Cytotoxic effect of evodiamine in SGC-7901 human gastric adenocarcinoma cells via simultaneous induction of apoptosis and autophagy

AZHAR RASUL[1], BO YU[2], LILI ZHONG[1], MUHAMMAD KHAN[1], HONG YANG[2] and TONGHUI MA[1,2]

[1]Central Research Laboratory, Jilin University Bethune Second Hospital, Changchun 130041;
[2]School of Life Sciences, Liaoning Normal University, Dalian 116029, P.R. China

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Abstract. Evodiamine, an alkaloid isolated from *Evodia rutaecarpa*, possesses potent anticancer activity. Although many reports have elucidated the cytotoxic effects of evodiamine in a variety of cancer cells, little is known about the mechanism of evodiamine-induced cytotoxic activity in gastric cancer cells. To date, no report has addressed the synchronized role of autophagy and apoptosis in evodiamine-induced cytotoxic activity. This study was conducted to investigate the synchronized role of autophagy and apoptosis in evodiamine-induced cytotoxic activity on SGC-7901 human gastric adenocarcinoma cells and further to elucidate the underlying molecular mechanisms. The MTT assay was used to examine the cytotoxicity of evodiamine against SGC-7901 gastric adenocarcinoma cells. The effects of evodiamine on the cell cycle and apoptosis were measured by flow cytometry and cellular morphology was observed under a phase contrast microscope. Acridine orange (AO) staining was used to detect autophagy. The expression levels of Bcl-2 and Bax were detected by Western blotting. The expression level of Beclin-1 in SGC-7901 cells was monitored by reverse transcription-polymerase chain reaction (RT-PCR). Here, we found that evodiamine significantly inhibited the proliferation of SGC-7901 cells and induced G2/M phase cell cycle arrest. Furthermore, both autophagy and apoptosis were activated during the evodiamine-induced death of SGC-7901 cells. Evodiamine-induced autophagy is partially involved in the death of SGC-7901 cells which was confirmed by using the autophagy inhibitor 3-methyladenine (3-MA). Additionally, Beclin-1 is involved in evodiamine-induced autophagy and the pro-apoptotic mechanisms of evodiamine may be associated with down-regulation of Bcl-2 and up-regulation of Bax expression. The inhibitory effects on SGC-7901 cells were associated with apoptosis, autophagy and cell cycle arrest at the G2/M phase in a dose-dependent manner. These results suggest that evodiamine is an effective natural compound for the treatment of gastric cancer and may represent a candidate for *in vivo* studies of monotherapies or combined antitumor therapies.

Correspondence to: Dr Tonghui Ma, Central Research Laboratory, Jilin University Bethune Second Hospital, Changchun 130041, P.R. China
E-mail: math1080@gmail.com

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Introduction

Gastric cancer is one of the most frequently diagnosed cancers worldwide. It is the second most frequent cause of cancer-related deaths (1). The highest incidence rate of gastric cancer occurs in Eastern Asia. Gastric cancer ranked third among the most common malignancies in China (2). Presently, surgical treatment is the main therapy of gastric cancer, although the chance of survival is very low. Thus, there is a massive need for novel and promising agents for the treatment of gastric cancer. Plant-derived compounds have been an important source of anticancer agents and several anticancer compounds have been derived from natural sources in the last few decades (3,4).

Evodia rutaecarpa is a traditional Chinese medicinal plant known as Wu-Chu-Yu. It has been being used for the treatment of gastrointestinal disorders, headache and postpartum hemorrhage (5-7). Evodiamine is a naturally occurring quinolone alkaloid found in the fruit of *Evodia rutaecarpa*. The data of several studies concerning its cytotoxic activity in cancer cells demonstrated that evodiamine inhibited the growth of a wide variety of tumor cells, including breast (8), thyroid (9), liver (10), prostate (11-14), leukemic T-lymphocyte (5,15), melanoma (6,16-18) cervical (19,20), colon (21-24), colorectal (25) and lung cells (26) through induction of apoptosis, regulation of cell cycle and reduction of invasion and metastasis.

The common modes of cell death are apoptosis and autophagy (27,28). Apoptosis is considered as programmed cell death (PCD), while autophagy is a catabolic process of the cell and its organelles as the result of starvation (29,30). Autophagic vacuoles (AVOs) or autophagosomes are formed as result of the sequestering of parts of the cytoplasm or entire organelles respectively, during the process of autophagy (31). Autophagy is not only involved in the recycling of proteins and organelles sustaining survival, it can also induce cell death.

Several natural compounds such as avicins (32), curcumin (33) and avicin D (34), can induce autophagic cell death. Many cellular stresses can also cause autophagy such as ROS and mitochondria dysfunction (35). Currently inducing autophagic cell death has been studied as a potential method for cancer therapy. Previously it was reported that evodiamine induced autophagy in human cervical carcinoma HeLa cells (20). Although, many reports elucidated the cytotoxic effects of evodiamine, yet little is known about the mechanism of evodiamine-induced cytotoxic activity in gastric cancer. So far, no report has addressed the synchronized role of autophagy and apoptosis in evodiamine-induced cytotoxic activity. This study was conducted to investigate whether or not autophagy is involved in the antitumor effects of evodiamine. Additionally, this study was conducted to explore the synchronized role of autophagy and apoptosis in evodiamine-induced cytotoxic activity in human gastric cancer SGC-7901 cells and further to elucidate the underlying molecular mechanisms. Here, we demonstrated that evodiamine significantly inhibited the proliferation of SGC-7901 cells and induced G2/M phase cell cycle arrest. Furthermore, both autophagy and apoptosis were activated during the evodiamine-induced death of SGC-7901 cells.

Materials and methods

Chemical and reagents. Evodiamine was purchased from the Beijing Institute of Biological products and its purity was determined to be about 98% by HPLC measurement. Evodiamine was dissolved in dimethylsulfoxide (DMSO) to make a stock solution and diluted by DMEM (Sigma) before the experiments. Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Culture medium (DMEM), propidium iodide (PI), acridine orange (AO) and DMSO were purchased from Sigma. The apoptosis detection kit (Beyotime Biotechnology, Shanghai, China) and the rabbit polyclonal anti-human Bcl-2 and Bax antibodies were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). Mouse anti-β-actin and secondary antibodies were purchased from Santa Cruz Biotechnology (USA).

Cell culture. SGC-7901 cells were cultured in DMEM media supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were maintained under logarithmic growth conditions and allowed to grow to ~70% confluence before experimentation.

Morphological observation under a phase contrast microscope. SGC-7901 cells were seeded in 12-well flat bottom microtiter plates and then treated with evodiamine at the concentration of 0, 3, 5 and 10 μM. After 24 h of treatment, the morphology of SGC-7901 cells was observed under a phase contrast microscope (Olympus, Tokyo, Japan).

Cell viability assay. Cell viability was measured using the MTT assay as previously described (36). Briefly, SGC-7901 cells were placed in 96-well plates. After the 24 hours exposure to the different concentrations of evodiamine, 10 ml of MTT (5 mg/ml) was added to each well and incubated for 4 h and then the medium from each well was removed carefully. DMSO (150 μl) was added to each well. The absorbance was measured in the microplate reader at a wavelength of 570 nm. The cytotoxicity of evodiamine was measured and rate of on cell growth inhibition (I%) (37) was calculated using the following equation: I% = [A570 (control) - A570 (treated)]/A570 (control) x100.

Flow cytometric analysis of apoptosis. SGC-7901 cells were treated with 3, 5 or 10 μM evodiamine for 24 h and the cells were collected, washed and resuspended in PBS. The apoptotic cell death rate was examined with Annexin V-FITC and PI double staining using the Annexin V-FITC Apoptosis Detection kit according to the manufacturer's instructions. After staining cells with Annexin V-FITC/PI, flow cytometric analysis was performed and data were analyzed using the CellQuest software.

Acridine orange staining. Staining of cells with AO was performed according to a published procedure (38). In brief, AO at a final concentration of 1 mg/ml was added to cells after treating cells with 10 μM of evodiamine for 24 h and with negative control group for a period of 20 min in the dark at 37˚C. Then, cells were washed twice with PBS. Images of cells were obtained under fluorescence microscopy.

Flow cytometric quantification of acidic vesicular organelles (AVOs). AVOs formation (autophagosomes and autolysosomes) is a characteristic feature of autophagy (39). For quantification of AVOs, we used flow cytometry after cells were stained by AO (40). AO is a weak base that accumulates in acidic spaces and gives bright red fluorescence (punctate staining (dots) in the cytoplasm is detected by fluorescent microscopy. The intensity of the red fluorescence is proportional to the degree of acidity. Thus, the formation of AVOs can be quantified. Briefly SGC-7901 cells were harvested after treatment of 10 μM of evodiamine for 24 h. The cell pellet was collected in an eppendorf tube and cells were resuspended in 1 ml PBS. The staining of the cells was performed with AO (1 mg/ml) for 20 min in the dark at 37˚C. Cells were centrifuged at 1000 rpm for 5 min; the cell pellet was rinsed twice with PBS, and then resuspended in 500 μl PBS and analyzed by flow cytometry using the PI staining assay. Cell death was measured by PI staining (41). Briefly SGC-7901 cells were trypsinized after the treatment with 10 μM of evodiamine in the presence of the autophagy inhibitor 3-methyladenine (3-MA) for 24 h and collected and resuspended with 1 ml PBS. Cells were stained with 0.5 ml of staining solution (50 mg/ml PI, 100 mg/ml RNaseA, 0.2% Triton-100) and cells were incubated at 37˚C for 30 min in the dark. Cell death was measured by flow cytometry.

Western blot analysis. For determination of the effect of evodiamine on the expression of apoptotic related proteins, Western blot analysis was performed. Briefly, SGC-7901 cells were incubated without (control) and with evodiamine (5 or 10 μM) for 24 h, cells were harvested, washed twice with PBS and cell lysates were prepared using lysis buffer. Protein estimation was performed using the NanoDrop 1000

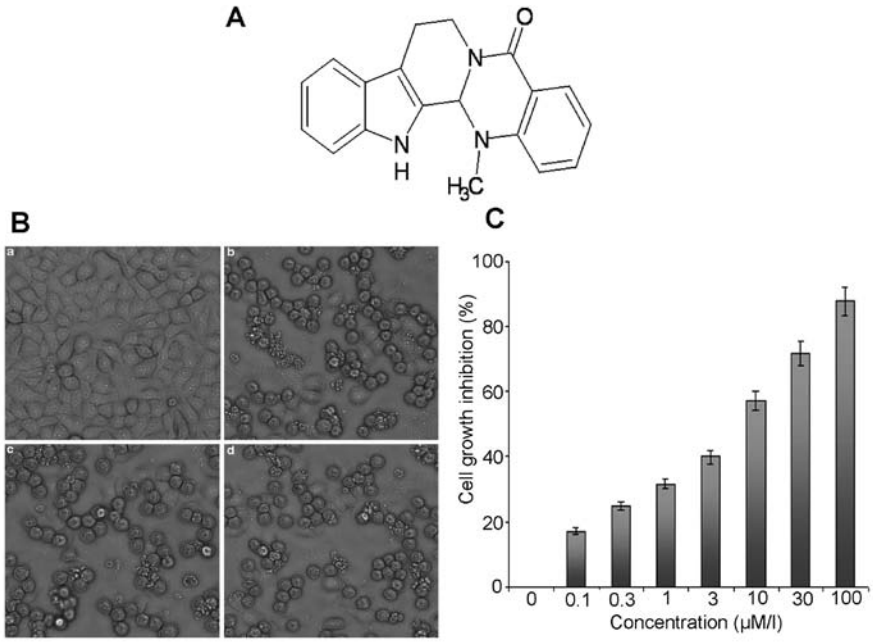

Figure 1. The effects of evodiamine on the morphological characteristics and the viability of SGC-7901 cells. (A) Chemical structure of evodiamine. (B) morphological changes of SGC-7901 cells observed under phase-contrast microscopy after treating cells with (a) control, (b) 3 μM, (c) 5 μM and (d) 10 μM of evodiamine for 24 h. (C) SGC-7901 cells were treated with various concentrations of evodiamine for 24 h. Cell death was measured by using the MTT assay. Data shown are means ± SD (n=3).

spectrophotometer (Thermo Fisher Scientific, USA). An equal amount of protein lysates of cells were subjected to SDS-PAGE followed by Western blotting. The membranes were soaked in blocking buffer (5% skimmed milk) for 2 h in TBST at room temperature. To probe for Bcl-2, Bax and β-actin, membranes were incubated overnight at 4˚C with relevant antibodies, followed by incubation with relevant secondary antibodies and signals were detected using the ECL plus chemiluminescence kit on X-ray film.

Reverse transcription-PCR analysis of Beclin-1. To elucidate the underlying mechanism of autophagy induced by evodiamine, RT-PCR was performed to evaluate the effect of evodiamine on the mRNA expression of Beclin-1. SGC-7901 cells were seeded in 12-well plates and allowed to attach overnight. After treating the cells without (untreated) and with evodiamine (5 or 10 μM) for 24 h, total RNA was extracted using the RNeasy RNA isolation kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using the Reverse Transcription System (Promega) with oligo(dT) primers. The resulting cDNA samples were amplified by PCR, using primers specific for Beclin-1 or GAPDH (primer sequences available upon request) and were separated by electrophoresis on a 2% agarose gel with DNA bands stained with ethidium bromide and photographed. GAPDH served as an endogenous control used to normalize expression data. Each sample was analyzed in quadruplicate.

Flow cytometric analysis of cell cycle. Flow cytometric analysis was performed to examine the effect of evodiamine on the cell cycle arrest as previously described (36).

Statistical analysis. The results were expressed as means ± SD and statistical differences were evaluated by the Student's t-test. P-values of <0.05 were considered significant.

Results and Discussion

We began our investigation with the screening of natural compounds against gastric adenocarcinoma SGC-7901 cells. We found that evodiamine could affect the growth of SGC-7901 cells. Evodiamine, a natural compound, belongs to the alkaloid family. The structure of evodiamine is shown in Fig. 1A. The effect of evodiamine on the growth of SGC-7901 cells was examined after treating the cells with 3, 5 or 10 μM of evodiamine for 24 h and photographs were captured under a microscope. These photographs showed that evodiamine induced the characteristic morphological changes in cells as compared to the control group (Fig. 1B). Moreover, the antiproliferative effect of evodiamine on SGC-7901 cells was examined by the MTT assay. The results showed that evodiamine inhibited the proliferation of SGC-7901 cells in a dosedependent manner (Fig. 1C).

In order to determine whether evodiamine induced apoptosis in SGC-7901 cells, flow cytometric analysis was performed after staining the cells with Annexin V-FITC/PI (propidium iodide). It was observed that there was a concentration-dependent and significant increase in the percentages of Annexin V-FITC positive cells. The results showed that the apoptosis rate was increased from 3.91 to 22.31, 27.57 and 34.29% at concentration of 0, 3, 5 and 10 μM of evodiamine respectively, after exposure of 24 h (Fig. 2). These results are in line with previous reports that evodiamine-induced cell death is associated with apoptosis in different cancer cell types such as breast (8), thyroid (9), liver (10), prostate (11-14), leukemic

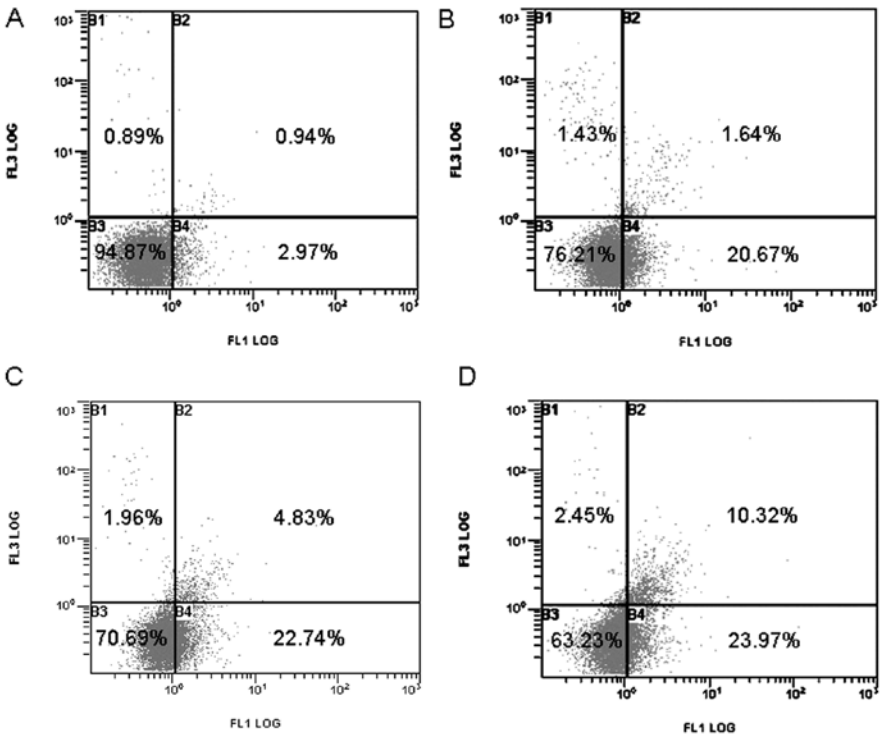

Figure 2. Apoptosis induced by evodiamine in SGC-7901 cells. SGC-7901 cells were treated with (A) 0 μM, (B) 3 μM, (C) 5 μM and (D) 10 μM of evodiamine for 24 h. Cells were stained with FITC-conjugated Annexin V and PI for flow cytometric analysis. The flow cytometry profile represents Annexin V-FITC staining in the x-axis and PI in the y-axis. The number represents the percentages of apoptotic cells in each condition. As shown, the cell populations in the lower right (Annexin V^+/PI^-) represent early apoptotic cells, while those in the upper right (Annexin V^+/PI^+) represent late apoptotic cells.

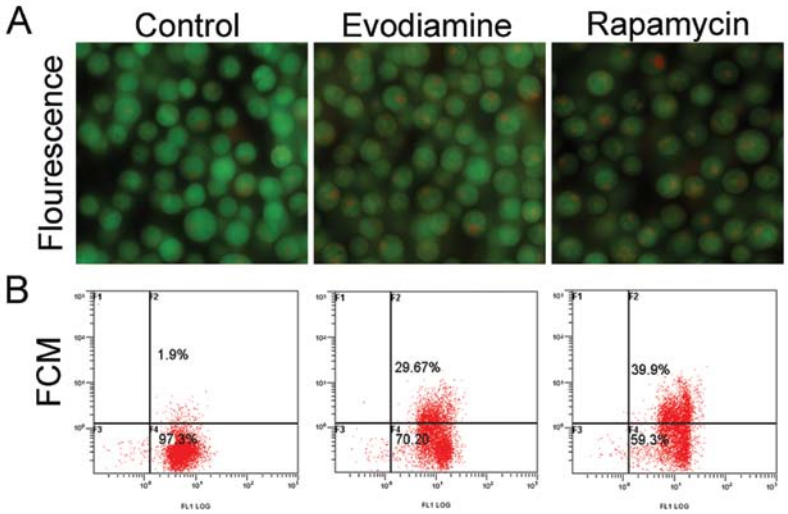

Figure 3. Acidic vesicular organelle (AVOs) formation was observed by fluorescence microscopy and quantified by flow cytometry using acridine orange staining. (A) Cells were treated with evodiamine for 24 h before stained with acridine orange. Cells were examined by fluorescence microscopy. Representative images of cells from three independent experiments are shown, (B) The number represents the percentage of AVOs formation in SGC-7901 cells in each profile after treating cells without (control) and with evodiamine (10 μM) or rapamycin (positive control group) for 24 h. Three independent experiments were performed.

T-lymphocyte (5,15), melanoma (6,16-18), cervical (19,20), colon (21-24), colorectal (25) and lung cancer cells (26).

Previously, it was reported that evodiamine induced autophagy in human cervical carcinoma HeLa cells (20). We hypothesized that evodiamine can induce the autophagy in gastric adenocarcinoma SGC-7901 cells. Autophagy is closely associated with tumors and plays an important role in human tumor suppression, so inducing autophagy is a potential therapeutic strategy in adjuvant chemotherapy (31,35). We investigated whether or not autophagy is involved in the cytotoxic effects of evodiamine on human gastric cancer SGC-7901 cells. For verification, cell death was analyzed by measuring the permeability of the cell membrane to AO staining as described in Materials and methods. The formation

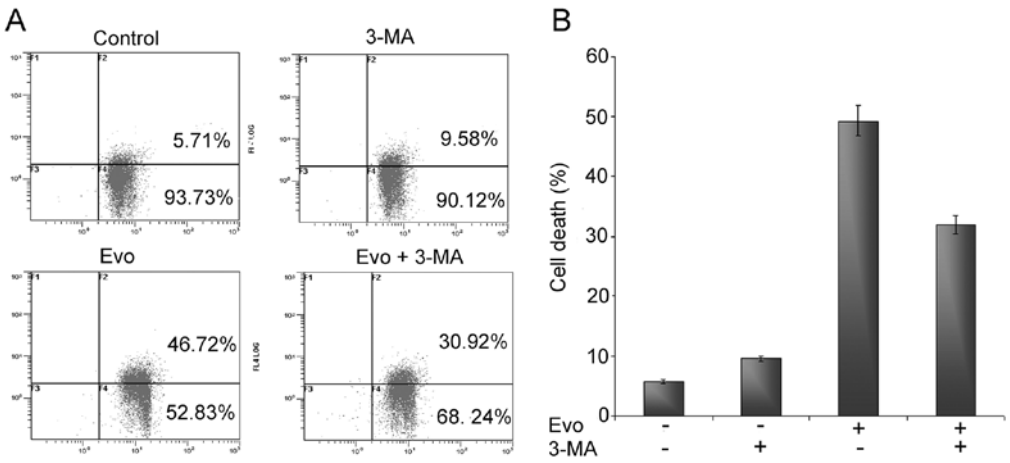

Figure 4. Effect of evodiamine-induced autophagy on cell death of SGC-7901 cells. (A, B) Cells were treated with evodiamine (10 μM) in the presence or absence of 3-MA. The number represents the percentage of dead cells in each profile after treating cells with 3-MA alone, evodiamine (10 μM) alone, evodiamine (10 μM) together with 3-MA and with negative control group for 24 h. Three independent experiments were performed.

of acidic vesicular organelles (AVOs), is one of the characteristic features of cells which passes through the process of autophagy after exposure of different autophagy inducer agents (42,43). Autophagic vacuoles (AVOs) or autophagosomes are formed as a result of sequestering the parts of the cytoplasm or entire organelles respectively during the process of autophagy (31). Thus, we observed the effect of evodiamine treatment on the formations of AVOs in SGC-7901 cells using fluorescence microscopy upon staining with the lysosomotropic agent AO. In fact, AO is a weak base that freely passes across the plasma membrane in a neutral state distinguished by green fluorescence. After entrance into acidic compartments, AO changes into the protonated form which is distinguished by bright red fluorescence while control cells primarily showed green fluorescence (Fig. 3A).

For the confirmation of autophagy, we quantified AVOs by flow cytometry using AO staining as described in Materials and methods. The results indicated that there was a significant formation of AVOs in the treated cells as compared to the normal cells. The percentages of AVOs were 29.67 and 39.9% after exposure of evodiamine (10 μM) and rapamycin (positive control) for 24 h as compared to the control group (1.9%), respectively (Fig. 3B). These findings indicate that the natural compound evodiamine has the potential to activate autophagy in gastric cancer SGC-7901 cells. This result is also consistent with that of the other studies that natural compounds can induce autophagy in various cancer cells (32-34). Currently inducing autophagic cell death has been studied as a potential method for cancer therapy. To determine the role of evodiamine-induced autophagy in the death of SGC-7901 cells, we added the autophagy inhibitor, 3-methyladenine (3-MA) which controlled the autophagy pathway at various points (44). Initially, the effects of 3-MA over cell growth inhibition were assessed. It was found that the viability of cells was >90% when cells were treated with 3-MA alone. Next, the SGC-7901 cells were treated with 10 μM of evodiamine together with the autophagy inhibitor, 3-MA and were analyzed for cell death by flow cytometry using the PI staining assay. Cells stained with PI were considered as dead cells (41). Evodiamine-induced cell death was partially suppressed when the cells were treated in combination with the specific autophagy inhibitor, 3-MA (Fig. 4). These results showed that evodiamine-induced autophagy was partially involved in the death of SGC-7901 cells.

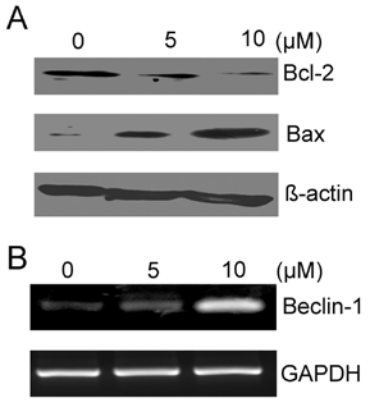

Figure 5. The effects of evodiamine over levels of apoptosis and autophagy-related proteins. (A) Expression levels of Bcl-2 and Bax in SGC-7901 cells treated without (control) and with evodiamine (5 or 10 μM) for 24 h were monitored by Western blot assay. β-actin was used as a loading control. Western blots are representative of three independent experiments. (B) Beclin-1 and GAPDH mRNA expression levels were assessed by standard RT-PCR. GAPDH served as a loading control.

In order to understand the molecular mechanism underlying apoptosis induced by evodiamine, expression of apoptosis related proteins were assessed though Western blotting which demonstrated that evodiamine down-regulated the expression of Bcl-2 and up-regulated the expression of Bax in SGC-7901 cells in a dose-dependent manner (Fig. 5A). The Bcl-2 family includes anti-apoptotic (e.g., Bcl-2) and pro-apoptotic proteins (e.g., Bax). The balance between these two groups is critical for induction of apoptosis and this balance determines the cells' decision to undergo apoptosis (45,46). Consistent with our previous results evodiamine induced apoptosis via modulation of Bcl-2 and Bax indicating that decreases in the level of Bcl-2 and increases in the level of Bax may trigger the evodiamine-induced apoptosis of SGC-7901 cells. These

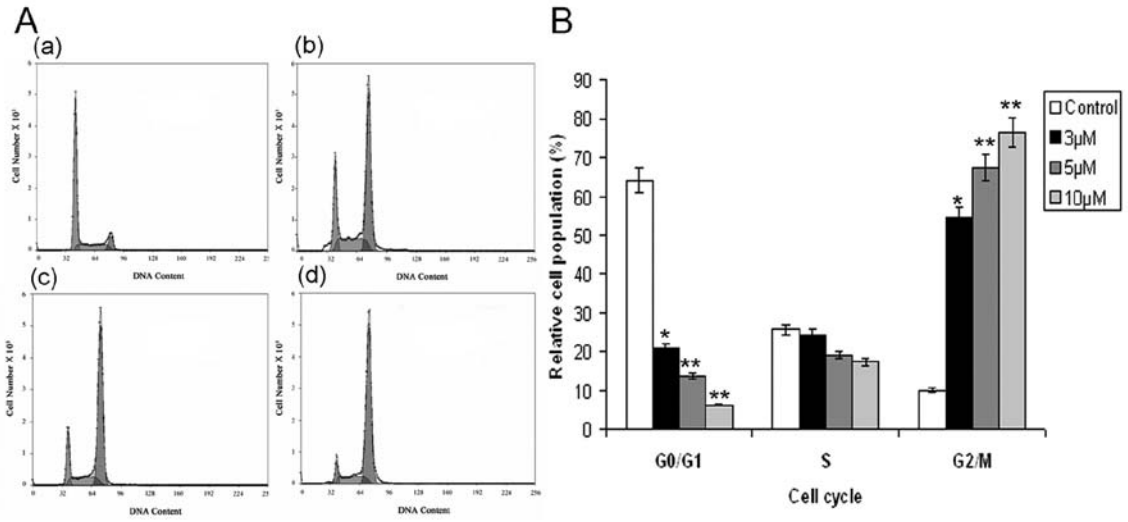

Figure 6. Effect of evodiamine on cell cycle distribution. (A) SGC-7901 cells were treated with (a) 0 μM, (b) 3 μM, (c) 5 μM and (d) 10 μM of evodiamine for 24 h and then they were stained with PI for flow cytometric analysis. Histograms show number of cells/channel (y-axis) vs. DNA content (x-axis). The values indicate the percentage of cells in the indicated phases of cell cycle. The data shown are representative of three independent experiments with similar results. *P<0.05 and **P<0.01 compared with the control.

results are in the agreement with previous findings (25). To elucidate the underlying mechanism of autophagy induced by evodiamine, RT-PCR was performed to evaluate the effect of evodiamine on the mRNA expression of Beclin-1 which plays a key role in autophagy (44). The results showed that evodiamine activated Beclin-1 gene expression in a dose-dependent manner (Fig. 5B). This increase in the level of Beclin-1 gene expression is similar with the previously reported induced autophagy by evodiamine in cervical cancer carcinoma HeLa cells (20). The results indicate that Beclin-1 is involved in evodiamine-induced autophagy in SGC-7901 cells. Although in the present study, for the first time it is reported that evodiamine simultaneously induced both apoptosis and autophagy in SGC-7901 cells, the relation between apoptosis and autophagy remains unknown. Further study is needed to analyze the relation between these two processes at the protein level.

Accumulating data indicate that cell cycle progression is tightly controlled by various checkpoints in normal cells while regulation of the cell cycle is altered due to abnormal cell growth in cancerous cells. These checkpoints become activated due to certain factors such as DNA damage, exogenous stress signals and defects during the replication of DNA or failure of chromosomes in attaching themselves to the mitotic spindle. The loss of this regulation is the hallmark of cancer. Several studies reveal that maintaining the proper cell cycle progression in cancer cells is a potential and effective strategy to halt tumor growth (47-49). We analyzed the effect of evodiamine over the cell cycle progression of SGC-7901 cells. It was observed that evodiamine arrested the cell cycle at the G2/M phase, which supported the previous reported results (50). The percentage of accumulation of cells in the G2/M phase was increased from 10.17% in the control group to 54.57, 67.27 and 76.41% in the cells treated with 3, 5 and 10 μM of evodiamine, respectively for 24 h (Fig. 6). Published studies have shown that evodiamine induces the cell cycle arrest at different phases. For example, evodiamine induced cell cycle arrest in human colon LoVo cells at the S phase (24). The result of evodiamine cell cycle arrest at the G2/M phase is also consistent with other studies that evodiamine arrests the cell cycle at G2/M phase (8,11,13). These findings suggest that G2/M phase arrest is one of the mechanisms through which evodiamine induces cytotoxicity in SGC-7901 cells. Numerous chemotherapeutic and chemopreventive agents have potential antiproliferative effects via arresting the cell division at certain checkpoints of the cell cycle (51,52).

In conclusion, these results provide experimental evidence that evodiamine significantly inhibits the proliferation of SGC-7901 cells and induces G2/M phase cell cycle arrest. Furthermore, both autophagy and apoptosis were activated during the evodiamine-induced death of SGC-7901 cells. Beclin-1 is involved in evodiamine-induced autophagy and the pro-apoptotic mechanisms of evodiamine may be associated with down-regulation of Bcl-2 and up-regulation of Bax expression. These findings indicate that evodiamine is a potent antitumor agent for treating gastric cancer. The ability of evodiamine to induce autophagy underlines its potential utility as a new gastric cancer treatment modality.

Acknowledgements

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