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Effects of quercetin on the apoptosis of the human gastric carcinoma cells

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ABSTRACT

Quercetin, a natural constituent abundantly present in grapes, red wine, and other food products, is known to possess potent antiproliferative effects against various malignant cells. The present study aims to investigate the effect of quercetin on the apoptosis and morphology of gastric carcinoma BGC-823 cells, as well as the probable mechanism, in an effort to identify an effective drug as a potential candidate for gastric cancer. Gastric carcinoma BGC-823 cells were treated with quercetin, and cell morphology was determined by light microscopy and transmission electron microscopy. Apoptosis and cell cycle were measured by flow cytometry, using propidium iodide staining. The apoptotic protein expression of caspase-3, Bcl-2 and Bax was detected by Western blot. Quercetin induced apoptosis in BGC-823 cell. Some morphologic features of apoptosis were found, such as cell shrinkage or even apoptosis body. Quercetin changed the apoptotic protein expression. These results indicate that quercetin can induce apoptosis of the BGC-823 cells. A decrease in Bcl-2/Bax ratio with the increased expression of caspase-3 provides evidence that quercetin-induced apoptosis may be mediated via the mitochondrial pathway.

1. Introduction

Quercetin, a bioflavonoid commonly present in a variety of plants, is known to have several biological effects, including anti-inflammatory, antiviral and antitumor activities, which have been described earlier. More attention has been paid, especially to its antitumor effect. One of the mechanisms could probably be through intervening the apoptic way. Apoptosis plays a critical role in homeostasis, development, neoplasm, and some degenerative diseases. Apoptosis regulators have emerged as key targets for the targetable drug delivery design of therapeutic strategies that modulate cellular life-and-death decisions (Fischer and Schulze-Osthoff, 2005). The pathways leading to apoptosis mainly include mitochondria and death receptors such as Fas, TNFR, and TNF-related apoptosis-inducing ligand (TRAIL) receptor (Gupta, 2001; Kroemer and Reed, 2000). Disrupting the potential and permeability of mitochondrial membranes causes proapoptotic proteins such as cytochrome C and apoptosis-inducing factor (AIF) to be released from the mitochondria to the cytosol. This process is regulated the Bcl-2 family members, including Bcl-2, Bcl-XL, Bak, Bax, Bid (van Gurp et al., 2003; Scorrano and Korsmeyer, 2003; Kuwana et al., 2002; Crompton, 2000; Wei et al., 2001). Cytochrome C triggers a cascade from the initiator to the effector caspase by activating apoptosis protease activating factor-1 (Apaf-1), whereas AIF induces caspase-independent apoptosis (Cain et al., 2002; Cande et al., 2002). Therefore, we measured the expression of Bcl-2 and caspase-3 by Western blot to clarify the probable mechanism.

Apoptosis can be induced by various chemotherapeutic drugs (Kaufmann and Earnshaw, 2000). Quercetin, with its antitumor activity characteristics, can induce apoptosis of various tumor cells including leukemic cells, pancreatic tumor, breast cancer, hepatoma cells and prostate cancer (Shen et al., 2003; Lee et al., 2002; Choi et al., 2001; Kawahara et al., 2009; Chang et al., 2009; Yuan et al., 2010). However, gastric cancer has seldom been investigated (Chen et al., 2008). Although there is a large volume of literature on the cytostatic and pro-apoptotic effects of quercetin, its mechanism of action has not yet been clearly defined. Therefore, further investigations were warranted on the probable mechanism of quercetin on gastric carcinoma BGC-823 cells. This study evaluated the effect and the mechanism of quercetin on gastric carcinoma BGC-823 cells. However, the actual mechanism of the molecular processes that characterizes the anti-cancer properties of this flavonoid need still further study.

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2. Materials and methods

2.1. Chemicals, reagents and media

Media and reagents included revolutions-per-minute indicator (RPMI) 1640, fetal bovine serum (FBS), phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), trypsin–EDTA solution, penicillin, streptomycin, dimethyl sulfoxide (DMSO; Invitrogen-Gibco BRL). A carbon dioxide (CO_2) incubator, a sterile bench, a phase-contrast microscope (Olympus, Tokyo, Japan), a centrifuge, a spectrophotometer, pipettes, culture flasks, and tubing were used. All the other reagents (such as quercetin, propidium iodide (PI), etc.) were obtained from Sigma (Sigma–Aldrich Corp, St. Louis, MO, USA), unless otherwise specified. Quercetin was dissolved in DMSO to a concentration of 12 mM. Further dilutions were performed in cell culture media (Wilson and Poellinger, 2002). DMSO was used as a vehicle control throughout the study.

2.2. Cell lines and culture

BGC-823, SGC-7901, MKN45, SW116, EC109, Ges-1 cells of gastric carcinoma were obtained from the cell bank of the Chinese Academy of Science and cultured at 37 °C in RPMI1640, supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere containing 5% CO_2. The cells were maintained in the medium for 24 h before exposure to the final indicated concentrations of quercetin and DMSO at 37 °C in 5% CO_2 environment.

2.3. Light microscope

BGC-823 cells placed in 2 ml of medium were seeded with 5×10^4 density per well in a 24-well plate, and allowed to attach for 24 h before the addition of quercetin. Later, the cells in a 24-well plate were incubated with different concentrations of quercetin (15, 30, 60, 90 and 120 μmol/L) or DMSO for 24, 48 and 72 h, and the BGC-823 cells were macroscopically observed everyday under the phase-contrast microscope, throughout the culture period. The results and photos were obtained using Winfast PVR software. Hematoxylin–Eosin (HE) staining was done for morphological study.

2.4. Electron microscopy (EM)

After overnight growth, the cells were incubated with different concentrations of quercetin (0, 60, 90 and 120 μmol/L). They were then harvested and washed once with Sorensen's phosphate buffer (pH 7.2) and fixed in 2.5% glutaraldehyde (freshly prepared in Sorensen's buffer) for 2–3 h. Following fixation, the cells were rinsed twice in buffer and dehydrated with graded concentrations of acetone. Then, they were oriented and embedded in flat embedding moulds. Ultrathin sections were cut with a diamond knife and mounted on 300-mesh copper grids, which were post-stained with uranyl acetate and lead citrate, and finally viewed under an electron microscope (JEM-1200EX, Japan).

2.5. Apoptosis and cell cycle analysis by flow cytometry

Control and quercetin-treated cells were collected, washed twice with ice-cold PBS and fixed in 70% ethanol at 4 °C for at least 4 h, and stained with 100 μl (50 μg/ml)of PI for 20 min in darkness. The cell cycle phase distribution was analyzed in three different experiments using flow cytometry (FACScan flow cytometry, Becton Dickinson Immunocytometry System, San Jose, CA, USA).

2.6. Western blotting

Western blotting was performed on the lines of a previously reported procedure (Difilippantonio et al., 2000). Gastric carcinoma BGC-823 cells (1×10^5) were incubated for 24 h at 37 °C in RPMI 1640 medium containing 10% FBS, Next, they were incubated using different concentrations of quercetin (15, 30, 60, 90, and 120 μmol/L) and a control group was set up. Finally, the cells were collected and rinsed twice in PBS supplemented with 0.05% BSA. The cell pellets were lysed at 4 °C for 15 min in a lysis buffer (150 mM NaCl, 1 mM KH_2PO_4, 1 mM EGTA, 1 mM Na_3PO_4, 5 mM $MgCl_2$, 0.1 mM phenylmethyl sulfonyl fluoride, 0.15 U/ml aprotinin, 1 μg/ml pepstatin, and 10% glycerol) and centrifuged at 18,000*g* for 20 min at 4 °C. The protein concentration in the supernatant was measured by the method followed by Bradford. Total proteins (30 μg) were separated by SDS–PAGE using a 12.5% polyacrylamide gel. The proteins in the gel were transferred to a 0.45 μm nitrocellulose membrane with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell. Membranes were blocked for 2 h with PBST (0.05% v/v Tween-20 in PBS, pH7.2) containing 1% nonfat milk. All additional immunostaining steps were performed in PBST, at room temperature. Membranes were incubated with primary antibody (Santa Cruz Biotechnology) for 2 h, and then with secondary antibody (Santa Cruz Biotechnology) for 1 h. Membranes were washed in PBST for 5 min, four times between each step, and were developed with diazobenzidine, and enhanced with nickel chloride. The experiment was repeated three times.

2.7. Statistical analysis

The data was presented as mean ± SD and analyzed by one-way or two-factor repeated measurements of the analysis of variance. Differences between the treatment groups were analyzed by Duncan's multiple-range test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Changes in the morphology of BGC-823 cells in response to quercetin treatments

3.1.1. Observation by light microscopy

BGC-823 cells were identified by their typical cobblestone configuration which adhibited the flask wall with longer pseudopods, irregular morphology and growth bloom; many tumor giant cells were observed, when viewed by light microscope in the control group. After treatment with quercetin, a variety of morphological changes were noted: loss of attachment, chromatin condensation, shrinkage, rounding, shortened to unordered pseudopods, relaxed or messy, the tumor giant cells decreased or disappeared. Nuclei condensed even form the apoptosis body with increased of concentration of quercetin and extended time (24, 48 and 72 h) (Fig. 1).

3.1.2. Observation by electron microscopy

The gastric carcinoma BGC-823 morphology treated with 60, 90 and 120 μmol/L of quercetin at 24 h was further studied under EM. We observed condensation of the cell nucleus. The condensed nucleus was observed to break into several fragments. The entire cell condensed and was reorganized into so-called 'apoptotic bodies' (Fig. 2).

3.2. Quantification of apoptosis and effects on cell cycle progression by flow cytometry

Following the above mentioned methods, the sub-G1 apoptotic fraction in untreated cells or quercetin-treated by flow cytometry

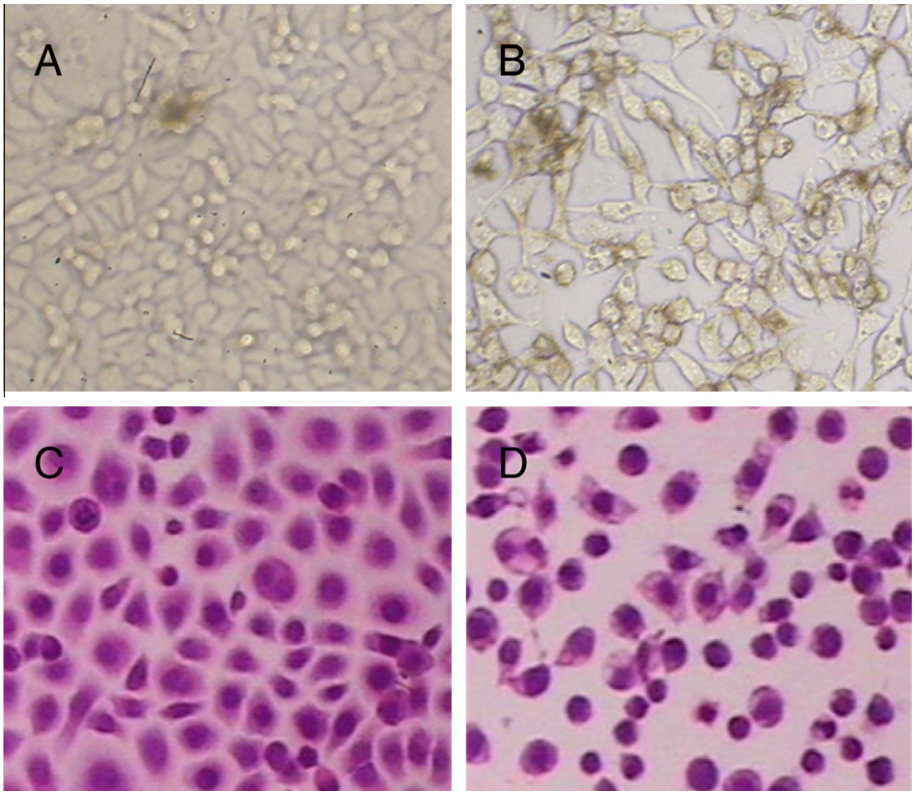

Fig. 1. Quercetin-induced morphological changes in gastric carcinoma BGC-823 cells (observations under a light microscope), images of the cultures recorded at 24 h (A and B) and after HE coloration at 24 h (C and D). (A and C) control; (B and D) BGC-823 cells treated with 60 μmol/L quercetin. (200×).

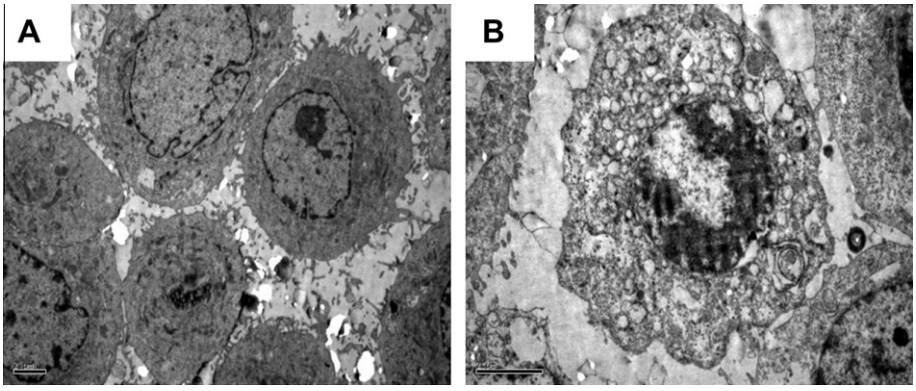

Fig. 2. Quercetin-induced apoptotic morphological changes of gastric carcinoma BGC-823 cells (observed with transmission electron microscopy), (A) control, BGC-823 cells with control showed that their surface morphology and structure had been maintained (10,000×); (B) BGC-823 cells treated with quercetin showed changes in their asymmetrical morphology and the condensation of the cell nucleus (60000×).

were examined, to study the effect of quercetin on BGC-823 apoptotic levels. The results indicated that a typical sub-G1 fraction, representing the apoptotic cell population, began to appear at 24 h. The apoptotic cell percentage increased in a concentration-related manner, reaching a 35% value with 120 μml/L (Fig. 3, Fig. 4), and the quercetin effect on cell cycle phase distribution was determined. Fig. 3 shows a representative histogram, and the data is summarized in Fig. 5. When compared with control, quercetin increased the population in the S phase from 10.04% to 25.5% at 24 h, consistent with its growth inhibitory effects and from 26.98% to 41.22% at 48 h, with a corresponding decrease in cells in the G1 phase, in a dose-dependent manner. This data corroborates the potent inhibitory effect of quercetin on DNA synthesis.

To strengthen the present work, investigation on the effects of quercetin in other gastric cancer cell lines SGC-7901 and MKN45, colorectal carcinoma cell line SW116, esophagus cancer cell line EC109 and non tumor gastric epithelium cell Ges-1 has been performed. Although there is apoptosis in some cell lines (Fig. 6), Sw116 also showed necrosis when treated with the same concentration of quercetin except for apoptosis. Little apoptosis was observed in non-tumorous gastric epithelium cell Ges-1 after treatment with quercetin. We noticed that the cell cycle phase distribution is different from that of BGC-823. We belive there must be some different mechanisms about the effects of quercetin on different cell lines which is our next research aim to be investigated.

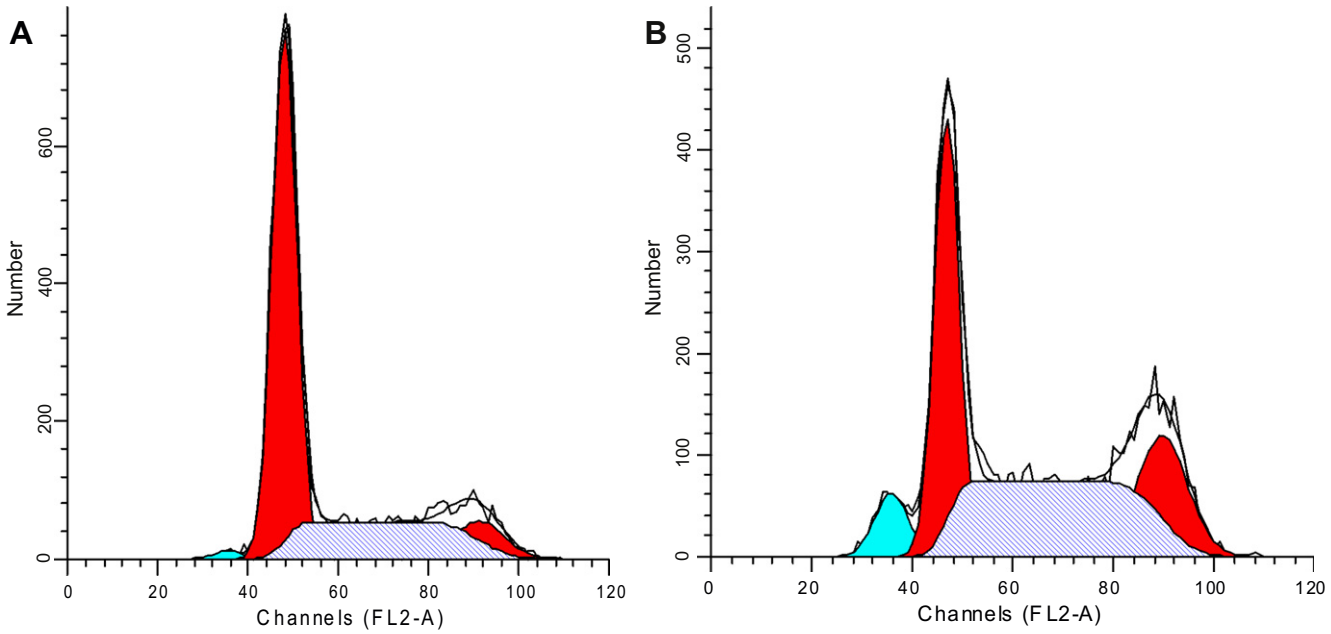

Fig. 3. Quercetin effect on apoptosis and cell cycle. BGC-823 cells were exposed to different concentrations (15, 30, 60, 90 and 120 μmol/L) of quercetin or 0.1% dimethyl sulfoxide (DMSO; control) for 24 and 48 h in complete medium, stained with propidium iodide (PI) , and then analyzed by flow cytometry. Culture images recorded at 24 h, (A) show cells that received DMSO treatment (control); cells in (B) received 60 μmol/L quercetin treatment.

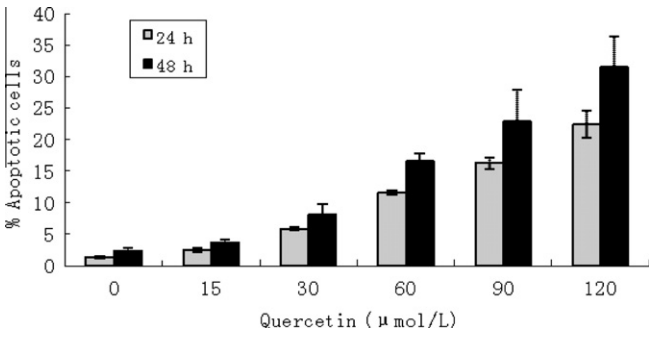

Fig. 4. Quercetin induces apoptosis of various carcinoma cell lines. Titration of quercetin on induction of apoptosis. Several cells were treated with quercetin at indicated doses for 24 h before assay of apoptosis by propidium iodide (PI) method using flow cytometry. Data are representative of three individual experiments.

3.3. Effects of quercetin on the expression of Bcl-2, Bax and caspase-3 protein in BGC-823 cells

The quercetin effect on the protein expression of Bcl-2 and Bax was examined by Western blot analysis. The Bcl-2 levels decreased in a dose-dependent manner in cells treated with quercetin for 24 h, whereas the Bax levels revealed only minimal change (Fig. 7A) The data is summarized in Fig. 7B.1 and Fig. 7B.2, respectively. Western blot analysis utilizing anti-caspase-3 antibodies revealed that the treatment of the BGC-823 cells, with increasing quercetin concentrations, led to an increase in the 17-kDa fragments of cleaved caspase-3 and a decrease in the 32-kDa pieces of uncleaved caspase-3 (Fig. 7A). This data is summarized in Fig. 7B.3. A drop in the Bcl-2/Bax ratio with increased expression of caspase-3, provides ample evidence that quercetin-induced apoptosis is probably mediated by the mitochondrial pathway.

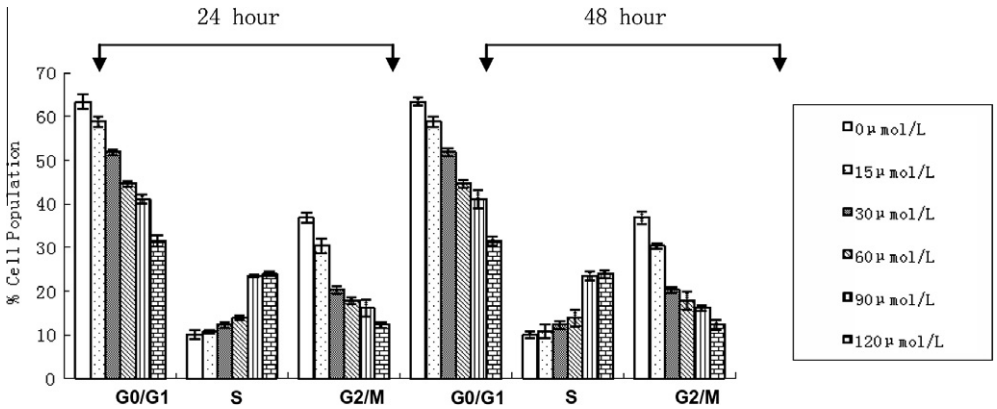

Fig. 5. Quercetin induces changes in cell cycle distribution of gastric carcinoma. Plot of summarized values from DNA histograms. Three experiments were performed in duplicate and gave similar results.

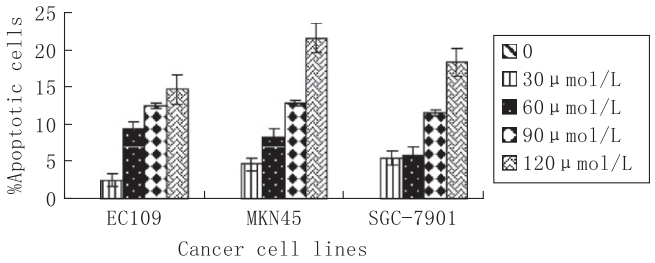

Fig. 6. Quercetin induces apoptosis of various carcinoma cell lines, Titration of quercetin on induction of apoptosis. Several cells were treated with quercetin at indicated doses for 24 h before assay of apoptosis by propidium iodide (PI) method using flow cytometry. Data are representative of three individual experiments.

4. Discussion

Gastric cancer is the fifth most common malignancy and the second leading cause of cancer mortality in the world, with approximately 930,000 new cases and more than 700,000 deaths, estimated in 2006 (Jemal et al., 2008). Despite improvements in early detection and chemotherapy, there has been little impact in terms of increased survival. Preventive strategies involving modifiable risk factors including diet, are less well understood. Induction of apoptosis is currently recognized as one of the active strategies to arrest cancer cell proliferation. Medicinal plants rich in antioxidant phytochemicals particularly, are receiving growing attention in recent years as potential chemopreventive and chemotherapeutic agents due to their antiproliferative and apoptosis-inducing effects.

Quercetin is an important constituent of the flavonoid family, found in many fruits and vegetables, as well as in olive oil, red wine, and tea. A recent report indicates that blood plasma concentrations may exceed 8 μmol/L quercetin and quercetin metabolites in response to quercetin-rich diets (Wiczkowski et al., 2008). Dietary flavonoid quercetin may be linked with reduced risk of developing cancers (Wilson et al., 2009; Kyle et al., 2010). Various pharmacological characteristics of quercetin have been demonstrated, including antioxidation by scavenging free radicals, prevention of cardiovascular diseases, and reduction of neurodegenerative disorders (Terao, 2009; Hwang et al., 2009; Han et al., 2009). Quercetin has also been reported to suppress tumor growth in vitro and in vivo (Du et al., 2009; Theodoratou et al., 2007; Chien et al., 2009; Tan et al., 2009.). However, the relationship between quercetin and development of stomach cancer is still nebulous. Detailed studies in future on the functions and the mechanisms of quercetin will help settle the controversies regarding quercetin consumption and gastric cancer development.

Therefore, in this study, quercetin was selected and tested for its apoptotic activity against the stomach cancer cell line BGC-823. The BGC-823 cells were observed to be sensitive to quercetin induced apoptosis, which caused the typical apoptotic alternations, including morphological changes assessed by the light microscope and apoptotic rate (%) by flow cytometry. Protein analysis of Bcl-2, Bax, caspase-3 indicated the apoptotic effect of quercetin on gastric cancer BGC-823 cells. Apart from the changes observed by light microscopy, changes in the organelles have also been described by electron microscopy. Following the condensation of the cell and nucleus, the nucleus is broken up and separates into a number of fragments which are consecutively packaged into apoptotic bodies. The molecular events responsible for this are unknown, but caspase-mediated cleavage of nuclear proteins may be involved.

Cell cycle analysis revealed that quercetin blocked the cells in the S phase, in a dose-dependent manner. S-phase arrest may occur at the expense of either G_0/G_1 or G_2/M. The apparent increase in the S phase with a slight decrease in the G_0/G_1 phase on treatment with quercetin suggests a cytostatic effect in response to a non-ideal growth environment, which might maintain the cells in the S phase. The normal cells are blocked in G_1 at a point called the 'restriction point', where the cell must make a commitment either to continue into S phase, or to stop in G_0/G_1 and wait until conditions are more favorable for cell replication. BGC-823 cells have likely lost this control mechanism, and continue through the critical phases of cell division (G_2, and M), and ultimately die due to apoptosis, in the presence of quercetin. Earlier flow cytometry studies demonstrated that quercetin blocked the division of cancer cells in S, G_2 or M phases (Nguyen et al., 2004; Shan et al., 2009; Yang et al., 2006), as seen in the BGC-823 cells. Other studies revealed that quercetin can induce cell cycle arrest either in the S phase (Cheong et al., 2004) or the G_1/S phase (Ye et al., 2007) depending on the cell type and treatment conditions. We demonstrated that quercetin induced arrest at the S phase from cell cycle analysis (Fig. 4C). Therefore, we inferred that quercetin inhibited proliferation through S arrest of the cell cycle and induced apoptosis via caspase-3 cascade in the human gastric cancer cell line BGC-823, in the following manner.

In this study, incubation of BGC-823 cell lines up to 24 and 48 h with different concentrations of quercetin, revealed apoptosis, to a different degree, potency and consistency course. The apoptotic cell percentage increased in a concentration- dependent manner, reaching 35% with 120μml/L.

Casepase-3, a key factor in apoptosis execution, is the active form of procaspase-3. Both the mitochondria- initiated intrinsic pathway and the death receptor-triggered extrinsic pathway can lead to caspase-3 activation. It further initiates downstream several apoptotic programs, culminating in typical biochemical and morphological alterations of the cell. Based on this hypothesis, caspase-3 activity was evaluated by Western blot, and the expression of active caspase-3 was significantly increased after treatment

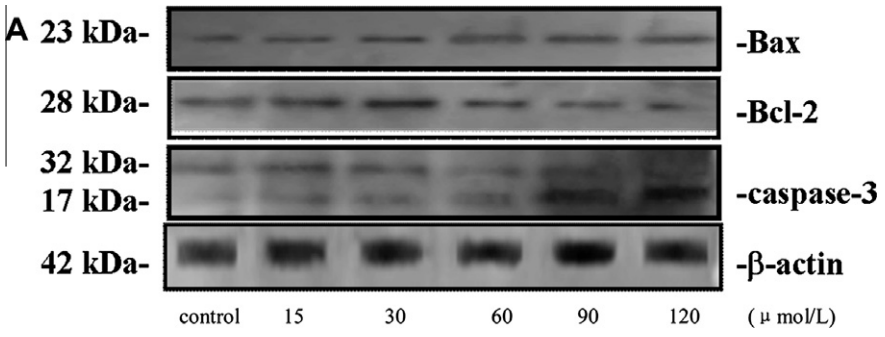

Fig. 7A. Quercetin effect of on apoptosis-related protein expression. Bcl-2, Bax and caspase-3 expressions assessed by Western blot. BGC-823 cells were treated with quercetin at the indicated doses for 24 h. Whole cell lysates were subjected to Western blot analysis. The data shown represent three individual experiments.

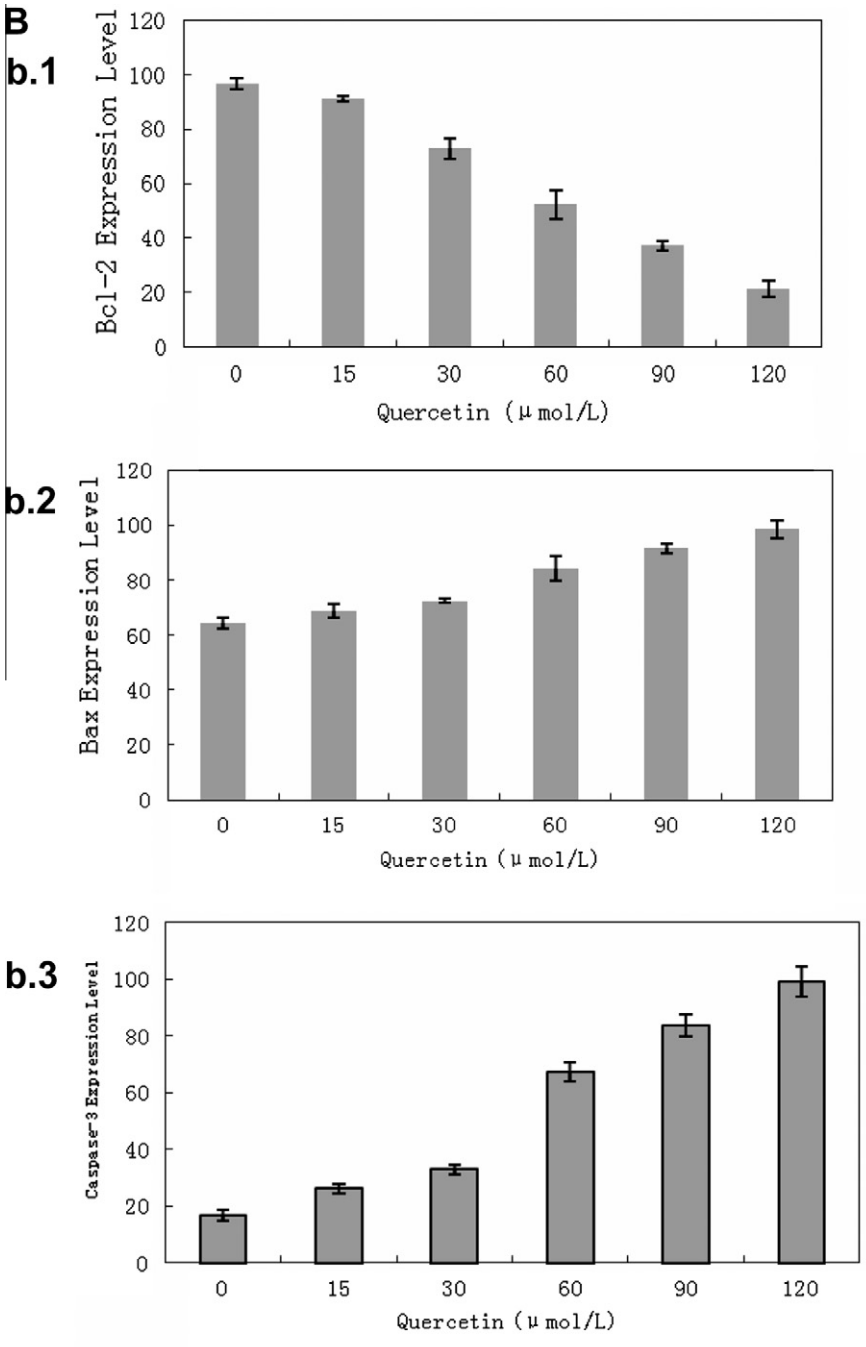

Fig. 7B. Quercetin effect of on apoptosis-related protein expression. The relative abundance of each band to its own control actin band was estimated by densitometric scanning of the exposed films. Each bar represents the mean ± S.D. (n = 3).

with quercetin. From the present study, the higher sensitivity of the BGC-823 cells to apoptosis, induced by adding quercetin particularly appears consistent with the simultaneous increase in caspase-3 activity.

Mitochondrial membrane permeability is regulated by various members of the Bcl-2 family, which constitute a critical cellular checkpoint in the intrinsic pathway of apoptosis (Dewson and Kluck, 2009). The Bcl-2 family interferes with the relocalization of cytochrome C, resulting in the inhibition of the binding of this protein to Apaf-1. The latter effect is observed consequent to the activation of Bax and/or Bak and the formation of openings in the outer membrane.

Mitochondria are the chief sites of reactive oxygen species (ROS) generation. ROS is involved in apoptosis by damaging the DNA. Park et al. (2003) and Chang et al. (2009) reported that quercetin shows antioxidant activity, and its induction of apoptosis is dependent on ROS production. Excessive ROS generation can result in the opening up of the mitochondrial permeability transition pore, consequently releasing cytochrome C from the intermembrane space into the cytosol, culminating in activating the caspase cascade and finally, apoptotic cell death.

Bcl-2, a 28-kDa integral membrane oncoprotein, was the first antiapoptotic gene product to be discovered. Over expression of Bcl-2 in tumor cells was reported to be able to prevent its

apoptosis. The major antiapoptotic protein of the Bcl-2 family inhibits ROS production, cytochrome c release, and caspase-3 activation, whereas Bax, a 23-kDa protein, is a pore-forming proapoptotic protein that facilitates cytochrome C release, triggering caspase-mediated apoptotic cell death. Bcl-2 and Bax are gaining respect as attractive targets to design new anticancer drugs, and agents that can lower the Bcl-2/Bax ratio are regarded as promising chemopreventive and chemotherapeutic agents (Youle and Strasser, 2008; Mackey et al., 1998).

On examining the expression level of Bcl-2 in BGC-823 cells, BGC-823 cells treated with quercetin were found to exhibit a lower level of Bcl-2 than those in the control cells (Fig. 7). A decrease in the Bcl-2/Bax ratio was also observed following the exposure of BGC-823 cells to quercetin in the present study. Along with the increased expression of caspase-3, provide evidence that quercetin-induced apoptosis is mediated by the mitochondrial pathway.

Besides, from these results, the mechanism of the morphological change of BGC-823 cells treated by quercetin can be inferred to probably be related to the loss of mitochondrial membrane potential and the ability to permeate through the outer mitochondrial membrane, facilitating the release of cytochrome C from the mitochondria to the cytosol, resulting in the condensation of BGC-823 cells or even the formation of apoptotic bodies.

In conclusion, the results of the present work demonstrate that quercetin can alter the morphology and induce apoptosis of gastric cancer BGC-823 cells. The mechanism involved is relevant to stimulating the activity of caspase-3 and decreasing the rate of Bcl-2/Bax, the inhibition of cell proliferation through cell cycle arrest at S phase, and the induction of mitochondria-mediated apoptosis in human gastric cancer cells (BGC-823). Although the detailed mechanism is still not clearly recognized, from the present study, a new hypothesis is suggested, in which the anti-cancer/toxicological potential of quercetin could correlate with changes in the mitochondrial energetic, similar to the reports of Dorta et al. (2005), which are based on the results of the expression of caspase-3, Bcl-2, and Bax, as this family is closely related to the mitochondrial pathway. Quercetin was reported to increase the expression of the death receptor (DR) 5 in non-small cell lung cancer cells (Chen et al., 2007; Jung et al., 2010), and caused the redistribution of DR4 and DR5 onto lipid rafts in colon adenocarcinoma cells (Psahoulia et al., 2007). Investigations of the direct interactions between quercetin and these receptors are important subjects for future research on the preventive mechanism of flavonoids in cancers. We showed that quercetin exerts a strong pro-apoptotic activity on BGC-823 cells. This property could have great physiological relevance in humans, depending on a dietary intake, rich in fruits and vegetables. Further studies are proceeding to define the concrete mechanism of quercetin-induced gastric cancer BGC-823 cell apoptosis to use the drug as a potential candidate in treating gastric cancer.

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