Reduction of apoptosis by proanthocyanidin-induced autophagy in the human gastric cancer cell line MGC-803

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Received September 10, 2015; Accepted October 20, 2015

DOI: 10.3892/or.2015.4419

Abstract. Proanthocyanidins are flavonoids that are widely present in the skin and seeds of various plants, with the highest content in grape seeds. Many experiments have shown that proanthocyanidins have antitumor activity both *in vivo* and *in vitro*. Autophagy and apoptosis of tumor cells induced by drugs are two of the major causes of tumor cell death. However, reports on the effect of autophagy induced by drugs in tumor cells are not consistent and suggest that autophagy can have synergistic or antagonistic effects with apoptosis. This research was aimed at investigating whether proanthocyanidins induced autophagy and apoptosis in human gastric cancer cell line MGC-803 cells and to identify the mechanism of proanthocyanidins action to further determine the effect of proanthocyanidins-induced autophagy on apoptosis. MTT assay was used to examine the proanthocyanidin cytotoxicity against human gastric cancer cell line MGC-803. Transmission electron microscopy and monodansylcadaverine (MDC) staining were used to detect autophagy. Annexin V APC/7-AAD double staining and Hoechst 33342/propidium iodide (PI) double staining were used to explore apoptosis. Western blotting was used to determine expression of proteins related to autophagy and apoptosis. Real-time quantitative PCR technology was used to determine the mRNA level of Beclin1 and BCL-2. The results showed that proanthocyanidins exhibit a significant inhibitory effect on the human gastric cancer cell line MGC-803 proliferation *in vitro* and simultaneously activate autophagy and apoptosis to promote cell death. Furthermore, when proanthocyanidin-induced autophagy is inhibited, apoptosis increases significantly, proanthocyanidins can be used together with autophagy inhibitors to enhance cytotoxicity.

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Key words: proanthocyanidins, gastric cancer, MGC-803, autophagy, apoptosis

Introduction

Gastric cancer is one of the most common malignant cancers. According to incomplete statistics, there are approximately one million new cases of gastric cancer diagnosed worldwide each year, and gastric cancer is the second leading cause of death, accounting for ~8% of cancer-related deaths (1). Since the early symptoms of gastric cancer are not obvious, the cancer is typically in the middle and advanced stages by diagnosis, and its five-year survival rate is less than 20% (2). In 2006 alone, there were 950,000 new cases of gastric cancer, which accounted for 9% of new cancer cases, behind only lung, breast and colon cancer (3). Therefore, gastric cancer is a malignant tumor that seriously endangers human health. Approximately 70% of gastric cancers occur in developing countries, which have less medical resources than developed countries (4). More than 40% of patients with gastric cancer are Chinese (5). Although research is committed to the development of emerging fields such as nano-medicine (6-8) and stem cell technology (9-11), surgical resection, radiotherapy and chemotherapy are still the main treatment methods for malignant tumors at present. However, current chemotherapy drugs have many issues. Although these drugs are able to delay tumor growth and extend survival, they are highly controversial in tumor treatment since of the lack of an ideal therapeutic effect, the frequent occurrence of drug resistance, and strong toxic side-effects (12-14). Therefore, the extraction of highly effective, low-toxicity active ingredients from natural products to replace or combine with existing chemotherapy drugs has become a new research trend.

Proanthocyanidins are flavonoids that are widely present in the skin and seeds of various plants, with the highest content in grape seeds (15,16). Their molecular formula is $C_{30}H_{26}O_{12}$, and their molecular weight is 578.52 Da. Currently, proanthocyanidins can be extracted from many natural compounds and are also a major component of many Chinese medicines (17-19). Long-term studies have shown that proanthocyanidins possess anti-inflammatory properties, decrease blood pressure, and inhibit platelet aggregation, atherosclerosis and oxidation, among other functions (20-24). Since they are widely available and exhibit low toxicity and few side-effects, proanthocyanidins have received wide attention. In recent years, many experiments have shown that

proanthocyanidins have antitumor activity both *in vivo* and *in vitro*, and proanthocyanidins were shown to inhibit or kill many types of tumors (25-29).

Autophagy and apoptosis of tumor cells induced by drugs are two of the major causes of tumor cell death. Autophagy is an important cellular metabolic process that is highly conserved throughout evolution and is widely present in eukaryotic cells. Autophagy is a programmed cell death that is different from apoptosis and is termed type II programmed cell death (30,31). Autophagy is characterized by the presence of a large number of autophagosomes in the cytoplasm, and various digestive enzymes in the lysosome digest and degrade the contents in the vacuoles to convert them into substances required by the body for energy (32,33). Several studies have confirmed that autophagy plays an important role in tumorigenesis and therapy (34-36). However, reports on the effect of autophagy induced by drugs in tumor cells are not consistent and suggest that autophagy can have synergistic or antagonistic effects with apoptosis (37,38). In the present study, we treated the human gastric cancer cell line MGC-803 with proanthocyanidins to determine whether proanthocyanidins induced autophagy and apoptosis in these cells and to identify the mechanism of proanthocyanidins action to further determine the effect of proanthocyanidin-induced autophagy on apoptosis.

Materials and methods

Reagents. Proanthocyanidins were bought from Nanjing Zelang Medical Technology Co. Ltd. (Nanjing, China), with a purity of over 98% as testified by high-performance liquid chromatography. SDS-proanthocyanidins GE Gel Preparation kit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Nanjing KeyGen Biology China (Nanjing, China), monodansylcadaverine (MDC) autophagy detection kit, Annexin V-APC/7-AAD apoptosis detection kit, First Strand cDNA Synthesis kit and Taq DNA polymerase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cytotoxicity assay kit, 3-methyladenine (3-MA), Hoechst 33342/propidium iodide (PI) double staining kit and MTT cell proliferation were purchased from Thermo Fisher Scientific (Shanghai, China).

Cell culture. The human gastric cancer cell line MGC-803 was purchased from Nanjing KeyGen Biology China. The cells were cultured in RPMI-1640 complete medium containing 10% calf serum (CS) at 37˚C in a 5% CO_2 incubator. Cells in the logarithmic growth phase were used for the experiments.

MTT assay for cell proliferation (IC_{50}). A cell suspension with a concentration of $5x10^4$ cells/ml was prepared, and 100 μl of the cell suspension was added to each well of a 96-well culture plate, incubated at 37˚C in a 5% CO_2 incubator (Sanyo XD-101; Sanyo, Osaka, Japan) for 24 h. Complete medium was used to dilute the drug to the desired concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 μg/ml), and 100 μl of the corresponding drug-containing medium was added to each well. A negative control and a positive control group were also included. The 96-well plate was incubated at 37˚C in a 5% CO_2 incubator (Sanyo XD-101) for 48 h. The plate was then subjected to MTT staining, and the OD value was measured at λ=490 nm. The inhibition rate and drug IC_{50} value of each group were calculated.

Annexin V APC/7-AAD double staining to detect apoptosis. Cells growing in the logarithmic phase were trypsinized and seeded into a 6-well plate. The corresponding drug-containing medium was added (100, 20 or 4 μg/ml) after the cells were attached to the plate and negative control group was included at the same time. After treatment with the drug for 48 h, 0.25% trypsin (without EDTA) was used to trypsinize and gather the cells. The cells were washed twice with phosphate-buffered saline (PBS) (centrifugation at 2,000 rpm, 5 min), and $5x10^5$ cells were collected. The cells were then resuspended in 500 μl of binding buffer. After 5 μl of Annexin V-APC was added and mixed well, 5 μl of 7-AAD was added and mixed well. The reaction was performed at room temperature for 5-15 min in the dark, and a flow cytometer (FACSCalibur; Becton-Dickinson, USA) was used to detect apoptosis.

Transmission electron microscopy. MGC-803 cells in the logarithmic growth phase were incubated in drug-containing medium (100, 20 and 4 μg/ml). A negative control group was included at the same time. All the cells were harvested 24 h later. Trypsin (0.25%) was used to remove the cells from the plate. The cells were then centrifuged at 1,000 rpm for 10 min. After the supernatant was discarded, the cells were washed twice with PBS, and 2.5% glutaric acid was added. The cells were fixed for 90 min at 4˚C. After the cells were embedded, sectioned and stained with uranyl acetate and lead citrate, the autophagosomes were observed under a transmission electronic microscope (JEM-1011, Japan).

MDC staining to detect autophagy. Cells in the logarithmic growth phase were trypsinized and seeded into a 6-well plate. The next day, after the cells attached to the walls, drug-containing medium was added (100, 20 and 4 μg/ml). A negative control group was included at the same time. After treatment with the drug for 48 h, 0.25% trypsin (without EDTA) was used to gather the cells. Wash buffer (1X; 300 μl) was used to wash the cells once, and an appropriate amount of 1X wash buffer was added to resuspend the cells, with the cell concentration adjusted to 10^6 cells/ml. A total of 90 μl of cell suspension was transferred to a new microfuge tube and 10 μl of MDC staining solution was added and gently mixed. After staining at room temperature for 15-45 min in the dark, the cells were gathered by centrifugation at 800 x g for 5 min. Wash buffer was used to wash the cells three times, and the cells were resuspended in 100 μl of gatherion buffer. The cell suspension was dropped onto a slide and covered with a coverslip. The slide was then observed under a fluorescence microscope (Olympus IX51; Olympus, Japan).

Western blotting to determine protein expression. Cells in the logarithmic growth phase were trypsinized and seeded onto a 6-well plate. The next day, after the cells attached, drug-containing medium was added (100, 20 and 4 μg/ml). A negative control group was included at the same time. Pre-chilled lysis buffer (200 μl) was added to each group.

After mixing, the lysate was incubated on ice for 30 min. After vortexing, the lysate was centrifuged at 13,000 x g for 10 min at 4˚C. The supernatant was saved, and the BCA method was used to measure the protein concentration of the samples. The proteins were resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane. After the membrane was blocked overnight with 5% non-fat milk, the primary antibody (1:200) was added and incubated overnight at 4˚C in a sealed bag. TBST was used to wash the membrane three times for 10 min, and the membrane was then incubated with the secondary antibody (1:4,000) for 1 h. Finally, the membrane was incubated with chemiluminescence solution and exposed to film.

Hoechst 33342/PI double staining to detect apoptosis. Cells in the logarithmic growth phase were trypsinized and seeded into a 6-well plate. The next day, after the cells attached, drug-containing medium was added. A negative control group was included at the same time. After treatment with the drug for 48 h, 0.25% trypsin (without EDTA) was used to gather the cells. A total of 10^5-10^6 cells was resuspended in 1 ml of medium, 10 μl of Hoechst 33342 staining solution was added to the cells and mixed well, and the suspension was incubated at 37˚C for 5-15 min. The cells were centrifuged at 500-1,000 rpm for 5 min at 4˚C, and the supernatant was discarded. Buffer A (1 ml) was used to resuspend the cells, and 5 μl of PI staining solution was added, and incubated at room temperature for 5-15 min in the dark. The suspension was mixed well and observed under a fluorescence microscope Olympus IX51.

Fluorescence quantitative PCR to detect gene expression. Total RNA was isolated from logarithmically growing MGC-803 cells, and the purity of the RNA was determined. The isolated RNA was reverse transcribed into cDNA using a kit from Thermo Fisher. Fluorescent staining and a quantitative PCR were used to perform real-time quantitative PCR (ABI StepOne Plus, USA). The primers were synthesized by Nanjing GenScript Technology Co., Ltd. with the sequences: GAPDH (101-bp product) [5'-ACAACTTTGGTATCGTGG AAGG-3' (sense), and 5'-GCCATCACGCCACAGTTTC-3' (antisense)]; Beclin1 [(140-bp product) (5'-ATGTCCACAGA AAGTGCCAA-3' (sense), and 5'-GGGTGATCCACATCTGT CTG-3' (antisense)]; and BCL-2 (114-bp product) [(5'-AAATC CGACCACTAATTGCC-3' (sense), and 5'-TGCTCTTCAGAT GGTGATCC-3' (antisense)]. The amplification conditions were 95˚C pre-denaturation for 5 min followed by 95˚C denaturation for 15 sec, 60˚C annealing for 20 sec, and 72˚C extension for 40 sec for a total of 40 cycles. The specificity of the amplified products was monitored by melting curves. Software was used to calculate the relative expression of the target genes in each group, and GAPDH was used as an internal reference to assess the expression of target genes.

Statistical methods. The data are presented as the mean ± standard deviation. The SPSS 16.0 statistical software was used for data analysis. Analysis of variance (ANOVA) was used to compare the difference between groups under different conditions, and $p<0.05$ was considered to indicate a statistically significant result.

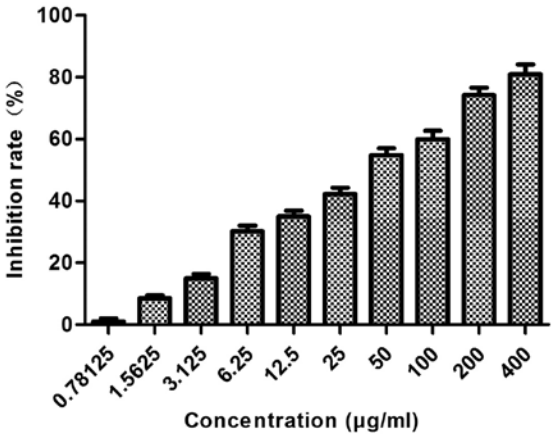

Figure 1. Concentration curve of the inhibition of MGC-803 cell proliferation by proanthocyanidins. A range of concentrations of proanthocyanidins was used to treat MGC-803 cells for 48 h, and a dose-dependent inhibition of cell proliferation was observed. The data are presented as the mean ± standard deviation (n=6).

Results

The inhibitory effect of proanthocyanidins on the proliferation of MGC-803 cells. As shown in Fig. 1, a range of concentrations of proanthocyanidins (400, 200, 10, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 μg/ml) was used to treat MGC-803 cells for 48 h, and the results showed that proanthocyanidins inhibited MGC-803 cell proliferation in a dose-dependent manner. At 48 h, the IC_{50} value was 40.654 μg/ml.

The effect of proanthocyanidins on the apoptosis of MGC-803 cells. To test whether the inhibition of cell proliferation by proanthocyanidins was associated with apoptosis, MGC-803 cells were treated with proanthocyanidins for 48 h, and then, flow cytometry was used to analyze the percentage of apoptotic cells. The results in Fig. 2 show that proanthocyanidins induced apoptosis in MGC-803 cells in a dose-dependent manner.

The effect of proanthocyanidins on the microstructural morphology of MGC-803 cells. To verify whether the cytoplasmic vacuoles observed by inverted microscopy were related to autophagy, transmission electron microscopy was used to observe autophagosomes in MGC-803 cells treated with proanthocyanidins. As shown in Fig. 3, untreated cells had normal nuclei, cytoplasm and organelles, whereas proanthocyanidin-treated cells showed a high number of autophagosomes of various sizes, and autophagosomes containing mitochondria were also observed by electron microscopy. This suggests that autophagy occurred in the cells after proanthocyanidin treatment.

MDC staining for autophagosome labeling. An inverted fluorescence microscope was used to observe MDC-labeled autophagic vacuoles, clear punctate structures were observed in the cytoplasm and perinuclear region, and the changes of the particles inside the cell were used to determine the level of autophagy. As shown in Fig. 4, compared with the control group, proanthocyanidins-treated cells showed stronger

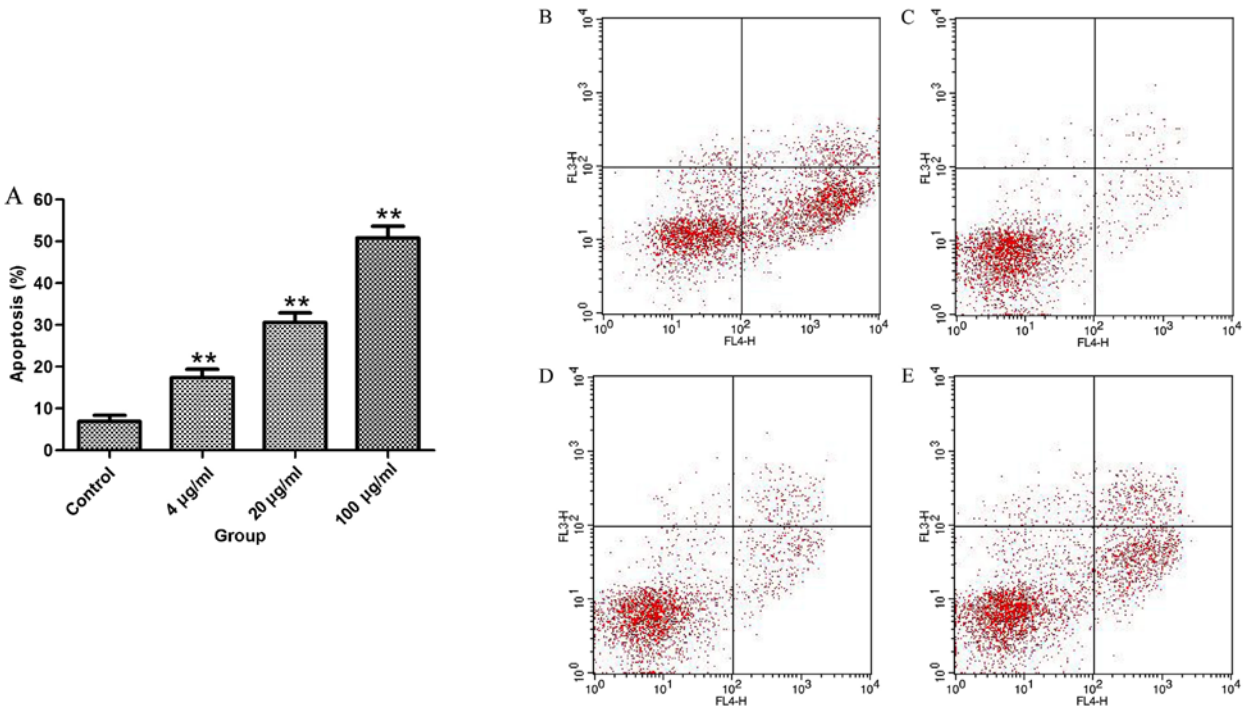

Figure 2. Curve of the effect of different concentrations of proanthocyanidins on the apoptosis of MGC-803 cells. (A) Compared with the control group, the percentage of apoptotic cells in the proanthocyanidins group significantly increased in a dose-dependent manner. The data are presented by the mean ± standard deviation (n=3). $^{**}p<0.01$. (B-E) Apoptotic profile of cells treated with different concentrations of proanthocyanidins (0, 4, 20 and 100 μg/ml).

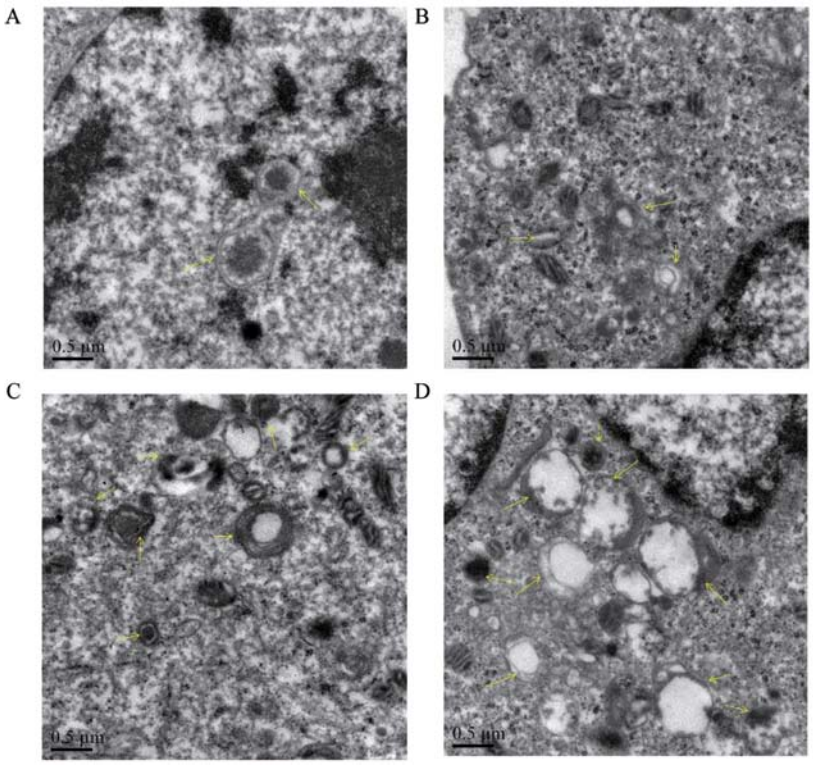

Figure 3. (A) Observation of the microstructure of MGC-803 cells treated with proanthocyanidins. Untreated MGC-803 cells had a normal nucleus, cytoplasm and organelle morphology. (B-D) Cells treated with different concentrations of proanthocyanidins (4, 20 and 100 μg/ml) showed a high number of various sizes of autophagic vacuoles, and some autophagosomes contained mitochondria and cytoplasm. (A-D, magnification, x20,000). The arrows indicate autophagic vacuoles and autophagosomes.

fluorescence intensity and more autophagic vacuoles labeled with MDC, and the number and staining intensity of the vacuoles increased with increasing proanthocyanidins dose. This suggests that proanthocyanidins induced autophagy.

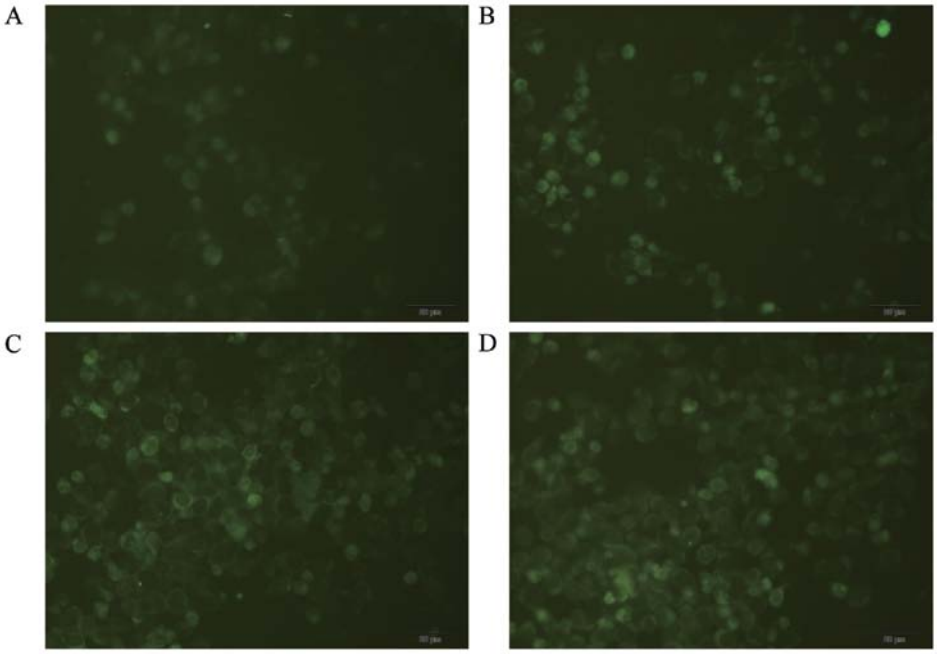

Figure 4. MGC-803 autophagic vacuoles labeled with MDC. Compared with the control group (B-D; magnification, x100), cells treated with different concentrations of proanthocyanidins (4, 20 and 100 μg/ml) exhibited increased number and fluorescence intensity of autophagic vacuoles labeled by MDC. (A) 0 μg/ml, magnification, x100.

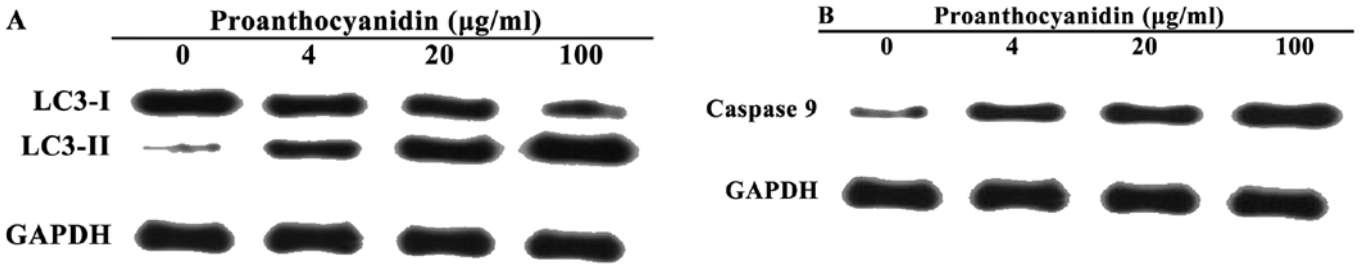

Figure 5. Proanthocyanidins induce the expression of LC3 and caspase 9 in MGC-803 cells. (A) Western blotting of the LC3 protein level in MGC-803 cells treated with different concentrations of proanthocyanidins. (B) Western blotting of the caspase 9 protein level in MGC-803 cells treated with different concentrations of proanthocyanidins.

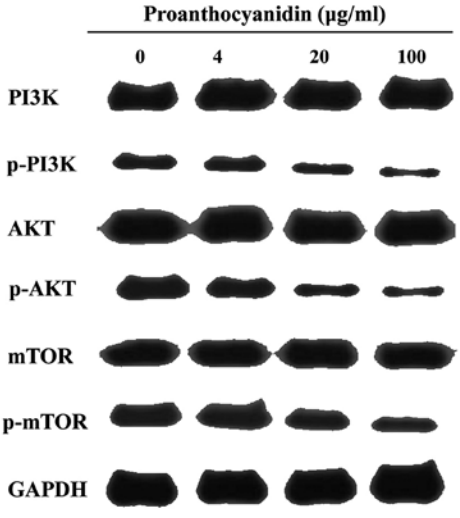

Figure 6. The effect of proanthocyanidins on the PI3K/AKT/mTOR signaling pathway. After proanthocyanidins treatment, the expression of PI3K, AKT, and mTOR did not significantly change, but the phosphorylation of PI3K, AKT and mTOR decreased in a dose-dependent manner. $^{**}p<0.01$.

Proanthocyanidins induces LC3 and caspase 9 expression in MGC-803 cells. Western blot analyses were used to detect changes in the levels of LC3 and caspase 9 after MGC-803 cells were treated with proanthocyanidins. On SDS-PAGE gels, LC3-II ran faster than LC3-I, producing two bands by western blotting. Fig. 5A shows that the untreated cells exhibited only a faint LC3-I band, whereas the LC3-II band was not detected. In contrast, after treatment with proanthocyanidins, the level of LC3-II increased significantly in a dose-dependent manner. Fig. 5B shows that treatment with proanthocyanidins significantly increased the expression of caspase 9 compared with the control group in a dose-dependent manner.

Effect of proanthocyanidins on the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (PKB/AKT)/mammalian target of rapamycin (mTOR) signaling pathway. The PI3K/AKT/mTOR signaling pathway is the canonical pathway that negatively regulates the initiation of autophagy. It has been reported that inhibition of this pathway induces cell autophagy. As shown in Fig. 6, western blot analyses showed

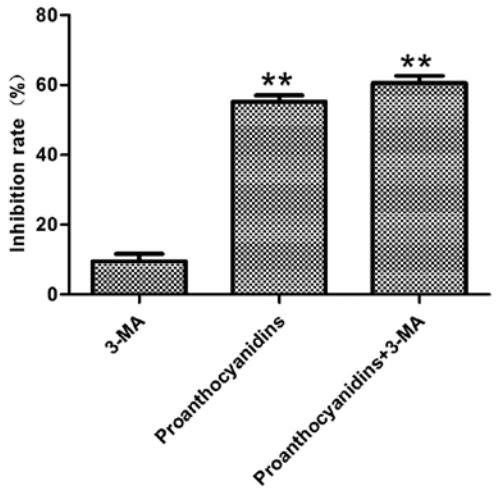

Figure 7. Proanthocyanidin cytotoxicity following inhibition of autophagy. MGC-803 cells were pretreated with 3-MA (5 mM) for 48 h, and the cells were then treated with proanthocyanidins (40.7 μg/ml) for 48 h. MTT assays were used to detect cell proliferation. The inhibition of cell proliferation by proanthocyanidins after the addition of 3-MA was significantly increased when compared with cells treated with proanthocyanidins alone. Data are expressed as the mean ± standard deviation (n=6) $^{**}p<0.01$.

that proanthocyanidins inhibited the phosphorylation of PI3K, AKT and mTOR in the PI3K/AKT/mTOR signaling pathway.

Inhibition of autophagy increased the cytotoxicity of proanthocyanidins. Preliminary experiments determined that proanthocyanidin treatment of MGC-803 cells activated both autophagy and apoptosis and that the inhibition of MGC-803 cell proliferation by proanthocyanidins occurred in a dose-dependent manner. To understand whether the cytotoxicity exhibited by proanthocyanidins was mediated by autophagy, the autophagy inhibitor 3-MA was added, and MTT assays were used to examine its cytotoxicity. The results showed that compared with cells treated with only proanthocyanidins, the addition of 3-MA significantly increased the percentage of apoptotic MGC-803 cells (Fig. 7).

Hochest 33342 and PI double fluorescence staining of live cells. Hochest 33342 and PI double staining is able to distinguish live and dead cells. When cells are in the late apoptotic stage or in the early necrotic stage, the nuclei are red in color, whereas the nuclei of live cells are blue. As shown in Fig. 8, MGC-803 cells treated with proanthocyanidins for 48 h exhibited nuclei with a bead-like shape, forming apoptotic bodies. There was no significant difference between treatment with 3-MA alone (Fig. 8B) and control cells (Fig. 8A). However, cells treated with proanthocyanidins (Fig. 8C) exhibited an increased percentage of apoptotic cells compared with the control cells (Fig. 8A) ($p<0.001$). Cells treated with proanthocyanidins + 3-MA (Fig. 8D) showed an increased percentage of apoptotic cells compared with cells treated with proanthocyanidins alone (Fig. 8C) ($p<0.01$). These results showed that apoptosis increased significantly in response to the inhibition of autophagy induced by proanthocyanidins.

The effect of proanthocyanidins on apoptosis following inhibition of autophagy. Since we found that the cytotoxicity

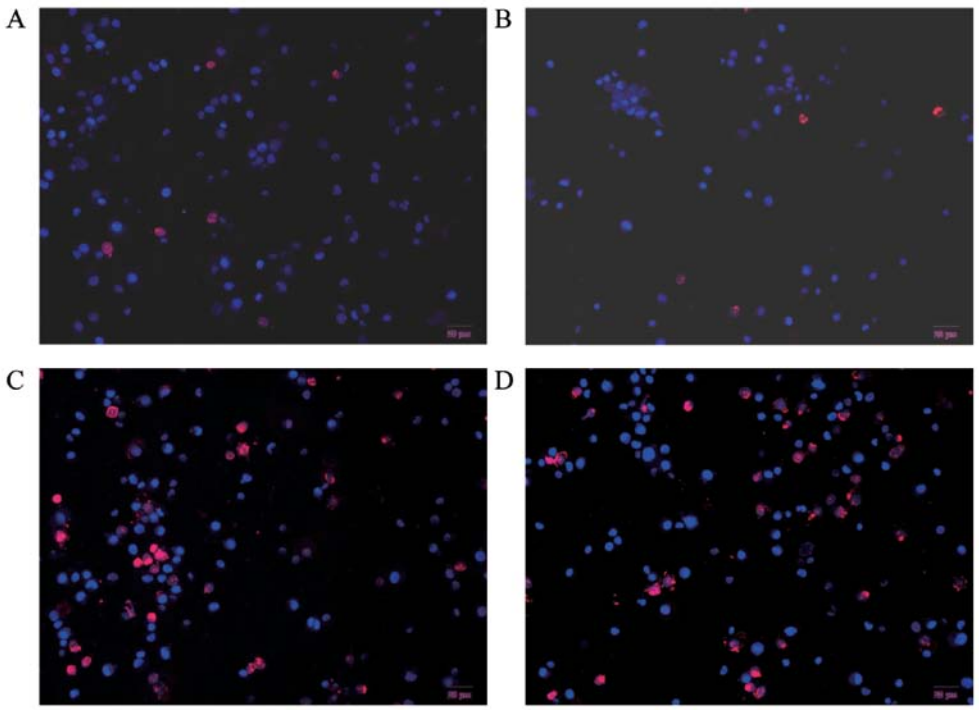

Figure 8. Hoechst and PI double fluorescence staining of live cells. After MGC-803 cells were treated with proanthocyanidins for 48 h, the nuclei showed a bead-like shape, forming apoptotic bodies. The percentage of apoptotic cells was 5.76±0.01% for the controls (A) and 34.42±0.02% for cells treated with proanthocyanidins (C). Compared with the controls, the percentage of apoptotic proanthocyanidin-treated cells increased significantly ($p<0.01$). The percentage of apoptotic 3-MA-treated cells (B) was 6.78±0.02%; compared with the control cells, there was no significant difference. The percentage of cells treated with proanthocyanidins + 3-MA (D) was 48.57±0.04%, which was significantly increased compared with cells treated with proanthocyanidins alone ($p<0.01$). Data are expressed as the mean ± standard deviation (n=3).

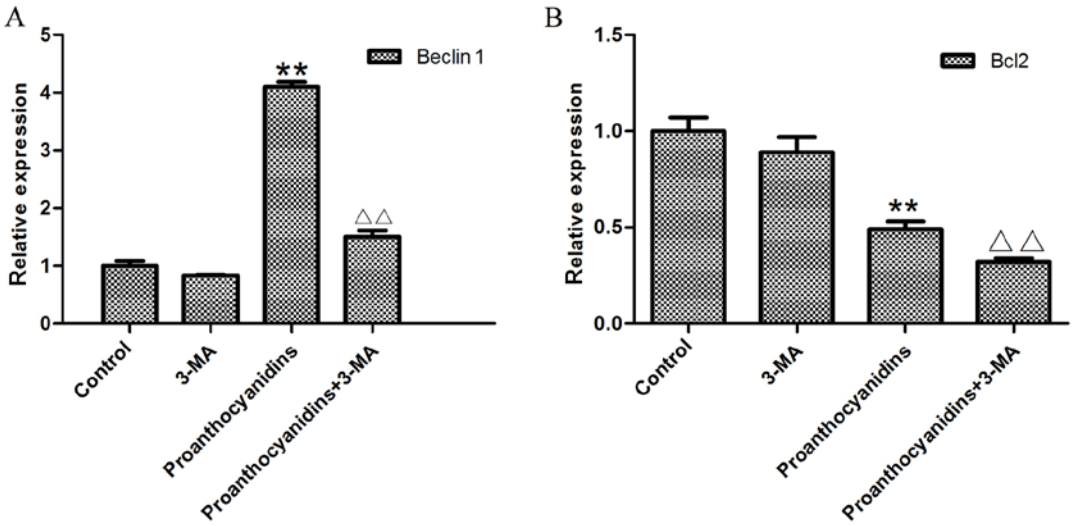

Figure 9. The effect of proanthocyanidins on autophagy and apoptosis when autophagy was inhibited. After treatment with 5 mM 3-MA, the expression of Beclin1 was decreased compared with that in control cells. After treatment with 40.7 μg/ml proanthocyanidins, the expression of Beclin1 increased significantly compared with that in control cells, $^{**}p<0.01$. However, when the proanthocyanidins were added after treatment with 3-MA, Beclin1 expression was significantly reduced compared with that in cells treated with proanthocyanidins alone, $^{\triangle\triangle}p<0.01$. (A) Data are expressed as the mean ± standard deviation (n=3). After treatment with proanthocyanidins, the level of Bcl2 decreased significantly compared with that in control cells, $^{**}p<0.01$, whereas simultaneous addition of 3-MA significantly decreased Bcl2 expression compared with that in cells treated with proanthocyanidins alone, $^{\triangle\triangle}p<0.01$. (B) Data are expressed as the mean ± standard deviation (n=3).

of proanthocyanidins was increased after the inhibition of autophagy, we speculated whether autophagy inhibited the effect of proanthocyanidins on apoptosis. Real-time quantitative PCR was used to investigate MGC-803 cells treated with proanthocyanidins. Using Beclin1 and BCL-2 as indicators, we investigated the effect of proanthocyanidins on autophagy and apoptosis when autophagy was inhibited. As shown in Fig. 9, after the addition of 5 mM 3-MA, the expression of Beclin1 decreased compared with that in control cells. After treatment with 40.7 μg/ml proanthocyanidins, the expression of Beclin1 increased significantly compared with that in control cells. However, when the proanthocyanidins were added after treatment with 3-MA, Beclin1 expression was significantly decreased compared with the level in cells treated with proanthocyanidins alone. After treatment with proanthocyanidins, BCL-2 decreased significantly compared with expression in the control cells, whereas the addition of 3-MA significantly decreased BCL-2 expression compared with that in cells treated with proanthocyanidins alone. Thus, when autophagy was inhibited, the apoptotic effect of proanthocyanidins was increased.

Discussion

In the present study, MTT assays were used to determine the effect of proanthocyanidins on the human gastric cancer MGC-803 cells and to calculate the IC_{50}. The results showed that proanthocyanidins significantly inhibit MGC-803 cells in a dose-dependent manner.

To test whether the inhibition of MGC-803 cells by proanthocyanidins is related to the induction of autophagy, we used MDC staining to label autophagic vacuoles and transmission electronic microscopy to observe autophagosomes, which are published methods to confirm the presence of autophagy (39). MDC staining showed that proanthocyanidin-treated cells exhibited increased autophagic vacuoles, and transmission electronic microscopy confirmed the existence of autophagosomes in the cells. Therefore, from the morphology, we can preliminarily confirm that proanthocyanidins induce autophagy in MGC-803 cells. Microtubule-associated protein 1 light chain 3 (LC3) plays a key role in autophagy in mammalian cells. LC3 consists of soluble LC3-I and lipidated LC3-II. Under various stresses, such as hypoxia and drug treatment, the cells initiate autophagy, and LC3-I undergoes a ubiquitination-like modification and processing to form LC3-II. Therefore, the level of LC3-II is positively correlated with the number of autophagic vacuoles. When autophagy occurs inside the cells, LC3-II increases significantly, and the detection of the change in LC3-II levels can accurately determine the amount of autophagy (40-43). When western blot analyses were used to determine the levels of the autophagy marker LC3, LC3-I levels decreased, whereas LC3-II increased in proanthocyanidin-treated MGC-803 cells, and both of these effects were dose-dependent. This confirmed that treatment with proanthocyanidin-induced autophagy in MGC-803 cells.

We next used flow cytometry to observe apoptosis of MGC-803 cells after treatment with proanthocyanidins. The proanthocyanidins significantly induced apoptosis in MGC-803 cells. According to published studies, proanthocyanidins induce apoptosis in multiple tumor cell types, and the above result is consistent with these reports (44,45). Apoptosis is initiated by p53, which activates relevant proteins to form a channel on the mitochondria to allow cytochrome *c* in the mitochondria to be released into the cytoplasm, which eventually activates caspase family proteases (46). Caspase family proteases are a type of aspartate-specific cysteine-containing proteases that are important for apoptosis. Caspases are

divided into apoptosis initiation factors and apoptosis effectors according to their function (47). Caspase 9 is an important initiation factor and can be activated by other proteins or by itself to activate a series of downstream effectors. Eventually, the cells undergo biochemical and morphological changes that lead to apoptosis (48). Our experiments have shown that proanthocyanidins activate caspase 9 and induce apoptosis in MGC-803 cells.

Molecular signaling pathways are closely involved in autophagy and apoptosis. Phosphatidylinositol 3 kinase (PI3K)/protein kinase B (PKB/AKT)/mammalian target of rapamycin (mTOR) is one of the currently most-studied pathways. This pathway is well accepted as being associated with cell autophagy and apoptosis. PI3K phosphorylates phosphatidylinositol (4,5) bisphosphate [PtdIns (4,5)P2] in the cytoplasmic membrane to generate phosphatidylinositol (3-5) triphosphate [PtdIns (3,4,5) P3], which recruits AKT to the inner side of the cytoplasmic membrane. AKT is then phosphorylated and activated by another protein kinase, 3-phosphoinositide-dependent protein kinase 1 (PDK1). Activated AKT further activates mTOR by inhibiting the tuberous sclerosis complex (TSC1/2), which is an inhibitor of mTOR. The inhibition of TSC1/2 activity by phosphorylated AKT leads to the activation of mTOR. mTOR is a serine/threonine kinase that inhibits autophagy when activated (49). Similarly, apoptosis is also affected by the PI3K/AKT pathway. Activated AKT binds to Ser184 of the BCL-2 family member BAX. After phosphorylation, BAX inactivates mitochondrial cytochrome *c*, blocking the activation of caspases and inhibiting apoptosis (50). Whereas, activated AKT also phosphorylates Ser196 of caspase 9, inactivating it and thus directly reducing apoptosis (51). Multiple studies have shown that autophagy and apoptosis induced by a variety of drugs are all associated with the PI3K/AKT/mTOR pathway (52-55). Western blot analyses showed that although proanthocyanidins did not affect the total amount of PI3K, AKT, and mTOR, these compounds did significantly reduce the amount of p-PI3K, p-AKT and p-mTOR; in other words, proanthocyanidins reduced the activation of the PI3K/AKT/mTOR pathway. This may be one of the reasons that proanthocyanidins induce autophagy and apoptosis and is also consistent with other reports (56).

The results above showed that both autophagy and apoptosis are activated when proanthocyanidins induce cell death in the human gastric cancer MGC-803 cells and that the activation mechanism is associated with the inhibition of the PI3K/AKT/mTOR pathway.

To further explore the relationship between proanthocyanidin-induced autophagy and apoptosis, we used the classical autophagy inhibitor 3-methyladenine (3-MA) to determine the effect of autophagy on apoptosis. MTT assays found that after the addition of 3-MA, the effect of proanthocyanidins increased. When Hoechst 33342/PI double fluorescence staining was used to observe apoptosis after the inhibition of autophagy, apoptosis increased significantly. These results suggest that the inhibition of autophagy may increase apoptosis. To verify this hypothesis, real-time quantitative PCR technology was used to determine the mRNA level of Beclin1 and BCL-2. Beclin1 plays a key role in autophagy of mammalian cells. Beclin1 is also the mammalian homologue of the yeast protein ATG6/Vps30 and is located on human chromosome 17q21 (57). Beclin1 promotes the formation of autophagosomes by forming a complex with class III PI3K (58). The expression of Beclin1 is positively correlated with autophagy in multiple malignant tumor cell types (59-61). BCL-2 is a key regulator of the known apoptosis proteins and is negatively correlated with apoptosis induced by various signals (62,63). When BCL-2 increases, the BCL-2/BAX heterodimer interferes with the release of cytochrome *c*, thereby blocking the activation of the upstream caspase protease and in turn inhibiting apoptosis (64-66). The inhibition of autophagy by 3-MA not only decreased Beclin1 mRNA but also increased BCL-2 mRNA, which further confirmed that apoptosis increases significantly when autophagy induced by proanthocyanidins is blocked.

In summary, proanthocyanidins exhibit a significant inhibitory effect on human gastric cancer cell (MGC-803) proliferation *in vitro* and simultaneously activate autophagy and apoptosis to promote cell death. The mechanism is associated with interference of the PI3K/AKT pathway by proanthocyanidins and a change in the amount of the downstream autophagy proteins LC3 and Beclin1, as well as in the apoptosis proteins BCL-2 and caspase 9. Furthermore, when proanthocyanidin-induced autophagy is inhibited, apoptosis increases significantly and tumor cells undergo cell death. Therefore, as an active ingredient of natural products with low toxicity, proanthocyanidins can be used together with autophagy inhibitors to enhance cytotoxicity.

Acknowledgements

The present study was supported by the State Administration of Traditional Chinese Medicine of Jiangsu Province (grant no. LZ13240).

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