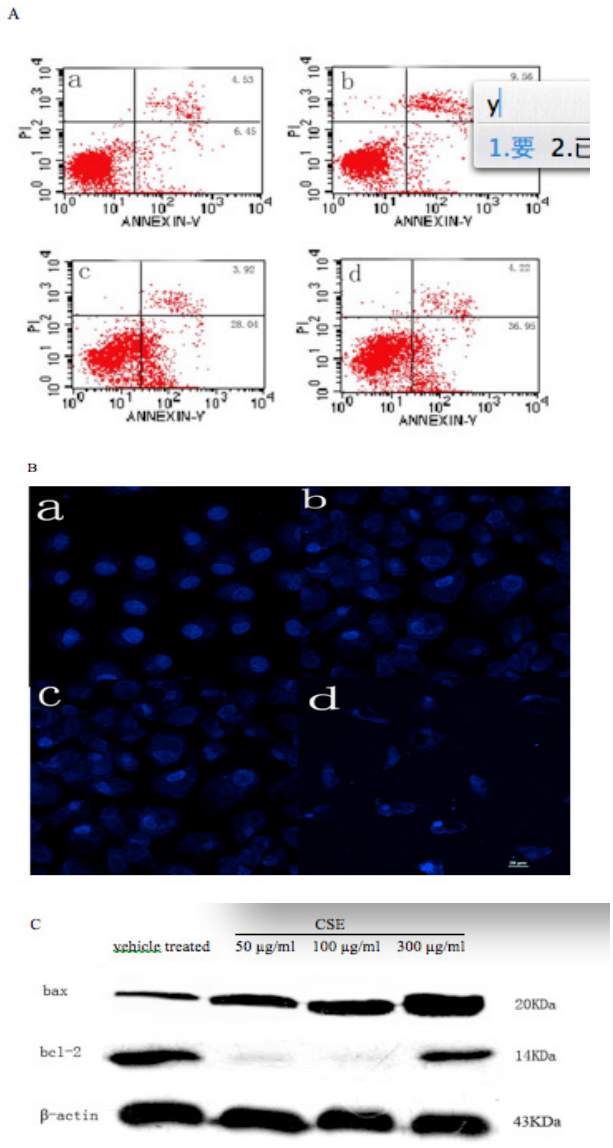

Figure 2. CSE Induced Apoptosis in BGC-823 Cells after 24h Following the Treatment. (A) Apoptotic cells determined with Annexin V/PI staining by flow cytometry assay. a: vehicle treated; b: 50 μg/ml; c: 100 μg/ml; d: 300 μg/ml. (B) Morphological changes of BGC-823 cells as determined with a LSCM at 600× labeled with DAPI. a: vehicle treated; b: 50 μg/ml; c: 100 μg/ml; d: 300 μg/ml. (C) Western blot analysis of the expressions of the level of bcl-2, bax and β-actin (internal control) protein in vehicle treated and CSE-treated BGC-823 cells

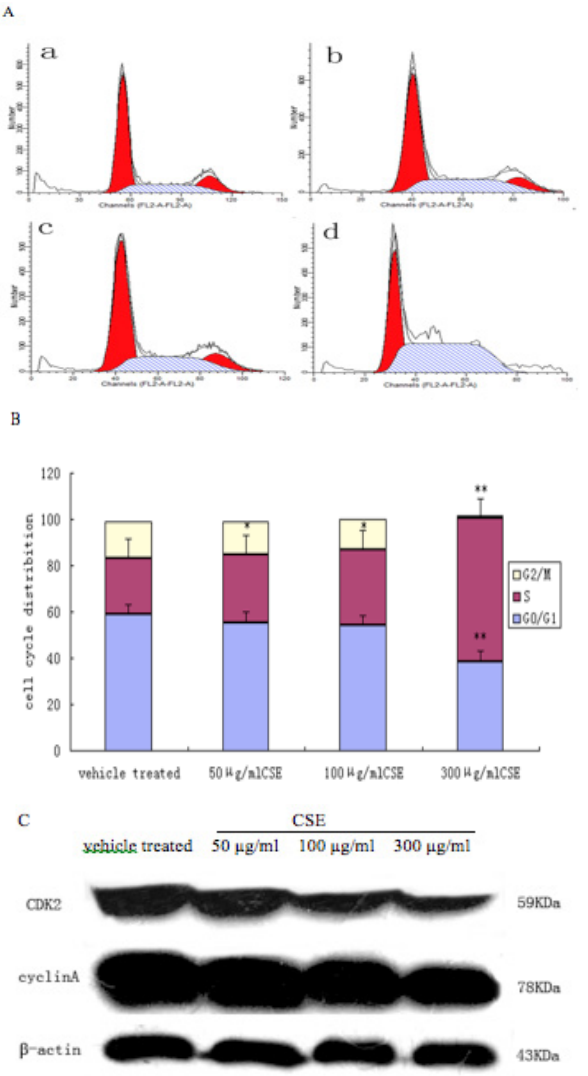

Figure 3. CSE Induces S Cell Cycle Arrest in BGC-823 Cells. (A) Cell cycle distribution was monitored by FCM using a propidium iodide staining assay. a: vehicle treated; b: 50 μg/ml; c: 100 μg/ml; d: 300 μg/ml. (B) Histogram of cell cycle distribution after treatment with CSE for 24 h. Cell cycle distribution was monitored by flow cytometry using a propidium iodide staining assay. Each histogram represents three parallel experiments, and each bar represents the mean±SEM (one-way ANOVA). *P<0.05 vs.control, **P<0.01 vs.control. (C) Western blot analysis of the expression of cyclin A and CDK2 with or without CSE treatment of BGC-823 cells

To determine whether apoptosis induced by CSE was due to a mitochondrial-dependent caspase pathway, we next investigated the levels of bcl-2 and bax, which was the core protein in the caspase cascade of BGC-823 cells after CSE treatment for 24 h. Figure 2C shows the expression of bax was increased and bcl-2 was decreased after CSE treatment for 24 h compared to the vehicle treated, which indicated that CSE indeced the apoptosis process in BGC-823 cells.

Analysis of cell cycle phase distribution by flow cytometry

Cell cycle phase distribution was analyzed by flow cytometry with PI staining (Figure 3A). The percentages of cells in G0/G1, S and G2/M phases were calculated using CellQuest and Modfit software and are indicated in the Figure 3B. Flow cytometry DNA analysis of the treated cultures revealed that CSE induced concentration-dependent cell cycle arrest in the S phase, which was accompanied by the G0/G1 phase cells were markedly decreased from 58.38% to 38.72%. A significant accumulation of cells in the S phase (30.51–61.54%) was observed at various CSE doses. This confirmed that CSE blocked DNA replication in the S phase of the cell cycle rather than G2/M phase arrest induction in BGC-823 cells, which contributed to the effects of CSE on decreasing viability against BGC-823 cells.

To gain insight into the mechanism of S phase cell cycle arrest induced by CSE, we examined the expression of cyclins A and CDK2, which are tightly related to S cell cycle progression, using the western blot assay. As shown in Figure 3C, the expression of cyclin A and CDK2 was

decreased after CSE treatment for 24 h.

Discussion

Natural products with anticancer properties could be valuable substances in cancer treatment. In this study, we assessed the inhibitory effects and molecular mechanisms of CSE using human gastric cancer BGC-823 cells. MTT showed that CSE inhibited the growth of BGC-823 cells in both time-dependent and concentration-dependent manners. To determine whether the cytotoxic activity of CSE was due to apoptosis, BGC-823 cells were treated for 0-72 h with indicated concentrations of CSE. Not only were morphological changes such as cytoplasmic shrinkage, detachment from each other, floating in the medium, distortion and some blurring under a fluorescence microscope observed, but marked chromatin condensation and apoptotic body formation in CSE-treated cells were also observed in cells stained with DAPI using an LSCM.

Membrane blistering and apoptotic bodies can be seen under the phase contrast microscope, consistent with previous studies (Sgonc et al., 1994). A major disadvantage in morphological detection of apoptosis is that it is only qualitative. The quantitative analysis of apoptosis mainly relies on flow cytometry with fluorescent markers. DAPI staining, which can detect cell apoptosis, is one of the most sensitive and ideal indicators (Dai et al., 2009).

The present annexin V-FITC/PI staining assay revealed that early and total apoptotic rates of CSE treatment (50, 100 and 300μg/ml) were significantly higher than those of vehicle treated. This may be because of the fact that early apoptosis was mainly revealed by DAPI staining assay. Moreover, DAPI staining assay and typical morphological changes with LSCM afterwords, such as the formation of apoptotic bodies, appeared after the cells treated by CSE, confirming that CSE induced apoptosis in BGC-823 cells.

Dysregulation of the cell cycle mechanism has also been shown to play an important role in the growth of various types of cancer cell, and the induction of cancer cell apoptosis is recognized as an important target in cancer therapy. In this study, CSE-inhibited BGC-823 cell proliferation partly as a result of an accumulation of cells in the S phase of the cell cycle. The S phase is associated with DNA synthesis and the mitotic preparation period, which plays a crucial role in cell cycle progression. The complex formation of cyclins with CDKs results in an active agent that phosphorylates substrates involved in cell cycle progression (Doree et al., 2002; Yu et al., 2007). The mitosis-promoting factor, which comprises a complex of CDK2 and cyclin A, is thought to be the key controller of the progression from S to mitosis (Ayad, 2005). In this study, CSE induced S phase cell cycle arrest the cells of S phase were present at 2.4 fold the typical concentration after 24 h treatment, and Cyclins A and CDK2 were downregulated indicating that cell cycle-related proteins were involved in the CSE-induced cell cycle arrest in BGC-823 cells.

CSE can inhibit the BGC-823 cells cultured in vitro and induce its apoptosis in a concentration- and time-dependent manner. We further confirmed that CSE could induce cellular apoptosis via a mitochondria-mediated pathway.

The accumulated data suggest that the mitochondria-initiated death pathway plays an important role in triggering apoptosis in response to those stimuli. In the mitochondria-initiated death pathway, mitochondria undergoing permeability transition release apoptogenic proteins or apoptosis-inducing factor from the mitochondrial inter membrane space into the cytosol to activate caspase-9, and activated caspase-9 in turn cleaves and activates executioner caspase-3.

The activities of caspase-3, -8, and -9 were up-regulated, indicating that caspases participated in this apoptotic process. Bax is a key component for cellular induced apoptosis through mitochondrial stress (Wei et al., 2001). Upon apoptotic stimulation, Bax forms oligomers and translocates from the cytosol to the mitochondrial membrane (Jürgensmeier et al., 1998; Marzo et al.,1998) and has been shown to induce cytochrome c release and the subsequent steps (including caspase-9 and caspase-3) in the execution phase of apoptosis (Lakhani et al., 2006; Zhao et al., 2006).

BGC-823 cells treated with CSE exhibited elevated levels of proapoptotic Bax expression, while anti-apoptotic Bcl-xL was down-regulated after the significant increase of caspase-3 activity. Based on these results we concluded that caspase and mitochondrial protein played important roles in the apoptotic pathway of CSE-treated BGC-823 cells.

In conclusion, the findings indicate that CSE could significantly inhibit the growth and induce apoptosis via the bcl-2 family related mitochondria-mediated pathway of human gastric cancer cell line BGC-823 in vitro. The promotion of the specific apoptosis pathway, key molecular targets that specifically initiate apoptosis of gastric cancer cells and quality standards of CSE should be further explored. Thus, CSE would be worth investigating as a novel therapeutic agent originating from a natural source, and the induction of apoptosis by CSE in other cancer cell lines is the subject of on-going investigations.

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