Induction of p53 contributes to apoptosis of HCT-116 human colon cancer cells induced by the dietary compound fisetin

Do Y. Lim and Jung Han Yoon Park

Department of Food Science and Nutrition, Hallym University, Chuncheon, Korea

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LARGE BOWEL CANCER is one of the leading causes of death in the United States (16), and the incidence of this disease is also increasing rapidly in Asian countries. Thus far, an insufficient degree of success has been achieved by dietary measures, chemical compounds, screening and surveillance colonoscopy, and colonoscopic polypectomy. Thus there is an urgent need to develop new strategies to reduce the incidence and prevalence of colorectal cancer. The hallmarks of cancer include tumor cell proliferation and survival and tumor angiogenesis and metastasis (11). According to currently available knowledge regarding the molecular aberrations that underlie carcinogenesis, several promising targets for the prevention and treatment of cancer appear to exist. A common denominator of many of these targeted strategies is the removal of malignant cells via the induction of apoptosis while sparing nontransformed cells (10, 14). Thus bioactive compounds with the ability to induce apoptosis in cancer cells may potentially be utilized as cancer chemopreventive and chemotherapeutic agents.

Fisetin, 3,3′,4′,7-tetrahydroxyflavone, is found in fruits and vegetables including cucumber, grape, strawberry, apple, persimmon, and onion (1) and was previously shown to evidence antioxidant (12), anti-inflammatory (13, 31), anti-invasive (13), and antiangiogenic (13) effects. In addition, fisetin has been previously reported to inhibit the proliferation of a variety of cancer cells, including hepatocarcinoma (20–80 μmol/l) (2), prostate cancer (IC50, 23–80 μmol/l) (9), and colon cancer (IC50, 55–90 μmol/l) (21) cells. Furthermore, it has been reported to induce apoptosis in SK-HEP-1 hepatocellular carcinoma (2), HL-60 myeloleukemic (22), and LNCaP prostate cancer (19) cells and has also been demonstrated to enhance tumor necrosis factor (TNF), doxorubicin, and cisplatin-induced cytotoxicity in H1299 human lung adenocarcinoma cells (31). However, to the best of our knowledge, the effects of fisetin on apoptosis in colon cancer cells and its underlying mechanisms have yet to be clearly elucidated.

Apoptosis is induced by two major pathways, which are referred to as the intrinsic and extrinsic pathways. The intrinsic pathway is induced via alterations in the Bcl-2 family of proteins, thus increasing the permeability of the mitochondrial membrane. This increased mitochondrial membrane permeability induces the release of cytochrome c and Smac/Diablo, which in turn results in the activation of caspase-9. The extrinsic pathway is mediated by cell surface death receptors, including Fas, TNF receptors, and receptors (DR4 and DR5) for TNF-related apoptosis-inducing ligand (TRAIL). Ligand stimulation of the death receptors induces the formation of a death-inducing signaling complex (DISC). The final step in this process is the recruitment and activation of one of the caspases, typically caspase-8, followed by the activation of effector caspases, including caspase-3 and caspase-7 (reviewed in Ref. 17). The two pathways of apoptosis converge on caspase-3 and subsequently on other proteases and nucleases that execute the final events of apoptosis.

The p53 tumor suppressor protein performs a critical role in inducing apoptosis (36). Many of the proapoptotic Bcl-2 family members, including PUMA, Bax, Bik, and Bid, have been reported to be transcriptional targets of p53 (15, 28, 35). In addition, p53 is translocated from the cytoplasm to the mitochondria and can directly induce the permeabilization of the outer mitochondrial membrane via the formation of complexes with the protective Bcl-XL and Bcl-2 proteins, thus resulting in cytochrome c release (25). Furthermore, p53 stimulates a
conformational change in Bax, favoring its migration to the mitochondria (3, 35). We have previously demonstrated that fisetin inhibits cell cycle progression at concentrations of 20–60 μmol/l in HT-29 colon cancer cells harboring a mutant p53 gene (24). In this study, we attempted to ascertain whether fisetin induces apoptosis in colon cancer cells, as well as the mechanisms of its action, using HCT-116 human colon cancer cells harboring the wild-type p53 gene. We have demonstrated that fisetin induces apoptosis at concentrations of 5–20 μmol/l, via the activation of both death-receptor- and mitochondrion-dependent pathways. In addition, we have determined that the induction of p53 contributes to fisetin-induced Bax translocation to the mitochondria and apoptosis.

MATERIALS AND METHODS

Materials. All reagents and chemicals were purchased from the following suppliers: fisetin, anti-β-actin, RIA-grade BSA, transferrin, 7-aminoactinomycin D, and Hoechst 33258 (Sigma, St. Louis, MO); phycoerythrin (PE)-conjugated annexin V (annexin V-PE), and antibodies against caspase-8, cytochrome c, and TRAIL (BD Pharmingen, Franklin Lakes, NJ); antibodies against cleaved caspase-9, cleaved caspase-7, cleaved caspase-3, poly (ADP-ribose) polymerase (PARP), Bcl-xL, Bid, Bik, Bak, Bok, and Bim (Cell Signaling, Beverly, MA); antibodies against α-tubulin, Smac/Diablo, Bcl-2, Bax (N-20), Bax (B-9) AF488, Fas, Fas ligand (Fas-L), p53, and MDM2, p53 small interfering RNA (siRNA), control A siRNA, and caspase-8 inhibitor (Z-IETD-FMK) (Santa Cruz Biotechnology, Santa Cruz, CA); 5,5’,6,6’-tetrachloro-1,1’3,3’-tetratetraethyl-imidacarbocyanine iodide (JC-1), and MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA); and Cell Death Detection ELISAPLUS kit (Roche, Mannheim, Germany).

Cell culture. HCT-116, HT-29, and IEC-6 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM/F12 containing 100 ml/l of FBS with 100,000 U/l of penicillin and 100 mg/l of streptomycin. HCT-116 cells between passages 23 and 33 were employed in these studies. To assess the effects of fisetin, the cells were plated with DMEM/F12 containing 10% FBS. Prior to fisetin treatment, the monolayers were rinsed and serum-starved and then pretreated for 4 h with 0 or 20 μmol/l of caspase-8 inhibitor (Z-IETD-FMK) prior to treatment with 0 or 20 μmol/l of fisetin for 24 h. The cells were trypsinized and incubated for 15 min in darkness with annexin V and 7-aminoactinomycin D at room temperature. Apoptotic cells were analyzed via flow cytometry, by use of a FACScan system. The data were analyzed by using Modfit version 1.2 software (Verity Software, Topsham, ME).

Expression of p53 expression by siRNA. To suppress p53 expression with siRNA, 2 × 106 cells were transfected with 2.3 μmol/l of p53 siRNA or control A siRNA (Santa Cruz Biotechnology) by using Amaxa Cell Lines Nucleofector kit R (Amaxa, Gaithersburg, MD) in accordance with the manufacturer’s recommended protocols.

Cell death detection ELISAPLUS assay. Cells transfected with p53 siRNA or control A siRNA were plated in 12-well plates at a density of 100,000 cells/well and treated for 24 h with 0 or 20 μmol/l of fisetin. The cell lysates were then assayed for the quantitative determination of mono- and oligonucleosomes released into the cytoplasm by using a Cell Death Detection ELISAPLUS kit (Roche) in accordance with the manufacturer’s recommended protocol.

Immunocytochemistry. HCT-116 cells transfected with p53 siRNA or control A siRNA were plated on four-well chamber slides and treated with fisetin as described above. After fisetin treatment, the cell monolayers were incubated for 40 min with 10 ml/l of MitoTracker Red CMXRos in DMEM/F12 supplemented with 5% FBS. The cells were fixed for 30 min with 4% formaldehyde buffer containing 0.5% saponin at room temperature, then blocked for 20 min with 10% goat serum at room temperature, then blocked for 20 min with 10% goat serum (Santa Cruz Biotechnology) in TBST. Bax (B-9) AF488 antibody (1:100) was applied for 1 h at room temperature, after which the cell monolayer was washed and mounted with Mounting Solution (Sigma). Photographs were taken with a Carl Zeiss AxiosImager microscope (Carl Zeiss, Jena, Germany).

Statistical analyses. The results are expressed as means ± SE and were analyzed via ANOVA. Differences among the treatment groups were assessed by Duncan’s multiple-range test, using the SAS system for Windows version 8.1. Differences were considered significant at P < 0.05.

RESULTS

Fisetin induces apoptosis of HCT-116 cells. In a previous work (24), we reported that fisetin dose dependently inhibited the growth of HT-29 cells harboring a mutant p53 gene (8), at concentrations of 20–60 μmol/l. To determine whether fisetin inhibits the growth of HCT-116 cells containing the wild-type p53 gene, the cells were treated with various concentrations (0–20 μmol/l). Fisetin induced a marked dose-dependent reduction in the number of viable HCT-116 cells (Fig. 1A). The same concentration (20 μmol/l) of fisetin caused only a slight reduction in HT-29 cell growth (24) and exerted no detectable
Fig. 1. Fisetin induces apoptosis and cleavage of caspases in HCT-116 cells. HCT-116 cells were plated on 24-well plates at a density of 50,000 cells/well (A), in 4-well chamber slides at a density of 50,000 cells/well (C), and in 100-mm dishes at a density of 2,000,000 cells/dish (D) with DMEM/F12 supplemented with 10% FBS. IEC-6 cells were plated in 24-well plates at a density of 50,000 cells/well (B). At 24 h after plating, the monolayers were serum starved in DMEM/F12 supplemented with 5 mg/l transferrin, 0.1 g/l BSA, and 5 µg/l selenium (serum-free medium) for 24 h and subsequently incubated in serum-free medium containing 0, 5, 10 or 20 µmol/l of fisetin. A and B: cells were treated with fisetin for the indicated times and the cell numbers were estimated via MTT assay. Each bar represents mean ± SE (n = 6). Means without a common letter differ, P < 0.05. C: HCT-116 cells were treated for 24 h with fisetin and stained with the DNA-specific dye Hoechst 33258. D: HCT-116 cells were treated for 24 h with fisetin and the total cell lysates were subjected to Western blotting with the indicated antibodies. Photographs of the chemiluminescent detection of the blots, which are representative of 3 independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified, and the control levels were set to 100%. The adjusted mean ± SE (n = 3) of each band is shown above each blot. Means without a common letter differ, P < 0.05.
effects on the viability of IEC-6 normal intestinal epithelial cells (Fig. 1B).

To determine whether fisetin induces apoptosis in HCT-116 cells, the cells were treated for 24 h with fisetin at 0–20 μmol/l, then stained with Hoechst 33258 dye. Fisetin increased the number of cells with condensed and fragmented nuclei, which is consistent with the morphological features of apoptosis (Fig. 1C). Also in a manner consistent with the morphological features of apoptosis, fisetin increased the cleavage of PARP, an enzyme that is crucial to the repair of DNA damage. The ability of PARP to repair DNA damage is abrogated after cleavage of PARP, and thus cleaved PARP is generally considered to be a useful marker for apoptosis (Fig. 1D). In the HT-29 cells, similar effects on cell death, caspase activation, and PARP cleavage were observed with higher concentrations (20–60 μmol/l) of fisetin (data not shown).

Fisetin induces depolarization of the mitochondrial membrane in HCT-116 cells. Since fisetin induced the activation of caspase-9, we subsequently attempted to determine whether fisetin increases mitochondrial membrane permeability in HCT-116 cells. Fisetin-treated cells were stained with JC-1, and the percentages of cells evidencing green-positive and red-negative fluorescence were scored as depolarized cells via FACS analysis. Fisetin reduced the number of cells with normal mitochondria and increased the number of cells with depolarized mitochondrial membranes, in a concentration-dependent manner (Fig. 2A). Fisetin also increased the levels of Smac/Diablo and cytochrome c in the cytoplasm and decreased those levels in the mitochondria (Fig. 2B).

Fisetin alters the levels of Bcl-2 family proteins and induces Bax translocation to mitochondria. Because fisetin increased the permeability of the mitochondrial membranes and because Bcl-2 proteins have been recognized to regulate the permeability of the mitochondrial membrane, we then attempted to determine whether fisetin alters the levels of the Bcl-2 family proteins. Fisetin induced a reduction in the levels of Bcl-xL and Bcl-2 proteins but increased those of Bak, BimEL, BimL, and BimS. Truncated Bid was not detected, whereas the levels of intact Bid were decreased in the fisetin-treated cells, thus suggesting that fisetin may have induced the cleavage of Bid. An alteration in the size of Bik was detected in the cells treated with 20 μmol/l of fisetin (Fig. 3A). Bax levels remained unchanged in the fisetin-treated cells (Fig. 3A), but Bax levels were reduced in the cytoplasm and increased in the mitochondria of the fisetin-treated cells (Fig. 3B).

Fisetin increases cleavage of caspase-8. Because the levels of intact Bid were reduced and the levels of cleaved caspase-3 were increased in the fisetin-treated cells, we attempted to determine the effects of fisetin on caspase-8 activation, via Western blot analysis. Fisetin induced a decrease in the levels of uncleaved caspase-8 and an increase in the levels of cleaved...
caspase-8. In addition, the levels of Fas-L, TRAIL, and DR5 increased in the fisetin-treated cells, but the levels of Fas remained unaltered (Fig. 4).

A caspase-8 inhibitor mitigates fisetin-induced apoptosis. Since caspase-8 was activated in the fisetin-treated HCT-116 cells, we attempted to determine whether the inhibition of caspase-8 would mitigate the fisetin-induced apoptosis. The pretreatment of cells with the caspase-8 inhibitor Z-IETD-FMK prior to fisetin treatment resulted in an increase in the number of intact cells and a reduction in the numbers of apoptotic cells, compared with what was observed with the cells treated only with fisetin (Fig. 5A). The fisetin-induced reduction in intact Bid levels and increase in caspase-3 cleavage were prevented in the presence of the caspase-8 inhibitor. The fisetin-induced cleavage of caspase-9 was suppressed slightly when the cells were pretreated with the caspase-8 inhibitor (Fig. 5B).

Inhibition of p53 expression results in the mitigation of fisetin-induced apoptosis and the translocation of Bax to the mitochondria. The induction of the p53 tumor suppressor protein can initiate either cell cycle arrest and DNA repair or caspase-8. In addition, the levels of Fas-L, TRAIL, and DR5 increased in the fisetin-treated cells, but the levels of Fas remained unaltered (Fig. 4).

**Fig. 3. Fisetin alters the levels of Bcl-2 family proteins in HCT-116 cells.** Cells were treated with fisetin as described in Fig. 1D. A: cell lysates were subjected to Western blotting with indicated antibodies. B: cells were subjected to subcellular fractionation as described in Fig. 2 and then analyzed via Western blotting with Bax antibody. Photographs of the chemiluminescent detection of the blots, which are representative of 3 independent experiments, are shown. The relative abundance of each band to its own β-actin (A) or α-tubulin (B) was quantified, and the control levels were set at 100%. The adjusted mean ± SE (n = 3) of each band is shown above each blot. Means without a common letter differ, P < 0.05.

**Fig. 4. Fisetin increases the levels of cleaved caspase-8, Fas ligand (Fas-L), TRAIL, and DR5 in HCT-116 cells.** Cells were treated with fisetin as described in Fig. 1D. Cell lysates were subjected to Western blotting with indicated antibodies. Photographs of the chemiluminescent detection of the blots, which are representative of 3 independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified, and the control levels were set at 100%. The adjusted mean ± SE (n = 3) of each band is shown above each blot. Means without a common letter differ, P < 0.05.
and annexin V, then analyzed via flow cytometry. The number of living cells
during h-incubation, the cells were trypsinized, loaded with 7-aminoactinomycin D
and the incubation was continued for another 24 h. A
edly increased levels in cells treated with 20 µmol/l of Z-IETD-FMK. Fisetin (0 or 20 µmol/l) was then
treated with 0 or 20 µmol/l of fisetin, as described in Fig. 1. After serum starvation, cells were
plated in 12-well plates at a density of 100,000 cells/well (A) and in 100 mm dishes at a density of 2,000,000
cells/dish (B), as described in Fig. 1. After serum starvation, cells were treated
for 4 h with 0 or 20 µmol/l of Z-IETD-FMK. Fisetin (0 or 20 µmol/l) was then added and the incubation was continued for another 24 h. A: after the 24 h-incubation, the cells were trypsinized, loaded with 7-aminoactinomycin D and annexin V, then analyzed via flow cytometry. The number of living cells and early apoptotic cells is expressed as a percentage of the total cell number. Each bar represents mean ± SE (n = 6). Means without a common letter differ, P < 0.05. B: total cell lysates were subjected to Western blotting with the indicated antibodies. The relative abundance of each band to its own β-actin was quantified, and the control levels were set to 100%. The adjusted mean ± SE (n = 3) of each band is shown above each blot. Means without a common letter differ, P < 0.05.

Fisetin induces apoptosis of colon cancer cells

Fig. 5. The caspase-8 inhibitor Z-IETD-FMK mitigates fisetin-induced apoptosis in HCT-116 cells. Cells were plated in 12-well plates at a density of 100,000 cells/well (A) and in 100 mm dishes at a density of 2,000,000 cells/dish (B), as described in Fig. 1. After serum starvation, cells were treated for 4 h with 0 or 20 µmol/l of Z-IETD-FMK. Fisetin (0 or 20 µmol/l) was then added and the incubation was continued for another 24 h. A: after the 24 h-incubation, the cells were trypsinized, loaded with 7-aminoactinomycin D and annexin V, then analyzed via flow cytometry. The number of living cells and early apoptotic cells is expressed as a percentage of the total cell number. Each bar represents mean ± SE (n = 6). Means without a common letter differ, P < 0.05. B: total cell lysates were subjected to Western blotting with the indicated antibodies. The relative abundance of each band to its own β-actin was quantified, and the control levels were set to 100%. The adjusted mean ± SE (n = 3) of each band is shown above each blot. Means without a common letter differ, P < 0.05.

The hallmarks of cancer include tumor cell proliferation and survival, as well as tumor angiogenesis and metastasis (11). In a previous study, we demonstrated that fisetin inhibits CDK activities, resulting in cell cycle arrest in HT-29 colon cancer cells (24) harboring a mutant p53 gene. Sung et al. (31) reported that fisetin downregulates nuclear factor-κB-regulated gene products associated with cell proliferation, antiapoptosis, and metastasis in human lung cancer cell lines. A more current report demonstrated that fisetin induces apoptosis and cell cycle arrest in LNCAp human prostate cancer cells (19). In this study, we have demonstrated that fisetin induces apoptosis in HCT-116 human colon cancer cells harboring the wild-type p53 gene and that the induction of p53 contributes to fisetin-induced apoptosis. Our results demonstrated that fisetin induced nuclear condensation, the cleavage of caspases 9, 3, and 7, and the cleavage of PARP (Fig. 1) at low concentrations (5–20 µmol/l) relative to those utilized in other studies on the subject. In vivo animal model tests to determine whether fisetin induces apoptosis in colon cancer cells have yet to be conducted.

Caspases are a family of proteases that are collectively known as one of the principal executioners of apoptosis, and their cleavage and subsequent activation are considered the primary hallmarks of apoptosis. In the death receptor-dependent pathway, death receptors and their ligands (e.g., Fas/Fas-L and DR5/TRAIL) induce caspase-8 activation; activated caspase-8 in turn cleaves Bid and/or directly triggers the activation of caspase-3. When cleaved, truncated Bid migrates to the mitochondria, where it increases the permeability of the mitochondrial membrane and induces cytochrome c release and caspase-9 cleavage (17). Our investigations have revealed that fisetin treatment induced an increase in the levels of cleaved caspase-8. The protein levels of Fas-L, DR5, and TRAIL were also shown to have increased in the fisetin-treated cells (Fig. 4). Furthermore, the caspase-8 inhibitor, Z-IETD-FMK, mitigated fisetin-induced apoptosis, the cleavage of caspases-3 and -9, and an increase in intact Bid levels (Fig. 5). These findings show that the activation of the death receptor pathway is one of the mechanisms by which fisetin induces apoptosis in HCT-116 cells.

Caspase-9 is activated principally by the mitochondrion-dependent pathway, in which apoptotic stimuli increase the permeability of mitochondrial membranes and the subsequent release of cytochrome c into the cytoplasm. The released cytochrome c then binds with procaspase-9 and apoptosis protease-activating factor 1 (Apaf-1) to form an apoptosisosome, in which procaspase-9 is activated to its active form. Activated caspase-9, in turn, causes the subsequent cleavage of caspase-3 (23). In the present study, fisetin treatment induced the cleavage of caspases 9, 3, and 7 (Fig. 1). Additionally, cytochrome c was released into the cytoplasm and the depolarization of the mitochondria was observed by immunocytochemistry.
Fig. 6. Inhibition of p53 induction results in a decrease in fisetin-induced apoptosis. A: cells were treated with fisetin as described in Fig. 1. Total cell lysates were subjected to Western blotting with antibodies raised against p53, MDM2, or β-actin. B–E: cells were transfected with p53 small interfering RNA (siRNA) or control A siRNA. B: transfected cells were treated with 0 or 20 μmol/l fisetin. The total cell lysates were subjected to Western blotting with antibodies raised against p53, cleaved poly(ADP-ribose) polymerase (PARP), or β-actin. C: transfected cells were plated in 12-well plates at a density of 100,000 cells/well and treated with 0 or 20 μmol/l of fisetin. The cell lysates were assayed using Cell Death Detection ELISAPLUS kit to detect apoptotic cells. The mono- and oligonucleosomes released into the cytoplasm were quantified, and enrichment factor was calculated. Enrichment factor = absorbance of the sample/absorbance of the control (control A siRNA transfected, 0 μmol/l fisetin). Each bar represents mean ± SE (n = 12). Means without a common letter differ, P < 0.05. D: transfected cells were treated with fisetin and subjected to subcellular fractionation as described in Fig. 2B. The resulting mitochondrial fractions were analyzed via Western blotting with Bax antibody. The relative abundance of each band to its β-actin (A and B) or α-tubulin (D) was quantified, and the control levels were set to 100%. The adjusted mean ± SE (n = 3) of each band is shown above each blot. Means without a common letter differ, P < 0.05. E: transfected cells were treated with fisetin and subjected to immunocytochemistry with Bax-AF488 antibody. Mitochondria were stained with Mitotracker Red (red color) and Bax probed with anti-Bax-AF488 antibody (green color). Bright yellow coloration of the merged image represents Bax translocated from the cytosol to the mitochondria.
mitochondrial membrane was induced in the fisetin-treated cells (Fig. 2). The addition of the caspase-8 inhibitor, Z-IETD-FMK, abrogated the fisetin-induced reduction in the levels of intact Bid (Fig. 5). These findings indicate that fisetin-induced caspase-8 cleavage may have induced Bid cleavage, which might have in turn contributed to the cleavage of caspase-9. However, the caspase-8 inhibitor mitigated fisetin-induced caspase-9 activation to a relatively small degree, which suggests that the contribution of the death receptor pathway to fisetin-induced caspase-9 activation is relatively less profound than that of the mitochondrion-dependent pathway. These results indicate that fisetin induces the activation of caspase-9 primarily via the mitochondrion-dependent pathway.

The Bcl-2 family of proteins performs critical roles in the regulation of mitochondrial membrane permeability. Anti- or proapoptotic Bcl-2 family proteins reside in the cytoplasm or on the mitochondrial outer membrane. In response to apoptotic stimuli, they form homo- or heterodimers in the mitochondria (32). In the present study, fisetin was shown to alter the levels of Bcl-2 family member proteins. The levels of anti-apoptotic Bcl-xL and Bcl-2 and intact Bid proteins were decreased and proapoptotic Bak and Bim levels were increased in the fisetin-treated cells. In addition, fisetin treatment resulted in the translocation of Bax from the cytoplasm to the mitochondria (Fig. 3). These results show that the alterations in Bcl-2 family proteins induced the increase in the permeability of the mitochondrial membrane and the subsequent activation of caspase-9 in the fisetin-treated cells.

In the present experiment, levels of p53 were increased markedly in the fisetin-treated HCT-116 cells (Fig. 6A) and the inhibition of p53 expression with transfection of p53 siRNA inhibited fisetin-induced apoptosis (Fig. 6, B and C). p53 is known to induce apoptosis via the transcriptional stimulation of a variety of apoptosis-inducing genes, including Bax, Bid, DR5, Fax, Noxa, and PUMA (29, 37). Fisetin increased p53 transcriptional activities but p53 siRNA did not alter the fisetin-induced changes in the levels of the Bcl-2 proteins, including Bax, Bak, and Bcl-xL (D. Y. Lim and J. H. Y. Park, unpublished results). The p53 protein has been demonstrated to bind to and inhibit Bcl-2 and Bcl-xL or to activate Bax directly (4, 25). In the present study, fisetin-induced Bax translocation to the mitochondria was attenuated in the p53 siRNA-transfected cells (Fig. 6, D and E), thus indicating that p53 activation contributes to the mitochondrial translocation of Bax via a transcription-independent pathway. Yamaguchi et al. (35) have also reported that p53 is required for microtubule-damaging agent-induced Bax conformational changes in HCT-116 cells. Collectively, the results of this study show that the induction of p53 is responsible, at least in part, for the fisetin-induced mitochondrial translocation of Bax and for apoptosis in HCT-116 cells.

In the present study, we noted an alteration in the size of Bik in the cells treated with 20 μmol/l of fisetin (Fig. 3A), but did not attempt to determine whether and how this is related to fisetin-induced apoptosis. Bik is a BH3-only, proapoptotic member of the Bcl-2 family (26). Once activated, Bik initiates apoptosis by binding to and neutralizing Bcl-2 antiapoptotic proteins via its BH3 domain (34). There is a paucity of information regarding the size changes and activation of Bik. It has been reported that Bik existed as a phosphoprotein and migrated as a doublet when the Bik protein was labeled with 35S. Comparison with Bik labeled with 32P revealed that the slower moving band might be phosphorylated and that this phosphorylation may have increased the potency of Bik (33).

In our study, the treatment of cells with fisetin induced a more rapidly migrating band, suggesting that the change in the size of Bik may not be associated with the induction of apoptosis in fisetin-treated cells. Further study will be required to determine the nature of the change in the size of Bik, and whether this is associated with the activation of Bik.

Fisetin may not be absorbed well and, even after being absorbed, it may not exist as the parent compound, but rather as metabolites such as glucuronides, sulfates, or other conjugate species. To date, the absorption, metabolism, blood concentrations, half-life, and biological activity of fisetin have not been well characterized, except that one study has reported that fisetin is methylated in the human liver (6). The present study utilized fisetin at concentrations of 5–20 μmol/l, and other investigators have assessed the effects of fisetin at higher concentrations than those utilized in our studies. At the present time, we do not know whether fisetin can be delivered effectively to the target tissue, so that the concentrations of fisetin in the target tissue as high as those used in the present cell culture studies can be achieved. However, if fisetin is not absorbed well, it remains possible that colonocytes will be exposed to high concentrations of unabsorbed fisetin in the lumen. In an effort to determine whether the concentrations used in the cell culture studies are physiologically relevant, the concentrations of fisetin and its metabolites in human serum, as well as in the colonic lumen, should be determined in the future.

In conclusion, we have demonstrated that fisetin induces apoptosis via the activation of caspases in HCT-116 human colon cancer cells. Our results reveal that fisetin alters the expression of several Bcl-2 family member proteins and death receptor signals, ultimately resulting in the induction of the caspase-9/-3 cascade. Fisetin also causes an increase in the protein levels of the p53 tumor suppressor, which stimulates the migration of Bax to the mitochondria. In the present study, we provide some of the molecular basis for using fisetin as a potential apoptosis stimulatory agent. Further in vivo evaluations of the potential of fisetin as an antitumorigenic agent are clearly warranted.

GRANTS

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Fisetin induces apoptosis of colon cancer cells


