

Apigenin-induced apoptosis is enhanced by inhibition of autophagy formation in HCT116 human colon cancer cells

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Abstract. Apigenin (4',5,7-trihydroxyflavone) is a natural flavonoid, shown to have chemopreventive and/or anticancer properties in a variety of human cancer cells. The involvement of autophagy in apigenin-induced apoptotic cell death of HCT116 human colon cancer cells was investigated. Apigenin induced suppression of cell growth in a concentration-dependent manner in HCT116 cells. Flow cytometric analyses indicated that apigenin resulted in G2/M phase arrest. This flavone also suppressed the expression of both cyclin B1 and its activating partners, Cdc2 and Cdc25c, whereas the expression of cell cycle inhibitors, such as p53 and p53-dependent p21^{CIP1/WAF1}, was increased after apigenin treatment. Apigenin induced poly (ADP-ribose) polymerase (PARP) cleavage and decreased the levels of procaspase-8, -9 and -3. In addition, the apigenin-treated cells exhibited autophagy, as characterized by the appearance of autophagosomes under fluorescence microscopy and the accumulation of acidic vesicular organelles by flow cytometry. Furthermore, the results of the western blot analysis revealed that the levels of LC3-II, the processed form of LC3-I, was increased by apigenin. Treatment with the autophagy inhibitor 3-methyladenine (3-MA) significantly enhanced the apoptosis induced by apigenin, which was accompanied by an increase in the levels of PARP cleavage. These results indicate that apigenin has apoptosis- and autophagy-inducing effects in HCT116 colon cancer cells. Autophagy plays a cytoprotective role in apigenin-induced apoptosis, and the combination of apigenin and an autophagy inhibitor may be a promising strategy for colon cancer control.

Introduction

Apigenin (4',5,7-trihydroxyflavone; Fig. 1A), a naturally occurring flavone, it is widely distributed in many fruits and vegetables such as parsley, onions, apples, tea and chamomile. In recent years, apigenin has been increasingly recognized as a cancer chemopreventive agent. The chemopreventive aspects of apigenin have been evaluated both *in vitro* and *in vivo*. Apigenin has been shown to be growth inhibitory in a variety of human cancer cell lines including colon, pancreatic, oral squamous, lung and leukemia cells (1-5). An important effect of apigenin is to increase the stability of the tumor suppressor p53 gene in normal cells. It has been shown that apigenin induced G2/M cell cycle arrest in colon cancer cells (1), and *in vivo* it is involved in p21^{CIP1/WAF1}-independent pathway for inhibitory phosphorylation of p34 (Cdc2) and concomitant G2/M arrest in mouse keratinocytes (6). Apigenin was shown to induce apoptosis in a variety of cancer cells (3,4,7,8). Apigenin has shown to inhibit tumor cell invasion and metastases by regulating the hypoxia-inducible factor 1- α protein level and to inhibit transforming growth factor β 1-induced vascular endothelial growth factor expression in human prostate cancer cells (9). Moreover, apigenin has been reported to potentiate the effect of tumor necrosis factor-related apoptosis-inducing ligand, paclitaxel, ABT-263, 5-fluorouracil (5-FU) and cisplatin against various human cancers (10-13).

Autophagy, an evolutionarily conserved process, sequesters and degrades long-lived cellular proteins and organelles through the lysosomal machinery (14,15). The purpose of autophagy is the recycling of cellular components to sustain metabolism under stress conditions such as nutrient deprivation and to prevent accumulation of damaged proteins and organelles (16). The first evidence for a role of autophagy in cancer was found by Liang *et al* (17). The autophagy-promoting activity of beclin-1 in human breast cancer cells is associated with inhibition of MCF7 cellular proliferation (17). It is reported that beclin-1, a phylogenetically conserved protein that is essential for autophagy, can inhibit tumorigenesis and is expressed at decreased levels in human breast carcinoma. Recent evidence indicates that the phosphoinositide 3-kinase (PI3K), Akt and mammalian target of rapamycin (mTOR) pathway, which is activated in many types of cancer (18) is important in autophagy regulation, especially through activating mTOR kinase, leading to suppression

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of autophagy (15). Recently, increasing evidence indicates that autophagy is closely associated with tumors. It participates as a tumor suppressor in tumor development in the early stages and as a proto-oncogene in advanced stages (19). Autophagy has been shown to increase as a result of chemotherapy, leading the cancer cells to autophagic cell death (programmed cell death) (20). However, although autophagy is a potential therapeutic target in adjuvant chemotherapy, the exact role and the relationship of autophagy with cancer development and progression, autophagic cell death, and apoptosis in cancer are still unclear.

Colorectal cancer (CRC) is the third most common incident cancer among men and the second most frequent among women in worldwide (21). Although specific causes of colon cancer are not known, nutritional and environmental factors have been associated with the development of colon cancer. In Korea, incidence of CRC has significantly increased. Annual percentage changes in age-standardized incident rates were 6.2% in men and 6.8% in women between 1999 and 2009 using the world standard population as a reference population. It is the second common cancer after stomach among men and the third most common after cancers of thyroid and breast among women in Korea (22,23). Surgical resection currently remains the only curative treatment for CRC, however, it is unsatisfactory. This is because only 70% of colorectal tumors are resectable, 75% of which are curable, and many patients have to receive adjuvant chemotherapy (24). Due to the incomplete therapeutic options for colon cancer, there is a need to develop preventive treatment approaches for this malignancy. This study was conducted to investigate the ability of apigenin to induce apoptotic cell death and the ability of apigenin to induce autophagy in HCT116 human colon cancer cells, and whether inhibition of autophagy could potentiate the proapoptotic effect of apigenin.

Materials and methods

Reagents. Apigenin, acridine orange, 3-methyladenine (3-MA), and monoclonal antibody against β -actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Apigenin was dissolved in dimethylsulfoxide (DMSO) and stored at -20°C before the experiments and dilutions were made in culture medium. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Amresco (Solon, OH, USA). Antibodies against beclin-1, Cdc2, Cdc25c, cyclin B1, p21, p53, poly (ADP-ribose) polymerase (PARP), caspase-3, caspase-8 and caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibody against LC3B was obtained from Cell Signaling Technology (Danvers, MA, USA). RPMI-1640, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from HyClone (Logan, UT, USA).

Cell culture and apigenin treatment. The human colorectal cancer cell line HCT116 was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37°C in a humidified 95% air and 5% CO_2 in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Assays for cell viability. The cell growth was determined by MTT assay. Cells were seeded in 6-well culture plate and incu-

bated at 37°C for 24 h. Then, cells were treated with different concentration of apigenin (0-50 μM) for 24 h. The cells were incubated in the dark with MTT reagent (0.5 mg/ml) at 37°C for 2 h. Medium was removed, formazan was dissolved in DMSO and the absorbance at 540 nm was measured by using an ELISA plate reader.

Hoechst staining and observation of nuclear structure. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4 $\mu\text{g}/\text{ml}$ Hoechst 33342 for 20 min at room temperature. The stained cells were washed twice with PBS and analyzed by a fluorescent microscope.

Cell cycle analyses by flow cytometry. Cells were harvested with trypsinization and washed once with PBS. After centrifugation, the cells were fixed in 70% ethanol at -20°C overnight. Fixed cells were prepared for flow cytometry analysis by washing twice with PBS and then stained in propidium iodide (PI) (Sigma-Aldrich) solution (50 $\mu\text{g}/\text{ml}$ in PBS) for 30 min at room temperature in the dark. Flow cytometry analysis was performed on a Cytomic FC500 (Beckman Coulter, Istanbul, Turkey).

Western blot analysis. After apigenin treatment, cells were harvested and washed with cold PBS. Total cells were lysed in lysis buffer [40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin and 100 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride (PMSF)]. After centrifugation, the supernatant was collected and protein concentration was determined by protein assay reagents (Bio-Rad, Hercules, CA, USA). Equal amount of protein extracts were denatured by boiling at 100°C for 5 min in sample buffer (Bio-Rad). The proteins were separated on subject to 6-15% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 buffer (TBS-T) (20 mM Tris, 100 mM NaCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature. The membranes were washed 10 min, 4 times with TBS-T buffer each for 10 min and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The membranes were washed once for 10 min, 4 times with TBS-T buffer. Antigen-antibody complex were detected by the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA).

Annexin V staining. The Annexin V-FITC is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. Cells harvested were washed twice with cold PBS, suspended the cells in 1X binding buffer (BD Biosciences, San Diego, CA, USA). The counted cells were stained in PI and Annexin V-FITC solution (BD Pharmingen FITC Annexin V Apoptosis Detection kit, BD Biosciences) for 15 min at room temperature in the dark, and then, the stained cells were analyzed by flow cytometry.

Detection of autophagy with acridine orange staining. The formation of acidic vesicular organelles (AVOs) is a well-known feature of autophagy. Cells were seeded in 6-well culture plate and incubated at 37°C for 24 h. Then,

cells were treated with different concentration of apigenin (0-50 μM) for 24 h, and stained with acridine orange (1 $\mu\text{g}/\text{ml}$) for 15 min. Subsequently, cells were washed with PBS and examined under a fluorescent microscope. Depending on intracellular acidity, autophagic lysosomes appeared as orange/red fluorescent cytoplasmic vesicles, while the nuclei were displayed bright green. Alternatively, to quantify the development of AVOs, apigenin-treated cells were stained with acridine orange (1 $\mu\text{g}/\text{ml}$) for 15 min, trypsinized, and then washed with PBS. The stained cells were then analyzed using a flow cytometer.

GFP-LC3 assay. HCT116 cells were transfected with LC3-GFP plasmid using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Cells were treated with 3-MA (5 mM), apigenin (25 μM) or apigenin + 3-MA for 24 h and the formation of punctuate LC3-positive structures was observed by confocal microscopy.

Statistical analysis. Results are expressed as the mean \pm SD of three separate experiments and analyzed by Student's t-test, and considered significantly different at $p < 0.05$ or $p < 0.01$.

Results

Apigenin suppresses the growth of HCT116 cells. To investigate the growth inhibitory effect of apigenin (Fig. 1A), the MTT assay was performed in HCT116 cells. As shown in Fig. 1B, apigenin inhibited effectively cell proliferation of HCT116 cells in a concentration-dependent manner. The lower concentrations of apigenin (6.25 μM) did not affect cell viability; however, the higher concentrations (25 and 50 μM) of apigenin significantly reduced the cell viability of HCT116 cells. Additional experiment was done to confirm the growth inhibitory activity of apigenin on HCT116 cells when analyzed by microscopic observations. As shown in Fig. 1C, cells treated with apigenin displayed distinct morphological changes compared with untreated cells. In particular, cells were rounded and cell number was decreased in a concentration-dependent manner after apigenin treatment.

Apigenin modulates cell cycle progression in HCT116 cells. To identify the mechanism responsible for apigenin-induced cell growth inhibition, cell cycle progression was evaluated by flow cytometry analysis, as shown in Fig. 2A, apigenin treatment caused a significant cell cycle arrest in the G2/M phase concentration-dependently. An accumulation of cells in G2/M phase of 9.99, 20.1, 27.2 and 29.5% was observed for 6.25, 12.5, 25 and 50 μM , respectively, when compared with untreated cells (7.12%). Next, we examined whether apigenin can modulate the expression of G2/M cell cycle regulators. Cells were treated with various concentrations of apigenin for 24 h and then the level of G2/M cell cycle regulatory proteins were examined by western blot analysis. As indicated in Fig. 2B, after the cells were exposed to apigenin, the levels of p53 and its downstream protein p21^{CIP1/WAF1}, a potent cyclin-dependent kinase (CDK) inhibitor in G1 and G2/M phases, were increased in a concentration-dependent manner. Therefore, in agreement with previous findings

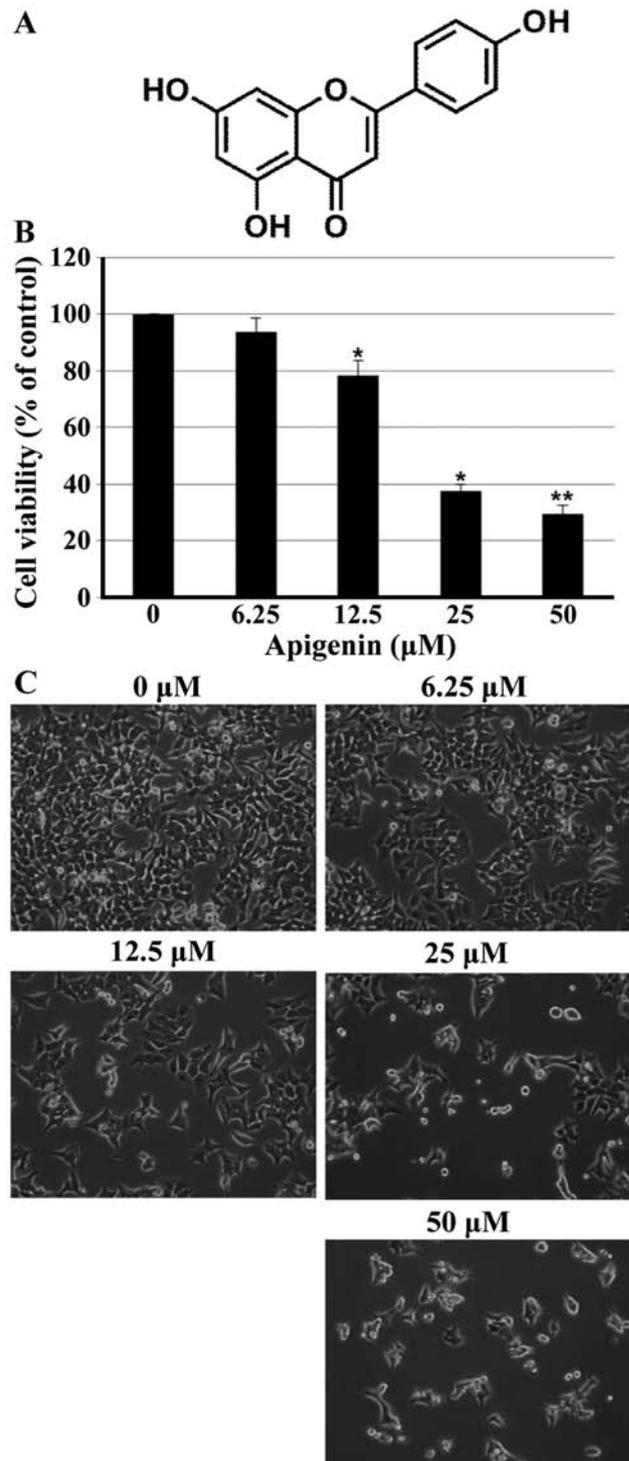


Figure 1. Effect of apigenin on cell viability of HCT116 cells. (A) Chemical structure of apigenin. (B) Cells were treated with increasing concentration of apigenin for 24 h, and then MTT assay was performed to measure cell viability. Cell viability (%) was expressed as a percentage compared to the untreated cells. The results are expressed as mean \pm SD (n=6) of three separate experiments. The significance was determined by Student's t-test * $p < 0.05$; ** $p < 0.01$, compared with untreated cells. (C) Cells were treated with the indicated concentrations of apigenin for 24 h, and then photographed by phase contrast microscopy at x400 magnification.

(Fig. 2A), apigenin treatment decreased Cdc25c and its regulatory protein Cdc2 as well as cyclin B1 (Fig. 2B). These data

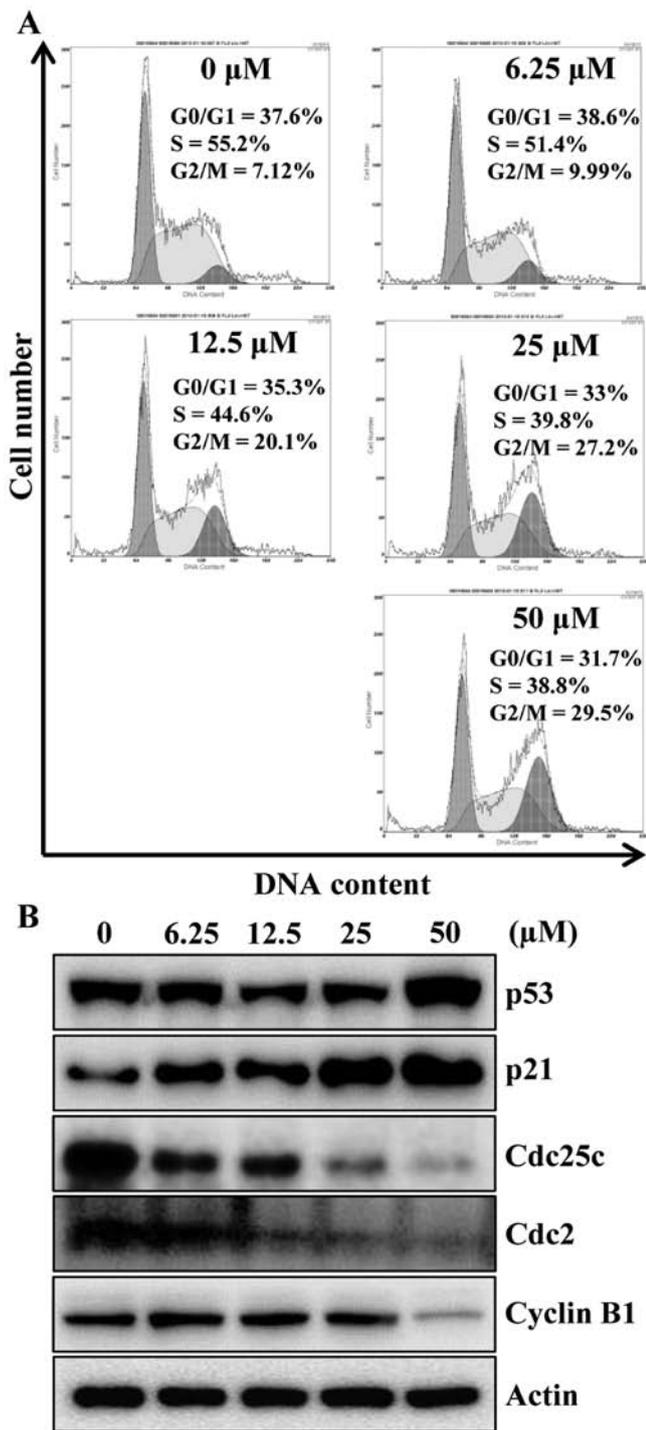


Figure 2. Effects of apigenin on the cell cycle and the levels of cell cycle regulator proteins in HCT116 cells. (A) Exponentially growing cells at 60-70% confluence were treated with apigenin (0-50 μM) for 24 h. The cells were fixed and digested with RNase, and then cellular DNA was stained with PI and analyzed by flow cytometry. The percentages of cells in G0/G1, S and G2/M phases of cell cycle were calculated and are displayed within each histogram. (B) Cells were treated with apigenin (0-50 μM) for 24 h. Total cell lysates were prepared and subjected to 10-12% SDS-PAGE and electrophoretically transferred to PVDF membrane. Western blot analysis was conducted with indicated antibodies and ECL kits. Actin was used as an internal control. Representative results from three independent experiments are shown.

suggest the possibility that apigenin-induced growth inhibition of HCT116 cells was the result of G2/M arrest.

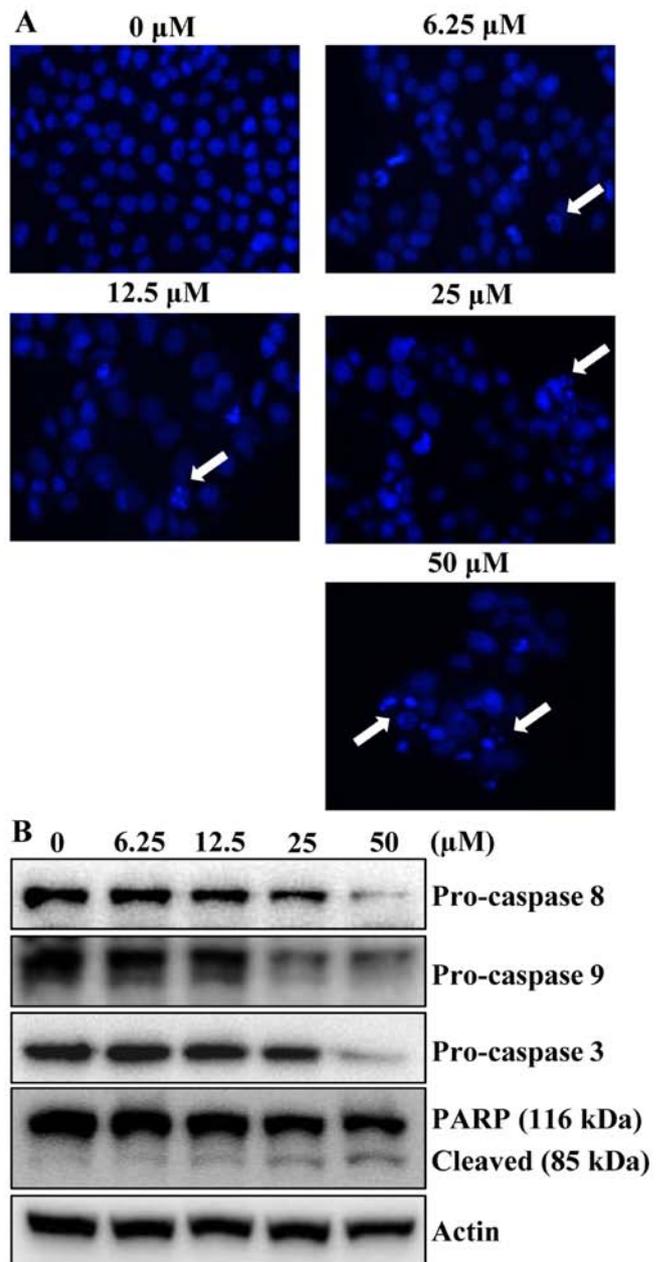


Figure 3. Effect of apigenin on apoptotic cell death in HCT116 cells. (A) HCT116 cells were treated with indicated concentration of apigenin for 24 h, and then stained with a fluorescent DNA-binding dye, Hoechst 33342. The cells were observed and photographed under a fluorescence microscope using blue filter. Apoptotic cells (arrows). Magnification, x400. (B) HCT116 cells were treated with apigenin for 24 h and lysates were used to measure the levels of caspases and PARP (116 kDa). Representative results from three independent experiments are shown. Actin was used as an internal control.

Apigenin induces apoptosis in HCT116 cells. To determine whether the growth inhibitory effects of apigenin were due to apoptotic cell death, the morphological changes of cellular structures were assessed with Hoechst 33342 staining. The untreated control cells displayed intact nuclear structure, while cells were treated with apigenin resulted in chromosomal condensation and formation of apoptotic bodies, which are characteristics of programmed cell death (Fig. 3A, arrows). The effect of apigenin on apoptosis was further verified by western blot analysis. Apigenin treatment decreased the protein levels

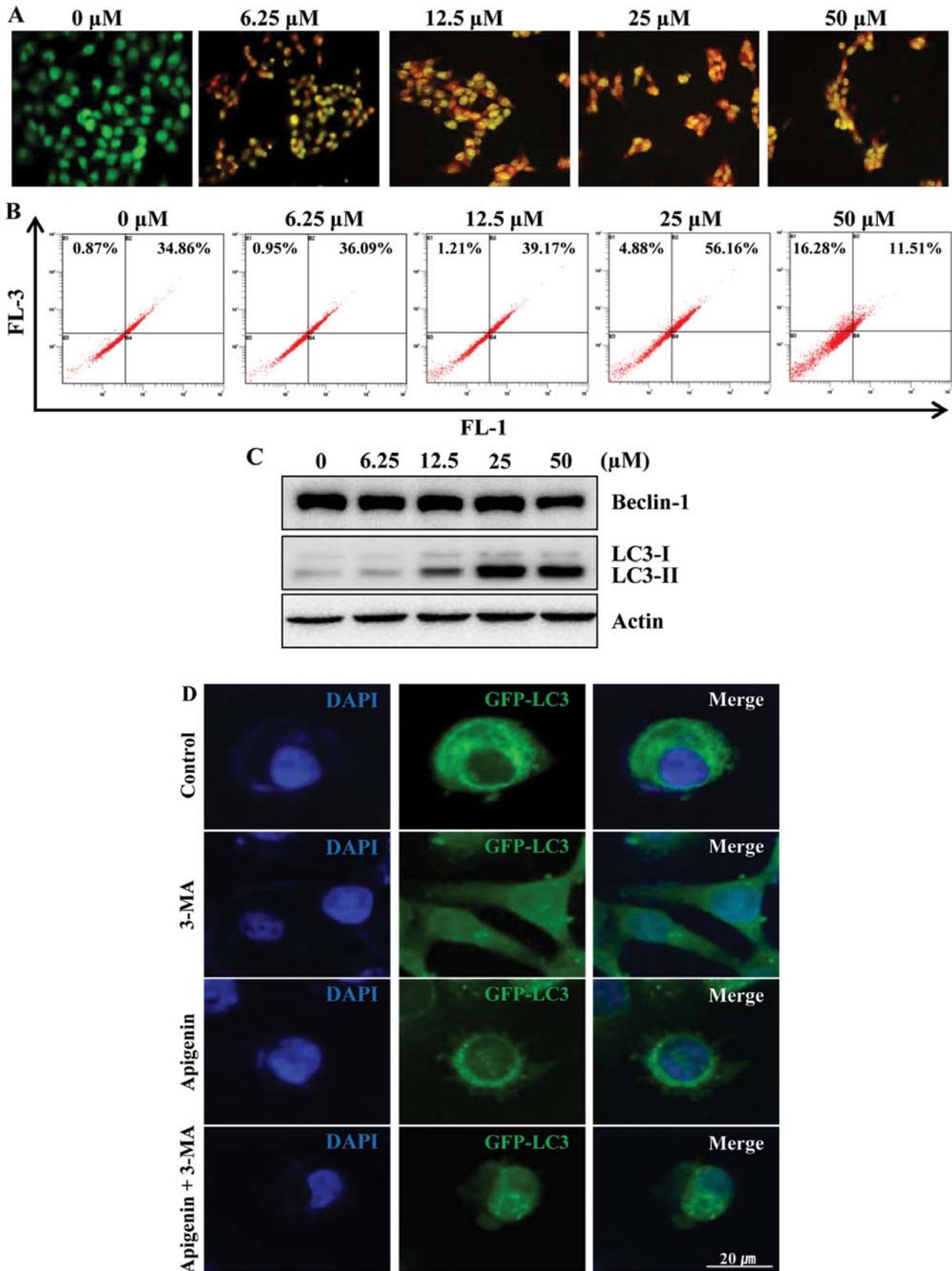


Figure 4. Induction of autophagy by apigenin in HCT116 cells. (A) Cells were treated with apigenin (0-50 μ M) for 24 h, stained with acridine orange and then imaged by fluorescence microscopy to detect the acidic vesicular organelle (AVO) formation. (B) Flow cytometric analysis of the apigenin-treated cells after staining with acridine orange for the quantification of AVOs. (C) HCT116 cells were treated with various concentration of apigenin for 24 h. Cells were lysed and the level of beclin-1 and LC3 proteins were analyzed by western blot analysis. Actin was used as an internal control. (D) After HCT116 cells were transfected with the LC3-GFP plasmid for 24 h, the medium was replaced with media containing 25 μ M apigenin plus the presence or absence of 3-MA (5 mM) and incubated for another 24 h. The GFP-LC3 puncta formation was observed by confocal microscopy. Representative results from three independent experiments are shown. Bar, 20 μ m.

of procaspase-8, -9 and -3 in concentration-dependently. In accordance with the decrease of procaspases with consequent increase of the levels of active forms of caspases, the cleavage of PARP was increased in a concentration-dependent manner (Fig. 3B). These results suggested that apigenin induced apoptotic cell death in HCT116 cells.

Apigenin induces autophagy in HCT116 cells. Autophagy, an evolutionally conserved self-digestive process, is known as a cell survival mechanism during nutrient depletion and is essential to cellular homeostasis maintenance by facilitating the disposal of unfolded proteins and cellular constituents (25). It has been reported that inhibition of autophagy has potential to promote apoptosis induced by several anticancer agents, which suggested that autophagy may play a protective role in cancer cells (26,27). Recently, apigenin has been reported to induce autophagy in human breast cancer cells (28). To determine whether apigenin induces autophagy in HCT116 cells, we examined the effect of apigenin on the formation of acidic vesicular organelles (AVOs) in HCT116 cells. For this, we used acridine orange (AO), which accumulates in acidic cell compartments and displays bright red fluorescence (29). The formation of AVOs can be detected by fluorescence microscopy and quantified by flow cytometry. As shown in Fig. 4A, apigenin treatment caused progressive increase of AVO formation in HCT116 cells. In agreement with microscopic observation, flow cytometry analysis also showed a concentration-responsive increase in AVOs in apigenin-treated cells as compared with their respective untreated control cells (Fig. 4B).

It is known that the beclin-1 level and LC3 conversion (LC3-I to LC3-II) are selective markers of autophagy. As shown in Fig. 4C, apigenin treatment markedly increased the expression of LC3-II, suggestive of autophagy induction. However, apigenin did not affect the expression of beclin-1 significantly. To further confirm the induction of autophagy by apigenin, we utilized fluorescence microscopy to examine autophagosome formation in HCT116 cells transiently expressing GFP-LC3 protein. Due to the conversion of LC3-I (a soluble form) to the LC3-II (a lipidized form), which associates with the membranes of autophagosomes (30), this conversion can be detected by observing the formation of punctate structures. Treatment of these cells for 24 h with apigenin resulted in relocalization of the GFP-LC3 protein to punctate cytoplasmic dots, indicators of autophagosome formation. Furthermore, co-treatment with apigenin and 3-MA, inhibits autophagy at initiation of double membrane encapsulation, significantly inhibiting GFP-LC3 dot formation in HCT116 cells (Fig. 4D).

Autophagy inhibition enhances apigenin-induced apoptosis in HCT116 cells. Accumulating data suggest that inhibition of autophagy may enhance chemosensitization in human cancer cells (31). Thus, we determined whether inhibition of autophagy could potentiate apigenin-induced apoptotic cell death. For this, we first performed Annexin V/PI staining to evaluate the effects of 3-MA on apigenin-induced apoptosis. Results indicated that apoptosis induction was 25% by apigenin, 23% by 3-MA, and 45.3% by the combination of the two agents (Fig. 5A). Furthermore, the alteration in the protein levels of LC3 and apoptotic proteins procaspase-8, -9 and -3 and PARP

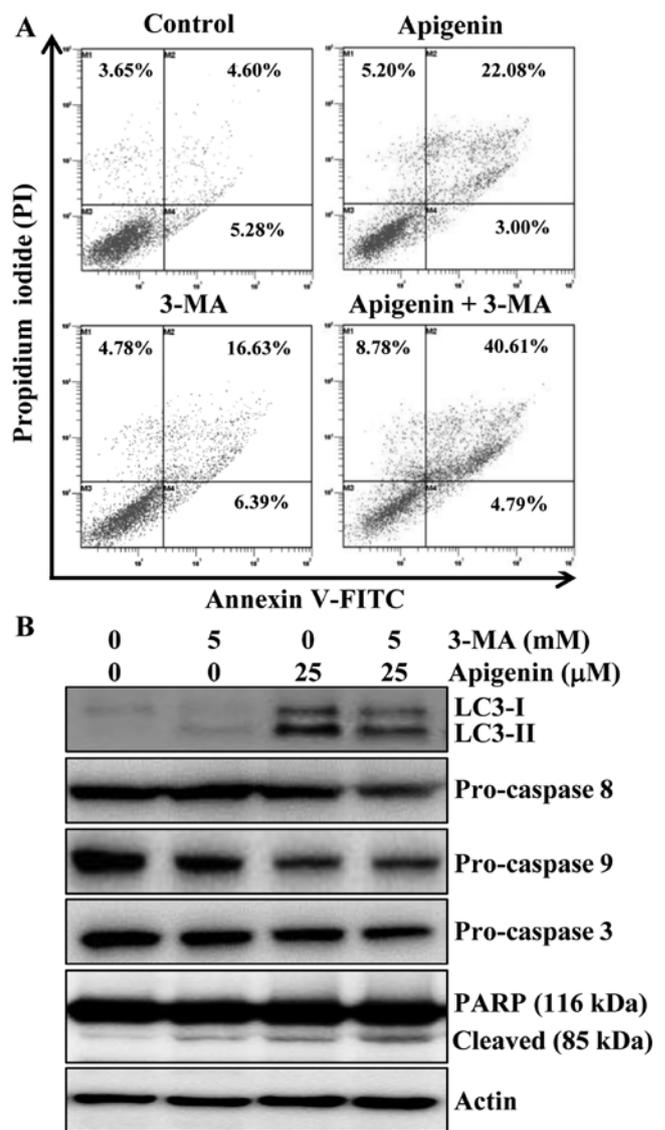


Figure 5. Effect of 3-MA on the activation of apoptosis by apigenin. (A) Cells were treated with apigenin (25 μ M), 3-MA (5 mM) alone or in combination for 24 h, stained with Annexin V/PI, and the apoptotic cell death was analyzed using flow cytometry. (B) Cells were exposed to apigenin, 3-MA alone or in combination for 24 h. Whole-cell lysates were prepared and analyzed by western blot analysis using antibodies against LC3, PARP, procaspase-3, -8 and -9. Representative results from three independent experiments are shown. Actin was used as an internal control.

cleavage were also observed by western blot analysis. The protein level of LC3-II was increased by apigenin, however, addition of 3-MA abrogated the effect of apigenin on LC3-II induction (Fig. 5B). We also found that the combination of 3-MA and apigenin resulted in a significant increase in the level of PARP cleavage and decrease in procaspase-3, -8 and -9 compared to apigenin treatment alone (Fig. 5B). Altogether, these results indicated that inhibition of autophagy by 3-MA enhanced apigenin-induced cell death in HCT116 cells.

Discussion

Many studies have demonstrated that apigenin exhibits chemopreventive effects on various cancer cells. Apigenin has been

reported to act via several mechanisms, including promotion of cell cycle arrest (1,32), apoptosis and suppression of signal transduction (33). One of most obvious mechanisms of apigenin is to induce p53 tumor suppressor protein at the translational level, followed by p21 induction (34). In the present study, apigenin effectively suppressed the growth of HCT116 cells by cell cycle arrest and this growth inhibition was attributed to apoptotic cell death. Furthermore, an alteration in the ratio of Bax to Bcl-2 has been proposed to play an important role in anticancer agent-induced cell death (35). The effect of apigenin on Bcl-2 family protein is controversial. Several reports indicated that apigenin could upregulate Bax and down-modulate Bcl-2 expression in numerous cancer cell lines including lung and breast cancer (4,28). However, in agreement with the report of Shao *et al* (12), we could not find any significant changes in levels of pro-apoptotic protein, Bax and anti-apoptotic protein, Bcl-2, in HCT116 cells (data not shown). The precise reason for this difference is not clear but cell type and conditions for experiment used may count for this discrepancy.

We found that apigenin induces autophagy in colon cancer cells. In the autophagic pathways, there are two molecular regulation mechanisms, PI3K/protein kinase B (Akt)/mTOR and class III PI3K signal transduction pathways. In the process of autophagy, the complex of beclin-1 and class III PI3K-dependent autophagy has an important function in mediating the localization of autophagy-related proteins to the preautophagosomal membrane (15). Autophagy has been shown by several antineoplastic agents in a great number of cancer cells, such as cervical carcinoma (36), glioma (37) or colon cancer cells (38). Our result showed progressive increase of LC3-II protein levels compared with the control cells by apigenin. At 25 μ M concentration, apigenin dramatically increased LC3-II, but higher concentration of apigenin (50 μ M) decreased LC3-II and beclin-1 levels. It is likely 50 μ M apigenin induced more cell death than 25 μ M. The knockdown of beclin-1 caused slow cell growth compared with cells with normal expression of beclin-1 (39). Thus, we observed that apigenin induced both apoptosis and autophagy. This phenomenon may be explained by the effect of apigenin on the PI3K/Akt/mTOR pathway. It has been indicated that the anticancer effect of apigenin is related to the inhibition of the PI3K/Akt/mTOR pathway (32,40), which is also an essential pathway that negatively regulates autophagy (41).

Furthermore, we found that autophagy inhibition using 3-MA pronounced apigenin-induced cell death. Adjusting autophagy appropriately may increase the cytotoxicity of anticancer drugs in tumor cells (42). A recent report indicated that 3-MA worked well with chemotherapeutic drugs by triggering apoptosis in some cancer cells (39). For example, autophagy inhibition by using 3-MA augmented chemotherapeutic effect of 5-FU in several *in vitro* models of human colon cancer (43). Therefore, we hypothesized that treatment of autophagy inhibitors might sensitize HCT116 cells to apigenin chemotherapy by improving the rate of apoptosis cell death induced by apigenin or by converting the autophagy process to an apoptotic process. It has been indicated that, inhibition of autophagy by 3-MA enhances the apoptosis in many cancer cells. The mitochondrial apoptotic pathway is a relatively important death receptor pathway for the induction of apoptosis by chemotherapeutic drugs (24).

In conclusion, apigenin suppressed growth of HCT116 cells in a concentration-dependent manner by causing G2/M cell cycle arrest. In addition, apigenin-treated cells exhibited inductions of apoptosis and autophagy. The inhibition of autophagy by 3-MA enhanced the apoptosis induced by apigenin through activations of pro-caspases-8, -9 and -3 and cleavage of PARP. These results provide evidence that inhibition of autophagy may be an effective way to improve the chemotherapy of anti-cancer agents against human colon cancer.

Acknowledgements

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