Abstract. Myricetin is a flavonol found in various berries, herbs, and walnuts. Previous studies have demonstrated that myricetin has anticancer effects against several types of cancer, including hepatocarcinoma, skin carcinoma, and pancreatic cancer. However, the anticancer activity of myricetin on human colon cancer has not been yet established. In the present study, we investigated the anticancer effects of myricetin on HCT-15 human colon cancer cells. We found that myricetin induces cytotoxicity and DNA condensation in human colon cancer cells in a dose-dependent manner. We also determined that myricetin increases the BCL2-associated X protein/B-cell lymphoma 2 ratio, but not cleavage of caspase-3 and -9. In addition, myricetin induced the release of apoptosis-inducing factor from mitochondria. These results suggest that myricetin induces apoptosis of HCT-15 human colon cancer cells and may prove useful in the development of therapeutic agents for human colon cancer.

Most colorectal cancer is associated with lifestyle and increasing age, with only a minority of cases associated with an underlying disorder (1). Moreover, colorectal cancer is the second most common cause of cancer death in men and women. This corresponds to approximately 10% of all patients with cancer-related death. However, the marked increase of survival in patients with metastatic disease in the past five years stems from a better understanding of the molecular aspects of colon cancer and metastasis, the development of anticancer therapeutic agents and active surgical approaches (2). For this reason, the continuous development of new anticancer agents is essential for human colon cancer therapy.

Flavonoids are a group of more than 4,000 polyphenolic compounds that occur naturally in foods of plant origin. These compounds possess a common phenylbenzopyrone structure and are categorized according to the saturation level and opening of the central pyran ring, mainly into flavonols, flavones, isoflavones, flavanols, and flavanonols (3, 4). These polyphenolic compounds display a remarkable spectrum of biological activities including antioxidant, anti-inflammatory, anti-carcinogenic, and modulation of enzymatic activities that might be able to influence processes that are dysregulated during cancer development (5, 6).

Myricetin, a member of the flavonol class of flavonoids, is found in berries, vegetables, herbs, and walnuts in the form of glycosides (7, 8). Recently, studies have suggested that myricetin has anticancer effects against several types of cancer (9, 10). However, the effect of myricetin on human colon cancer cells has not been established.

In the present study, we investigated the effect of myricetin on HCT-15 human colon cancer cells.

Materials and Methods

Reagents. Myricetin and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC) Annexin V apoptosis Detection Kit II was purchased from BD bioscience (San Jose, CA, USA).

Cell culture and myricetin treatment. HCT-15 human colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Briefly, cells were cultured at 37°C in the presence of 5% CO₂ in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 200 IU/ml penicillin, 200 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. Myricetin was reconstituted in Dimethyl sulfoxide (DMSO) and then diluted to the desired concentration in RPMI-1640 (final DMSO concentration 0.2% v/v). In the control (untreated) samples, an equal amount of DMSO was added.
Cell viability assay. Cell viability was measured by colorimetric MTT assay. The cells (2×10^4 cells/well) were seeded in 96-well culture plates with RPMI-1640. Cells were treated with the different concentrations of myricetin (5 μM to 100 μM) and incubated at 37°C for 24 h. After the treatment, medium containing myricetin was removed and MTT (0.5 mg/ml) in phosphate-buffered saline (PBS) was added to each well. After incubation at 37°C for 4 h, MTT solution was removed and formazan product was dissolved in solubilization solution (1:1 DMSO:ethanol) into a colored solution. Absorbance of the formazan solution was quantified by an enzyme-linked immunosorbent assay (ELISA) microplate reader at 570 nm.

DNA condensation assay. HCT-15 human colon cancer cells were first seeded on round coverslip in 12-well cell culture plate. After exposure to different concentrations of myricetin for 24 h, the cells were fixed by 100% cold methanol. After washing with PBS three times, the cells on coverslip were mounted on glass slides with permanent mounting solution with 4',6-diamidino-2-phenylindole (DAPI). DNA condensation in the cells was then observed with a fluorescence microscope (Nikon, Tokyo, Japan).

Annexin-V/PI binding assay. The cells were seeded into 6-well cell culture plate and then treated with different concentrations of myricetin. After 24 h, both floating and attached cells were collected and pooled into a round-bottom tube. The collected cells were washed with PBS and stained with FITC-labeled annexin-V and propidium iodide (PI). A total of 10,000 events for stained cells were analyzed using a FACS Calibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blot analysis. Myricetin-treated HCT-15 human colon cancer cells were lysed in ice-cold whole-cell lysate buffer (RIPA) containing 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride. The mixture was incubated at 4°C for 30 min. Cell debris were removed by microcentrifugation, followed by quick freezing of the supernatant. The protein concentration of each sample was determined using a micro bicinechonic acid assay kit (Pierce, Rockford, IL, USA). Equivalent protein samples (20 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically-transferred to polyvinylidene difluoride (PVDF) transfer membrane. The membrane was placed into a blocking solution (5% non-fat milk) at room temperature for 1 h. After blocking, antibodies against β-actin, caspase-9, caspase-3, B-cell lymphoma 2 (BCL2), BCL2-associated X protein (BAX), BCL2 homologous antagonist killer (BAK), and apoptosis-inducing factor (AIF) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the primary antibodies. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Santa Cruz Biotechnology) were used as secondary antibodies. Band detection was performed using the enhanced chemiluminescence (ECL) detection system and exposure to radiographic film. Pre-stained blue markers were used for molecular weight determination.

Statistical analysis. The results are presented as the mean±standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe’s post-hoc test using IBM SPSS Statistics (SPSS Inc., Delaware, IL, USA). The differences were considered statistically significant at p<0.01.

Results

Myricetin induces death of HCT-15 human colon cancer cells. In preliminary studies, the cytotoxic effect of myricetin (Figure 1A) on HCT-15 human colon cancer cells was measured by the MTT assay. The treatment of myricetin induced death of HCT-15 human colon cancer cells in a dose-dependent manner. In comparison to controls (treated with only 0.2% DMSO), treatment with 100 μM of myricetin induced about 70% reduction in cell viability on HCT-15 human colon cancer cells (Figure 1B).

Myricetin induces nuclear condensation and apoptotic death of HCT-15 human colon cancer cells. As described in the previous section, myricetin significantly reduced cell viability of HCT-15 human colon cancer cells at 50 and 100 μM. Therefore, these concentrations of myricetin were used for studying the major mechanisms underlying myricetin-induced cell death of HCT-15 human colon cancer cells. To investigate
Figure 2. Myricetin induced DNA condensation and apoptotic cell death of HCT-15 human colon cancer cells. A: HCT-15 human colon cancer cells were treated with myricetin (5, 25, 50, and 100 μM) for 24 h and then DAPI staining was performed as described in Materials and Methods. The lower panels show the corresponding fields of DAPI fluorescence. Original magnification was ×200 and ×400. B: Human colon cancer cells were treated with myricetin for 24 h and stained with annexin-V-FITC and PI-phycobiliproteins (PE). Fluorescence intensity was measured by flow cytometry and presented as a percentage of each quadrant.
DNA damage and apoptotic cell death of human colon cancer cells, human colon cancer cells were treated with myricetin at different concentration from 5 to 100 μM for 24 h. The nuclear condensation assay was used to assess the morphological changes of apoptosis in HCT-15 human colon cancer cells. As shown in Figure 2A, DAPI staining revealed that treatment of HCT-15 with 100 μM of myricetin induced significant nuclear rounding and shrinkage of HCT-15 human colon cancer cells in comparison to controls (Figure 2A). In addition, the flipping of phosphatidyl serine from the inside to the outside of the cell membrane, a major event in the apoptotic process, was detected by annexin-V binding assay after treatment of HCT-15 human colon cancer cells with myricetin.

As shown in Figure 2B, we found that myricetin induced apoptotic cell death in a dose-dependent manner. Moreover, treatment with myricetin led to 44.6% apoptotic (annexin-V+PI- and annexin-V+/PI+) HCT-15 human colon cancer cells. Myricetin treatment induced some secondary necrotic cell death followed by apoptosis. These data indicate that treatment of myricetin certainly induces apoptosis of HCT-15 human colon cancer cells.

Myricetin increases the BAX/BCL2 ratio and BAK expression in HCT-15 human colon cancer cells. Activation of the caspase cascade and the increase in the BAX/BCL2 ratio are important for induction and processing of apoptosis. For this reason, the downstream events of myricetin-induced apoptosis in HCT-15 human colon cancer cells were characterized by activation of caspase-3, -9, and BAX/BCL2 using western blot analysis. As shown in Figure 3A, myricetin increased the ratio of BAX/BCL2 and expression of BAK. Mitochondrial dysfunction, an essential early event in cell death, requires either BAX or BAK, both of which are negatively-regulated by BCL2 (11). We found that treatment of myricetin increased the BAX/BCL2 ratio as well as BAK expression in a dose-dependent manner. However, the expression of procaspase-3 and caspase-9 were hardly changed in HCT-15 human colon cancer cells following 100 μM of myricetin treatment (Figure 3B). These data indicate that myricetin might induce human colon cancer cell death via increasing the BAX/BCL2 ratio and mitochondrial dysfunction.

Myricetin induces AIF release. Dysfunction of mitochondria can result in the release of pro-apoptotic molecules such as AIF and cytochrome c into the cytosol. As shown in Figure 4. Myricetin induced AIF release from mitochondria into cytosol in human colon cancer cells. Human colon cancer cells were incubated with different myricetin concentration for 24 h, subsequently lysed, and equal amounts of cytosolic fraction proteins were separated by SDS-PAGE and transferred against PVDF membranes. Membranes were probed with indicated antibodies to AIF, cytochrome c, and β-actin and detected by ECL solution. β-Actin was used as the internal control. The results are from one experiment representative of four performed that showed similar patterns.
4A, treatment with myricetin induced the release of AIF into the cytosol in HCT-15 human colon cancer cells in a dose-dependent manner. Moreover, a major caspase-independent mechanism of cell death is regulated by AIF (12). However, myricetin treatment did not lead to release of cytochrome c in HCT-15 human colon cancer cells (Figure 4B). These results indicate that myricetin can induce apoptotic cell death of human colon cancer cells by inducing AIF release.

Discussion

Myricetin is found in fruits, vegetables, and edible berries usually used in red wines. Recently, myricetin has been reported to induce apoptotic cell death of HepG2 human hepatocarcinoma cells via increasing the BAX/BCL2 ratio, and activation of caspase-3, -9, and cytochrome c release (13). Moreover, myricetin had beneficial effects such as anticancer, antioxidant, and antibacterial activity on 1,2 dimethylhy-drazine-induced rat colon carcinogenesis (14). However, the effects of myricetin on HCT-15 human colon cancer cells were still unknown. This study focused on the anticancer activity of myricetin on human colon cancer cells in vitro.

As shown in Figure 1, myricetin induced human colon cancer cell death in a dose-dependent manner. After 24 h of incubation, at the concentrations of 50 and 100 μM of myricetin treatment significantly reduced the viability of human colon cancer cells in comparison to the control.

The two primary modes of cell death are apoptosis and necrosis. To investigate which type of cell death myricetin induces in human colon cancer cells, we determined apoptotic and necrotic cell death by annexin-V/PI staining. The flipping of phosphatidyl serine from the inside to the outside of the cell membrane is a major event in the apoptotic process and was detected by annexin-V binding assay after treatment of HCT-15 cells with myricetin. We found that myricetin induced apoptotic cell death of human colon cancer cells in a dose-dependent manner.

The mitochondrial pathway is relatively more important than the death-receptor pathway for induction of apoptosis by chemotherapeutic agents (15). Mitochondrial dysfunction induces an increase in the BAX/BCL2 ratio, and caspase activation, and these two major pathways are essential for apoptosis of cells. Previous studies have shown that myricetin induces cell death via the mitochondrial pathway. Myricetin increased expression of cleaved caspase-3 and -9, and the BAX/BCL2 ratio in hepatocarcinoma HepG2 cells (13). Treatment of myricetin also led to apoptotic cell death of UVB-induced skin cancer cells via induction of BCL2-associated death promoter (BAD)-mediated apoptosis (16). To investigate whether myricetin induced human colon cancer cell death through the activation of mitochondria-mediated apoptotic pathway, human colon cancer cells treated with different concentrations of myricetin for 24 h. As shown in Figure 3, 100-μM myricetin treatment significantly increased the BAX/BCL2 ratio and BAX level. However, cleavage of caspase-3 and caspase-9 were barely induced by myricetin.

These results indicate that myricetin treatment induced human colon cancer cell death activity through the BAX/BCL2-dependent pathway (Figure 3A and B). AIF and cytochrome c release are typical signals downstream of BAX/BCL2. This study demonstrated human colon cancer apoptosis by myricetin treatment via enhancement of AIF release from mitochondria into the cytosol (Figure 4). Previous studies reported that the cytochrome c is involved in the caspase-dependent pathway, but AIF release occurs via a caspase-independent pathway in apoptosis (17, 18). Therefore, our results indicate that myricetin induced apoptosis of HCT-15 human colon cancer cells via BAX/BCL2-dependent and caspase-independent pathway.

In summary, myricetin treatment reduced cell proliferation and induced apoptotic death of HCT-15 human colon cancer cells. Myricetin induced apoptosis through an increase in BAX/BCL2 ratio and by AIF release from mitochondria into the cytosol. These results suggest that myricetin-induced apoptosis was mediated by mitochondrial dysfunction and the BAX/BCL2-dependent pathway, which may prove useful in the development of therapeutic agents for the treatment of human colon cancer.

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References