Lignans Are Involved in the Antitumor Activity of Wheat Bran in Colon Cancer SW480 Cells

Hongyan Qu, Ronald L. Madl,* Dolores J. Takemoto,† Richard C. Baybutt, and Weiqun Wang3

Department of Human Nutrition, *Department of Grain Science, and †Department of Biochemistry, Kansas State University, Manhattan, KS 66506

ABSTRACT Wheat bran was shown to provide protection against colorectal cancer in human intervention and animal studies. Our recent study showed, however, that antitumor activities of wheat bran from various wheat cultivars differed significantly even when wheat fiber was equal in diets. We hypothesized that phytochemical lignans in wheat bran may account for the differences among wheat cultivars in cancer prevention. The concentration of a major lignan, secoisolariciresinol diglycoside, was determined by HPLC in 4 selected wheat cultivars (i.e., Madison, Ernie, Betty, and Arapahoe). The lignan concentrations and their antitumor activities, previously determined in APC-Min mice, were correlated (r = 0.73, P < 0.02). The cancer preventive mechanisms of 2 prominent lignan metabolites (enterolactone and enterodiol) were further studied in human colon cancer SW480 cells. Treatment with enterolactone and enterodiol, alone or in combination, at 0–40 μmol/L resulted in dose- and time-dependent decreases in cell numbers. Although the cytotoxicity as measured by trypan blue staining in adherent cells was not affected, DNA flow cytometric analysis indicated that the treatments induced cell cycle arrest at the S-phase. Western blot analysis for cyclin A, a required protein for S/G2 transition, showed that the cyclin A protein levels decreased after treatment with enterodiol or the combination of enterolactone and enterodiol at 40 μmol/L for 72 h. Apoptosis analysis by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay showed an increased percentage of apoptotic cells in the floating cells after enterodiol alone or combined treatments. These results suggest for the first time that lignans may contribute, at least in part, to the cancer prevention by wheat bran observed in APC-Min mice. Inhibition of cancer cell growth by lignan metabolites seems to be mediated by cytostatic and apoptotic mechanisms.


KEY WORDS: • lignans • wheat bran • SW480 colon cancer cells • cell cycle arrest • apoptosis

Adequate dietary fiber intakes from whole grains were shown to reduce colon cancer risk in human intervention and animal studies (1–5), but experimental evidence concerning colon cancer prevention obtained using different fiber sources is controversial (6). A recent study in the Takemoto laboratory (7) demonstrated that antitumor activities of various wheat cultivars were significantly different, even when the wheat fiber content was equal in diets fed to adenomatous polyposis coli (APC)4 truncated Min mice. Wheat bran contains many phytochemicals that may play an important role in colon cancer prevention (8–13).

Lignans are a group of diphenolic compounds that are present in the outer layers of grains. A considerable number of studies were conducted in flaxseed, which has a large content of lignans (14,15). In wheat, lignans were found in the bran layer, and the major lignan in wheat bran is secoisolariciresinol diglycoside (SDG) (16,17). When consumed, SDG is converted by intestinal microflora to 2 lignan metabolites, enterodiol and enterolactone (18–20) (Fig. 1). Lignan metabolites act as antioxidants and free radical scavengers, which may decrease the risk of cancer development (21). They were shown to reduce epithelial cell proliferation and the number of aberrant crypt foci in animal models (22,23). In addition, they were reported to modulate the effects of estrogen by binding competitively with estrogen to estrogen receptors, thus reducing the risk of estrogen-dependent cancer (24,25). We reported previously that enterolactone was a strong antioxidant against human LDL oxidation (26). We also demonstrated that enterolactone was capable of inhibiting colon cancer cell growth by inducing phase II detoxification enzyme activity (27).

Cancer progression has been thought to lead to a loss of cell cycle regulation (28). We and other workers reported that some chemopreventive agents inhibited tumor growth by interrupting cell cycle progression (29–31). The objectives of

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3 To whom correspondence should be addressed. E-mail: wwang@ksu.edu.
4 Abbreviations used: APC, adenomatous polyposis coli; DMSO, dimethyl sulfoxide; SDG, secoisolariciresinol diglycoside.

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this study were to determine the lignan concentrations in the bran of 4 selected wheat cultivars (Madison, Ernie, Betty, and Arapahoe) and then compare the concentrations with their antitumor activities. The effects of lignan metabolites on colonic cancer cell growth and the potential underlying mechanisms of cell cycle perturbation and induction of apoptosis were further investigated.

MATERIALS AND METHODS

SDG extraction. Four wheat cultivars, including Madison (soft red winter cultivar), Ernie (soft red winter cultivar), Betty (hard white winter cultivar), and Arapahoe (hard red winter cultivar) were obtained from the Kansas Crop Improvement Association. Wheat was first tempered to 15% moisture, then milled through a laboratory mill (UZWIL BUHLER 120767) in the Department of Grain Science, Kansas State University. Bran was separated using a 64-grit gauze cloth. Wheat bran (20 g) was mixed with 200 mL of 70% v/v aqueous methanol containing flavone as an internal standard at 5 μmol/L. The mixture was refluxed at 70°C for 4 h. The supernatant obtained by centrifuging at 200 × g for 10 min was evaporated with a rotary evaporator in a vacuum until completely dry. The dried sample was subjected to a base hydrolysis with 20 mL of 0.3 mol/L NaOH for 2 d at 40°C, and then acidified with 2 mol/L H2SO4 to pH 3. An aliquot of 5 mL of sample was passed through a SPE-C18 column (5 μL, Alltech), eluted with 5 mL methanol, and 5 mL deionized water. After being washed with 10 mL deionized water, SDG was eluted with 10 mL of 50% methanol.

HPLC quantitation of SDG. HPLC quantitation of SDG was adopted from the published methods (32–34) with a minor modification. Briefly, SDG was separated by HPLC chromatography on a C18 column (5 μm, 250 × 4.6 mm, Alltech), eluted with a 5% acetonitrile in pH 2.8, 0.01 mol/L phosphate buffer (solvent A) over 100% acetonitrile (solvent B) at a flow rate of 1 mL/min and a gradient condition. The SDG peak was identified according to both retention time and spectrum by comparison with a standard SDG provided by Dr. Alister D. Muir from the Agriculture-food Canada (Saskatchewan, Canada). SDG was dissolved in dimethyl sulfoxide (DMSO) and diluted in methanol. A linear HPLC calibration curve for standard SDG was obtained for the concentrations at 0–200 μmol/L. The recovery of SDG from the extracted samples was ~35–40% as determined by the internal standard flavone.

Cell treatment and cell growth assay. Human colon cancer cell line SW480 was purchased from ATCC and cultured in DMEM (Sigma) with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were then cultured in 6-well plates at 37°C with 5% CO2 and treated with enterolactone and enterodiol, alone or in combination, at 0–40 μmol/L for 24–72 h, at which time the cells reached <100% confluence. Both enterolactone and enterodiol were purchased from Sigma-Aldrich. They were dissolved in DMSO and mixed with fresh medium to achieve the desired concentration. The final DMSO concentration in all cultures was 0.2%, a concentration that did not alter cell growth or cell cycle measurements compared with the vehicle-free media. After treatment, the cells were detached with trypsin-EDTA, and the cell number was counted with a hemacytometer. All four 1-mm corner squares of the hemacytometer were counted, and an average was calculated.

Cytotoxicity assay. Cell viability was measured in adherent cells by trypan blue staining. The viable cell numbers in treated cells were compared with that in vehicle controls.

Cell cycle analysis. The cells were fixed in 70% ice-cold ethanol and stored at 4°C until analysis. Fixed cells were centrifuged at 200 × g for 5 min. The supernatant was discarded, and the pelleted was washed with 0.01 mol/L phosphate buffer saline solution, pH 7.4. The cells were resuspended in phosphate buffer containing 20 g/L of propidium iodide and 5000 U/L of RNase (Promega) at 37°C for 30 min. DNA flow cytometric analysis (FACSCalibur, Becton Dickinson) was performed with an excitation at 488 nm and an emission at 630 nm.

Western blot analysis. Treated cells were harvested and suspended in Triton lysis buffer. Protein concentration was measured in the supernatant and 60 μg of whole cell protein was electrophoresed on 12% SDS polyacrylamide gels and transferred to pure nitrocellulose membrane with a semidyed transfer cell (Trans-blot SD cell, Bio-Rad). The membrane was blocked in 5% nonfat dry milk overnight at 4°C and washed with 0.5% PBS-Tween (x:v) 6 times for 5 min each time. The membrane was incubated with a 1:200 dilution of rabbit polyclonal IgG against cyclin A (Santa Cruz Biotechnology) for 1 h at room temperature. β-Actin was used as loading control. After washing, the membrane was incubated in donkey anti-rabbit IgG of cyclin A-horseradish peroxidase-conjugated (1:5000; Santa Cruz Biotechnology) and β-actin (1:2000, Santa Cruz Biotechnology) for 1 h at room temperature. The blot was scanned and the band was detected using FluoroChem™ 8800 Advanced Imagine System (Alpha Innotech). Based on the molecular weight of cyclin A and β-actin, we identified the 55-kDa band as cyclin A and the 43-kDa band as β-actin. Cyclin A protein levels were normalized as ratios of the density of β-actin in the same sample and then presented as the percentages of the vehicle controls.

Apoptosis analysis. Both adherent and floating cells were collected separately and fixed by 1% paraformaldehyde and 70% ice-cold ethanol at concentration of 1 × 10⁶ cells/L. Fixed cells were analyzed for apoptosis by the APO-BrdUTM terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay according to commercial instructions (BD Biosciences Pharmingen).

Statistical analysis. All of the data were analyzed using SAS, version 8.1. The cell growth and cell cycle data were analyzed by 2-way ANOVA using the General Linear Model procedure followed by Fisher’s protected Least Square Difference. The cyclin A data and apoptosis data were analyzed by 1-way ANOVA followed by Fisher’s protected Least Square Difference. Covariate-adjusted means were compared by the levels of the independent variable. Linear regression was used to determine the relation between lignan concentrations and antitumor activities in 4 wheat cultivars by Pearson’s correlation coefficients (r). A probability < 0.05 was considered significant.

RESULTS

Lignan concentrations and antitumor activities in wheat bran from 4 wheat cultivars. The concentrations of SDG were 82.9 ± 27.2 μg/g in Madison (n = 3), 52.3 ± 31.3 μg/g in Ernie (n = 2), and 42.7 ± 0.1 μg/g in Betty (n = 2). SDG was undetectable in Arapahoe. Based upon the published data in APC-Min mice (7), we calculated antitumor activities using the following formula: (total tumor numbers in mice fed basal diet – total tumor numbers in mice fed wheat bran diet)/total tumor numbers in mice fed basal diet. The antitumor activities were 58.6 ± 1.8 in Madison, 36.1 ± 3.5 in Ernie, 23.7 ± 4.5 in Betty, and 27.2 ± 2.6 in Arapahoe. SDG concentrations and antitumor activities were correlated (r = 0.73, P < 0.02).

Cell growth. Treatment of SW480 cells with enterolactone and enterodiol, alone or in combination, at 0–40 μmol/L
resulted in dose- and time-dependent decreases in cell numbers compared with the vehicle control (Fig. 2). At 20 and 40 μmol/L, the inhibition by the combined treatment appeared more severe than that of each lignan metabolite alone.

**Cytotoxicity.** Cell viability was generally >95% in adherent cells, and the treated cells did not differ from the vehicle controls (data not shown).

**Cell cycle.** Treatment with enterolactone, enterodiol, or their combination induced cell cycle arrest at S phase. Representative flow cytometry profiles of SW480 cells after treatment with enterolactone at 40 μmol/L for 24, 48, and 72 h are presented in Figure 3 (upper panel). The percentage of cells in S-phase was significantly increased after treatment with each lignan metabolite or a combination of both at the higher concentrations (≥20 μmol/L) at various time points (Fig. 3, lower panel). As the percentage of cells in S phase increased, the percentage of cells in both G1 and G2/M phases decreased correspondingly.

**Cyclin A protein levels.** Cyclin A protein levels were significantly lower in cells treated with enterolactone and the combination compared with the vehicle control (Fig. 4).

**Apoptosis.** The treatments did not affect apoptosis in adherent cells compared with the vehicle controls (data not shown). However, the percentage of apoptotic cells in floating cells was significantly increased after treatment with enterodiol or the combination (Fig. 5).

**DISCUSSION**

Many data suggest that lignans in flaxseed or rye bran contribute to cancer prevention (35–40). However, Kranen et al. (41) found that some lignan precursors from flaxseed or rye bran did not inhibit the development of intestinal benign tumors in APC mutated Min Mice. Using the same Min mouse model, we found previously that wheat bran did inhibit the development of intestinal neoplasia, but the degree of inhibition differed significantly among the various wheat cultivars (7). Using those data and cultivar SDG concentrations measured in the present study, we found that the concentrations of SDG in the wheat cultivars were correlated with their antitumor activities. SDG concentrations in the 4 wheat lines...
varied from undetectable in Arapahoe to 83 μg/g in Madison, explaining in part the controversial results in the literature concerning anticancer properties of wheat lignans.

To confirm the cancer preventive activities, we further assessed the effects of lignan metabolites in human colon cancer SW480 cells. The SW480 cell line was shown previously to be very sensitive to the effects of phytochemical flavones because of specific genetic mutations (31,42). In this study, we found that SW480 cells growth was significantly inhibited by both lignan metabolites, individually or combined. The reduction in cell number did not seem to be due to toxicity because cell viability was not changed by any of the treatments. Our treatment of SW480 cells with enteroceptone, enterodiol, or a combination of both, blocked cell cycle progression in S-phase, which was further confirmed by Western blot analysis for cyclin A protein, a required protein for S/G2 transition. Cyclin A decreased significantly after treatment with the lignan enterodiol or the combination of enterolactone and enterodiol.

Cell cycle arrest may trigger the DNA repair machine, leading to apoptosis, as suggested by our data from apoptotic analysis. Treatment of SW480 cells with enterodiol, or the combination of enterolactone and enterodiol significantly increased the numbers of apoptotic cells compared with the vehicle controls. The induction of apoptosis in colon cancer cells by lignans is in agreement with the work of Haukost et al. (43), which showed that naturally occurring lignans induced apoptosis in colorectal tumor cells through changing mitochondrial membrane potential and downregulation of the antiapoptotic protein, bcl(x).

The concentrations of enterolactone and enterodiol used in this study were <40 μmol/L, which are high relative to plasma levels in humans or animals (usually in the nanomolar range) (44). In addition to a low plant lignan intake, the low levels of lignan metabolites in plasma are likely due to their efficient enterohepatic circulation reported by others (44). This suggests that large amounts of the lignan metabolites could be taken up by the liver, by-passing the systemic circulation, and then be secreted into the intestinal lumen via bile. A high concentration of lignan metabolites, therefore, might be achievable in the enterocytes of the gut lumen.

In conclusion, lignan SDG concentrations in wheat bran from 4 selected wheat cultivars were significantly different, but correlated with their anticancer activities, suggesting that SDG may contribute to the cancer preventive activity of wheat bran. Treatment of human colon cancer cells with the SDG metabolites, enterolactone and enterodiol, alone or in combination, resulted in dose- and time-dependent decreases in cell numbers. The inhibition of cell growth by lignan metabolites seems to be mediated by cytostatic and apoptotic mechanisms.

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