Wheat Type (Class) Influences Development and Regression of Colon Cancer Risk Markers in Rats

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We previously found red wheat more effective than white wheat in reducing colon cancer risk in rats when fed during initiation and postinitiation stages. Here we examine the effect of wheat on colon cancer risk in early and late postinitiation stages in carcinogen-treated rats. Four groups were fed a basal diet, 1 group a red wheat diet, and 1 group a white wheat diet. After 6 wk, 1 basal, the red and white groups were killed (early postinitiation stage). Of the remaining basal groups, 1 continued on the basal diet, 1 was switched to red and another to white wheat for 8 more wk (late postinitiation stage). Red and white wheat significantly reduced morphological and biochemical (β-catenin accumulated crypts) markers in both early and late postinitiation stages. Both wheat diets reduced dysplasia markers (sialomucin-expressing ACF and mucin depleted foci), compared to the basal diet, during the late postinitiation stage, but red wheat more so. Only red wheat significantly reduced the number of metallothionein-positive crypts, a stem cell mutation marker, in both stages. Overall, red wheat flour reduced risk markers more than white wheat flour, and this was more pronounced in the late post-initiation stage.

INTRODUCTION
Epidemiological studies have reported an association between whole grain consumption and reduced colon cancer risk (1,2). A recent epidemiological study found that whole grain wheat, but not whole grain rye or oats, was associated with a lower incidence of colorectal cancer (3). As wheat is the major cereal consumed in western cultures, this points to wheat as the cereal of greatest interest in terms of chemoprevention.

However, the epidemiological studies suggesting that whole grain consumption reduces risk of colorectal cancer are open to an alternative interpretation. There are several types (classes) of wheat, each used in different types of foods. Red wheats (primarily hard red) are the class of wheat used in breads whereas white wheats (primarily soft white) are used in cakes, pastries, and cookies. Durum wheat is used exclusively to make pasta. Most whole grain products are whole grain breads, and therefore made with red wheats. Most refined grain products are made with white wheats. Thus, studies focusing on differences in whole versus refined grains are largely examining differences between red and white wheat consumption.

We have found that diets containing hard red wheat reduce colonic precancerous lesions [aberrant crypt foci (ACF)] relative to diets containing soft white wheat, but that the state of refining had no influence (4). That is, only the class of wheat (hard red vs. soft white) influenced ACF number, not whether the wheat was whole or refined. In this study the wheat-containing diets were fed prior to, during, and after carcinogen administration. Therefore, at what stage of carcinogenesis wheat is acting at, the initiation stage or postinitiation stage, could not be determined. In the present study our first objective was to examine the effect of red and white wheats on colon cancer risk when fed only in the post-initiation stage.

Individuals consuming a Western-style dietary pattern appear to have a greater susceptibility for colon cancer relative to those consuming a prudent diet that is lower in red meat and higher in vegetables (5). Consequently, many individuals in Western societies are presumably in a high risk state for colon cancer. Thus, the second objective was to examine whether wheat diets would regress risk in animals who are already in a high risk state.

To ascertain colon cancer risk, a variety of different markers have been used. ACF are colonic precancerous lesions frequently used as a morphological indicator of colon cancer risk, with large ACF (≥4 AC/ACF), that correlate with eventual tumor formation, of particular interest (6). ACF may be broadly categorized as either dysplastic or hyperplastic. Dysplastic ACF, which appear to be more advanced towards tumorigenesis, can be distinguished from hyperplastic ACF based on mucin production (7). Normal colonic crypts and hyperplastic ACF produce sulfomucin, whereas dysplastic crypts produce either a mix of sulfomucin and sialomucin or only sialomucin (8). A strong trend has been noted between the number of sialomucin-producing ACF and development of
colonic tumors (9). Mucin-depleted foci (MDF), which may be the most dysplastic type of ACF, are suggested to be the most tumorigenic type of ACF (10). In addition, flat ACF, which are not elevated above the mucosal surface, appear to be severely dysplastic and may also be closely related to tumorigenesis (11). Finally, most dysplastic crypts accumulate severely dysplastic and may also be closely related to tumorigenic type of ACF (10). In addition, flat ACF, which are suggested to be premalignant lesions (13).

Considerable evidence indicates that many, perhaps most, cancers are a disease of stem cells. Colon cancer in particular has substantial evidence supporting a cancer stem cell etiology (see Ref. 14 for review). Stem cells, being long-lived and self-renewing, can persist sufficiently long to accumulate the number of mutagenic events thought necessary to transform to a cancer cell. As with all stem cells, cancer stem cells (CSC) have self-renewal ability and high proliferative activity, which are maintained by the Wnt signaling pathway (15, 16). In colon cancer development, when this pathway becomes dysregulated, crypt stem cells lose their normal regulatory controls on cell division and undergo proliferation and neoplastic transformation, resulting in an expansion of the CSC population, which eventually fill the entire crypt (17). There is strong evidence that the persistent overexpression of metallothionein (MT) in colonic crypts indicates mutations in colon crypt stem cells (18). The number of MT-positive crypts shows a dose response to colon carcinogen administration and correlates with ACF number (18–20). Further, MT immunoreactivity in patients with colonic adenomas correlates inversely with immunoreactivity of the tumor suppressor p53 (21). Finally, MT overexpression in colon tumors is associated with a poor prognosis in humans (22). As immunohistochemical studies suggest that an increase in crypt cells displaying a stem cell phenotype is associated with colon carcinogenesis, quantification of this marker would likely be useful as a marker of colon cancer risk (23).

The final objective of this study was to examine the correlations among the different markers of colon cancer risk measured in this study: AC and ACF, flat ACF, sialomucin expression within ACF, MDF, BCAC, and MT-positive crypts.

MATERIALS AND METHODS

Animals and Diets

Male Wistar rats, weighing between 50 and 75 g, were purchased from Harlan Sprague Dawley (Indianapolis, IN). All rats were housed individually in a room maintained at 20°C and a 12-h light/dark cycle. Throughout the study, diet and distilled water were given ad libitum. Animal handling and housing followed National Institutes of Health guidelines and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee. The basal diet was a modification of the AIN-93G purified diet (24). The 2 wheat-based diets consisted of 61.5% of wheat flour by weight, either as a refined soft white or refined hard red flour. The refined hard red and refined soft white wheat flours were a gift of ConAgra Foods (Commerce City, CO). As the wheat flours were a commercial grade, each class of flour (i.e., soft white and hard red) represents a mixture of varieties within that class. The composition of the diets is shown in Table 1. The concentration of total protein and dietary fiber was constant for all diets, and the concentration of digestible carbohydrate and fat was similar.

Experimental Design

Rats were adapted to the basal diet for 10 days, then administered the colon carcinogen 1,2-dimethylhydrazine (50 mg/kg body weight) subcutaneously, 2 times, a week apart. Five days after the second carcinogen administration rats were divided into 6 groups (n = 15/group). Two groups were fed either the refined hard red or refined soft white wheat diets. The remaining 4 groups continued to be fed the basal diet. After 6 wk of feeding, the hard red, soft white, and 1 basal diet group were killed to represent the early postinitiation stage of colon cancer. The remaining 3 basal diet groups were divided into basal, hard red wheat, and soft white wheat groups and fed their corresponding diets for 8 more wk. These were considered the late postinitiation stage groups. The experimental design is illustrated in Fig. 1. Body weights were recorded weekly and food intake was recorded biweekly.

Colon Sample Preparation

Colon samples were removed and fixed as previously described (25). The most distal 2.5 cm of colon was used for

<table>
<thead>
<tr>
<th>TABLE 1 Composition of the diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet constituent(^a) (g/kg)</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Flour(^b)</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>Corn starch</td>
</tr>
<tr>
<td>Casein</td>
</tr>
<tr>
<td>Cellulose</td>
</tr>
<tr>
<td>Soybean oil</td>
</tr>
<tr>
<td>% Carbohydrate</td>
</tr>
<tr>
<td>% Protein</td>
</tr>
<tr>
<td>% Fiber</td>
</tr>
<tr>
<td>% Fat</td>
</tr>
</tbody>
</table>

\(^a\) Constant ingredients include (g/kg): AIN-93G mineral mix, 35; AIN-93G vitamin mix, 10; L-cystine, 3; choline bitartrate, 2.5; cholesterol, 1.24; butylated hydroxytoluene, 0.014.

\(^b\) Obtained from ConAgra Food Ingredients (Commerce City, CO). Organic bread flour was the product used for refined hard red flour and Famous Pastry flour was the product used for refined soft white flour.
FIG. 1. Experimental design. All animals were adapted to the basal diet for 10 days, followed by 2 s.c. injections of the colon-specific carcinogen 1,2-dimethylhydrazine (50 mg/kg body weight), 1 wk apart. Five days after the second injection of carcinogen, 4 groups of rats were fed the basal diet, 1 group the red wheat diet, and 1 the white wheat diet. One basal group, the red wheat group, and the white wheat group were killed 6 wk later, to represent an early postinitiation stage. The remaining 3 basal groups, 1 continued on the basal diet, 1 switched to the red wheat diet, and 1 switched to the white wheat diet. Eight weeks later, those animals were killed, to represent a late postinitiation stage. N = 15 per group.

determination of aberrant crypt parameters, as described below. The next most distal 2.5 cm portion of the colon was divided into half lengthwise (approximately 1.25 cm of each), embedded in paraffin, and 4 μm thick en face serial sections of mucosa cut for histological analysis. Sections from one half were used for determination of β-catenin accumulated crypts and the other half for MT-positive crypts, as described below. The colons were randomly encoded to allow for unbiased evaluation.

Determination of AC, ACF, Flat ACF, Sialomucin-Producing ACF, and MDF

AC, ACF, and flat ACF were enumerated on 0.2% methylene blue stained formalin fixed colon tissue. Total AC and ACF number were recorded as described by Bird (26). Flat ACF were recorded according to criteria described by Paulsen et al. (11). After ACF determination, colons were stained with high-iron diamine alcian blue solution (HID-AB) for visualization of the presence or absence of mucin and type of mucin and scored according to the criteria described by Caderni (9). Both methylene blue and HID-AB stained colon tissues were examined at 100× magnification under a light microscope (BX40, Olympus, Tokyo, Japan).

Immunohistochemical Determination of BCAC and MT-Positive Crypts

Paraffin embedded sections were heated at 65°C for 30 min, deparaffinized in xylene, and rehydrated through graded alcohol solutions. Antigen retrieval was carried out by heating sections in a pressure cooker in antigen unmasking solution (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer’s instruction for BCAC or by microwave treatment for 10 min in 0.01 M citrate buffer for MT-positive crypts. To prevent nonspecific staining, the sections were treated with 2% serum albumin for 30 min. Sections were incubated with mouse monoclonal anti-β-catenin antibody (BD Transduction Laboratories, Lexington, KY) or with anti-metallothionein antibody (Dako, Carpinteria, CA) at 4°C overnight. Negative control sections were incubated with normal horse serum at the same dilution as the primary antibody. After overnight incubation, sections were incubated with secondary antibody biotinylated anti-mouse immunoglobulin (for BCAC) or horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Carpinteria, CA; for MT-positive crypts) for an hour, and thereafter treated with 0.3% hydrogen peroxide to quench endogenous peroxidase activity. Staining of the tissue for BCAC was performed using avidin-biotin reagent (Vectastain ABC reagent, Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized by treatment with hydrogen peroxide and diaminobenzidine (Vector Laboratories, Burlingame, CA). Sections were counter-stained with hematoxylin. β-Catenin expression was evaluated at 40× magnification based on the presence of staining in cytoplasm only (C-BCAC) or cytoplasm and nuclei (C+N-BCAC). MT-positive colonic crypt cells were scored as 10%, 20%, 40%, and >50% of positive staining for MT in the colonic crypts. The percent of MT-positivity was determined by the intensity of staining of the crypts.

Statistical Analyses

The data were analyzed using SAS (version 9.3, SAS Institute, Cary, NC). Two-way analysis of variance was used to examine the effect of diets and of postinitiation stage (early vs. late). Differences among group means were inspected using the Student-Newman-Keuls test. Pearson correlation analysis was used to determine the associations between colon cancer risk markers. A probability of P ≤ 0.05 was used as the critical level of significance.

RESULTS

Body Weight and Food Intake

No differences were found in average daily food intake or final body weight among diet groups (data not shown). Neither weekly body weight nor weekly food intake differed among the groups (data not shown).

Effect of Wheat Diets on Morphological Markers

As shown in Fig. 2A and 2B, both wheat diets significantly reduced the number of AC, ACF, and large ACF compared to
the basal diet in both the early and late postinitiation stages. Although the hard red wheat diet group generally had lower values for the risk markers compared to the soft white group, these differences did not achieve statistical significance.

Overall, the number of ACF decreased from the early to late post-initiation stage (7.02 ± 0.43 vs. 5.04 ± 0.32 respectively, \( P < 0.001 \)), whereas the number of large ACF increased (0.70 ± 0.08 vs. 1.14 ± 0.11, \( P < 0.001 \)).

**Effect of Wheat Diets on Dysplasia Markers**

In the early postinitiation stage, there were no differences among the groups in flat ACF or MDF number (Fig. 2C). However, SiM-ACF number was significantly lower in the hard red wheat diet group relative to the basal diet. SiM ACF number in the soft white wheat diet group did not differ from the basal or hard red wheat groups. In the late postinitiation stage, flat ACF, SiM ACF, and MDF number was least in the hard red wheat diet group, followed by the soft white wheat diet and the basal group (Fig. 2D). The differences between the hard red and soft white groups were statistically significant for SiM ACF and MDF.

Flat ACF and MDF number significantly increased overall from the early to late post-initiation stage (\( P < 0.001 \) for both), whereas SiM ACF number remained unchanged between stages.

**Effect of Wheat Diets on BCAC**

In the early postinitiation stage, both wheat diet groups had significantly fewer C-BCAC and C+N-BCAC compared to the basal diet group (Fig. 3A). In the late postinitiation stage, the hard red wheat diet group had significantly fewer C-BCAC and C+N-BCAC than the soft white wheat diet and the basal groups (Fig. 3B). In the late postinitiation stage, C+N-BCAC were essentially absent in the hard red wheat group. Fig. 4A shows a representative image of BCAC.

Overall, both C-BCAC and C+N-BCAC significantly decreased between the early and late post-initiation stages (\( P = 0.007 \) and \( p < 0.001 \), respectively).

**Effect of Wheat Diets on MT-Positive Crypts**

As shown in Fig. 3C and 3D, in the early postinitiation stage, the number of crypts displaying approximately 20% MT-positivity (MT2) and the total number of MT-positive crypts were significantly fewer in the hard red wheat diet group compared to the soft white wheat and basal groups.
Similarly, in the late post-initiation stage, the number of crypts displaying approximately 10% MT positivity (MT1) and the total number of MT-positive crypts were significantly fewer in the hard red wheat diet group compared to the soft white wheat and basal groups. The soft white wheat diet did not differ from the basal group in MT-positivity in either the early or late post-initiation stage. Fig. 4B shows a representative image of MT-positive crypts.

Overall, MT2, MT3, and MT4 significantly decreased between the early and late postinitiation stages ($P < 0.001$,

FIG. 3. $\beta$-catenin accumulated crypts (BCAC) and metallothionein-positive (MT positive) crypts in the distal colon. Cytoplasmic (C-BCAC) and cytoplasmic + nuclear (C+N-BCAC) in the early (A) and late (B) postinitiation stage. MT-positive crypts in the early (C) and late (D) postinitiation stage. MT1, MT2, MT3, and MT4 indicate 10%, 20%, 40%, and >50% crypt immunopositivity for metallothionein (MT), respectively. MT total indicates the total number of crypts immunopositive for metallothionein.

FIG. 4. Immunohistochemical staining of paraffin sections of colonic crypts for $\beta$-catenin accumulated crypts (BCAC) (A) and metallothionein-positive crypts (B) under light microscopy (400×). A: arrowhead indicates the nuclear accumulation of $\beta$-catenin; adjacent crypts out of view showed minimal staining.
$P = 0.012$, and $P = 0.035$, respectively), and there was a strong trend for a decrease in total MT between the early and late postinitiation stages ($P = 0.055$).

**Regression of Colon Cancer Risk by Wheat Diets**

Comparing the risk factor values of the basal group from the early postinitiation stage with the values in the groups from the late postinitiation stage allows determination of whether the risk factor progressed (increased), showed no change, or regressed (decreased) with time. Table 2 summarizes changes in risk factors for the late postinitiation stage diet groups from the early postinitiation stage basal diet. With the exception of ACF and C+N-BCAC, the basal diet risk factors showed either no change or a significant progression from the early post-initiation stage. In contrast, the soft white wheat group, with the exception of an increase in MDF, showed either no significant change (large ACF, flat ACF, MT1, MT total) or a significant regression (AC, ACF, SiM ACF, C-BCAC, C+N-BCAC) from the early postinitiation stage basal group. The hard red wheat group showed either no significant change (large ACF, flat ACF, MDF) or a significant regression (AC, ACF, SiM ACF, C-BCAC, C-BCAC, C+N-BCAC, MT1, MT total) from the early postinitiation stage basal group. In all cases where regression was noted in the wheat diet groups, the magnitude of the regression was greater, sometimes much greater, in the hard red wheat group compared to the soft white wheat group (e.g. MDF, C-BCAC, MT1, and MT total).

### TABLE 2

Influence of wheat diets on changes in colon cancer risk factors

<table>
<thead>
<tr>
<th>Change from early post-initiation basal diet</th>
<th>distal colon</th>
<th>Basal</th>
<th>Hard red</th>
<th>Soft white</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>NC</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>ACF</td>
<td>↓↓</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Large ACF</td>
<td>↑↑</td>
<td>NC*</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Flat ACF</td>
<td>↑↑</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>SiM ACF</td>
<td>NC</td>
<td>↓↓*</td>
<td>↑</td>
<td>↓↓</td>
</tr>
<tr>
<td>MDF</td>
<td>↑↑</td>
<td>NC</td>
<td>↑</td>
<td>↓↓</td>
</tr>
<tr>
<td>C-BCAC</td>
<td>NC</td>
<td>↑↑</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>C+N-BCAC</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>MT1</td>
<td>NC</td>
<td>↓↓</td>
<td>↓↓</td>
<td>NC</td>
</tr>
<tr>
<td>MT total</td>
<td>NC</td>
<td>↓↓</td>
<td>↓↓</td>
<td>NC</td>
</tr>
</tbody>
</table>

*NC = no significant difference from the early post-initiation basal group; AC = aberrant crypts; ACF = aberrant crypt foci; SiM = sialomucin; MDF = mucin-depleted foci; C-BCAC = cytoplasm only β-catenin accumulated crypts; C+N-BCAC = cytoplasm and nuclei; MT = metallothionein; significantly different from the early postinitiation basal group = ↓ (P < 0.05), ↓↓ (P < 0.01).

*Significantly less compared to the soft white group, $P < 0.05$, indicating greater regression from the early postinitiation basal diet.

**Correlations Among Colon Cancer Risk Markers**

As shown in Table 3, the number of AC, ACF, large ACF, and SiM ACF were strongly and significantly correlated with one another. MDF number correlated strongly with number of AC, large ACF, and flat ACF, but not with ACF or SiM ACF number. C-BCAC significantly correlated only with AC, ACF, and large ACF number. MT total correlated weakly but significantly with AC, ACF, and somewhat more strongly with C-BCAC. Thus, as would be expected, the morphological markers (AC, ACF, and large ACF) were strongly correlated with each other. The markers of ACF dysplasia (flat ACF, SiM ACF, and MDF) showed modest and inconsistent correlations with one another. The biochemical marker (C-BCAC) showed modest but significant correlations with the morphological markers and the stem cell mutation marker.

**DISCUSSION**

Previously, we reported that in carcinogen-treated rats fed whole or refined hard red or soft white wheat flour during both the initiation and post-initiation stages, there was a reduction in aberrant crypt foci due to the class of wheat, not the state of refining, with red wheat more protective than white wheat (4). Therefore, in the present study, we chose to investigate the effect of wheat class when fed only during the postinitiation stage of colon cancer, using several different types of markers of colon cancer risk. Because in our previous study we found that wheat refining had no influence on ACF number, and because wheat is mostly consumed as refined wheat, we chose to compare refined wheat flours.

All colon cancer risk markers used in the present study have been reported previously, but not all together in the same study, to our knowledge. This allowed us to determine the correlation between these different markers of colon cancer risk. We further chose to examine the effect of the wheat diets only in the postinitiation stage, thus eliminating any effect of the wheat flours on carcinogen metabolism or other initiation-related events. Finally, we examined 2 postinitiation periods: an early stage, beginning 5 days after carcinogen administration and lasting for 6 wk, and a late stage, beginning at the end of the early stage and lasting for 8 wk. By comparing the early postinitiation basal group with the late post-initiation groups, we could determine for each diet group whether a risk marker increased over time (progression), decreased (regression), or stayed the same.

We observed that both the hard red and soft white wheat diets significantly reduced the number of AC, total ACF, and large ACF compared to the basal diet during both the early and late promotion stage of colon cancer. This finding is not consistent with our previous study, where we found fewer AC and ACF in the hard red wheat group compared to the soft white wheat group (4). However, in that study, rats were fed the wheat-containing diets before, during, and for 9 wk after carcinogen administration. Using a feeding period that...
including both initiation and postinitiation stages may account for the divergent results from the current study.

ACF are heterogeneous, with only a minority of ACF displaying dysplasia (27,28). These dysplastic ACF are believed to be within the adenoma-carcinoma sequence (29). For flat ACF and MDF, considered markers of crypt dysplasia, there were no differences among the diets in the early post-initiation stage. However, very few flat ACF or MDF were detected, making it difficult to detect a statistically significant difference. In contrast, SiM ACF, a more prevalent marker of dysplasia, a reduction was found in the wheat groups relative to the basal group. SiM ACF number in the soft white group was intermediate between the basal and hard red wheat groups and did not differ from either one. In the late postinitiation stage, when there were greater numbers of flat ACF and MDF, all 3 markers of dysplasia showed significant reductions in the wheat diets compared to the basal diet. Further, there were significantly fewer SiM ACF and MDF in the hard red wheat group compared to the soft white group. Thus, overall, hard red wheat was more effective at reducing markers of crypt dysplasia than was soft white wheat.

β-catenin, a transcriptional coactivator in the Wnt signaling pathway, serves a critical role in cell proliferation, differentiation, and migration. However, accumulation of β-catenin, either through activating mutations of the β-catenin gene or changes in other components of the Wnt-β-catenin signaling pathway, is strongly associated with colon carcinogenesis (30). Dysplastic ACF often display cytoplasmic accumulation of β-catenin, whereas such accumulation is uncommon in hyperplastic ACF (11,31). Both wheat diets greatly reduced the number of C-BCAC and C+N-BCAC in the early postinitiation stage, and to approximately the same degree, whereas in the late postinitiation stage the hard red wheat diet reduced the C-BCAC to a significantly greater degree than the soft white wheat, and only the hard red wheat reduced C+N-BCAC relative to the basal group. This suggests that one or more components in the wheat is acting to antagonize Wnt signaling. In Wnt signaling, in the absence of secreted Wnt proteins, β-catenin forms a destruction complex with the cytoplasmic proteins Axin, APC, GSK3, and CK1, referred to as the Axin complex assembly, and is subsequently phosphorylated by CK1, leading to its ubiquitination and degradation. However, Wnt proteins may bind to transmembrane receptors Frizzled and LRP5/6, leading to phosphorylation of Frizzled, which then recruits Axin, GSK3, and CK1, preventing their interaction with β-catenin. The result is accumulation of β-catenin in the cytosol and translocation to the nucleus, where it serves as a coactivator, bound to TCF/LEF, to activate Wnt-responsive genes (see Ref. 32 for review). Several studies using colon cancer cells have demonstrated the ability of compounds found in natural products to suppress Wnt signaling. Polyphenolic flavonoids such as genistein and kaempferol appear to decrease signaling by reducing binding of the β-catenin/TCF complex to DNA (33). Anthocyanins decreased β-catenin mRNA expression, likely due to changes in methylation of Wnt inhibitors as a result of decreased activity of DNA methyltransferases (34). However, as red and white wheats contain little or no anthocyanins, and genistein and kaempferol are not present in wheat, other compounds must be involved (35). Thus, the component of wheat responsible for the reduced accumulation of β-catenin is uncertain, although it is likely present in greater amounts in red than white wheat.

### Table 3

Correlation coefficients between colon cancer risk markers

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>MT total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. AC</td>
<td>0.919</td>
<td>0.819</td>
<td>0.315</td>
<td>0.684</td>
<td>0.339</td>
<td>0.332</td>
<td>0.235</td>
<td></td>
</tr>
<tr>
<td>2. ACF</td>
<td>0.545</td>
<td>0.182</td>
<td>0.643</td>
<td>0.186</td>
<td>0.317</td>
<td>0.229</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Large ACF</td>
<td>0.422</td>
<td>0.518</td>
<td>0.448</td>
<td>0.294</td>
<td>0.187</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Flat ACF</td>
<td>0.276</td>
<td>0.481</td>
<td>0.125</td>
<td>0.083</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. SiM ACF</td>
<td>-0.099</td>
<td>-0.061</td>
<td>0.047</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. MDF</td>
<td>0.351</td>
<td>0.574</td>
<td>0.670</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>7. C-BCAC</td>
<td>0.919</td>
<td>0.819</td>
<td>0.315</td>
<td>0.684</td>
<td>0.339</td>
<td>0.332</td>
<td>0.235</td>
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<tr>
<td>8. ACF</td>
<td>0.545</td>
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<td>0.643</td>
<td>0.186</td>
<td>0.317</td>
<td>0.229</td>
<td></td>
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<tr>
<td>9. Large ACF</td>
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<td>0.518</td>
<td>0.448</td>
<td>0.294</td>
<td>0.187</td>
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<td></td>
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<tr>
<td>10. Flat ACF</td>
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<td>0.125</td>
<td>0.083</td>
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<td></td>
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<tr>
<td>11. SiM ACF</td>
<td>-0.099</td>
<td>-0.061</td>
<td>0.047</td>
<td></td>
<td></td>
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<tr>
<td>12. MDF</td>
<td>0.351</td>
<td>0.574</td>
<td>0.670</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>13. C-BCAC</td>
<td>0.919</td>
<td>0.819</td>
<td>0.315</td>
<td>0.684</td>
<td>0.339</td>
<td>0.332</td>
<td>0.235</td>
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</table>

Values are correlation coefficients (upper values) and probability values for statistical significance of the correlation (lower values), n = 85–90.
has been proposed as a marker of colonic crypt stem cell mutation, based on a high correlation with crypt-restricted loss of glucose-6-phosphate dehydrogenase (G6PDH) activity (18), known to result from somatic mutation of the G6PDH gene in crypt stem cells (36), as well as elevated MT expression in cancers displaying microsatellite instability (37), a high correlation with ACF number (20), and reduction by efficacious chemotherapy agents such as 5-fluorouracil (38). We observed that the hard red wheat diet had significantly fewer MT-positive crypts compared to the basal diet groups at the end of both the early and late postinitiation stages. The soft white wheat diet did not reduce the number of MT-positive crypts relative to the basal group. Curcumin, a spice found to reduce ACF number and protect against colon tumors in rats, was found to reduce MT gene expression in HT29 cells, a colon cancer cell line (39,40). To our knowledge, our findings are the first report of reduction in a putative stem cell mutation marker by a dietary component in an animal model of colon cancer.

Current evidence indicates that enhanced Wnt signaling activity is characteristic of colon cancer stem cells (41). The statistically significant correlation between the number of C-BCAC and crypts showing MT-positivity is consistent with this finding. However, our dietary intervention, coming after carcinogen administration, would not have influenced activating mutations of the \(\beta\)-catenin. This raises the question of how red wheat could reduce the number of MT-positive crypts. Possibly one or more components from red wheat is antagonizing binding of Wnt proteins to its receptor or preventing disassociation of the Axin complex assembly, both of which would promote \(\beta\)-catenin destruction. The stem cell microenvironment appears to regulate Wnt activity (41). In particular, paracrine signaling by hepatic growth factor, secreted from myofibroblasts, seems critical for maintaining the stemness of stem cells (41). Thus, the dramatic reduction in MT-positivity in the red wheat groups relative to the basal groups could reflect an influence of a red wheat component on the stem cell microenvironment that would inhibit Wnt signaling. Further study of the mechanism by which red wheat component(s) influence cancer stem cell development seems warranted.

Given that many individuals in Western societies are probably at high risk for colon cancer, dietary interventions that could reduce their risk would be highly desirable. To that end, we examined whether the wheat-based diets could regress an established high risk state. With the basal diet most risk markers either increased, indicating progression of risk, or did not change. In contrast, several markers decreased in the white wheat group, and the majority of markers decreased in the red wheat group, indicating regression to a lower risk state. For the majority of markers, values measured in the red wheat group were lower than in the white group, suggesting that red wheat is more effective at inducing regression or stopping progression. Few studies have examined the ability of a dietary agent to regress colon cancer risk. A diet containing 5% polyethylene glycol, a nondigestible polymer, was found to greatly regress ACF number (42). Chlorogenic acid, a phenolic acid present in many fruits and vegetables, regressed ACF by 51%; however, large ACF showed no significant regression (43). Curcumin, ferulic acid, and perillyl alcohol have been examined for their ability to regress ACF. Of the 3, only curcumin caused a significant regression of ACF (44). The failure of ferulic acid to cause regression is of particular interest, as it is the major phenolic acid present in wheat (45). Previous studies on colon cancer risk regression have used only ACF as the marker of risk and have fed isolated compounds. Our study measured risk regression with markers in addition to ACF, and thus is a much more comprehensive examination of the effect of diet on regression. Further, we used a food item—wheat flour—as opposed to an isolated compound, which thus represents a more realistic situation.

Our study allows calculations of correlations among a number of markers of colon cancer risk. Not unexpectedly, AC, ACF, and large ACF number all correlated with one another. ACF number significantly correlated with SiM ACF, C-BCAC, and MT total, whereas large ACF showed highly significant correlations with flat ACF, SiM ACF, MDF, and C-BCAC, but only a trend toward a correlation with MT total. Overall, both ACF and large ACF correlate with other risk markers, but to somewhat different degrees. A high (42%) coincidence has been reported between flat ACF and MDF in carcinogen-treated rats (46). Although we did not determine coincidence of these 2 markers in the present study, our finding of a highly significant correlation between these 2 measures is consistent with this. MDF have also been reported to have greater \(\beta\)-catenin staining in the cytoplasm and nucleus than do ACF (47). This is not consistent with the present study, in which we found a highly significant correlation between ACF and C-BCAC, but no correlation between MDF and C-BCAC. A study by Donnelly and colleagues observed a linear correlation between total MT-positive crypts number and ACF number (\(r = 0.732, P < 0.01\)) or large ACF (\(r = 0.84, P < 0.01\)) in carcinogen-treated Balb/c mice (20). We also found a correlation between total MT-positive crypts and both ACF and large ACF, although not as strong as reported by Donnelly et al. Recently, evidence has been reported that increased Wnt signaling activity is characteristic of colon cancer stem cells (41). Again, this is consistent with our finding of a highly significant correlation between C-BCAC and MT total, the cancer stem cell mutation marker used in the present study. Surprisingly, SiM ACF did not correlate with either MDF, C-BCAC, or MT total, suggesting perhaps that siamomucin expression represents a less useful marker of risk than the other markers examined.

Overall, both wheat diets reduced certain markers of colon cancer risk, and this reduction was more marked in the late post-initiation stage, where the effect of the hard red wheat diet was generally much greater relative to the soft white wheat. This greater reduction by red wheat in markers of colon cancer risk is broadly consistent with the results from other
studies, in which hard red wheat diet reduced the number of ACF or tumor incidence when compared to white wheat-containing diet (4,48). Further, it is consistent with our hypothesis that the epidemiological observations of a protective effect of whole grains, relative to refined grains, is actually due to greater consumption of red wheat and is not due to the state of refining (whole vs. refined) of the grains. However, why red wheat shows a stronger chemopreventive effect than white wheat is unclear. The concentration of major classes of potentially chemopreventive compounds and antioxidant activity does vary among wheat varieties, but only modestly. Neither total phenolics, total flavonoids, xanthophylls, ferulic acid, or total antioxidant activity varied among red and white wheats in a way that can explain the observed effect of red wheat (49). Regardless, the finding that such a commonly consumed food item as red wheat has significant chemopreventive activity against colon cancer presents a significant public health opportunity for reducing colon cancer incidence and warrants further investigation.

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REFERENCES