Flavonoids isolated from *Citrus platymamma* induce mitochondrial-dependent apoptosis in AGS cells by modulation of the PI3K/AKT and MAPK pathways

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Abstract. *Citrus platymamma* hort. ex Tanaka (Rutaceae family) has been widely used in Korean folk medicine for its wide range of medicinal benefits including an anticancer effect. In the present study, we aimed to investigate the molecular mechanism of the anticancer effects of flavonoids isolated from *Citrus platymamma* (FCP) on AGS cells. FCP treatment significantly inhibited AGS cell growth in a dose-dependent manner. Furthermore, FCP significantly increased the percentage of cells in the sub-G1 phase (apoptotic cell population), and apoptosis was confirmed by Annexin V double staining. Chromatin condensation and apoptotic bodies were also noted in the FCP-treated AGS cells. Moreover, immunoblotting results showed that FCP treatment significantly decreased the expression of procaspase-3, -6, -8 and -9, and PARP and increased cleaved caspase-3, cleaved PARP and the Bax/Bcl-xL ratio in a dose-dependent manner. In addition, the phosphorylation of AKT was significantly decreased, whereas extracellular signal-related kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs) were significantly increased in the FCP-treated AGS cells. Taken together, the cell death of AGS cells in response to FCP was mitochondrial-dependent via modulation of the PI3K/AKT and MAPK pathways. These findings provide new insight for understanding the mechanism of the anticancer effects of FCP. Thus, FCP may be a potential chemotherapeutic agent for the treatment of gastric cancer.

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Introduction

Gastric cancer is the second most common cancer worldwide next to lung cancer (1), and is a major public health issue in Korea. Currently, the available treatments for gastric cancer are inadequate. With advances in recent techniques, the overall 5-year survival rate of gastric cancer patients ranges from 10 to 30% (2,3). However, gastric cancer patients in advanced stages have limited treatment options. Hence, there is an urgency to identify novel therapeutic agents that can reduce the mortality of cancer patients with few side effects.

Over the past few years, flavonoids from dietary sources have attracted interest in preventing cancer with low toxicity. Flavonoids are abundantly present in fresh fruits and vegetables and have various health benefits (4,5). Korean *Citrus platymamma* hort. ex Tanaka (Byungkyul in Korean), a member of the Rutaceae family, has been used in traditional herbal medicines in Korea. It contains abundant flavonoids which have been reported to have various properties that regulate the inflammatory response and halt carcinogenesis and cancer progression (6). It is also speculated that the intake of flavonoids reduces the risk of most types of cancer (7,8). Our previous studies indicated that flavonoids isolated from Korean *Citrus aurantium* L. effectively inhibited the proliferation of various cancer cells by inducing cell cycle arrest and apoptosis and suppressed inflammatory mediators in L6 skeletal muscle cells (9-12). However, the anticancer effects and the related mechanisms of flavonoids from *C. platymamma* (FCP) have not yet been elucidated.

Apoptosis is a critical cell death mechanism with a distinctive phenotype and plays an important role in the mechanism of chemotherapies against various types of carcinoma (13). Apoptosis signaling pathways mainly function through two major pathways (the intrinsic pathway - mitochondria-mediated apoptosis and the extrinsic pathway - death receptor-mediated

apoptosis) (14,15). In addition, anticancer agents activate the PI3K/Akt signaling pathway which is critical in regulating cell proliferation and apoptosis (16). In addition, mitogen-activated protein kinases (MAPKs) such as extracellular signal-related kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (p38 MAPKs) are also involved in survival, proliferation and apoptosis (17). Thus, apoptosis plays crucial roles in the anticancer properties of many anticancer molecules by preventing or controlling abnormal cell development (18).

Based on the above evidence, in the present study, we investigated the anticancer activity and the related mechanism of FCP in AGS cells. The present study provides new insight for understanding the mechanism of the anticancer effects of FCP in AGS cells.

Materials and methods

Isolation of flavonoids from Korean Citrus platymamma Hort. ex Tanaka. The fruit of *C. platymamma* hort. ex Tanaka was obtained from the Animal Bio-Resources Bank (Jinju, Korea). The flavonoids were isolated, and high-performance liquid chromatography (HPLC) was performed at the Department of Chemistry, Gyeongsang National University by Professor Sung Chul Shin as described previously (19). The FCP samples were stored at -70˚C until further use.

Materials and chemicals. RPMI-1640 medium, fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin) were purchased from Gibco (BRL Life Technologies, Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to Bcl-xL, Bax, caspase-3, -6, -8 and -9, cleaved caspase-3, poly(ADP ribose) polymerase (PARP), cleaved PARP, p-Akt, JNK, p-JNK, p38, p-p38, ERK1/2 and p-ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA, USA). The Akt and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Millipore (Billerica, MA, USA), respectively. Horseradish peroxidase (HRP)-coupled goat anti-mouse IgG and anti-rabbit IgG were purchased from Enzo Life Sciences. Muse™ Cell Cycle kit and Annexin V and Dead Cell kit were purchased from Millipore. Materials and chemicals used for electrophoresis were obtained from Bio-Rad (Hercules, CA, USA).

Cell culture and viability assay. Human gastric cancer AGS cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The AGS cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C. To assess the effect of FCP on AGS cell growth, the cells were seeded at $10x10^4$ cells/ml in a 12-well plate and were treated with FCP at various concentrations (25, 50, 75, 100, 125 and 150 μg/ml). After 24 h of incubation at 37˚C, 100 μl of MTT (0.5 mg/ml) was subsequently added to each well and incubated for 3 h at 37˚C. The culture medium was then removed, and 500 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. After mixing, absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad).

Cell cycle distribution and analysis of cell apoptosis. The AGS cells were incubated without or with FCP at concentrations of 75 and 150 μg/ml for 24 h at 37˚C, and the cells were collected, washed with cold PBS, and then centrifuged. The pellet was fixed in cold 70% ethanol (v/v) for 3 h at -20˚C. The cells were washed once with PBS, and 200 μl was transferred to fresh tube. Muse Cell Cycle kit reagent (200 μl) was added to each tube and incubation was carried out for 30 min at room temperature in the dark. For analysis of apoptosis, the pellet was resuspended in 1 ml media and 100 μl was transferred to a new tube. Then, 100 μl of Muse Annexin V and Dead Cell kit reagent was added to each tube and incubation was carried out for 20 min at room temperature in the dark. Then, stained samples were analyzed using a Muse™ Mini FACS machine (Millipore).

Morphological change and DAPI fluorescent staining. The AGS cells were treated with the indicated concentrations of FCP for 24 h at 37˚C, and the cells were washed with cold PBS and fixed with 37% formaldehyde (1:4 dilution with 95% ethanol) for 10 min at room temperature. The fixed cells were washed with PBS and stained with a 4',6-diamidino-2-phenylindole (DAPI, Vectashield H-1500; Vector Laboratories, Inc., Burlingame, CA, USA). The nuclear morphology of the cells was examined by fluorescence microscopy (x400 magnification; Leica, Germany).

Western blot analysis. For the western blot analysis, the AGS cells were treated with the indicated concentrations of FCP for the indicated times at 37˚C and the cells were lysed in ice-cold RIPA buffer [1% (w/w) NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2, 2 mM EDTA, and 50 mM NaF (as phosphatase inhibitor) and protease inhibitors]. The protein concentrations were determined using a Bradford assay (Bio-Rad) method (20). Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldene fluoride (PVDF) membrane (Immobilon-P, 0.45 μm; Millipore) using the TE 77 Semi-Dry Transfer Unit (GE Healthcare Life Sciences, Buckinghamshire, UK). The membranes were incubated with the primary antibodies overnight followed by a conjugated secondary antibody to peroxidase. Blots were developed under an ECL detection system (GE Healthcare Life Sciences). The bands were quantitatively analyzed using the Image J program (http://rsb.info.nih.gov).

Statistical analysis. The statistical analysis was calculated by the Student's t-test, using SPSS version 10.0 for Windows (SPSS, Chicago, IL, USA). The results are expressed as the mean ± standard deviation (SD) of at least three independent experiments. The statistical significance was accepted as $P<0.05$.

Results

Quantitative analysis and characterization of FCP. The flavonoids were isolated from the fruit of *C. platymamma* by using HPLC-MS/MS. Totally 13 peaks were identified based on

Table I. List of identified flavonoids from *C. platymamma* and the quantification data.

	Compound	RT (min)	$[M-H]^-/[M-H]^+$	MS/MS	Mean ± SD
1	Naringin	16.93	579/-	459, 313, 271, 193, 151	2,483.5±1.6
2	Hesperidin	18.45	609/-	608, 325, 301	1,163.2±1.6
3	Hydroxypentamethoxyflavone	39.88	/389	374, 359, 341, 165	2,785.2±10.9
4	Hydroxypentamethoxyflavone	39.88	/389	374, 359, 341, 165	393.4±2.3
5	Sinensetin	42.57	-/373	373, 358, 343, 339, 329, 320, 312, 283, 181, 151	384.7±4.2
6	Pectolinarigenin	43.84	/313	313, 285, 181, 156, 153, 135	525.8±13.2
7	Dihydroxytetramethoxyflavone	44.76	375	375, 360, 345, 342, 314, 302, 299, 285, 271, 227, 212, 197, 169, 166, 149	370.2±4.2
8	Nobiletin	45.32	-/403	388, 373, 355, 327, 211, 165	3,911.9±5.5
9	Heptamethoxyflavone	46.15	/433	418, 403, 385, 211, 165	674.5±4.4
10	Tetramethyl-O-isoscutellarein	47.99	-/343	343, 328, 313, 299, 285, 211, 181, 135, 133	3,417.4±11.8
11	Hydroxypentamethoxyflavone	49.35	/389	374, 359, 341, 165	1,258.3±7.7
12	Hydroxyhexatamethoxyflavone	50.56	/419	404, 389, 373, 361, 343, 328, 315, 283, 227, 165	154.5±3.5
13	Hydroxypentamethoxyflavone	52.67	359	359, 344, 329, 311, 298, 286, 241, 224, 227, 211, 197, 183, 179, 135	258.5±1.7

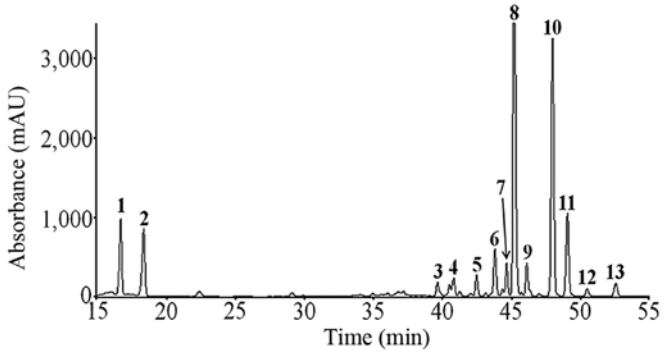

Figure 1. HPLC chromatogram patterns of *C. platymamma* at 280 nm. The list of identified flavonoids from the peaks included (1) naringin, (2) hesperidin, (3, 4, 11 and 13) hydroxypentamethoxyflavone, (5) sinensetin, (6) pectolinarigenin, (7) dihydroxytetramethoxyflavone, (8) nobiletin, (9) heptamethoxyflavone, (10) tetramethyl-O-isoscutellarein, and (12) hydroxyhexatamethoxyflavone. HPLC, high-performance liquid chromatography.

the HPLC retention time and the ultraviolet-visible spectra of standard compounds in a library (Fig. 1). The flavonoids were identified according to the peaks of the HPLC chromatogram and the mass-spectral and quantification data are provided in Table I.

FCP inhibits the growth of AGS cells. To determine the appropriate inhibitory concentrations of FCP, firstly AGS cells were treated with various concentrations (0-150 μg/ml) for 24 h and cell viability was evaluated by MTT assay. As shown in Fig. 2A, FCP showed a dose-dependent inhibitory effect at 24 h when compared to the control (DMSO only), and the 50% inhibitory concentration (IC_{50}) value was ~150 μg/ml ($P<0.01$ for the FCP-treated group compared with the control). Hence, we used FCP at concentrations of 0, 75 and 150 μg/ml for the subsequent experiments. Microscopic examination revealed changes in cell shape, such as cell shrinkage and a decrease in cell numbers was also observed in the FCP-treated cells (Fig. 2B).

FCP induces apoptosis in AGS cells. Next, flow cytometry was performed to determine cell cycle distribution and the population of cell death in the FCP-treated AGS cells. FCP treatment increased the percentage of the sub-G1 cells (apoptotic cell population) by 18, 41 ($P<0.01$) and 45% ($P<0.01$) at 0, 75 and 150 μg/ml, respectively. Meanwhile, FCP substantially decreased the G0/G1, S and G2/M populations (Fig. 3). We also assessed the effect of FCP on the induction of apoptosis in AGS cells by Annexin V-FITC/PI double-labeled staining and flow cytometry. As shown in Fig. 4A and B, FCP significantly increased the early apoptotic cell proportion and the late apoptotic cell proportion of AGS cells in a dose-dependent manner. Moreover, apoptotic changes such as nuclear fragmentation and apoptotic bodies were also observed in the FCP-treated AGS cells at 75 and 150 μg/ml by Hoechest 33342 staining (Fig. 4C). These results suggest that FCP could induce cell death in the AGS cells.

FCP induce caspase activation and subsequent cleavage of PARP in AGS cells. Western blotting was performed to determine whether FCP-induced cell death was caspase-dependent. In addition, we examined the expression of apoptosis-related proteins, such as Bax and Bcl-2, in the FCP-treated AGS cells. The results showed that the expression of procaspase-3, -6, -8 and -9 was significantly decreased while cleaved caspase-3 and cleaved PARP were significantly increased in a dose-dependent manner (Fig. 5). No significant changes were found in PARP expression. FCP also increased the Bax/Bcl-xL ratio in the

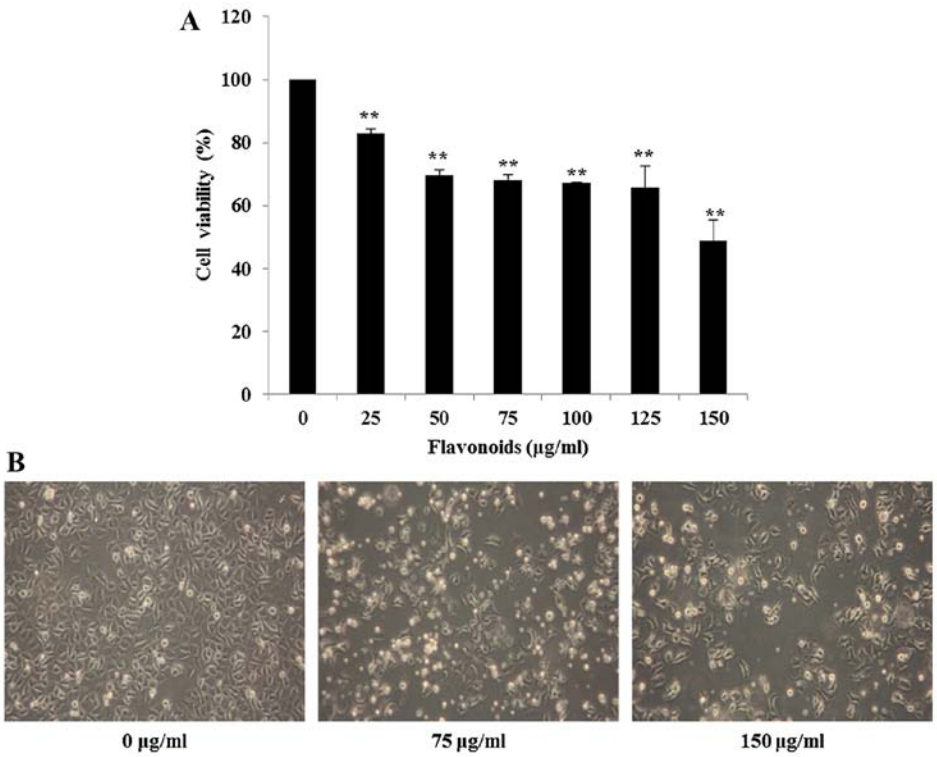

Figure 2. Inhibitory effects of FCP on AGS cells. The AGS cells were treated with the indicated concentrations of FCP for 24 h. (A) Cell viability was determined by an MTT assay. The data are expressed as the mean ± standard deviation (SD) of at least three independent experiments (*P<0.05, **P<0.01 compared to the control). (B) Morphology of the cells was examined under light microscopy (x400 magnification).

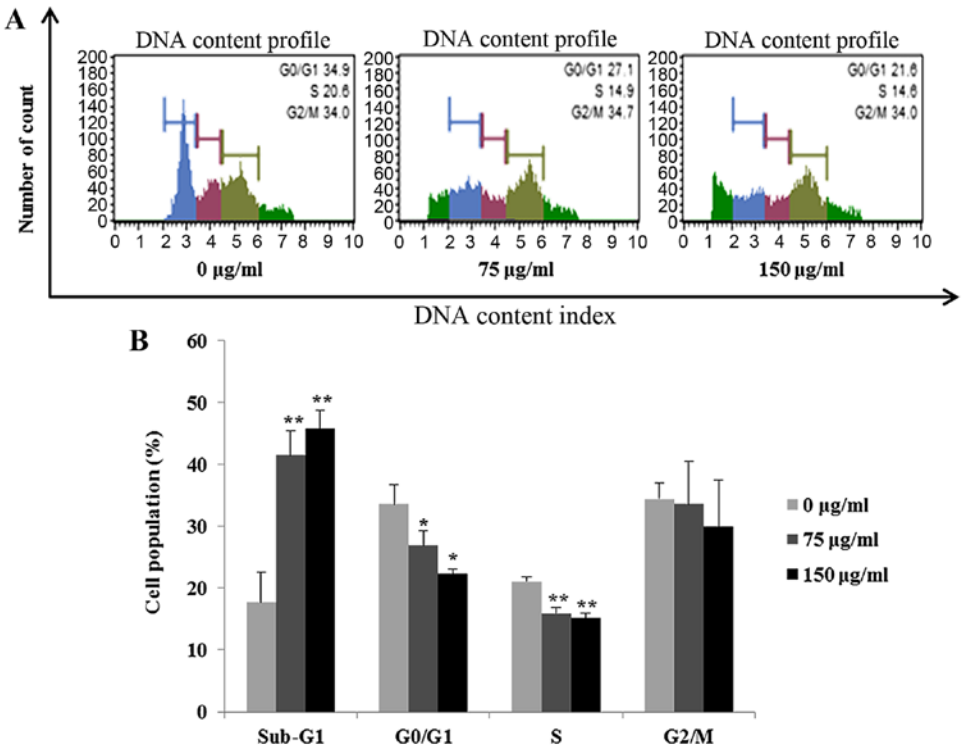

Figure 3. Regulatory effect of FCP on cell cycle progression of AGS cells. The AGS cells were treated with the indicated concentrations of FCP for 24 h. (A and B) Cell cycle distribution was determined by using the Muse Cell Cycle kit, and stained samples were analyzed with the Muse™ Mini FACS machine. The data are expressed as the mean ± standard deviation (SD) of at least three independent experiments (*P<0.05, **P<0.01 compared to the control).

AGS cells in a dose-dependent manner. These results suggest that FCP induced caspase-dependent apoptosis in AGS cells.

FCP modulates the PI3K/AKT and MAPK pathways in the AGS cells. The PI3K/AKT and MAPK signaling pathways

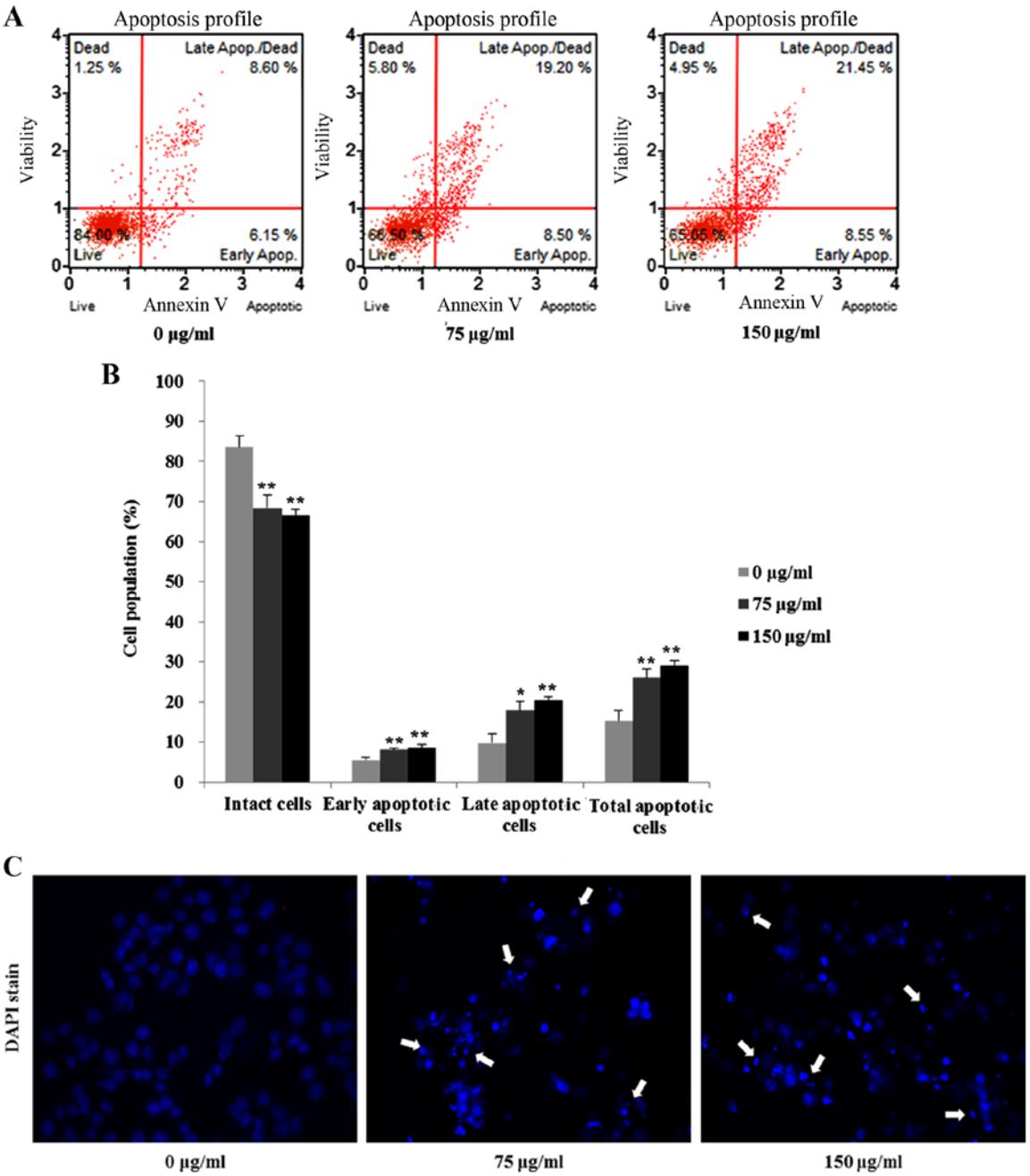

Figure 4. Regulatory effect of FCP on the apoptosis of AGS cells. The AGS cells were treated with the indicated concentrations of FCP for 24 h. (A and B) Apoptosis was assessed by Annexin V-PI double staining using the Muse™ Mini FACS machine. The data are expressed as the mean ± standard deviation (SD) of at least three independent experiments ($^{*}P<0.05$, $^{**}P<0.01$ compared to the control). (C) The cells were stained with DAPI and analyzed by fluorescence microscopy (white arrows indicate fragmented or condensed nuclei).

play an important role in regulating cell proliferation and apoptosis. Since the activity of AKT is regulated by phosphorylation, we examined the phosphorylation status of PI3K/AKT and MAPKs by immunoblotting during the FCP-induced apoptosis in AGS cells. FCP significantly dephosphorylated AKT at 75 and 150 μg/ml but no effects were found in total AKT (Fig. 6). Moreover, the phosphorylated forms of ERK1/2, JNK and p38 MAPK were significantly increased at 75 and 150 μg/ml, but no effects were found on total ERK1/2, JNK and p38 MAPK (Fig. 6). These findings suggest that FCP induced the apoptosis in AGS cells by modulating the PI3K/AKT and MAPK pathways.

Discussion

The present study was designed to determine whether FCP induces cell death and to further investigate the underlying mechanisms of the FCP-induced apoptosis of AGS cells. Flavonoids are naturally occurring botanical polyphenols present in plant foods and can safely modulate the physiological function and enhance the anticancer activity against various human cancer cell lines (21). Furthermore, the pharmacological activities of FCP against inflammation, allergies, viruses, cancer, and other ailments have been reported (22). In addition, flavonoids and polyphenols from various herbal plants such as

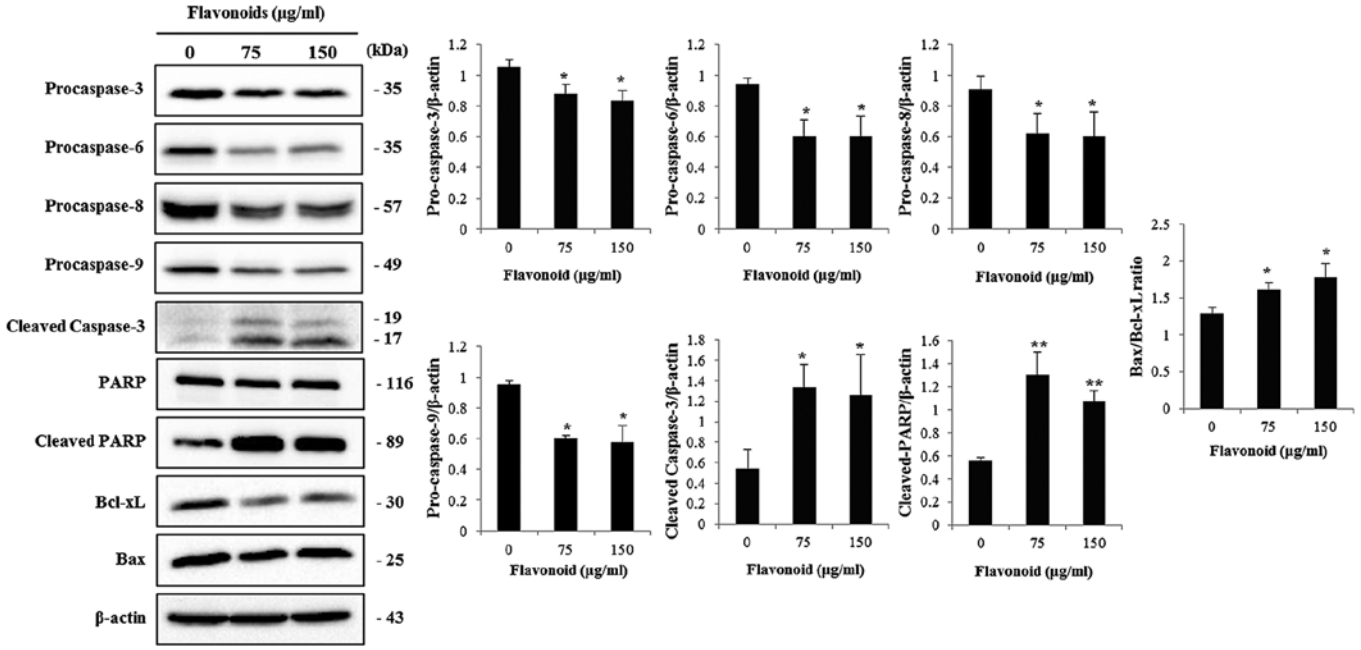

Figure 5. Caspase activation and subsequent cleavage of PARP in the FCP-treated AGS cells. The AGS cells were treated with the indicated concentrations of FCP for 24 h. The cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting. Densitometric analyses of procaspase-3, -6, -8, and -9, cleaved caspase-3, PARP and cleaved PARP and the Bax/Bcl-xL ratio are expressed as mean ± SD of three independent experiments (*P<0.05, **P<0.01 compared with the control).

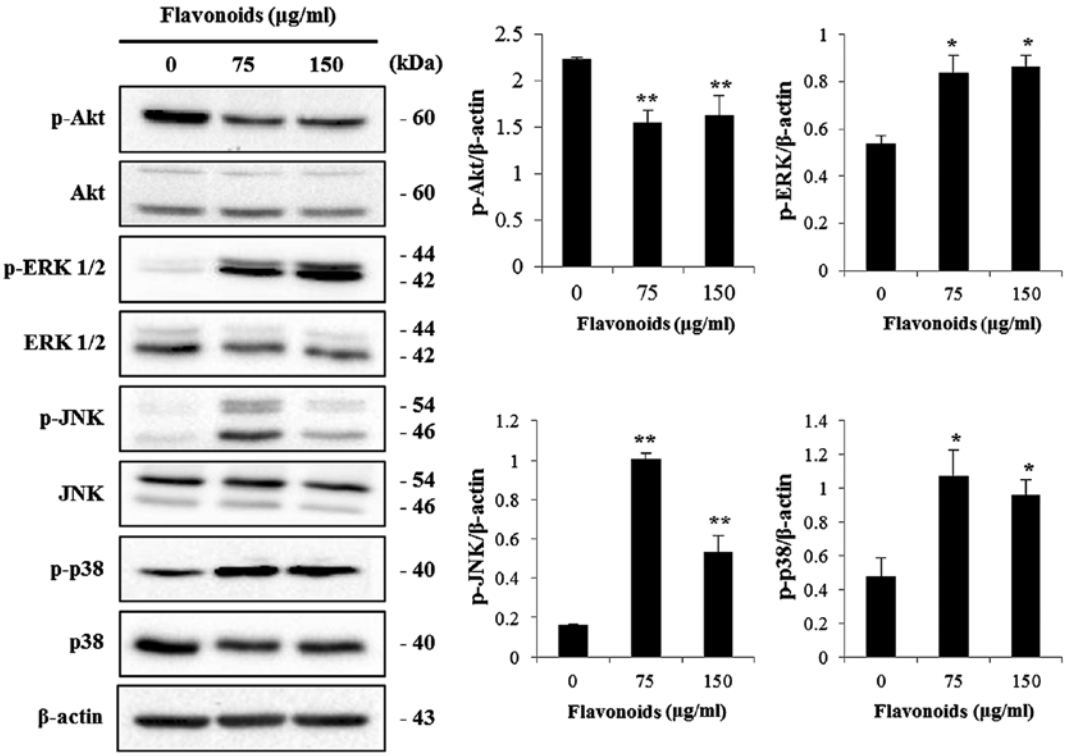

Figure 6. FCP modulates the PI3K/AKT and MAPK signaling pathways in the AGS cells. The AGS cells were treated with the indicated concentrations of FCP for 24 h. The cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting. Densitometric analyses of AKT, p-AKT, ERK1/2, p-ERK1/2, JNK, p-JNK, p38, and p-p38 MAPK are expressed as mean ± SD of three independent experiments (*P<0.05, **P<0.01 compared with the control).

Scutellaria baicalensis G., *Lonicera japonica* T. exhibit anti-inflammatory and anticancer activities by inducing cell cycle arrest and apoptosis in various cancer cell lines (23,24). In addition, monomers such as naringin, nobiletin and hesperetin exhibit anticancer effects by cell cycle arrest and the apoptosis pathway in human cancer cell lines (25,26). In the present study, we investigated the anticancer activity of FCP on AGS human gastric cancer cells.

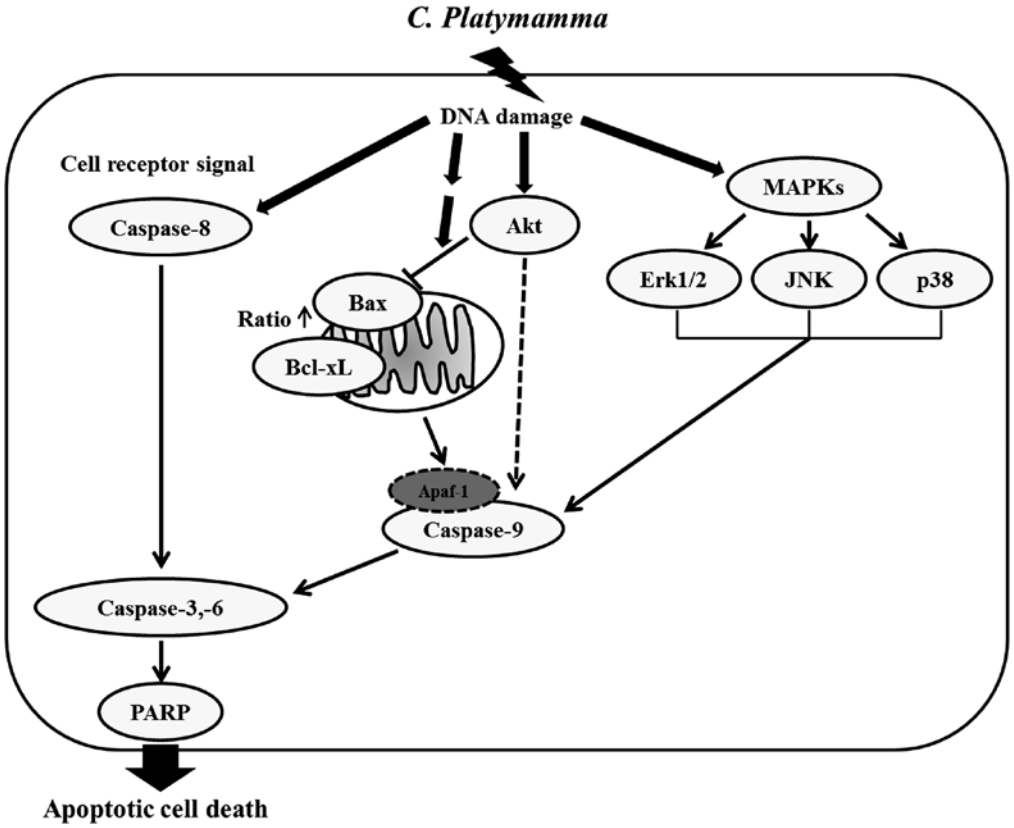

Figure 7. Schematic diagram representing the anticancer mechanism of the FCP-induced apoptosis in AGS cells. FCP induced mitochondrial-dependent apoptosis by upregulation of the Bax/Bcl-xL ratio, caspase-3 activation and subsequent cleavage of PARP. Furthermore, FCP activates PI3K/AKT and MAPK signaling pathways in AGS cells. Taken together, the PI3K/AKT and MAPK pathways are involved in FCP-induced apoptosis in AGS cells, and FCP may have chemotherapeutic potential for the treatment of gastric cancer (→ indicates activation, ⊥ indicates inhibition, --- indicates indirect or multiple pathways).

Firstly, FCP significantly suppressed the growth of AGS cells in a dose-dependent manner. Evidence suggests that apoptosis (type I programmed cell death) is the most popular underlying mechanism by which various anticancer and chemopreventive agents including natural compounds exert anticancer effects (27). Previous studies have demonstrated that the mechanism of cell apoptosis is through the caspase signaling pathway. Recently, our studies demonstrated that cell cycle aberrations often lead to apoptosis in various cancer cell lines (11,12). Presently, accumulation of sub-G1 phase cells (indication of apoptosis) was found in the FCP-treated AGS cells in a dose-dependent manner (Fig. 3). Furthermore, apoptosis was confirmed by FITC-Annexin V and PI double staining (Fig. 4A and B). Similar results have been reported on the induction of apoptosis in various cancer cell lines (11,12,28). In addition, cleaved nuclei and apoptotic bodies were found in the FCP-treated cells (Fig. 4C). These results revealed that FCP effectively suppressed the growth of AGS cells and induced apoptosis.

For the evaluation of their underlying mechanisms, immunoblotting was performed. The results showed that the expression of procaspase -3, -6, -8 and -9 was significantly downregulated in a dose-dependent manner. Caspase-3 is a crucial executioner caspase that activates cleavage of PARP which results in apoptosis. In our study, the increased expression of cleaved caspase-3 simultaneously induced PARP cleavage (Fig. 5). These results indicate that FCP induce apoptosis in a caspase-3-dependent manner. Moreover, the Bcl-2 family plays an important role in apoptosis and are apoptotic regulatory proteins which control the mitochondrial apoptotic process. The pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family in the cell, determines whether a cell lives or dies (29). Bcl-xL interacts with the mitochondrial plasma membrane and protects from other apoptotic factors, such as Bax and Bak that prevents induced cytochrome *c* from the plasma membrane. A previous study demonstrated that nobiletin induces apoptosis in various tumor cell lines via inhibition of overexpression of the Bcl family of proteins (26). The ratio of Bax/Bcl-xL appears to be a determining factor of apoptosis. In the present study, Bcl-xL was significantly downregulated, whereas Bax protein was unchanged while the ratio of Bax/Bcl-xL was upregulated in the FCP-treated AGS cells (Fig. 5). Cytochrome *c* can bind to APAF-1 when it is released from the mitochondria into the cytosol by increasing the Bax/Bcl-xL ratio, thus leading to the activation of caspase-3 and finally apoptosis.

We further examined the phosphorylation status of PI3K/AKT and MAPKs by immunoblotting to elucidate the molecular mechanism and pathways involved in FCP-induced apoptosis. We demonstrated that FCP inhibited the constitutive level of PI3K and its downstream target AKT (Fig. 6), which have been reported to regulate cell proliferation and apoptosis. Similarly to our results, the inhibition of the PI3K/Akt signaling pathway can induce the apoptosis of various types of cancer cells (30,31). Moreover, the MAPK signaling pathway is also involved in survival, proliferation and apoptosis and consists of three major groups: ERKs, JNKs and the p38 MAPKs (32). Even though activation of the ERK1/2 pathway is generally

associated with cell proliferation and survival, it has also been reported to stimulate apoptosis in T cells through Fas ligand expression (33). In addition, ERK1/2 induces apoptosis via prevention of the inactivation of a member of the pro-apoptotic Bcl-2 family, BAD (34). Moreover, JNK is a downstream kinase of the MAPK family which has been reported to regulate the expression of receptors such as Fas and the Fas ligand in apoptosis (35). JNK is also involved in the intrinsic apoptosis pathway where activated JNK modulates the expression of pro-apoptotic proteins such as Bid and Bax and stimulates the release of cytochrome *c* from mitochondria into the cytosol (36). Activated JNK also downregulates the expression of Bcl-2, an anti-apoptotic protein (37). It has also been demonstrated that activated p38 MAPK stimulates apoptosis in various cell lines in response to a variety of stimuli (38). Similar expression patterns were observed in our experiments. Phosphorylated forms of ERK1/2, JNK and p38 MAPK were increased in the FCP-treated AGS cells (Fig. 6). These results revealed that PI3K/AKT and MAPKs are involved in the apoptosis induced by FCP in AGS cells.

In conclusion, we demonstrated that FCP suppressed cell viability and induced caspase-dependent cell death in the AGS cells. The induction of apoptosis triggered in the FCP-treated AGS cells was modulated by the PI3K/AKT and MAPK signaling pathways (Fig. 7). To our knowledge, this is the first study to elucidate the anticancer properties of FCP in AGS cells. Thus, FCP may be a potential chemotherapeutic agent for the treatment of human gastric cancer.

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