Short-Chain Fructo-oligosaccharides Reduce the Occurrence of Colon Tumors and Develop Gut-associated Lymphoid Tissue in Min Mice

Fabricre Pierre, Pascale Perrin, Martine Champ, Francis Bornet, Khaled Meflah, and Jean Menanteau

Introduction

The incidence of colon cancer varies between different populations. Although genetic factors play a role in its pathogenesis, it is widely recognized that environmental and dietary factors are of high importance in 85–90% of all cases. The observations of Burkitt in Africa, where the incidence of cancer is low, framed the hypothesis that high-fiber diets are protective against colon cancer (1). However, animal studies of dietary fiber in chemically induced colon carcinogenesis have yielded conflicting results, showing a protective role in some experiments but not in others (2–7). This could reflect the variety of experimental designs relative to differences in carcinogens, amount and type of dietary fiber, stage of carcinogenesis, and mucosal status when the diet was administered. Furthermore, colon cancers in these models differ in some respects from those observed in humans. They arise from flat foci of dysplasia rather than adenomatous polyps and are not relevant to the genetic alterations generally depicted in early stages of human colon carcinogenesis (8). One particular type of genetic change that is thought to predispose to colon cancer is germ-line mutation in the Apc gene on chromosome 5q, which is responsible for FAP (9). A high frequency of APC mutations in sporadic adenomas and adenocarcinomas of the colon has also been reported (10). It is likely that APC mutations play a role in the early stages of colorectal tumorigenesis. Recently, a mouse model (Min mice) has been developed in which the animals are heterozygous for a non-sense mutation of the Apc gene, the murine homologue of APC (11). These mice have numerous adenomas throughout the small intestine and colon, which develop into adenocarcinomas. In our study, gut tumors and small intestine lymphoid nodules were counted in Min mice fed fiber-enriched diets for 6 weeks. Neither starch-free wheat bran nor resistant starch modified the number of tumors. However, short-chain fructo-oligosaccharides dramatically reduced the incidence of colon tumors and concomitantly developed gut-associated lymphoid tissue. Our experiment shows that short-chain fructo-oligosaccharides counteract advanced stages of colon carcinogenesis, possibly via stimulation of antitumoral immunity by modulation of the colonic ecosystem.

Materials and Methods

Animals. C57BL/J-Min/+ mice (Min mice) age 5 or 6 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME). Males and females were studied to determine whether the diet effect was sex related. The animals (n = 40) were divided randomly into eight groups and housed one group per cage: four groups of eight females age 5 or 6 weeks (F5 and F6); and four groups of two males age 5 or 6 weeks (M5 and M6).

Diet. Mice were fed controlled, energy-balanced, powdered experimental diets (Table 1). The CD was a low-fiber diet containing 2% cellulose Arbocel type B00 (Durieux, Marne-la-Vallée, France). The three high-fiber diets were formulated to provide 5.8% total dietary fiber from retrograded corn starch, starch-free WB, or short chain fructo-oligosaccharides. RS was a retrograded high-amylose corn starch (Cerestar, Vilvoorde, Belgium). WB (Sofalia, Ennezat, France) was treated with amylglucosidase and then washed abundantly with water on a 620 μm sieve to remove entrapped starch. sc-FOSs (GFn, n ≤ 4) were produced from sucrose using a fungal fructofuranosyltransferase (Aaclight P, Béhign Meiji Industries, Neulilly sur Seine, France).

Protocol. Mice were age 6 or 7 weeks at the beginning of the nutritional intervention. Each group was fed ad libitum either the control low-fiber diet [n = (4 F5 + 4 F6) + (1 M5 + 1 M6)] or one of the three high-fiber diets [n = (3 F5 + 5 F6) + (1 M5 + 1 M6)]. Diet was distributed ad libidum in protected feeders and renewed daily. Because the life span of these mice is short (119 ± 31 days), feeding was conducted for 42 days to minimize the risk of death before the end of the nutritional intervention. Animals were weighed globally per cage every week throughout the experiment.
Tissue Sampling and Scoring of Tumors and Lymphoid Nodules. At the end of the nutritional intervention, mice were sacrificed by cervical dislocation. Colon and small intestine were removed quickly, opened longitudinally and washed with ice-cold PBS. Both intestinal and colon tumors were classified as small (diameter ≤ 1 mm) or large (diameter >1 mm). Mucus and villi in the small intestine were removed with a wet paintbrush. Macroscopic examination of the small intestine allowed tumors and lymphoid nodules to be counted without resorting to tissue fixation. For colon, the tissue was fixed in 10% buffered formalin, washed in deionized water, and finally stained for 10 min in a 0.1% methylene blue solution (13). Counts for colon tumors and aberrant crypt foci were performed under light microscopy. All large colon tumors were excised and embedded in paraffin. Sections were stained with hemalun-eosin-safran and examined under light microscopy for structural analysis.

Statistical Analyses. All data were studied by ANOVA. As the F test allowed rejection of the null hypothesis, comparisons between each fiber-enriched diet (enriched with RS, WB, or sc-FOS) and the low-fiber diet (CD) were performed with Bonferroni’s test. To satisfy the conditions required for ANOVA, transformed values were used for colon tumor counts (rare events, with the count being sometimes 0), as follows: $\sqrt{y} + \sqrt{y} + 1$, where $y$ is the tumor count (19), and ranked data for the lymphoid nodule count (unequal variances; Ref. 20). Lymphoid nodule counts were analyzed with two-way ANOVA, using a mixed model in which diet was the fixed factor and mouse age the random factor, to account for a possibly different state of immunological energy when the diets were begun. Results were considered statistically significant when $P < 0.05$. The Systat (version 5.2.1) package (Evanston, IL) was used for all statistical analyses.

Results and Discussion

Animal growth was unaffected by differences in diet content (Fig. 1), and no age- or sex-related effects on gut tumors were noted. Regardless of diet, tumors occurred mainly in the small intestine, and aberrant crypt foci were extremely rare in the colon. These results are in agreement with those of Jacoby et al. (13). However, a significant reduction ($P < 0.01$) in colon tumors, which were largely associated with the distal colon, was observed in mice receiving the diet supplemented with sc-FOSs (Table 2; Fig. 2A). Furthermore, four animals in this group were totally free of colon tumors, a condition not found with any of the other diets. Interestingly, the sc-FOSs effect was limited to the colon; there were no significant differences between diets in the number of tumors found in the small intestine. The fact that dietary interventions may differently affect the upper and lower gastrointestinal tract should be born in mind, because adenomas and adenocarcinomas in the upper gastrointestinal tracts, in addition to colonic polyps, are also observed frequently in FAP patients (21). The presence of adenomas in this group, whereas adenocarcinomas predominate in the other three groups.

The fact that a protective effect was obtained with sc-FOSs in the colon but not the small intestine suggests strongly that events specific to the colon were involved. sc-FOSs and RS are fermentable fibers that provide protection against earlier stages of colon carcinogenesis: they both reduce the number of azoxymethane-induced aberrant crypt foci in rats, in conjunction with high butyrate production. The inefficiency of RS in the present experiment suggests that fermentation either is not involved in the protective effect or is not sufficient by itself. In vivo and in vitro studies indicate that fructo-oligosaccharides can be used successfully as prebiotics to enhance the population of *Bifidobacterium* in the large intestine (15, 16). Direct evidence that *Bifidobacterium* stimulate murine antitumor immunity and modify cytokine expression has also been reported (17). It is well documented that these bacteria as well as *Lactobacillus* and other lactic acid-producing bacteria affect local and systemic immune response (18). A bifidogenic effect probably occurred in our experiment, because Howard et al. have shown that dietary supplementation with these same sc-FOSs enhanced the population of *Bifidobacterium* in mouse colon as soon as 14 days (15). To obtain an insight into the GALT response to changes in the colonic ecosystem, we looked at the lymphoid tissue of the small intestine. Examination of the colon for that purpose was omitted, because treatment of this tissue, to study colon tumors, made it impossible to accurately evaluate colon lymphoid follicles. Studies have shown that intestinal microflora are important in stimulating the postnatal development of Peyer’s patches, because there is an obvious reduction in the overall size of lymphoid follicles in germ-free animals (24). Furthermore, it is well established that upon antigenic stimulation, the immune cells, the differentiation and maturation of which are initiated in the GALT, migrate through lymphatic vessels to the draining mesenteric lymph nodes, the thoo.

Table 1 Composition of the four energy-balanced powdered experimental diets

<table>
<thead>
<tr>
<th>Components</th>
<th>CD</th>
<th>RS</th>
<th>WB</th>
<th>FOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrograd high-amylase corn starch</td>
<td>0</td>
<td>18.82</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Starch-free WB</td>
<td>0</td>
<td>0</td>
<td>7.14</td>
<td>0</td>
</tr>
<tr>
<td>sc-FOS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.83</td>
</tr>
<tr>
<td>Pre-gelatinized starch</td>
<td>64.27</td>
<td>47.47</td>
<td>59.33</td>
<td>59.43</td>
</tr>
<tr>
<td>Casein</td>
<td>20.00</td>
<td>18.87</td>
<td>18.77</td>
<td>19.45</td>
</tr>
<tr>
<td>D,L-methionine</td>
<td>0.40</td>
<td>0.38</td>
<td>0.38</td>
<td>0.39</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2.00</td>
<td>1.89</td>
<td>1.88</td>
<td>1.94</td>
</tr>
<tr>
<td>Corn oil</td>
<td>2.00</td>
<td>1.89</td>
<td>1.88</td>
<td>1.94</td>
</tr>
<tr>
<td>Lard</td>
<td>6.33</td>
<td>5.97</td>
<td>5.94</td>
<td>6.15</td>
</tr>
<tr>
<td>Minerals</td>
<td>4.50</td>
<td>4.25</td>
<td>4.22</td>
<td>4.38</td>
</tr>
<tr>
<td>Vitamins</td>
<td>0.50</td>
<td>0.47</td>
<td>0.47</td>
<td>0.47</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

- Providing 5.8% total dietary fiber (type III RS from retrograd corn starch).
- INRA formula No. 102.

* sc-FOSs REDUCE COLON TUMORS IN Min MICE

**Fig. 1. Growth of female Min mice during the nutritional intervention.** Because mice were weighed globally per cage, individual mouse weight was estimated as the weight of all mice receiving a given diet divided by the number of mice in the group ($n = 8$). Growth is not indicated for male mice, because estimations based on the weight of cages containing only two animals would not have been representative.

42 or 49 when tumors may already have arisen. Among these tumors, the largest would have escaped the sc-FOS diet effect and continued to grow in the same way as with the other diets. However, histopathological examination of the large tumors within the four groups suggested a delay in the transition from adenomas to carcinomas associated to the sc-FOS diet, because adenomas were as numerous as adenocarcinomas in this group, whereas adenocarcinomas predominated in the other three groups.

The fact that protective effect was obtained with sc-FOSs in the colon but not the small intestine suggests strongly that events specific to the colon were involved. sc-FOSs and RS are fermentable fibers that provide protection against earlier stages of colon carcinogenesis: they both reduce the number of azoxymethane-induced aberrant crypt foci in rats, in conjunction with high butyrate production. The inefficiency of RS in the present experiment suggests that fermentation either is not involved in the protective effect or is not sufficient by itself. In vivo and in vitro studies indicate that fructo-oligosaccharides can be used successfully as prebiotics to enhance the population of *Bifidobacterium* in the large intestine (15, 16). Direct evidence that *Bifidobacterium* stimulate murine antitumor immunity and modify cytokine expression has also been reported (17). It is well documented that these bacteria as well as *Lactobacillus* and other lactic acid-producing bacteria affect local and systemic immune response (18). A bifidogenic effect probably occurred in our experiment, because Howard et al. have shown that dietary supplementation with these same sc-FOSs enhanced the population of *Bifidobacterium* in mouse colon as soon as 14 days (15). To obtain an insight into the GALT response to changes in the colonic ecosystem, we looked at the lymphoid tissue of the small intestine. Examination of the colon for that purpose was omitted, because treatment of this tissue, to study colon tumors, made it impossible to accurately evaluate colon lymphoid follicles. Studies have shown that intestinal microflora are important in stimulating the postnatal development of Peyer’s patches, because there is an obvious reduction in the overall size of lymphoid follicles in germ-free animals (24). Furthermore, it is well established that upon antigenic stimulation, the immune cells, the differentiation and maturation of which are initiated in the GALT, migrate through lymphatic vessels to the draining mesenteric lymph nodes, the thoo.

Table 2  Effect of the different diets on the occurrence of small (diameter, ≤1 mm) and large (diameter >1 mm) tumors in the small intestine and colon and on the development of GALT

<table>
<thead>
<tr>
<th>Diet</th>
<th>Small intestine</th>
<th>Large intestine</th>
<th>GALT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mice</td>
<td>Total tumor</td>
<td>Small tumor</td>
</tr>
<tr>
<td></td>
<td>with tumors</td>
<td>count</td>
<td>count</td>
</tr>
<tr>
<td>CD</td>
<td>10 of 10</td>
<td>50.3 (19.6)</td>
<td>28.1 (10.1)</td>
</tr>
<tr>
<td>RS</td>
<td>10 of 10</td>
<td>49.7 (32.0)</td>
<td>27.2 (16.5)</td>
</tr>
<tr>
<td>WB</td>
<td>10 of 10</td>
<td>47.2 (16.2)</td>
<td>24.6 (11.7)</td>
</tr>
<tr>
<td>FOS</td>
<td>10 of 10</td>
<td>46.7 (23.5)</td>
<td>21.0 (14.5)</td>
</tr>
</tbody>
</table>

*a* Comparisons between fiber-enriched diets and the CD were all nonsignificant.

*b* Lower number of total tumors (*P* < 0.01) and small tumors (*P* = 0.01) with sc-FOSs than CD (transformed data, see "Materials and Methods").

*c* Higher number of lymphoid nodules (*P* < 0.05) with sc-FOSs than CD (ranked data).

**A significantly higher number** (*P* < 0.05) of macroscopically detectable lymphoid nodules were noted in the small intestine with the sc-FOS diet (Table 2). This suggests that the immune system may play a role in inhibiting tumor formation by eliminating cells that express tumor antigens if they are immunogenic enough to allow the expansion of immune cells specific for these antigens. Indeed, even if a precancerous lesion succeeds in generating a T-cell response, the presence of immunosuppressive or regulatory mechanisms may not allow the antitumor effector T cells to expand to the critical number required to eliminate abnormal cells. Changes induced in the pattern of biological response modifiers *in situ* may contribute to potentiation immunosurveillance. This has been demonstrated for interleukin 2, which can reverse the tumor-induced anergy of the immune response. There is no doubt that a qualitative study is needed to examine the patterns and the activation status of immunocompetent cells, which would permit a deeper interpretation of our data. Furthermore, the role of GALT in colon cancer has not yet been elucidated. Although lymphoid aggregates are rich in specific and nonspecific cytotoxic cells, they are also rich in immunosuppressive cells in relation to tolerance to luminal antigens. Moreover, colon cancer cells are poorly immunogenic, which is presumably why they do not respond to immunotherapy. Interestingly, we succeeded previously in reversing a later stage of carcinogenesis (established i.p. carcinomatosis) in a rat colon cancer model by using a combination of butyrate and interleukin 2, when neither of these substances alone proved effective. Butyrate can enhance the immunogenicity of colon cancer cells, and this mechanism is synergized with the stimulation of immune cells by interleukin 2 (26). In the case of *Min* mice, in which the nutritional intervention was made on advanced carcinogenesis, it is likely that a similar synergistic boost of the immune system to counterbalance immunocompetent cell anergy is required in addition to the mechanisms classically involved in the protective effects induced by fermentable fiber. Although WB was ineffective in our experiment, it has been shown to provide protection in animal models and to reduce the recurrence of rectal polyps in patients with FAP (27). Some hypotheses may account for this discrepancy: (a) WB appears to exhibit its effects at early stages of carcinogenesis and not at later stages, at least in some animal models (7); (b) our experimental diet was not balanced to produce the high amounts of secondary bile acids, the adsorption of which constitute the main protective mechanism of this fiber; and (c) as the length of the nutritional intervention is dependent on the short life span of *Min* mice, possible long-term effects of WB are excluded.

To our knowledge, the present study provides evidence, for the first time, that preneoplastic colon lesions may be highly sensitive to dietary intervention. This observation is important if dietary intervention is to be used in adult individuals who may have preneoplastic lesions in their colons. It appears that the use of synergistic properties...
may be a promising strategy in dietary modulation of colon carcinogenesis, the multiple effects originating either from a unique product as in our study, or from a combination of agents as proposed by Alabaster et al. (7).

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References

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