Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound luteolin

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Lim, Do Y., Yoonhwa Jeong, Angela L. Tyner, and Jung H. Y. Park. Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound luteolin. Am J Physiol Gastrointest Liver Physiol 292: G66–G75, 2007. First published August 10, 2006; doi:10.1152/ajpgi.00248.2006.—Luteolin is 3’,4’,5,7-tetrahydroxyflavone found in celery, green pepper, and perilla leaf that inhibits tumorigenesis in animal models. We examined luteolin-mediated regulation of cell cycle progression and apoptosis in the HT-29 human colon cancer cell line. Luteolin decreased DNA synthesis and viable HT-29 cell numbers in a concentration-dependent manner. It inhibited cyclin-dependent kinase (CDK)4 and CDK2 activity, resulting in G1 arrest with a concomitant decrease of phosphorylation of retinoblastoma protein. Activities of CDK4 and cyclin D1 levels decreased after luteolin treatment, although no changes in expression of cyclin A, cyclin E, cyclin D1, or cyclin D2 were detected. Luteolin also promoted G1 progression by downregulating cyclin B1 expression and inhibiting cell division cycle (CDC)2 activity. Luteolin promoted apoptosis with increased activation of caspases 3, 7, and 9 and enhanced poly(ADP-ribose) polymerase cleavage and decreased expression of p21CIP1/WAF1, survivin, Mcl-1, Bcl-xL, and Mdm-2. Decreased expression of these key antiapoptotic proteins contributed to the increase in p53-independent apoptosis that was observed in HT-29 cells. We demonstrate that luteolin promotes both cell cycle arrest and apoptosis in the HT-29 colon cancer cell line, providing insight about the mechanisms underlying its antitumorigenic activities.

FLAVONOIDS ARE POLYPHENOLIC compounds found in numerous plants and are promising candidates for cancer prevention. Chemoprotective effects of various flavonoids have been observed in animal cancer models (reviewed in Ref. 1). Luteolin is a flavone that is found in celery, green pepper, chamomile tea, and perilla leaf, and there is some evidence showing the anticancer effect of this compound. Topical application of luteolin decreased tumor incidence and multiplicity of 7,12-dimethylbenz[a]anthracene-induced and 12-O-tetradecanoyl-phorbol-13-acetate-promoted skin papillomas in mice (34). In vitro studies have reported that luteolin induces apoptosis in a variety of culture models (4, 14, 29, 31). These results suggest that luteolin may be a potential chemopreventive and chemotherapeutic agent. The prevention and treatment of cancer by dietary factors that inhibit cell proliferation is an exciting prospect. The flavonoids, which are remarkably nontoxic (27), appear to have promise as anticancer agents.

Deregulated cell cycle progression, driven by activation of growth-stimulating oncogenes, is one of the primary characteristics of cancer cells. Cell cycle progression is tightly controlled by the regulation of expression and activity of cyclin/cyclin-dependent kinase (CDK) complexes (reviewed in Ref. 22). Cyclins interact with specific CDKs to regulate their activity and substrate specificity. CDK4/6 in association with D-type cyclins and CDK2 in association with cyclins E and A sequentially phosphorylate the retinoblastoma (Rb) protein, and regulate the G1/S phase transition and progression through S phase (25). A key regulator of the G1/S transition of the cell cycle is a complex of cell division cycle (CDC)2 (CDK1) and a B-type cyclin (23). Cyclin B-CDC2 complexes are regulated by phosphorylation and protein interaction events that tightly control the timing and extent of CDC2 activation.

In addition to regulation of the cell cycle, apoptosis plays an important role in the maintenance of tissue homeostasis. It is important for getting rid of damaged cells, and impaired apoptosis contributes to development of cancer (reviewed in Ref. 21). Apoptosis is carried out by the coordinated actions of several caspases. It is also tightly regulated by the balance of pro- and antiapoptotic proteins in the cell, which include members of the Bcl-2 family and inhibitors of apoptosis (IAPs) (13).

Luteolin has been shown to inhibit growth and to induce apoptosis in a variety of systems, but its mechanisms of action are not well understood. It inhibits growth of human thyroid cancer cell lines (39), HL-60 myeloid leukemia cells (17), MiaPaCa-2 pancreatic tumor cells (18), and HepG2 human hepatocellular carcinoma cells (38). It has also been reported that luteolin induced a G1 cell cycle arrest in OCM-1 human melanoma cells (3). In addition, Wang et al. (36) reported that the percentage of SW480 colon cancer cells at G0/M phase increased when cells were treated with between 5 and 30 μmol/l luteolin. Luteolin has been reported to induce apoptosis of different cultured cells, including hepatoma cells (29), leukemia HL-60 cells (4), and pancreatic cancer cells (18), and can enhance death receptor-induced apoptosis (14, 31). Luteolin was also reported to induce DNA damage leading to the apoptosis of human lung squamous carcinoma CH27 cells (19).

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The present study was performed to examine how luteolin regulates cell cycle progression and apoptosis in HT-29 human colon cancer cells. We demonstrate that luteolin-induced HT-29 cell growth arrest occurs at least partly because luteolin modulates the cell cycle machinery, resulting in cell cycle blockage at the G1/S and G2/M phases. In addition, luteolin downregulates a number of antipapoptotic proteins leading to an increase in apoptosis of HT-29 cells.

MATERIALS AND METHODS

Materials. Reagents were purchased from the following suppliers: luteolin (3',4',5,7-tetrahydroxylavone), anti-β-actin, RIA grade bovine serum albumin (BSA), and transferrin (Sigma, St. Louis, MO); horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (Amersham, Arlington Heights, IL); [γ-32P]ATP (NEN-Life Sciences, Boston, MA); antibodies against cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, cleaved poly(ADP-ribose) polymerase (PARP), cyclin B1, phospho-CDC2 (Tyr15), Bcl-xl, and phospho-Rb (Ser807/811; Cell Signaling, Beverly, MA); antibodies against p21CIP1/WAF1, cyclin A (c-19), cyclin E (M-20), CDK2 (M-2), CDK4 (c-22), Rb (c-15), CDC2 p34, proliferating cell nuclear antigen (PCNA, PC10), survivin, and Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell culture. We acquired the HT-29 cell line from the American Type Culture Collection (Manassas, VA) and maintained the cells in DMEM-F-12 containing 100 g/l of FBS, with 100,000 U/l of Type Culture Collection (Manassas, VA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (Amersham, Arlington Heights, IL); [γ-32P]ATP (NEN-Life Sciences, Boston, MA); antibodies against cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, cleaved poly(ADP-ribose) polymerase (PARP), cyclin B1, phospho-CDC2 (Tyr15), Bcl-xl, and phospho-Rb (Ser807/811; Cell Signaling, Beverly, MA); antibodies against p21CIP1/WAF1, cyclin A (c-19), cyclin E (M-20), CDK2 (M-2), CDK4 (c-22), Rb (c-15), CDC2 p34, proliferating cell nuclear antigen (PCNA, PC10), survivin, and Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell culture. We acquired the HT-29 cell line from the American Type Culture Collection (Manassas, VA) and maintained the cells in DMEM-F-12 containing 100 g/l of FBS, with 100,000 U/l of penicillin and 100 mg/l of streptomycin. We used HT-29 cells between passages 137 and 148 in these experiments. To determine the effects of luteolin, we plated the cells with DMEM-F-12 containing 10% FBS. Before luteolin treatment, the cell monolayer was subjected to serum starvation, and treated for 2 h with luteolin, as described above. For cell cycle analysis, the cells were trypsinized and fixed with 70% ethanol in PBS for at least 1 h at 4°C. The fixed cells were then washed with PBS and incubated with 50 g/l of RNase for at least 15 min at room temperature. The nuclei were then stained with 0.5 g/l propidium iodide and subjected to fluorescence-activated cell sorting analysis using FACScan (Becton Dickinson, Franklin Lakes, NJ).

To estimate the number of apoptotic cells, cells were trypsinized and then incubated in the dark with phycoerythrin-conjugated annexin V (BD Pharmingen) and 7-aminoactinomycin for 15 min at room temperature. Apoptotic cells were analyzed by flow cytometry, utilizing FACScan. The data were analyzed by use of Modfit version 1.2 software (Verity Software, Topsham, ME).

Immunoprecipitation and immunoblot analyses. Cells were lysed as described previously (7), and the protein contents were determined by using a BCA protein assay kit (Pierce, Rockford, IL). The cell lysates (750 μg protein) were immunoprecipitated with 1 μg of anti-CDK2, anti-CDK4, or anti-CDC2 antibody, as described previously (6). The total cell lysates (50 μg protein) or immunoprecipitated proteins were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBS-T and incubated with the appropriate primary antibodies for 1 h at room temperature. After washing with TBS-T, the blots were incubated with the secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The proteins were detected using horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Membranes were exposed to X-ray films. The expression levels of each protein were determined by the Quantity One software (Verity Software, Topsham, ME).
resolved on SDS-PAGE (4–20% or 10–20%) and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The blots were blocked for 1 h with 5% nonfat dry milk dissolved in 20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, and 0.1% Tween 20 and then incubated for 1 h with anti-p21CIP1/WAF1 (1:500), anti-p27KIP1 (1:1,000), anti-p53 (1:500), anti-cyclin D1 (1:200), anti-cyclin A (1:1,000), anti-cyclin E (1:1,000), anti-cyclin B1 (1:1,000), anti-CDK2 (1:1,000), anti-CDK4 (1:1,000), anti-CDK2 (1:1,000), anti-phospho-Rb (1:1,000), anti-Rb (1:1,000), anti-PCNA (1:1,000), anti-cleaved caspase-3 (1:1,000), anti-cleaved caspase-7 (1:1,000), anti-cleaved caspase-9 (1:1,000), anti-cleaved PARP (1:1,000), or anti-β-actin (1:2,000) antibodies. The blots were then incubated with anti-mouse or rabbit horseradish peroxidase-conjugated antibody.

Signals were detected via enhanced chemiluminescence, using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Densitometric analyses were conducted using the Bio-profile Bio-ID application (Vilber-Lourmat, France). The expression levels were normalized to β-actin, and the control (0 μmol/l luteolin) levels were set to 100%.

**RT-PCR.** Total RNA was isolated with TRI reagent (Sigma), and the cDNA was synthesized using 2 μg of total RNA and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), as described previously (16). The cDNA was amplified in accordance with the previously described procedure (20). For each combination of primers, the PCR amplification kinetics were determined, the number of cycles corresponding to the plateau was counted, and PCR was conducted within an exponential range. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. Bands corresponding to each of the specific PCR products were quantitated via the densitometric scanning of the exposed film, by use of the Bio-profile Bio-ID application. The mRNA levels were normalized to β-actin, with the control (0 μmol/l luteolin) level set to 100%.

**Analysis of CDK activity in luteolin-treated HT-29 cells.** The cell lysates (750 μg protein) were immunoprecipitated by using polyclonal antibody against CDK2, CDK2, or CDK4, washed in 20 mmol/l Tris-HCl (pH 7.5) and 4 mmol/l MgCl2 (CDK2 and CDK2 kinase buffer) or 50 mmol/l HEPES, 10 mmol/l β-glycerophosphate, 1 mmol/l NaF, 0.1 mmol/l sodium orthovanadate, and 10 mmol/l MgCl2 (CDK4 kinase buffer). After being washed, the beads were incubated for 30 min at 15 μl of kinase buffer with 2 μg of histone H1 (Roche, Basel, Switzerland) and 3 μCi of [γ-32P]ATP at 37°C (CDK2 and CDK2 kinase assay) or for 30 min in 30 μl of kinase buffer with 1 μg of Rb (769, Santa Cruz) and 10 μCi of [γ-32P]ATP at 30°C (CDK4 kinase assay). The reaction was halted via the boiling of the samples for 5 min in SDS sample buffer. The 32P-labeled histone H1 or Rb was resolved on SDS-PAGE, and the gel was dried and subjected to autoradiography. The signals were quantitated via the densitometric scanning of the exposed film.
Assessment of luteolin effect on CDK2 activity in cell-free system. Serum-starved HT-29 cells were lysed as described previously (7), after which 750 μg of total cellular protein were used to immunoprecipitate the active CDK2 complexes with anti-CDK2 antibody. After capturing with protein A-Sepharose and subsequent washes, the active immune complexes were used to estimate CDK2 activity in the presence of increasing luteolin concentrations in CDK2 kinase buffer containing 3 μCi [γ-32P]ATP and 2 μg histone H1. Reactions were incubated for 30 min at 37°C and then terminated via the addition of SDS loading dye and resolved on SDS-PAGE, after which the dried gels were subjected to autoradiography.

Statistical analysis. 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.

RESULTS

Luteolin induces cell cycle arrest in HT-29 cells. To study whether luteolin inhibits HT-29 cell growth, we examined the effect of luteolin on viable cell numbers over a 3-day period of treatment. Cells in monolayer culture were treated with luteolin (0–60 μmol/l) in serum-free medium, and the viable cell number was estimated by the MTT assay. As illustrated in Fig. 1A, luteolin decreased the viable HT-29 cell numbers in a dose-dependent manner with an 83% decrease in cell number within 72 h of addition of 60 μmol/l luteolin.

Incorporation of [3H]thymidine into the DNA of HT-29 cells was next measured 2 h after simultaneous addition of luteolin and [3H]thymidine to the media. Luteolin inhibited [3H]thymidine incorporation in a dose-dependent manner (Fig. 1B). To determine how luteolin regulates cell cycle progression of HT-29 cells, cells were treated with 0 or 60 μmol/l luteolin for 2 or 24 h, and DNA was stained with propidium iodide followed by FACS analysis. Within 2 h after the addition of luteolin, we observed an increase in the percentage of cells in G1, and the G1 phase accumulation was accompanied by a corresponding reduction in the percentages of cells in S phase (Fig. 1C). However, following a 24-h luteolin treatment the percentage of cells in G1 decreased and that of G2/M phase increased (by 42%) (Fig. 1D). These data indicate that luteolin promotes both G1 and G2/M arrest in HT-29 cells.

Luteolin decreases the activities of CDK4 and CDK2. We next examined the effects of luteolin on expression and activities of the CDKs and cyclins that regulate the G1/S phase transitions of the cell cycle. Treatment of cells with 60 μmol/l luteolin had no apparent effect on CDK4 protein levels during the 12-h period, but it decreased cyclin D1 levels within 2 h, and the effect lasted during the 12-h period (Fig. 2A). When HT-29 cells were incubated for 2 h with various concentrations of luteolin, there was a dose-dependent decrease in cyclin D1 levels with an 86% decrease in protein levels in cells treated with 60 μmol/l luteolin (Fig. 2B). In vitro kinase assays were performed to examine CDK4 activity. Total cell lysates were immunoprecipitated with CDK4 antibody followed by in vitro kinase assays using the GST-Rb COOH-terminus as a substrate (Fig. 3A). A decrease in CDK4 activity of 31.3 ± 6.2% (Fig. 3B) and a decrease in CDK2 activity of 42.8 ± 6.1% (Fig. 3C) were observed with 60 μmol/l luteolin.

Fig. 3. Luteolin inhibits CDK4 activity in HT-29 cells. A: luteolin suppresses CDK4 activity within 2 h. HT-29 cells were treated with 0 or 60 μmol/l luteolin for the indicated periods. Total cell lysates (750 μg protein) were immunoprecipitated with an anti-CDK4 antibody and protein A-Sepharose, and in vitro kinase assays were performed using glutathione S-transferase (GST)-retinoblastoma (Rb) as a substrate. An autoradiograph of the dried gel (CDK4 activity) and a photograph of chemiluminescent detection of the immunoblot (CDK4 protein) are shown. The relative abundance of 32P-Rb (CDK4 activity) to its own CDK4 was quantified, and the control levels were set to 100%. The adjusted mean ± SE (n = 3) for CDK4 activity is shown above the blot. *Different from 0 μmol/l luteolin at a time, P < 0.05. B and C: luteolin induces a dose-dependent decrease in CDK4 activity. HT-29 cells were treated for 2 h with various concentrations of luteolin. CDK4 immune complexes were analyzed by an in vitro kinase assay using GST-Rb as a substrate (CDK4 activity) or by Western blotting (cyclin D1 and CDK4). The relative abundance of each band to its own band was quantified, and the control levels were set at 100%. The adjusted mean ± SE (n = 3) for cyclin D1 is shown above the blot (B) and that for CDK4 activity (32P-Rb) was shown in the bar graph (C). Decreased CDK4 activity correlates with a decrease in cyclin D1 levels.
The effect was persistent during the 12-h incubation period. The decreased CDK4 activity following the 2-h luteolin treatment was concentration dependent and correlated with decreased binding of cyclin D1 to this enzyme (Fig. 3, B and C).

Levels of CDK2, cyclin A, and cyclin E measured by immunoblotting were not changed by addition of 60 μmol/l luteolin over a 12-h period (data not shown). However, when CDK2 was immunoprecipitated with CDK2 antibody and its activity was measured by using in vitro kinase assays with histone H1 as a substrate, there was a significant inhibition of CDK2 activity following addition of luteolin (Fig. 4, A and B). At 2 h, a significant (38%) decrease in CDK2 activity (P < 0.05) was observed in cells treated with 40 μmol/l luteolin (Fig. 4B). To determine whether luteolin could modulate activity of CDK2 by direct interaction with this kinase, total cell lysates obtained from serum-starved cells were immunoprecipitated with CDK2 antibody, and the immune complex was incubated with increasing concentrations of luteolin in the kinase reaction. Luteolin dose dependently decreased CDK2 activity in cell-free system (Fig. 4C). These results indicate that the CDK2 inhibition may be the result of a direct interaction of luteolin with this kinase.

Luteolin decreases phospho-Rb and increases hypophosphorylated Rb levels. Members of the Rb protein family are phosphorylated and inhibited by CDK4 and CDK2, leading to...
activation of gene expression required for cell cycle progression. Since the activities of CDK2 and CDK4 were inhibited by luteolin, we examined whether addition of luteolin led to decreased phosphorylation of endogenous Rb in HT-29 cells. Immunoblotting was performed with total cell lysates and phospho-Rb antibody. Phospho-Rb levels decreased by 53% within 30 min after addition of luteolin (Fig. 5A), and a dose-dependent reduction was observed at 2 h after addition of luteolin (Fig. 5B). When immunoblotting was performed using total Rb antibody, two bands were detected with an increase in the intensity of the lower band (hypophosphorylated Rb) (Fig. 5). No changes were detected in expression of PCNA, which performs essential roles in DNA replication and repair and is often used as a marker of proliferation (reviewed in Ref. 33) (Fig. 5).

**Luteolin decreases CDC2 activity.** To elucidate mechanisms underlying the G2/M arrest observed after addition of luteolin, expression and activity of CDC2 (CDK1) kinase and cyclin B1, proteins that regulate the G2-M transition, were investigated in cells that were treated for 24 h with various concentrations of luteolin. Immunoblotting of total cell lysates revealed that luteolin significantly decreased cyclin B1 levels in a dose-dependent manner with a 70% decrease in protein levels after addition of 60 μmol/l luteolin, whereas it did not alter either CDC2 or phospho-CDC2 levels (Fig. 6A). In vitro kinase assays were performed to examine CDC2 activity. CDC2 was immunoprecipitated and its ability to phosphorylate histone H1 was examined. CDC2 kinase activity decreased in a dose-dependent manner following the addition of luteolin (Fig. 6B). An 80% decrease in the enzyme activity was observed in cells treated with 60 μmol/l luteolin. Luteolin-mediated negative regulation of CDC2 would contribute to increased G2/M arrest.

**Luteolin treatment leads to decreased p21 and p27 expression.** The activity of CDK-cyclin complex is controlled by CDK-inhibitory proteins. We examined the effect of luteolin on p21cip1/waf1 and p27kip1 protein levels. p21cip1/waf1 levels decreased in HT-29 cells at 0.5 h after treatment with 60 μmol/l luteolin, which lasted until 12 h. A more modest decrease in p27kip1 levels was also observed after luteolin treatment (Fig. 7A). Although it is well known that p21 and p27
are negative regulators of CDK activity, these proteins may also act as positive regulators of cyclin D-dependent kinases (5).

To examine whether luteolin regulates p21^{CIP1/WAF1} expression at the RNA level, RT-PCR analyses were performed. Treatment of HT-29 cells with increasing concentrations of luteolin led to a concentration-dependent decrease in p21^{CIP1/WAF1} mRNA levels, with a 51% decrease in p21^{CIP1/WAF1} transcripts after the addition of 60 μmol/l luteolin (Fig. 7B).

Luteolin induces apoptosis and downregulation of antiapoptotic proteins. The number of viable cells decreased following addition of luteolin to HT-29 cells. To determine whether cells were undergoing apoptosis in addition to cell cycle arrest, changes in phosphatidylserine membrane localization were analyzed by use of annexin V. A significant increase in the number of cells undergoing apoptosis was observed at 48 h after addition of 60 μM luteolin (Fig. 8A). Further evidence of enhanced apoptosis was provided by increased activation of caspases 3, 7, and 9 and enhanced PARP cleavage (Fig. 8B).

Apoptosis is regulated by a variety of proapoptotic and antiapoptotic proteins. We examined the expression of several antiapoptotic proteins including Bcl-xL, survivin, and Mcl-1 and found that levels of these proteins significantly decreased after luteolin treatment for 2 h (Fig. 8C). Interestingly, p53 levels increased, probably owing to the downregulation of the p53 negative regulator Mdm-2. However, apoptosis is p53 independent in HT-29 cells because these cells have mutant p53 (35).

**DISCUSSION**

Understanding how dietary components regulate proliferation and cell survival could play a critical role in development of new agents that can prevent and treat cancer with low toxicity. Previously, luteolin was reported to induce G2/M cell cycle arrest in SW489 cells (36). We observed that luteolin promoted both G1 and G2/M arrest in HT-29 colon cancer cells. Luteolin inhibited the activities of CDK4 and CDK2 leading to G1 arrest within 2 h after treatment. Addition of luteolin to HT-29 cells led to a dose-dependent decrease in CDK2 activity, which contributed to G2/M arrest at 24 h.

Members of the Rb family are important substrates of the CDKs, and cell cycle progression at the G1 check point coincides with the phosphorylation and consequent inactivation of Rb proteins (reviewed in Ref. 26). Cyclin D-dependent kinases initiate Rb phosphorylation in mid-G1 phase, after which cyclin E-CDK2 becomes active and completes this process by additional phosphorylation of Rb (30, 32, 37). In the present study, luteolin decreased levels of phospho-Rb and increased levels of hypophosphorylated Rb in a dose-dependent manner, indicating that luteolin inhibition of CDK activity resulted in reduced phosphorylation of CDK substrates. These results indicate that the increased hypophosphorylated Rb (or decreased phospho-Rb) contributes to G1/S arrest observed in luteolin-treated cells.

In addition to demonstrating that luteolin inhibits a variety of CDKs, we show that luteolin treatment leads to the downregulation of several genes including cyclin D1 and cyclin B1 that are required for CDK4/6 and CDC2 activation and progression through the G1 and G2/M checkpoints. The negative regulation of these positive regulators of cell cycle progression would impair CDK activities and contribute to the increase in G1 and G2/M arrest following addition of luteolin.
We found that luteolin promotes apoptosis of HT-29 cells and luteolin treatment led to decreased expression of multiple antiapoptotic proteins including Bcl-xL, Mcl-1, survivin, p21, and Mdm-2. Bcl-xL and Mcl-1 are antiapoptotic members of the Bcl-2 family, and their elimination played an important role in apoptosis in Hela cells (24). Survivin is a member of the IAP family of antiapoptotic proteins that bind and inhibit caspases, and IAP overexpression is often a poor prognostic marker in cancers (28). Expression of p21 often leads to inhibition of apoptosis, and its downregulation may be important for efficient apoptosis (10–12). Mdm-2 is a negative regulator of p53 that plays a critical role in apoptosis after DNA damage (9). The ability of luteolin to downregulate all of these different antiapoptotic proteins would contribute to the increase in apoptosis observed in the HT-29 cells.

The anticancer drug flavopiridol, currently being evaluated in clinical trials, is a CDK inhibitor that inhibits activity of the positive transcription elongation factor-b (P-TEFb), which is composed of CDK9 and cyclin T1 (2). Like luteolin, flavopiridol downregulates numerous cell cycle and antiapoptotic proteins including cyclin D, cyclin B, Bcl-2, Mcl-1, survivin, p21, p27, and Mdm-2. These proteins and the RNAs that encode them have short half-lives (reviewed in Ref. 2). In flavopiridol-treated cells, expression of short-lived proteins is rapidly downregulated in the absence of transcription. Our data indicate that luteolin inhibits multiple CDKs and that treatment of HT-29 cells with luteolin leads to decreased expression of multiple proteins with short half-lives. We also show that decreased expression of p21 is regulated at the level of mRNA expression. Future studies are needed to examine the intriguing possibility that luteolin may be a global transcriptional inhibitor like flavopiridol, targeting pTEFb (8).

In summary, luteolin induces G1 and G2/M cell-cycle arrest, which is mediated by inhibition of the activities of CDK2, CDK4, and CDC2 in HT-29 human colon cancer cells. In addition, luteolin promotes apoptosis through downregulation of several antiapoptotic proteins. Cell cycle check points and apoptosis play critical roles in the molecular pathogenesis of cancer and can influence the outcome of chemotherapy and radiotherapy. Because of this, dietary compounds such as luteolin hold considerable promise for cancer treatment. Our study provides some of the molecular basis for using luteolin as a possible antitumorigenic agent.

**GRANTS**

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Fig. 8. Luteolin regulates apoptosis in HT-29 cells. A: luteolin decreases cell viability and induces apoptosis. HT-29 cells were treated for 48 h with 0 or 60 μmol/l of luteolin. Cells were trypsinized, loaded with 7-amino-actinomycin D and annexin V, and then analyzed by flow cytometry. The numbers of living cells and early apoptotic cells are expressed as a percentage of total cell number. Each bar represents mean ± SE (n = 6). *Significantly different from 0 μmol/l luteolin, P < 0.05. B: increased caspase activation and poly(ADP-ribose) polymerase (PARP) cleavage are detected after luteolin treatment. HT-29 cells were treated for 48 h with 0 or 60 μmol/l of luteolin. The total cell lysates were subjected to immunoblotting with antibodies against cleaved caspase-9, cleaved caspase-3, cleaved caspase-7, cleaved PARP, or β-actin. C: luteolin treatment leads to a rapid decrease in levels of antiapoptotic proteins. HT-29 cells were treated with 0 or 60 μmol/l luteolin for 2 h. Total cell lysates were subjected to immunoblotting with antibodies against p53, Mdm-2, Bcl-xL, survivin, Mcl-1, or β-actin. For β and C, photographs of chemiluminescent detection of the bands, 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. *Different from 0 μmol/l luteolin, P < 0.05.

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