Dietary Flavone Is a Potent Apoptosis Inducer in Human Colon Carcinoma Cells

Uwe Wenzel,1 Sabine Kuntz, Mathias D. Brendel, and Hannelore Daniel

Institute of Nutritional Sciences, University of Giessen, 35392 Giessen [U. W., S. K., H. D.], and Third Department of Internal Medicine, University of Giessen, 35385 Giessen [M. D. B.], Germany

ABSTRACT

Flavonoids are polyphenolic compounds that occur ubiquitously in plants. They are discussed to represent cancer preventive food components in a human diet that is rich in fruits and vegetables. To understand the molecular basis of the putative anticancer activity of flavonoids, we investigated whether and how the core structure of the flavones, 2-phenyl-4H-1-benzopyran-4-one (flavone) affects proliferation, differentiation, and apoptosis in HT-29 human colon cancer cells. Moreover, the effects of flavone in transformed epithelial cells were compared with those obtained in nontransformed primary mouse colonocytes. Proliferation, differentiation, and apoptosis in transformed as well as nontransformed colon cells were measured by fluorescence-based techniques. Apoptosis was also determined by changes in membrane permeability, FACSscan analysis, and detection of DNA fragmentation. Semiquantitative reverse transcription PCR was performed to assess the effects of flavone on transcript levels. Flavone was found to reduce cell proliferation in HT-29 cells with an EC_{50} value of 54.8 ± 1.3 μM and to potently induce differentiation as well as apoptosis. The flavonoid proved to be a stronger apoptosis inducer than the clinically established antitumor agent camptothecin. The effects of flavone in HT-29 cells were associated with changed mRNA levels of cell-cycle- and apoptosis-related genes including cyclooxygenase-2 (COX-2), nuclear transcription factor κB (NF-κB), and bcl-2. Moreover, flavone, but not camptothecin, displayed a high selectivity for the induction of apoptosis and of growth inhibition only in the transformed colonocytes.

In conclusion, the plant polyphenol flavone induces effectively programmed cell death, differentiation, and growth inhibition in transformed colonocytes by acting at the mRNA levels of genes involved in these processes. Because these genes play a crucial role in colon carcinogenesis, flavone may prove to be a potent new cytostatic compound with improved selectivity toward transformed cells.

INTRODUCTION

Although a diet rich in fruits and vegetables is generally recognized as preventive with regard to the development of colorectal cancer, the dietary compounds responsible for this biological effect have not been identified as yet (1, 2). Flavonoids are a class of more than 4000 phenylbenzopyrones that occur in many edible plants, like fruits and vegetables. These polyphenolic compounds display a remarkable spectrum of biochemical activities including those that might be able to influence processes that are dysregulated during cancer development. These include, e.g., antioxidant activities (3), the scavenging effect on activated carcinogens and mutagens (4, 5), the action on proteins that control cell cycle progression (6), and altered gene expression (7). Because the biochemical activities are dependent on the individual flavonoid structure, each compound needs to be studied systematically to assess its individual biological potency. As part of a screening program, we have recently (8) shown for more than 30 flavonoids of the flavone, flavonol, flavanone, and isoflavone subgroups in three cancer cell lines that the potency and selectivity of the antiproliferative activities are strongly dependent on the particular structure of the compound. Several members of the flavone subgroup displayed strong antiproliferative activity in the human colon carcinoma cell line HT-29. Here, we tested whether the core structure of the flavone subgroup, 2-phenyl-4H-1-benzopyran-4-one (flavone), is able to potently inhibit proliferation of HT-29 as well. To get insight into the mechanism of growth-inhibition, we assessed whether flavone affects differentiation and apoptosis, which are impaired during colon cancer development (9, 10). A semiquantitative RT²-PCR approach was used to associate the effects of flavone on cell cycle progression, differentiation, and apoptosis with altered transcript levels of genes having central functions in these processes. Alterations of m-RNA levels were determined for COX-2, NF-κB, p53, p21, cyclin E and B as well as for bak, bax, and bcl-2 as members of the apoptosis-associated bcl-2 gene family. To establish that the effects of flavone are carcinoma-cell-specific, we investigated whether proliferation, differentiation, and apoptosis are also altered by the flavonoid in nontransformed mouse colonocytes in culture.

MATERIALS AND METHODS

Animals

C57BL/6OlaHsd inbred 9-week-old mice were purchased from Harlan Winkelman (Borchen, Germany) and were housed under standard conditions with free access to food and water.

Cell Culture

HT-29 Cells. HT-29 cells (passage 106) were provided by American Type Culture Collection and were used between passage 150 and 200. Cells were cultured and passaged in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine (all from Life Technologies, Inc., Eggenstein, Germany). Antibiotics added were 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc.). The cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells were passaged at preconfluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA (Life Technologies, Inc.).

Murine Colonocytes. Cultures of colonic primary epithelial cells from adult mice were established based on the method described by Booth et al. (11) with slight modifications. Therefore, the colons of seven mice that were at least 12 weeks old were cut into pieces of approximately 2 mm² and washed five or six times with 10 ml of DMEM, containing 25 mM HEPES, 1% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml gentamicin (all from Life Technologies, Inc.), by vigorous shaking and subsequent removal of the supernatant after sedimentation of the fragments. The colon pieces were minced to a pulp and digested in the DMEM with 150 units/ml collagenase (Sigma, Deisenhofen, Germany) and 20 μg/ml dispase (Boehringer, Mannheim, Germany) for 1–3 h at 37°C with gentle shaking until isolated crypts were visible under the microscope. The suspensions were transferred into 50-ml tubes and centrifuged for 3 min at 400 g to separate crypts and digestive enzymes. The pellets obtained were resuspended in 10 ml of DMEM, containing HEPES, 10% FCS, antibiotics, and 1.6% sorbitol, and the large crypt fragments were allowed to settle under gravity for 1 min, whereas single crypts remained in solution. The upper 5 ml of the suspensions were carefully taken by a pipette and transferred into new 50-ml tubes. The remaining loose

Received 12/20/99; accepted 5/16/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Institute of Nutritional Sciences, Hochfeldweg 2, D-63530 Freising-Weihenstephan, Germany. Phone: 49-8161-71-3997; Fax: 49-8161-71-3999; E-mail: wenzel@pollux.weihenstephan.de.

2 The abbreviations used are: Ac-DEVD, acetyl-aspartyl-glutamyl-valyl-aspartate; AP, alkaline phosphatase; CHAPS, 3-[[(cholamidopropyl)-dimethylammonium]-1-propansulfonate; CDK, cyclin-dependent kinase; COX-2, cyclooxygenase-2; FACS, fluorescence activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-κB, nuclear transcription factor κB; RT, reverse transcription.
pellet was extracted twice with 6–10 ml of sorbitol containing medium by vigorous agitation. The collected crypts were centrifuged for 5 min at 60 × g, and the supernatants containing bacteria and fibroblasts were removed. The extraction and centrifugation steps were repeated 4–5 times until the supernatant was clear. The final crypt pellet was suspended in 100 ml of DMEM containing 25 mM HEPES, 2.5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml gentamicin, and 0.25 units/ml human insulin (Hoechst Marion Roussel, Bad Soden, Germany). Crypt numbers were determined by pipetting 10 μl on a cover slide and counting the crypts by using the microscope. Crypts were seeded onto culture plates and cultured in a humidified atmosphere of 9% air and 9% CO2 at 37°C. The culture plates were coated with collagen type I (Vitrogen 100; 3.1 mg/ml from Collagen GmbH, Ismaning, Germany) by diluting the collagen with 50 mM sterile filtered acetic acid at a ratio 1:9 and pipetting 200 μl of this solution into each well. The solution was allowed to evaporate by placing the plates, uncovered, in a laminar flow hood overnight.

**Cell Proliferation and Acute Cytotoxicity**

For determination of proliferation, HT-29 cells were seeded at a density of 5 × 10^3 per well onto 24-well cell culture plates (Renner, Dannstadt, Germany) and allowed to adhere for 24 h. Mouse colonic crypts were seeded at a density of 50 crypts/well on collagen-coated 24-well plates and allowed to adhere for 48 h. Thereafter, medium was replaced by fresh culture medium containing the test compounds, and cells were allowed to grow for another 72 h. In case of the crypt cells only one-half of the medium was removed to maintain the growth factors that were secreted by the cells and that are necessary for optimal growth. Flavone and camptothecin (both from Sigma) were applied in DMSO, and the solvent reached a concentration not higher than 2% in all experiments. Controls were always treated with the same amount of DMSO as used in the corresponding experiments. Total cell counts were determined by SYTOX-Green (Bioprobes, Leiden, Netherlands), which becomes fluorescent after DNA binding. Therefore, cells were lysed by 1% Triton X-100 in isotonic NaCl, and cell numbers were determined based on a calibration curve. The calibration curve was measured using cell numbers between 1 × 10^4 and 1.5 × 10^5 cells, which had been adjusted after the determination of cell numbers in a Neubauer chamber, and fluorescence of the corresponding cell numbers was measured at 538 nm after excitation at 485 nm using a fluorescence multiwell-plate reader (Fluoroskan Ascent, Labsystems, Bornheim-Hersel, Germany).

Acute cytotoxicity was assessed by SYTOX-fluorescence with 5 × 10^4 adherent HT-29 or crypt cells per 24 wells, which had been exposed for 3 h to test compounds (150 μM). The percentage of dead cells in a cell population was determined by SYTOX-fluorescence prior to cell lysis in relation to the fluorescence measured after the solubilization of cells.

**Determination of Apoptosis Markers**

**Early Markers**

Apopain Activity. Apopain activity was measured as described previously (12). In brief, HT-29 cells were seeded at a density of 5 × 10^3 per well and crypts at a density of 1000 per well onto 6-well plates (Renner) and allowed to adhere for 24 h. Cells were then exposed to the test compounds for 72 h after 24 h of adherence. Cell numbers were determined after cells had been washed twice with PBS and trypsinized, and the harvested cells were pelleted for 10 min at 1500 × g. The pellets were resuspended in 550 μl of 1 M diethanolamine buffer (pH 9.8) with 0.5 mM MgCl2 and were homogenized. Homogenate (500 μl) was mixed with 500 μl of 0.1 mM fluorescein-diphosphate (Bioprobes) in diethanolamine buffer, and AP activity as a marker for differentiation was determined by the release of fluorescein using the multwell plate reader with excitation at 485 nm and emission at 538 nm. Fluorescein (Sigma) was used as the standard to determine AP-activity on the basis of the two phosphate residues released by cleavage of one fluorescein-diphosphate molecule.

**GROWTH INHIBITION OF COLON CANCER CELLS BY FLAVONE**

Fig. 1. A, proliferation of HT-29 cells as a function of flavone (○) and camptothecin (▲) concentration. Cells (5 × 10^3 per well) were incubated for 72 h with medium alone (control) or containing the compounds at various concentrations, and cell numbers were determined subsequently using SYTOX-Green nucleic acid stain. Cell counts for controls were 73,500 ± 4,500. B, AP activity after 72-h incubation with medium alone (□) or containing 50 μM flavone (●) or 50 μM camptothecin (▲). Forty % confluent cells were incubated with or without the compounds before AP activity was determined using fluorescein-diphosphate as a substrate. AP activity in control cells was determined as 25.2 ± 1.4 pmol/min × 10^6 cells.

*P < 0.05; ***P < 0.001 (n = 3).

Fig. 2. A, time-dependent apopain-activity in HT-29 cells incubated with medium alone (○) or containing in addition 150 μM flavone (●) or 50 μM camptothecin (▲). Cells (5 × 10^3) were incubated for the time indicated before the cytosol of the cells was prepared, and apopain-activity was determined by the use of the fluorogenic substrate Ac-DEVD-amino-4-methylcoumarin. B, apopain-activity as a function of flavone (○) and camptothecin (▲) concentration as determined after 24-h incubation. Apopain-activity in control cells was 4.5 ± 1.6 pmol/min × 10^6 cells.

*P < 0.05; ***P < 0.001.

**Fig. 1.** A, proliferation of HT-29 cells as a function of flavone (○) and camptothecin (▲) concentration. Cells (5 × 10^3 per well) were incubated for 72 h with medium alone (control) or containing the compounds at various concentrations, and cell numbers were determined subsequently using SYTOX-Green nucleic acid stain. Cell counts for controls were 73,500 ± 4,500. B, AP activity after 72-h incubation with medium alone (□) or containing 50 μM flavone (●) or 50 μM camptothecin (▲). Forty % confluent cells were incubated with or without the compounds before AP activity was determined using fluorescein-diphosphate as a substrate. AP activity in control cells was determined as 25.2 ± 1.4 pmol/min × 10^6 cells.

**Fig. 2.** A, time-dependent apopain-activity in HT-29 cells incubated with medium alone (○) or containing in addition 150 μM flavone (●) or 50 μM camptothecin (▲). Cells (5 × 10^3) were incubated for the time indicated before the cytosol of the cells was prepared, and apopain-activity was determined by the use of the fluorogenic substrate Ac-DEVD-amino-4-methylcoumarin. B, apopain-activity as a function of flavone (○) and camptothecin (▲) concentration as determined after 24-h incubation. Apopain-activity in control cells was 4.5 ± 1.6 pmol/min × 10^6 cells.

**Differentiation Assay**

After having reached 40% confluency on 25-cm² culture flasks (Renner), HT-29 cells were incubated for 72 h with the test compounds. Crypts were seeded at a density of 250 per well on 24-well plates and were incubated with the test compounds for 72 h after 24 h of adherence. Cell numbers were determined after cells had been washed twice with PBS and trypsinized, and the harvested cells were pelleted for 10 min at 1500 × g. The pellets were resuspended in 550 μl of 1 M diethanolamine buffer (pH 9.8) with 0.5 mM MgCl2 and were homogenized. Homogenate (500 μl) was mixed with 500 μl of 0.1 mM fluorescein-diphosphate (Bioprobes) in diethanolamine buffer, and AP activity as a marker for differentiation was determined by the release of fluorescein using the multiwell plate reader with excitation at 485 nm and emission at 538 nm. Fluorescein (Sigma) was used as the standard to determine AP-activity on the basis of the two phosphate residues released by cleavage of one fluorescein-diphosphate molecule.
precipitated by the addition of 1 ml of ethanol. The probe was inverted 5–8 times and was left for 3 min at room temperature before it was pelleted by centrifugation at 5000 \( \times g \) and 4\(^\circ\)C for 5 min. The DNA precipitate was washed by removal of the supernatant, the addition of 95\% ethanol, and centrifugation at 1000 \( \times g \) and 4\(^\circ\)C for 2 min. The pellet was dissolved in aqua bidest and adjusted to 0.2–0.3 \( \mu \)g DNA/\( \mu l \) by measuring the UV absorbance at 260 and 280 nm. The DNA was separated on a 1.5% agarose gel and visualized by UV after ethidium-bromide staining.

### Cell Cycle Analysis

HT-29 cells were seeded at a density of 1 \( \times 10^6 \) onto 25-cm\(^2\) culture flasks and were incubated for 24 or 48 h in the presence or the absence of the test compounds after having reached 40\% confluency. The cells were trypsinized, pelleted by centrifugation at 500 \( \times g \) for 5 min, washed twice with PBS, and adjusted to 2 \( \times 10^6 \) cells/ml PBS. Subsequently the cells were treated with 1 unit of DNase free RNase (MBI Fermentas, Heidelberg, Germany) per ml of PBS for 30 min at 37\(^\circ\)C before they were centrifuged again. The cells were fixed by suspending the pellet for 1 h at 4\(^\circ\)C in ethanol. Thereafter, the suspension was centrifuged in a microfuge for 0.5 min, and the pellet was resuspended in 0.9\% NaCl containing 1 \( \mu \)M SYTOX-Green. After a 15-min incubation, cell cycle analysis was performed using a FACScan (Becton Dickinson, San Jose, CA) and the Lysis II Ver. 1.1 software.

### Semiquantitative RT-PCR

RNA from HT-29 cells was isolated at the times indicated in Figs. 7–10 according to the method described by Chomczynski and Sacchi (13) with slight modifications. Reverse transcription was done with 5 \( \mu \)g of isolated RNA. First-strand cDNA synthesis was accomplished with an oligo-dithymide\(_{14}\) primer (MBI Fermentas). Amplification of sequence-specific fragments (Taq polymerase was from Sigma) was performed with 30 cycles (95\(^\circ\)C-denaturation for 1 min, 55\(^\circ\)C-hybridization for 2 min, 72\(^\circ\)C-extension for 2 min; Personal Cycler; Biometra, Göttingen, Germany). RT-PCR products were separated on a 1% agarose gel and visualized by ethidium bromide. The amount of first strand used to amplify specific sequences was derived from the linear range of amplification. The amplified GAP-DH sequence was used as a constitutively expressed control. The amplified products were photographed, and the intensity of the bands was analyzed by the SigmaGel software. No products were obtained for any genes without reverse transcription indicating the specificity of mRNA determination. A 3′-DNA/EcoRI + HindIII marker (MBI Fermentas) was used in all PCR experiments as a size control of the amplified products. Amplified cDNA sequences (primers were custom-synthesized by Eurogentec, Seraing, Belgium) were: GAP-DH, bp 558–1010; COX-2, bp 1366–1870; NF-\( \kappa \)B, bp 2832–3401; p53, bp 1878–19376 (bp 189–777 of exon 11); p21, bp 95–562; cyclin B, bp 40–893; cyclin E, bp 42–732; bax, bp 40–570; bak, bp 320–763; and bcl-\( X_1 \), bp 255–674.

### Calculation and Statistics

To derive the EC\(_{50}\) values for growth inhibition, a nonlinear approximation model by the least square methods based on a competition curve using one component was applied (GraphPad Prism, GraphPad). For statistical analysis Student’s \( t \) test (GraphPad Prism) was used. For each variable, at least three independent experiments were carried out. Data are given as the mean ± SE.

### RESULTS

#### Effects of Flavone on Proliferation, Differentiation, and Apoptosis in HT-29 Cells

Flavone reduced proliferation of the human colon carcinoma cell line HT-29 dose-dependently with an EC\(_{50}\) value of 54.8 ± 1.3 \( \mu \)M. Under the same experimental conditions, the antitumor agent camptothecin led to one-half of a maximal growth inhibition at a concentration of 20.5 ± 1.0 \( \mu \)M (Fig. 1A). At concentrations at which cell growth was completely abolished, i.e., at about 50 \( \mu \)M camptothecin and 100 \( \mu \)M flavone, neither compound revealed any cytotoxic effects (95.3 ± 5.7\% living cells at

**Fig. 3. Determination of early and late apoptosis events by HT-29 cell-staining with Hoechst 33342 (A–D) and Hoechst 33258 (E–H).** Cells (3 \( \times 10^6 \)) were seeded onto 24-well plates and exposed to flavone or camptothecin for the times given in graphs D and H. A–C, Cells stained with Hoechst 33342 after 16 h of exposure: E–G, cells stained with Hoechst 33258 after 24 h of incubation either with medium alone (A, E), with 50 \( \mu \)M flavone (B, F), or with 50 \( \mu \)M camptothecin (C, G). Cells that accumulated Hoechst 33342 (D) or that displayed nuclear fragmentation (arrows) by Hoechst 33258 staining (H) were counted and expressed as the percentage of apoptotic cells under control conditions (C). After flavone exposure (●), or after camptothecin treatment (▲).
50 μM camptothecin; 102.4 ± 6.9% living cells at 100 μM flavone). However, camptothecin at concentrations of >50 μM proved to be cytotoxic (86.9 ± 4.1% living cells at 75 μM, 45.5 ± 8.5% at 100 μM; 32.6 ± 7.5% at 150 μM). In contrast, flavone at a concentration of 150 μM was not toxic (97.4 ± 1.0% living cells) and exerted only moderate cytotoxic effects at concentrations >250 μM (82.9 ± 1.1% living cells at 250 μM; 73.4 ± 4.5% living cells at 500 μM). When applied at the highest nontoxic concentrations (150 μM flavone, 50 μM camptothecin) flavone was found to cause a 6-fold increase of AP-activity serving as a marker for cell differentiation (Fig. 1B), whereas camptothecin increased AP activity only by 2-fold (Fig. 1B). At a concentration of 250 μM, flavone caused an additional increase of AP activity to reach 853.8 ± 47.3% of control cells (data not shown).

Both, camptothecin as well as flavone, were found to activate the caspase-3 apopain as the most downstream enzyme in the apoptotic pathway, considered to serve as a valid marker of apoptosis (14, 15). Flavone (150 μM) increased apopain activity at 24 h and 48 h of exposure by a factor of 8.4 and 8.5, respectively, above that in control cells. Camptothecin (50 μM) led to a 7.1- and 10.5-fold stimulation, respectively, of the caspase-3 activity (Fig. 2A). Whereas this represents the maximal achievable stimulation of caspase activity by camptothecin without acute cytotoxic effects, flavone, when used at a concentration of 250 μM, further enhanced apopain activity to reach a 17-fold stimulation (Fig. 2B). Induction of apoptosis by 150 μM flavone or 50 μM camptothecin became also evident by increased accumulation of the Hoechst dye 33342 (Fig. 3, A–D). Cell-staining by this dye parallels apopain activation, and this is thought to be attributable to very early changes in membrane permeability after the initiation of apoptosis (16).

That the apoptotic signal given by activation of apopain is transmitted to the end point of the apoptotic pathway, i.e., the fragmentation of DNA, could be demonstrated for both compounds. Whereas nuclear fragmentation after 24 h of exposure of cells to camptothecin or flavone was detectable in about 25% of the cells (Fig. 3, E–H), it was more prominent at 48 h (Fig. 3H). When DNA was isolated from flavone- or camptothecin-treated cells, DNA fragmentation became evident in adherent cells and even more pronounced in floating apoptotic cells only after 48 h (Fig. 4A).

FACScan analysis of HT-29 cells exposed to flavone for 24 h suggested that flavone arrests the cells in a post-G1-phase. Exposure of cells for 48 h revealed a pool of cells in a sub-G1 phase that correlated with the enhanced DNA fragmentation (Fig. 4B) observed under these conditions. When cells were treated with higher flavone concentrations, the percentage of cells undergoing apoptosis was further increased from about 40% at 150 μM flavone (Fig. 3H) to 78.5 ± 6.9% at 250 μM (data not shown).

Effects of Flavone on Proliferation, Differentiation, and Apoptosis in Nontransformed Murine Colonocytes. To assess the cell specificity of flavone, we compared its effects on proliferation, differentiation, and apoptosis in HT-29 cells with colonocytes of a nontransformed genetic background. Isolation of crypts from mouse colon and growth of the isolated colonocytes are shown in Fig. 5.

Although camptothecin reduced proliferation of the primary cells with an EC50 of 50.0 ± 1.3 μM, flavone did not show any effects on cell growth (Fig. 6A). Growth inhibition by camptothecin at concentrations above 50 μM again was—at least in part—attributable to cytotoxic effects (16.1 ± 8.0% dead cells versus controls at 75 μM, 32.9 ± 9.1% at 100 μM, 57.4 ± 4.7% at 150 μM). Although 150 μM flavone or 50 μM camptothecin induced differentiation in HT-29 cells, both compounds failed to increase AP activity in murine primary colonocytes (Fig. 6B). Camptothecin, however, increased apopain activity 6.7-fold at 24 h and 3.6-fold at 48 h of exposure (Fig. 6C). In contrast, 150 μM flavone did not cause significant alterations in apopain activity neither after 24 h nor after 48 h of incubation (Fig. 6C).
Flavone Effects on Gene Expression in HT-29 Cells. To assess whether the effects of flavone on cell-cycle arrest and apoptosis observed in HT-29 cells were mediated by altered gene expression, mRNA levels of genes known to play a key role in cancer-related processes were determined. COX-2, which has become a target for colon cancer prevention and its pharmacotherapy (17), was found to be diminished in its transcript levels drastically when cells were exposed to flavone for 48 h (Fig. 7A). The mRNA of NF-κB, a transcription factor that has been recently shown to be involved in the chemoresistance of tumor cells by the inhibition of apoptosis (18), was also reduced dramatically by flavone treatment (Fig. 7B). That these effects on gene expression occurred selectively was shown by unaltered mRNA levels of the tumor suppressor p53 (Fig. 8A). The observed increase of p21-mRNA, a CDK-inhibitor that has been shown to be one of the major transcriptional targets of p53 (19), suggests that flavone affects p21 in HT-29 cells completely independently of p53 (Fig. 8B). Moreover, we observed pronounced down-regulation of the mRNA of CDK-regulatory proteins such as cyclin E and cyclin B (Fig. 9).

Induction of apoptosis by flavone was found to be associated also with altered transcript levels of pro- and antiapoptotic genes. Whereas transcript levels of the pro-apoptotic bax was not affected during the 48 h of flavone exposure of cells (Fig. 10A), mRNA levels of the pro-apoptotic bak were increased at 48 h of incubation by 3-fold (Fig. 10B). The mRNA levels of the antiapoptotic bcl-XL showed a rapid and significant reduction to 25% of that in control cells (Fig. 10C).

DISCUSSION

Epidemiological studies provided evidence that the high dietary intake of flavonoids with fruits and vegetables could be associated with a low colon cancer prevalence in humans (20, 21). Animal studies and investigations using different cellular models suggested that certain flavonoids can inhibit tumor initiation as well as tumor progression (22, 23). In a previous study, we showed that, in cancer cell lines including HT-29 and Caco-2 cells, the effects of flavonoids of different subclasses on cell cycle are strongly dependent on the specific structure of the compounds. Extending these studies, we here provide evidence that the nonhydroxylated core structure of the flavones, flavone, is a potent and selective inhibitor of proliferation of HT-29 cells. Moreover, it promotes differentiation and apoptosis in this human colon cancer cell line. Flavone, occurring in many cereal grains as well as in dill weed (24), inhibits proliferation of HT-29 in a concentration-dependent manner and was found to be as effective as the classical antitumor agent camptothecin. This topoisomerase I inhibitor is usually applied as a second-line pharmacotherapeutic in advanced colorectal cancers to promote apoptosis (25, 26). In our studies camptothecin also proved to be a strong inducer of apoptosis in HT-29 cells leading to DNA fragmentation. Flavone turned out to be even more potent than camptothecin in the activation of caspase-3 and nuclear fragmentation in HT-29 cells without cytotoxicity. Moreover, flavone restored a differentiated phenotype of the cells as judged by a 6-fold increase in AP activity, whereas camptothecin increased activity of this differentiation marker only by a factor of 2 at nontoxic concentrations.

Flow-cytometric analysis revealed that flavone can arrest HT-29 cells in a post-G1-phase before apoptosis occurs or differentiation is initiated in apoptosis-resistant cells. This inhibition of cell-cycle progression was associated with an altered expression of cell-cycle-relevant genes including the CDK-inhibitor p21. Transcript levels of...
p21 were found to be significantly increased by treating HT-29 cells with flavone. Development of sporadic tumors is generally associated with reduced expression of p21 (27), mainly as a result of loss-of-function mutations of p53 (28, 29). Moreover, p21 expression is directly related to terminal differentiation (28) and increased expression of p21 has been demonstrated to inhibit proliferation of malignant cells in vitro and in vivo (30). However, the role of p21 in the control of apoptosis is controversial. Whereas Chinery et al. (31) demonstrated that increased p21 expression promoted apoptosis in colorectal cancer cells, others reported p21 to be responsible for cell-cycle arrest and differentiation but not for apoptosis (30). Prevention of apoptosis induced by antitumor drugs has even been attributed to increased expression of p21 (32). However, Poljak et al. (33) showed that although the p21-mediated growth-arrest can protect cells from apoptosis, certain trans-acting factors can overcome these protective effects.

That the increased p21-expression contributes directly to the observed effects of flavone on apoptosis in our studies is unlikely because apoptosis attributable to increased p21 levels is generally preceded by a G1 arrest of cells (30), whereas flavone was found to arrest HT-29 cells in G2-M. Moreover, this G2-M arrest, already shown to occur by camptothecin in HT-29 cells (34), seems to be crucial for apoptosis induction in cells that do not express a functional p53, such as HT-29 (29, 35).

Nevertheless, flavone may prove to be a valuable tool for inhibition of CDKs in colorectal tumors for the following reasons: (a) induction of p21 by flavone was found to be independent of p53 mRNA levels, an effect of flavone that has recently been described in human lung adenocarcinoma cells (36); and (b) the inhibition of CDK2 by p21 may be amplified by the simultaneous decrease of cyclin E because S-phase entry in mammalian cells is induced by CDK2 complexed with S-phase cyclins such as cyclin E (37).

Not only cyclin E but also cyclin B has been shown to control cell-cycle progression in colon cancer cells (38, 39) and a reduction of cyclin B and cyclin E expression is associated with an improved cell-cycle control and cell-growth inhibition (40). In contrast to the decreased transcript levels of cyclin E, reduction in cyclin B-mRNA may contribute to the observed cell-cycle arrest induced by flavone because cyclin B complexed with CDK1 is important for the transition of G2-M phases (40).

Another gene product that has been linked to the loss of growth-control of colorectal tumors is COX-2 (41). This enzyme is over-expressed in almost 90% of all colorectal tumors (41) and has been shown to contribute to diminished apoptosis in colon cancer cells (42). Consequently, COX-2 has become the key target of pharmacotherapy (17). COX-2 mRNA levels were strongly reduced in cells exposed to flavone. Similarly, flavone exposure reduced the transcript levels of NF-xB, a transcription factor that is able to inhibit apoptosis in cancer cells and, thereby, contributes significantly to chemoresistance (18).

Besides the possibility of promoting apoptosis by down-regulation of NF-xB, flavone may prevail over apoptotic processes also by altering expression of other apoptosis-relevant genes such as bak, bax, and bcl-XL. bcl-XL has especially been associated with a reduced sensitivity to apoptotic signals in developing colon cancers (43), and its mRNA levels change rapidly. Because the mRNA of the pro-apoptotic bak was found to be up-regulated by flavone exposure only after 48 h when a large number of cells already had undergone apoptosis, it seems unlikely that bak is significantly involved in the apoptotic pathway activated by flavone. Nevertheless, it could contribute to apoptosis control because bak levels are generally decreased in primary colorectal adenocarcinomas (44).

As for all of the agents used or developed for cancer treatment, selectivity toward cancer cells is an important criterion. We, therefore, compared the flavone effects on HT-29 cells with that on primary murine colonocytes. Whereas the classical antitumor agent camptothecin induced apoptosis also in nontransformed cells, flavone failed to significantly affect caspase-3 activation in mouse colonocytes. This suggests that flavone is much more selective than camptothecin and may have the potential for reducing side effects compared with drugs that possess limited selectivity and that can cause mucosal damage (45). Moreover, the restricted selectivity of classical apoptosis-inducing antitumor drugs and, thereby, the increased apoptosis rates in normal cells of the human colorectum, can accompany neoplastic transformation (46).

In conclusion, we have demonstrated that the naturally occurring plant polyphenol, flavone, restores apoptosis sensitivity and differentiation in the human colon cancer cell line HT-29. The effects are likely to occur in vivo as well, because flavone has been shown to accumulate in intestinal cells (47). Flavone exerts its effects most likely by changing expression of genes known to play a crucial role in...
apoptosis, differentiation, and proliferation. Despite directing the dysregulated gene expression of HT-29 cells toward an expression pattern of nontransformed colonic cells, it needs to be elucidated which of the genes are affected causally and which are regulated secondarily.

Moreover, it remains to be determined whether all of the gene products remain unaffected in nontransformed cells or whether some are also responsive in the primary colonic cells without altering the phenotype.
Fig. 9. Determination of cyclin B (A) and cyclin E (B) mRNAs. HT-29 cells were treated for the indicated times with or without (control) flavone. Right, cyclin B and E:GAP-DH ratios from control (C) and flavone (●)-treated cells (n = 4). *, P < 0.05; **, P < 0.01.

Fig. 10. Amplification of cDNA sequences of bax (A) bak (B) and bcl-XL (C) and GAP-DH (constitutively expressed control) subsequent to incubation of HT-29 cells with or without (control) flavone for the indicated times. Bottom panels, the target gene:GAP-DH ratios from control (C) and flavone (●)-treated cells (n = 4). *, P < 0.05.
GROWTH INHIBITION OF COLON CELL CULTURES BY FLAVONE

ACKNOWLEDGMENTS

We greatly acknowledge Prof. Dr. K. Gietzen and Dr. L. Nei for teaching us the method of murine crypt preparation.

REFERENCES

Dietary Flavone Is a Potent Apoptosis Inducer in Human Colon Carcinoma Cells

Uwe Wenzel, Sabine Kuntz, Mathias D. Brendel, et al.

Cancer Res 2000;60:3823-3831.