Gossypol induces cell death by activating apoptosis and autophagy in HT-29 cells

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Abstract. Gossypol is a polyphenolic, yellowish compound derived from cottonseed extract. The present study examined the effects of gossypol on the apoptosis and autophagy of HT-29 cells. A Cell Counting Kit-8 assay, Annexin V-FITC, JC-1 staining and western blotting were used to identify the viability of cells, stages of apoptosis and the expression levels of the signaling proteins. Gossypol promoted apoptosis and induced the loss of mitochondrial membrane potential. Further investigation of the apoptotic mechanism revealed that gossypol increased the ratio of B-cell lymphoma 2 (Bcl-2)-associated X protein/Bcl-2 protein levels and upregulated the expression of caspase-3. Gossypol also enhanced the activity of microtubule-associated protein light chain 3 LC3-II and Beclin-1 and downregulated LC3-I, in a dose-dependent manner. Together, these finding suggested that gossypol may be a novel and potential antitumor agent.

Introduction

Colorectal cancer, including cancerous growths in the colon, appendix and rectum, is the third most commonly diagnosed cancer in men and the second most common in women, with an estimated 1,400,000 cases and a mortality rate of 693,900 worldwide in 2012 (1). Current treatment for colon cancer includes surgery, radiation, physical rehabilitation, nutritional therapy and chemotherapy using cytotoxic drugs (2). However, these treatments are not curative. Therefore, effective anticancer drugs for colon cancer are required.

Gossypol is a polyphenolic, yellowish compound derived from cottonseed extract. It is extensively used as a contraceptive agent in men (3,4). Gossypol has been identified as a promising anticancer agent. Moon *et al* (3) demonstrated that gossypol effectively inhibited tumor necrosis factor-α-induced expression of intercellular adhesion molecule 1 by activating the suppression of nuclear factor-κB, and *in vitro* invasion and adhesion in human breast cancer cells. Another study demonstrated that gossypol reduced the viability of three prostate cancer cell lines with a half-maximal inhibitory concentration (IC_{50}) of between 3 and 5 μmol/l. In addition, gossypol effectively inhibited prostate tumor-initiating cell-driven tumor growth in a NOD/SCID xenograft model, in addition to inducing DNA damage and activating p53 (5). Knowledge of the molecular mechanisms underlying the anticancer effects of gossypol against HT-29 human colonic cancer cells is limited. Autophagy and apoptosis are two evolutionarily conserved programmed cell death mechanisms, which occur in several physiological conditions (6). Autophagy and apoptosis are dysregulated in cancer cells (6); therefore, the present study examined the effects of gossypol on the apoptosis and autophagy of HT-29 cells.

Materials and methods

Compounds and reagents. Gossypol (>98% pure) was purchased from LKT Laboratories, Inc. (Hanzhou, China) and reconstituted in DMSO (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The Cell counting Kit-8 (CCK-8), 5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidalyl-carbocyanineiodide (JC-1) and Annexin V/PI apoptosis detection kits were purchased from Beyotime Institute of Biotechnology (Nanjing, China). Antibodies against Caspase-3, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), microtubule-associated protein light chain 3 (LC3), Beclin-1, Cytochrome *c* (Cyt-c) and β-actin were commercially available from MBL International Co. (Boston, MA, USA).

Cell culture. The HT-29 human colon cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco;

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Thermo Fisher Scientific, Inc.) in a humidified atmosphere in a 5% CO_2 incubator at 37˚C.

CCK-8 assay. The growth-inhibitory effect of gossypol was determined using a CCK-8 assay. Briefly, the HT-29 human colon cancer cells were seeded at a density of $1x10^4$ cells/100 μl/well in 96-well plates and were cultured overnight for attachment. Following 24 h of incubation, the HT-29 cells were incubated with gossypol (5, 10, 20, 40 or 80 μM/l) in 96-well plates for 24 h at 37˚C. Following incubation, 10 μl CCK-8 solution was added to each well and incubated at 37˚C for 4 h, and the absorbance was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

Analysis of cell apoptosis. Apoptosis kits were used to detect the effect of gossypol on the HT-29 cells. The cells ($5x10^4$ cells/well) were treated with gossypol (20 and 40 μM/l) for 24 h at 37˚C. The cells were harvested and washed with ice-cold PBS, resuspended with binding buffer, and simultaneously stained with Annexin-V-FITC and PI for 5-15 min. The cells were then analyzed immediately using flow cytometry.

Mitochondrial membrane potential (ΔΨm) assay. JC-1 staining was used to detect changes in ΔΨm. The cells ($2x10^5$ cells/well) were seeded in 6-well plates and treated with or without gossypol (20 and 40 μM/l) for 24 h. Following incubation; the collected cells were incubated with 5 μg/ml JC-1 in culture medium for 15 min at 37˚C. The staining solution was removed and the cells were washed twice with JC-1 staining buffer. The cell-associated fluorescence was analyzed using a FACSCalibur flow cytometer.

Western blot analysis of Caspase-3, Bax, Bcl-2, LC3, Beclin-1, Cyt-c and β-actin. The HT-29 cells were seeded at a density of $1x10^5$ cells/well in 6-well plates and, following incubation for 24 h, the cells were treated with gossypol (20 and 40 μM/l) for 24 h and then harvested. The expression levels of Bcl-2, Caspase-3, Bax, LC3 and Beclin-1, and the release of mitochondrial Cyt-c were then assessed using western blot analysis. Proteins were extracted from HT-29 cells using a total protein extraction kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the instructions of the manufacturer. Protein concentrations were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Aliquots of cell lysates containing 20 μl of protein were separated on a 12% SDS-PAGE gel, and transferred onto PVDF membranes. The membranes were blocked with TBST buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween-20, containing 5% skimmed milk, and were then incubated with polyclonal antibodies against Bcl-2 (cat. no. 2876; 1:500), Bax (cat. no. sc-493; 1:500), caspase-3 (sc-16647; 1:1,000), Cyt-c (cat. no. 250621; 1:1,000), LC3 (cat. no. 4108; 1:1,000), beclin-1 (cat. no. 4122; 1:500) and β-actin (cat. no. 4967; 1:1,000) overnight at 4˚C, respectively. The membrane was then incubated with either HRP-conjugated goat anti-rabbit IgG (cat. no. sc-2005; 1:10,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or goat anti-mouse IgG (cat. no. sc-2004; 1:10,000; Santa Cruz Biotechnology, Inc.) secondary antibodies for 1.5 h at room temperature. Peroxidase activity was detected via ECL visualization of the bands. The images were analyzed and quantified using a Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc.).

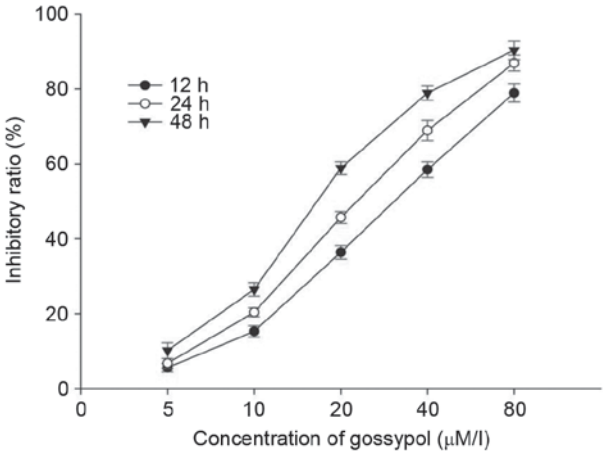

Figure 1. HT-29 cells were treated with various concentrations of gossypol for 12, 24 and 48 h, and the inhibitory effects were measured using a Cell counting kit-8 assay. Values are presented as the mean ± standard deviation of three independent experiments.

Statistical analysis. Statistical analyses were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Statistical comparisons were performed using Student's t-test. $P<0.05$ was considered significant a statistically significant difference between groups.

Results

Inhibitory effects of gossypol on HT-29 cells. A CCK-8 assay was performed to assess the inhibitory effects of gossypol on the HT-29 cells. The cells were cultured with 5-80 μM/l gossypol for 12, 24 and 48 h. As shown in Fig. 1, gossypol inhibited the growth of HT-29 cells in a time- and dose-dependent manner, with a 12 h IC_{50} of 31.20 μM/l, 24 h IC_{50} of 23.60 μM/l and 48 h IC_{50} of 17.97 μM/l. The two concentrations of 20 and 40 μM/l were selected for the following experiments.

Effect of gossypol on apoptosis. To determine the induction of cell apoptosis by gossypol, an Annexin V and PI staining assay based on flow cytometry was used. As shown in Fig. 2, there were significant changes in the proportions of early and late apoptotic, or necrotic HT-29 cells following exposure to gossypol for 24 h. Compared with the control group, the proportion of early apoptotic cells increased from 5.88 to 46.24% and the proportions of late apoptotic cells increased from 4.67 to 28.55%.

Effects of gossypol on ΔΨm. A loss of ΔΨm was detected by the fluorescent dye, JC-1. As shown in Fig. 3, treatment with gossypol (20 and 40 μM/l) for 24 h induced a loss in the ΔΨm of the HT-29 cells, suggesting damage to the mitochondria.

Expression of proteins of the apoptotic pathway. To investigate the mechanism underlying gossypol-induced apoptosis, the effects of gossypol on the modification of apoptosis-associated

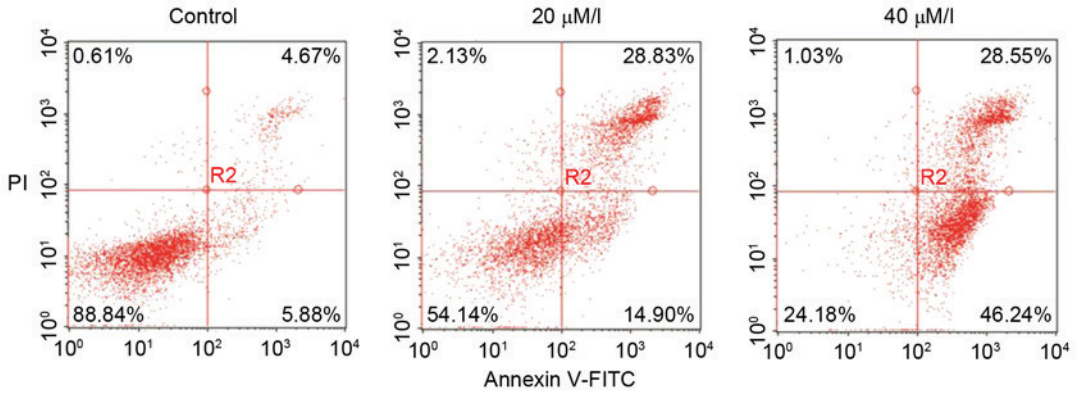

Figure 2. Apoptotic effects of gossypol on HT-29 cells. Apoptosis of HT-29 cells following treatment with gossypol (20 and 40 μM/l) for 24 h. Representative results from the fluorescence activated cell sorting assays of three independent experiments are presented.

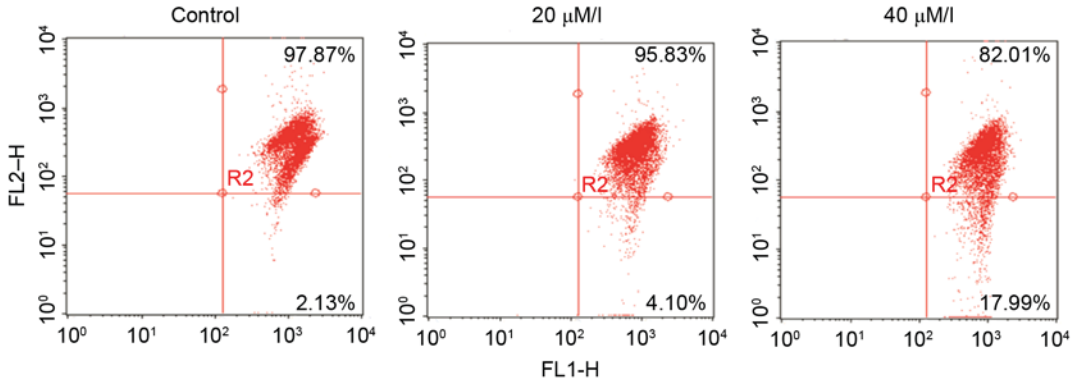

Figure 3. Effect of gossypol on the ΔΨm in HT-29 cells when treated for 24 h. The ΔΨm was detected using the fluorescent dye, JC-1, and analyzed using flow cytometry. The results are representatives of three independent experiments. ΔΨm, mitochondrial membrane potential.

proteins were examined. The results of the western blot analysis demonstrated that gossypol led to an increase in the protein levels of Bax, and decrease in the levels of Bcl-2 in the HT-29 cells following treatment with gossypol at concentrations of 20 and 40 μM/l (Fig. 4A and B). Gossypol also promoted the activation of caspase-3 (Fig. 4C).

Autophagy is induced by gossypol in HT-29 cells. The activity of autophagy in HT-29 cells was evaluated following treatment with gossypol. Alterations in the levels of autophagy marker proteins, LC3-I and LC3-II, in HT-29 cells were detected using western blot analysis. As shown in Fig. 5, the ratio of LC3-II/LC3-I and Beclin-1 were enhanced in a dose-dependent manner following exposure to gossypol (20 and 40 μM/l) for 24 h.

Discussion

The aims of the present study were to evaluate the antitumor activities of gossypol and the possible underlying molecular mechanism. Gossypol inhibited the proliferation of HT-29 cells in a concentration-dependent manner. The loss of ΔΨm is a hallmark of the early-stage of apoptosis (7). Mitochondrial involvement in gossypol-induced apoptosis was assessed using JC-1 staining, and Annexin V and PI double-staining was used to differentiate early and late apoptosis. The proportions of early and late apoptotic cells were higher, compared with those in the control group.

The signal of apoptosis in several human cancer cells in response to antitumor agents is upregulation of the mitochondrial apoptotic pathway triggered by an alteration in the ratio of Bax/Bcl-2 and the activation of caspases (8,9). In the present study, proteins associated with mitochondria-dependent apoptosis were measured in the HT-29 cells. The expression of Bcl-2 was significantly decreased following gossypol exposure (20 and 40 μM/l) for 24 h, whereas the expression of Bax was markedly increased. Therefore, the ratio of Bax/Bcl-2 was increased. A high Bax/Bcl-2 ratio leads to the release of Cyt-c and activates caspase 3, which is the final step in apoptosis (10,11). In the present study, caspase-3 was significantly increased following gossypol exposure (20 and 40 μM/l) for 24 h. These results suggested that gossypol induced apoptosis via the mitochondrial apoptotic pathway.

In addition to apoptosis, the present study investigated the autophagic effects of gossypol on the proliferation of HT-29 cells. A number of studies have reported that autophagy eliminates cancer cells, thus autophagy is important in the fight against cancer. It has been found that autophagy suppresses the development of carcinogenesis (12,13). LC3 and Beclin 1 are the most well-known markers of autophagy. During autophagy, LC3 is transformed from LC3-I to LC3-II for movement onto isolated membranes and autophagosomes (14,15). In the

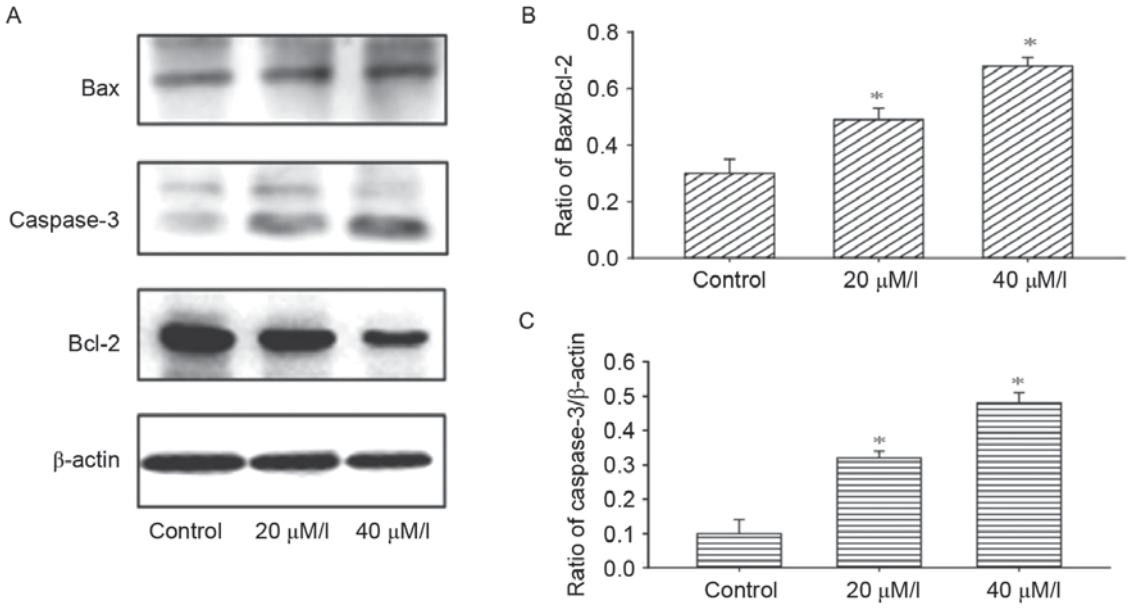

Figure 4. Effects of gossypol on proteins of the apoptotic pathway. (A) Effects on the protein expression levels of Bax, Bcl-2 and Caspase-3 were detected using western blot analysis following treatment with gossypol for 24 h. (B) Protein expression levels of Bax/Bcl-2 were detected using western blot analysis following treatment with gossypol for 24 h. (C) Protein expression levels of caspase-3 was detected using western blot analysis following treatment with gossypol for 24 h. Results are expressed as the mean ± standard deviation. Each experiment was performed in triplicate (n=3). Comparisons were made between the different concentrations of gossypol and the untreated control. *P<0.05, compared with the control. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

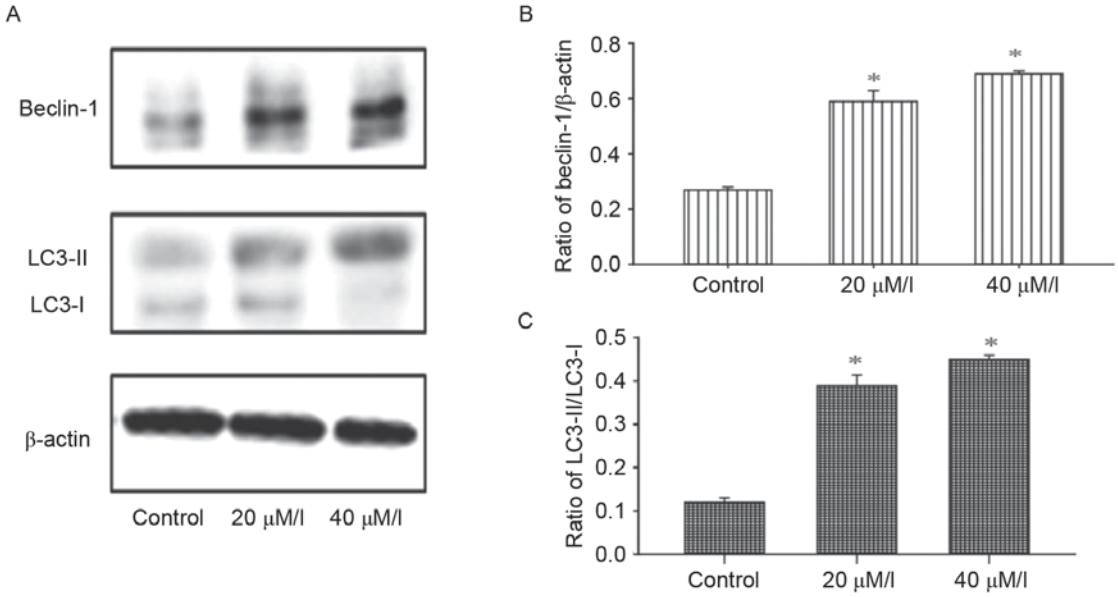

Figure 5. Effects of gosspol on the expression of markers of autophagy. (A) Effects on the protein expression levels of Beclin-1 and LC3 were detected using western blot analysis following treatment with gossypol for 24 h. (B) Protein expression level of Beclin-1 was detected using western blot analysis following treatment with gossypol for 24 h. (C) Protein expression levels of LC3-II/LC3-I were detected using western blot analysis following treatment with gossypol for 24 h. Results are expressed as the mean ± standard deviation. Each experiment was performed in triplicate (n=3). Comparisons were made between different concentrations of gossypol and the untreated group. *P<0.05, compared with the control. LC3, microtubule-associated protein light chain 3.

present study, the ratio of LC3-II/LC3-I was increased and the expression of Beclin 1 was upregulated following treatment with gossypol. Therefore, apoptosis and autophagy were induced in the HT-29 cells following gossypol treatment. Apoptosis is involved in eliminating damaged cells and tumor cells.

Although the molecular processes of apoptosis and autophagy differ, the end result of their actions is to remove unnecessary cells (16). The combinatorial use of anticancer agents, which induce autophagy and apoptosis, can be an effective therapeutic strategy and be used in treatment against cancer. The data presented in the present study demonstrated that gossypol induced apoptosis and autophagy, and can offer potential as a promising anticancer agent for the treatment of colorectal cancer due to its specific antitumor activity.

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