Cytokeratin, Lectin, and Acidic Mucin Modulation in Differentiating Colonic Epithelial Cells of Mice after Feeding Western-style Diets

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ABSTRACT

Several studies have recently reported the development of colonic epithelial cell hyperproliferation in rodents following the ingestion of Western-style diets. In this study, additional measurements related to differentiation and maturation of the colonic epithelial cells were made after feeding this type of diet. Two Western-style diets high in fat and phosphate content and low in calcium and vitamin D were fed to C57BL/6J mice for 12, 24, and 52 weeks. Diet A contained American Blend fat as a source of lipids, diet B contained corn oil, and control diet C was a standard AIN-76A semisynthetic diet which is lower in fat content and higher in calcium and vitamin D. Colonic epithelial cells were studied for three biomarkers: cytokeratin catalogue no. 18 (clone LE64) expression, soybean agglutinin carbohydrate lectin binding, and acidic mucins including sialo- and sulfomucins. Feeding of diets A and B revealed that colonic epithelial cells had increased expression of cytokeratin catalogue no. 18 and SBA carbohydrate lectin binding compared to controls (P = 0.0001 for diet A versus C and diet B versus C). Significant differences were found between diets B and C (P = 0.0001) and diets A and C (P = 0.0001) in total acidic mucins and in the ratio of sulfomucins to sialomucins (P = 0.0001). These findings demonstrate that both functional and structural modifications occurred in colonic epithelial cells under these dietary conditions, and further defined this rodent model for preclinical evaluation of nutritional and chemopreventive interventions.

INTRODUCTION

High-dietary fat and phosphate and low calcium and vitamin D have been considered risk factors for human colorectal cancer (1-3). In animal models, high-dietary fat magnified the development of chemically induced colonic carcinogenesis (4-6), and increased dietary calcium inhibited both colonic epithelial cell hyperproliferation and subsequent tumor formation (7-10). Recent studies have also shown that a Western-style diet containing high fat and phosphate content and low calcium and vitamin D, without any chemical carcinogen (11-13), induce hyperproliferation and hyperplasia of colonic epithelial cells.

To investigate alterations that might occur in differentiating colonic epithelial cells after feeding Western-style diets, cytokeratin, lectin, and acidic mucin biomarkers were studied. Cytokeratins are structural proteins of epithelial cells and are related to the differentiation of epithelial cells (14). Lectins characteristically bind to specific terminal carbohydrate molecules and have been used to analyze structural components and secretory functions in the human colon (15-18). Acidic mucins are indicators of colonic epithelial cell secretory function. Various studies have shown that only sulfomucin is present in the normal distal colon of humans and rodents (19, 20). We now report abnormalities that develop in differentiating colonic epithelial cells of mice maintained on Western-style diets during 52 weeks of study.

MATERIALS AND METHODS

Animals

Forty-five male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 3 weeks of age and fed with AIN-76A semisynthetic control diet for 3 weeks for acclimatization. At 6 weeks of age, these mice were randomly divided into three groups, each group receiving one of the three test diets: A (American Blend Fat), B (corn oil), or C (control) AIN-76A diet, respectively, for 12, 24, and 52 weeks. The mice were housed five per cage, with water ad libitum, in a controlled temperature room (21-24°C), with a 12-h light/dark cycle. After 12, 24, and 52 weeks on these diets, five mice from each group were sacrificed, and the rectum and distal colon were removed and prepared for histological and immunohistochemical studies.

Materials

Colonic tissues were fixed in 80% ethanol for 24 h and then transferred to 95% ethanol for processing. The tissues were dehydrated in absolute ethanol and embedded in paraffin. Four-μm tissue sections were cut for H&E staining. For histochemical staining, the sections were treated with periodic acid-silver methenamine and counterstained with methyl green. For immunohistochemical studies, the sections were subjected to microwave antigen retrieval and then incubated with the following antibodies: (a) anticytokeratin (clone LE64; cytokeratin catalogue no. 18) antibody from Amersham; (b) lectin SBA, a probe binding carbohydrate molecules of colonic epithelial cells using a biotinylated SBA purchased from Vector; and (c) acidic mucins, functional indicators of colonic mucous cells using the highIron diamine-Alcian blue method which stained both sulfomucin and sialomucin. The biotinylated secondary antibody and avidin-biotin kit were obtained from Vector.

Diets

The diets used were based on the semisynthetic rat and mouse diet developed by the American Institute of Nutrition, AIN-76A (19), which had been designed for optimal growth of both species. The complete form of the diet was used as a control diet. The Western-style diets were modified forms of the AIN-76A diet. The diet compositions are described in Table 1. Diet A used American Blend Fat as a source of fat, developed by the Institute of Shortening and Edible Oils, formulated to simulate the mixed lipid intake in the average American diet, with a polyunsaturated:saturated ratio of 0.55. It contained 27% beef tallow, 15% butter fat, 13% lard, 27% partially hydrogenated soybean and other vegetable oil, 12% partially hydrogenated soybean, 5% peanut oil, and 1% corn oil. Diet B used corn oil as the sole source of fat. Diet C was the control AIN-76A formulation (21). Diets A and B (Western-style diets) are similar to the Western-style diet described in previous studies (11-13), except for reduction of the vitamin D level to 0.2 units/g of feed (about 0.044 units/kcal) equivalent to about 100 units in a 2000-2400 kcal human diet based on nutrient density. This is higher than the actual adult dietary intake of vitamin D in the United States, which ranges from 60 to 80 units/day (22), despite the recommended dietary allowance for adults of 200 units/day (22).

The control diet had 3.6 kcal/g, whereas the stress diet had 4.5 kcal/g due to the high fat content. In previous related studies, rodents on ad libitum feeding tended to ingest about 20% less feed (weight) of the higher caloric density-Western-style diets. The dietary intake of these essential materials was thus roughly comparable in the different groups, with the exception of the altered items (fat, calcium, vitamin D), and phosphorus), which constitute the stress components.

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3 The abbreviation used is: SBA, soybean agglutinin.
these essential nutrients in all of these diets. Westem-style diet A and sucrose to give approximately equivalent nutrient densities of content, all essential nutrients (protein, methionine, choline, vitamins other than vitamin D, and minerals other than calcium and phosphorus) are present at 20% higher levels in Western-style diet 1 and sucrose to give approximately equivalent nutrient densities of these essential nutrients in all of these diets.

In this study, the main end points of interest were the biomarkers cytokeratin LE64 and lectin SBA. Cytokeratin LE64. The expression of cytokeratin LE64 normally occurs in cells on the mucosal surface and upper third of the colonic crypts (Fig. 1); no cytokeratin was expressed in the basal third of the crypts and little in the middle crypt. Under the effect of the Western-style diets, total cytokeratin LE64 expression in colonic epithelial cells increased significantly ($P = 0.0001$ for group diet A versus group C and group diet B versus group diet C). The LE64 score was 5.01 at 12 weeks in group diet A and 5.64 in group diet B compared weeks, and five mice from each group were sacrificed at 12, 24, and 52 weeks for analysis. The primary goal was to test for any differences in the major end points between the three groups of mice. For each end point, the data can be written either as a general linear model with fixed effects from diet, time, and a time X diet interaction (26), or written as a mixed model with fixed effects from diet, time, a time X diet interaction, and random effects (27). The two models differ in the presence of random effects in the mixed model. In this study, for each end point, we carried out both types of analysis and compared the results which were similar. Due to the fact that our main interest is in any diet different across time and the variations due to differences in mice are of less importance, we only present those results from the model with fixed effects. The fixed effects model used in this study can be written as $Y_{ijk} = \delta + t_i + \beta_j + \epsilon_{ijk}$, where $Y_{ijk}$ was the mean response of the k-th mouse, from the i-th diet group at time j, and $\delta$, $t_i$, $\beta_j$, and $\epsilon_{ijk}$ are the effects due to diet, time, diet X time interaction, and random variation, respectively. For each end point, pairwise comparisons between the three groups were performed. Bonferroni-type adjustment to the $P$ values was used to protect the level of type I error.

RESULTS

Cytokeratin LE64. The expression of cytokeratin LE64 normally occurs in cells on the mucosal surface and upper third of the colonic crypts (Fig. 1); no cytokeratin was expressed in the basal third of the crypts and little in the middle crypt. Under the effect of the Western-style diets, total cytokeratin LE64 expression in colonic epithelial cells increased significantly ($P = 0.0001$ for group diet A versus group C and group diet B versus group diet C). The LE64 score was 5.01 at 12 weeks in group diet A and 5.64 in group diet B compared

### Histological and Immunohistochemical Processing

**Techniques**

The avidin-biotin-peroxidase complex technique (23) was applied in the immunohistochemical studies of cytokeratin catalogue no. 18 (monoclonal antibody clone LE64; Amershams) and lectin SBA (Vector Laboratories). High-iron diamine-Alcian blue stain (24, 25) was used to investigate the acidic mucins.

### Scoring Methods Used for These Measurements

**Cytokeratin Catalogue No. 18.** Four compartments of the colonic crypt columns i.e., mucosal surface, upper third, middle third, and basal third, were separately scored for cytokeratin-positive or -negative cells. A compartment with more than one half of the epithelial cells positive was scored 2; with one half or less than one half of the epithelial cells positive, the compartment was scored 1; and with no epithelial cells positive, it was scored 0.

**Lectin SBA.** Each compartment of the colonic crypt column was scored for SBA from 0 (negative) to 4 (positive with goblet cells predominantly stained). A total score for a colonic crypt column was 16, maximum, and 0, minimum. 0, SBA negative; 1, Golgi area stained predominantly and was confined to the supranuclear zone of the columnar cells in the colonic crypts; 2, Golgi area staining increased and spread to a higher position in the cytoplasm of columnar cells in the colonic crypts; 3, goblet cells and Golgi area of columnar cells both stained; and 4, goblet cells predominantly stained.

**Sulfomucins and Sialomucins.** Sulfomucins and sialomucins were scored separately. When scored for sulfomucin, only the sulfomucin-positive cells were counted, and the remaining cells with or without sialomucin staining were regarded as negative cells. When sialomucin was measured, only the sialomucin-positive cells were scored, and the remaining cells with or without sulfomucin staining were regarded as negative cells. Each crypt column compartment was scored from 4 (maximum) to 0 (minimum) for sulfomucins or sialomucins. They were scored as follows: 0, mucins negative; 1, columnar cells predominantly stained; 2, goblet and columnar cells both stained; 3, goblet cells predominantly stained; and 4, goblet cells only stained. A total score for a crypt column for sulfomucins, sialomucins, or acidic mucins (sialomucin plus sulfomucin) was 16 (maximum) and 0 (minimum). Slides were scored without knowledge of the origin of the specimens.

### Biostatistical Analysis

In this study, the main end points of interest were the biomarkers cytokeratin LE64, lectin SBA, acidic mucin scores, and the ratio of sialomucins:sulfomucin in mouse colon. The data consist of three groups with 15 mice each on the control diet, Western-style diet A, and Western-style diet B. The study period was 52...
to 3.39 in the control group (Table 2 and Fig. 2). The cells with LE64 expression were expanded to lower crypt compartments (Fig. 3). Similar increases were also seen at 24 and 52 weeks. The difference between diet A and diet C was irrespective of time (time × diet interaction, \( P = 0.03 \), not significant after Bonferroni adjustment).

### Table 2. Cytokeratin LE64, lectin SBA-binding scores, and acidic mucins in mouse colon

<table>
<thead>
<tr>
<th>Control diet versus diet A or B.</th>
<th>Control Diet C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LE64</strong></td>
<td>Diet A Diet B</td>
</tr>
<tr>
<td>12 weeks</td>
<td>5.01 ± 0.25a</td>
</tr>
<tr>
<td>24 weeks</td>
<td>4.78 ± 0.27</td>
</tr>
<tr>
<td>52 weeks</td>
<td>5.07 ± 0.19</td>
</tr>
<tr>
<td>SBA</td>
<td>12 weeks</td>
</tr>
<tr>
<td>24 weeks</td>
<td>8.10 ± 0.33</td>
</tr>
<tr>
<td>52 weeks</td>
<td>8.02 ± 0.40</td>
</tr>
<tr>
<td><strong>Acidic mucins</strong></td>
<td>12 weeks</td>
</tr>
<tr>
<td>24 weeks</td>
<td>12.85 ± 0.56</td>
</tr>
<tr>
<td>52 weeks</td>
<td>11.65 ± 0.29</td>
</tr>
</tbody>
</table>

\( a \) Means ± SE.

There was a trend to increase LE64 score with time, mainly effected by increases in group C. Between diet group B and C, the differences were initially very high at 12 weeks, but moderated over time (\( P = 0.01 \) for diet × time interaction). The differences between diet groups B and C predominantly involved the middle crypt, as indicated in Fig. 3.

**Lectin SBA.** Lectin SBA binds normally to epithelial cells, especially goblet cells, at the mucosal surface and throughout the whole colonic crypt (Fig. 4). In the present study, SBA-binding scores were increased in mice fed with Western-style diets. There were highly significant difference in SBA scores between the Western-style diets and the control group (\( P = 0.0001 \) for group diet A versus group diet C and group diet B versus group diet C). These differences existed irrespective of the time (Tables 2, 3, and 4, and Figs. 2 and 5).

The means of the SBA-binding scores were 5.87 in the control group, 7.72−18.88 in the group consuming diet A, and 7.12−8.88 in the group on diet B, from 12 to 52 weeks (Table 2). The differences in SBA binding between Western-style and control diets were uniform over time in all four compartments (Fig. 5).

**Acidic Mucins.** Acidic mucins are mainly composed of sulfomucin and sialomucin. In normal distal colon and rectum, only sulfomucin is identified cytochemically (Fig. 6). Under the effects of Western-style diets, the scores of acidic mucins were increased in diet B and diet A over 52 weeks. The acidic mucin scores of diet B were 13.53,
MODULATION BY WESTERN-STYLE DIETS

DISCUSSION

High-dietary fat, low calcium, and low vitamin D have been associated with increased colon cancer risk in some studies (28). Additional studies have now shown that Western-style diets containing these risk factors fed to mice and rats induced hyperplasia and hyperproliferation in the colon (11–13). The findings reported here provide evidence that prolonged feeding of Western-style diets induces other cytochemical changes. Thus, increased carbohydrate binding by lectin SBA, overexpression of cytokeratin catalogue no. 18, and altered amounts and compositions of acidic mucins were found in the colonic epithelial cells of mice fed these diets. The changes began to appear by 12 weeks of feeding and persisted after 52 weeks of feeding in most of the biomarkers studied. These findings in differentiating colonic epithelial cells indicate functional and structural modifications as a response to the components of the Western-style diets.

Although these changes are not known to be specific for colonic tumor development, the overexpression of cytokeratin and an increased ratio of sialomucin:sulfomucins have been observed in individuals at increased risk for colonic cancer. Thus, cytokeratin AE1 expression, which is normally confined to the mucosal surface and the upper part of the crypt, expands to the middle and basal crypts in adenomas (14). Increased sialomucin has been found in adenomas and adenocarcinomas of the colon as well as in flat colonic tissue at increased risk (18, 29).

Table 3 Fixed effects model analysis among diet A, diet B, and control diet for four biomarkers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Diet effect (P)</th>
<th>Time × diet interaction (P)</th>
<th>Time effect (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between diet A and control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LE64</td>
<td>0.0001</td>
<td>0.03</td>
<td>0.008</td>
</tr>
<tr>
<td>SBA</td>
<td>0.0001</td>
<td>0.9</td>
<td>0.86</td>
</tr>
<tr>
<td>Acidic mucin</td>
<td>0.0001</td>
<td>0.26</td>
<td>0.25</td>
</tr>
<tr>
<td>Sialomucin/sulfomucin</td>
<td>0.0001</td>
<td>0.02</td>
<td>0.005</td>
</tr>
<tr>
<td>Between diet B and control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LE64</td>
<td>0.0001</td>
<td>0.01</td>
<td>0.0006</td>
</tr>
<tr>
<td>SBA</td>
<td>0.0001</td>
<td>0.09</td>
<td>0.1</td>
</tr>
<tr>
<td>Acidic mucin</td>
<td>0.0001</td>
<td>0.99</td>
<td>0.034</td>
</tr>
<tr>
<td>Sialomucin/sulfomucin</td>
<td>0.0001</td>
<td>0.03</td>
<td>0.001</td>
</tr>
</tbody>
</table>

12.27, and 12.21 compared to 11.75, 10.15, and 10.10 in the control diet group (Table 2 and Fig. 2). The difference between diet B and control diet C was highly significant irrespective of time (P = 0.0001).

The ratio of sialomucin:sulfomucin was increased with Western-style diet B at 24 weeks (0.56) and at 52 weeks (0.76) compared to the control group at 24 weeks (0.43) and at 52 weeks (0.36) (Table 4). For the group of diet B, the ratio of sialomucin:sulfomucin was significantly higher than the control group (P = 0.0001). In the control diet group, the ratio decreased at 24 and 52 weeks compared with 12 weeks.

A summary of the test results of the four markers between the two Western-style diet groups and the control group is given in Table 3.

Fig. 4. Lectin SBA bound to mucin of the goblet cells and the supranuclear region of columnar cells. ×400.

Fig. 5. Lectin SBA-binding scores in the colons of mice on Western-style diets A (■) and B (□) and control AIN-76A diet (●) for 12 (a), 24 (b), and 52 (c) weeks. Lectin SBA-binding increased in cells throughout the colonic crypts and not in the cells on the mucosal surface. Data are means. Bars, SE. S, surface; UC, upper crypt; MC, middle crypt; BC, basal crypt.
lial cell irritation or damage. The identification of these findings increases the utility of this rodent model for the preclinical evaluation of nutritional and other chemopreventive interventions in the colon.

REFERENCES


Table 4 Ratio of sialomucin to sulfomucin in mouse colon

<table>
<thead>
<tr>
<th>Weeks on diets</th>
<th>Diet A</th>
<th>Diet B</th>
<th>Control diet C</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.29 ± 0.02*</td>
<td>0.65 ± 0.06*</td>
<td>0.71 ± 0.08*</td>
</tr>
<tr>
<td>24</td>
<td>0.50 ± 0.03</td>
<td>0.56 ± 0.04</td>
<td>0.43 ± 0.02</td>
</tr>
<tr>
<td>52</td>
<td>0.31 ± 0.04</td>
<td>0.76 ± 0.20</td>
<td>0.36 ± 0.05</td>
</tr>
</tbody>
</table>

* Means ± SE.

Cytochemical changes in the colons of mice on the control diet also became modified during aging. Thus, increased expression of cytokeratin catalogue no. 18 (LE64) decreased total acidic mucins, and the ratio of sialomucins:sulfomucins differed significantly at 24 and 52 weeks compared to 12 weeks. However, these changes in mice on the AIN-76A control diet during aging occurred earlier in the Western-style diet groups. SBA binding was not modified with aging in the colons of mice on the control diet.

The increased cytokeratin LE64 expression, carbohydrate binding by lectin SBA, total acid mucins, and altered mucin composition after feeding the Western-style diets could reflect responses to chronic irritation by substances, including free fatty acid and bile acids (28, 30), present in the luminal contents of the colon after feeding the Western-style diets. The cytochemical changes induced by the Western-style diet in this study also appear comparable to changes reported in humans at increased risk (18, 29). It can therefore be suggested that measurements of these cytochemically identified components of colonic epithelial cells may be useful early indicators of colonic epithelial cells in normal distal colon. High-iron diamine-Alcian blue stain. X400.
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