Colitis-associated colon tumorigenesis is suppressed in transgenic mice rich in endogenous n-3 fatty acids

Introduction

Colorectal cancer (CRC) is one of the most common cancers in USA (1). Inflammation is thought to be one of the underlying etiologies of tumorigenesis in the colon. In fact, anti-inflammatory drugs have been used to prevent colon tumorigenesis (2–5). Furthermore, an inhibitor kappa B kinase beta knockout mouse with decreased activity of the pivotal pro-inflammatory transcription factor nuclear factor kappa B (NF-κB) showed reduced colon tumorigenesis induced by azoxymethane (AOM) (6). This was accompanied by lower expression of inducible nitric oxide synthase in the tumors of fat-1 mice versus wild-type (WT) control littermates. These transgenic mice express a Caenorhabditis elegans desaturase, leading to the formation of endogenously high levels of n-3 PUFA from n-6 PUFA, changing the n-6/n-3 PUFA ratio from values around 30/1 to ratios of approximately 1–5/1 (14). In contrast to feeding studies supplementing n-3 PUFA, this model eliminates confounding factors of diet (content of trace elements, fibers, antioxidant, etc.) that could have significant effects on colon carcinogenesis itself. As these confounding factors could compromise the elucidation of the molecular events underlying the impact of n-3 PUFA, we chose the fat-1 transgenic mice to directly test the role of an increased n-3 PUFA tissue content in colon tumorigenesis.

Materials and methods

Mice

Transgenic fat-1 mice were generated and maintained as described previously (14). Transgenic mice were backcrossed onto a C57bl background at least four times. Heterozygous fat-1 mice were then mated with WT mice to obtain WT and heterozygous fat-1 offspring. We used all the female offspring from several breeding pairs (19 fat-1 and 11 WT littersmates). In this study, all transgenic fat-1 mice were heterozygous. Animals were kept under specific pathogen-free conditions in standard cages and were fed a special diet (a modified AIN-76A diet in which 5% corn oil was substituted with 10% safflower oil, with an energy composition of protein 20%, carbohydrate 58% and fat 22%), high in n-6 and low in n-3 PUFA. Each cage housed 2 weight-matched female mice, combining WT and fat-1 transgenic mice.

PUPA analysis

Fatty acid profiles were analysed using gas chromatography as described previously (15). Briefly, 1 cm of mice tails (in order to perform the phenotyping of fat-1 mice) or blocks of colon tissue (5 × 5 mm) were ground to powder under liquid nitrogen. Samples were then subjected to extraction of total lipids and fatty acid methylation by heating at 100°C for 1 h under 14% boron trifluoride (BF3)–methanol reagent (Sigma, St. Louis, MO) and hexane (Sigma). Fatty acid methyl esters were analysed by gas chromatography using a fully automated 6890N Network GC System (Agilent Technologies) equipped with a flame-ionization detector. Peaks of resolved fatty acids were identified by comparison with fatty acid standards (Nu-chek-Prep), and area percentage for all resolved peaks was analysed using GC ChemStation Software (Agilent Technologies, Santa Clara, CA).

Tumor induction

Treatment scheme is summarized in Figure 1a. For tumor induction, female mice (fat-1 n = 16, WT n = 11) were injected intraperitoneally with the genotoxic carcinogen AOM (10 mg/kg, single dose, Wako Chemicals, Richmond, VA) followed by DSS (1.5%, for one week, MP Biomedicals, Solon, OH, LLC) in sterile, non-acidified drinking water. A body weight of 19 g was considered the threshold for starting the treatment. Previous studies authors from the n-3 PUFA eicosapentaenoic acid and docosahexaenoic acid (10,11). The n-3 PUFA-derived mediators have been implicated in the resolution of inflammation and therefore termed resolvins and protectins (11). It is known that resolvins and protectins can inhibit NF-κB activity (9). Our previous results in the fat-1 mouse model showed that an increased tissue status of n-3 PUFA led to formation of anti-inflammatory n-3 PUFA-derived resolvins and protectins and in turn to protection from acute DSS-induced colitis. This was associated with a significantly decreased activity of the pro-inflammatory transcription factor NF-κB (10). These observations, together with previous data demonstrating inhibition of chemically induced colon carcinogenesis by fish oil supplementation (12,13), led us to hypothesize a role for an increased n-3 PUFA tissue content in the prevention of colorectal tumorigenesis in a model of colitis-associated cancer.

The study presented here was designed to examine the effect of an endogenously increased n-3 PUFA status on the induction and development of colon tumors and its relation to inflammation in fat-1 mice versus wild-type (WT) control litters. These transgenic mice express a Caenorhabditis elegans desaturase, leading to the formation of endogenously high levels of n-3 PUFA from n-6 PUFA, changing the n-6/n-3 PUFA ratio from values around 30/1 to ratios of approximately 1–5/1 (14). In contrast to feeding studies supplementing n-3 PUFA, this model eliminates confounding factors of diet (content of trace elements, fibers, antioxidants, etc.) that could have significant effects on colon carcinogenesis itself. As these confounding factors could compromise the elucidation of the molecular events underlying the impact of n-3 PUFA, we chose the fat-1 transgenic mice to directly test the role of an increased n-3 PUFA tissue status in colon tumorigenesis.

Abbreviations: AOM, azoxymethane; CRC, colorectal cancer; DSS, dextran sodium sulfate; iNOS, inducible nitric oxide synthase; n-3 PUFA, omega-3 polyunsaturated fatty acids; NF-κB, nuclear factor kappa B; PCR, polymerase chain reaction; TGF-β, transforming growth factor beta; WT, wild-type.

1These two authors contributed equally to this study.

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have shown that similar treatment schemes result in colonic adenocarcinomas developing within 4 weeks and a 100% tumor incidence at week 6 in the ICR mouse strain, whereas C57BL6 mice showed a 50% adenocarcinoma incidence 18 weeks after treatment (1% DSS for 4 days) (16–18). Clinical assessment of all AOM/DSS-treated animals for body weight, stool consistency, rectal bleeding and general appearance was performed daily. To evaluate rectal bleeding, stool probes were tested using Hemoccult paper (Beckman Coulter, Fullerton, CA). Mice were anesthetized with isoflurane (Abbott Laboratories, Abbott Pak, IL) after 9 weeks and killed for chemical and pathological analyses.

Assessment of neoplasia and colitis

The colon was excised from the ileocecal junction to the anal verge, flushed several times with phosphate-buffered saline (Gibco, Carlsbad, Ca) and opened longitudinally. Gross examination was performed to measure colon length and to evaluate the pattern of tumor development, including quantity, size and position of each tumor within the large bowel. According to their localization, tumors were assigned to either the intermediate or distal third of the colon sample. In addition, the incidence (defined as number of mice with tumors/total mice in the group), the mean number of tumors/mouse ± standard deviation, as well as the mean tumor volume in the group ± standard deviation was calculated for each group. For tumor volume, we used a common approximated formula: \( V = \frac{0.5 \times (\text{length})^2 \times \text{width}}{C} \).

Tumors were excised separately for evaluation in Real Time (RT)–polymerase chain reaction (PCR) and part of the colon was used for fatty acid profiling by gas chromatography. Additionally, (inflamed) colonic tissue, as well as colon tumors, was processed for histopathological evaluation and further biochemical analyses. All procedures were performed in a blinded manner.

Colon tissue was stained with hematoxylin and eosin, and stainings were scored for inflammatory activity by an experienced pathologist in a blinded manner, according to the validated scoring system used previously (10,19): (i) severity of inflammation (0 no inflammation, 1 mild, 2 moderate and 3 severe) and (ii) thickness of inflammatory involvement (0 no inflammation, 1 mucosa, 2 mucosa plus submucosa and 3 transmural); epithelial damage (0 intact epithelium, 1 disruption of architectural structure, 2 erosion and 3 ulceration) and extent of lesions (0 no lesions, 1 punctuate, 2 multifocal and 3 diffuse). Colonic neoplasms were evaluated according to the criteria used by Suzuki et al. (18).

In order to perform the histological evaluation, we used an Olympus BX51 microscope with UPlanFI lenses and obtained representative photos with an Olympus QColor5 camera acquired using QCapturePro Software.

**Fig. 1.** Colon tumor induction in WT and fat-1 transgenic mice. (a) Tumor induction protocol. Mice were injected with AOM on day 1 followed by administration of 1.5% DSS in the drinking water for seven consecutive days, starting on day 8. Animals were killed for analysis 9 weeks later. (b) Photographs showing colons with tumors from a WT control mouse (upper) and from a fat-1 animal (lower). (c) Representative hematoxylin and eosin stain of inflamed colon and tumor from a WT mouse. The panel on the right is a higher magnification (2.5×) of the area indicated by the square frame. (d) Representative hematoxylin and eosin stain of inflamed colon and tumor from a fat-1 mouse. The panel on the right is a higher magnification (2.5×) of the area indicated by the square frame. (e) Tumor incidence in WT versus fat-1 mice in the distal (left), the intermediate (center) and the whole (right) colon. (f) Average number of tumor per mouse in WT versus fat-1 group (\( P < 0.05 \)). (g) Comparison of tumor size between WT and fat-1 animals with colon tumors (\( P < 0.01 \)).
Semiquantitative real-time PCR
Total RNA was isolated from whole colon tissue using RNeasy mini kit (Quiagen, Valencia, CA) following the manufacturer’s instructions. RNA concentrations and purity was determined spectrophotometrically by their absorbance at 260 nm in relation to the absorbance at 280 nm with a GeneQuant pro analyser (Amersham Biosciences, Piscataway, NJ). Reverse transcription of mRNA was performed using random primers (Promega, Madison, WI) to generate cDNA in a programmable Thermal Controller (PTC-100, MJ Research, Ramsey, Minnesota). Real-time PCR was carried out using Absolute QPCR SYBR Green Mix (ABgene, Rochester, NY) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol. All samples were processed in triplicates and means were standardized to guanosine adenine dinucleotide phosphate values. Results were expressed as a fold induction to WT animals. Primers were designed with Primer Select 5.00 Software (DNAsstar, Madison, WI) and purchased from Invitrogen, Carlsbad, CA.

NF-κB activation (enzyme-linked immunosorbent assay)
To quantify the activated p65/RelA protein, TransAM NF-κB p65 Activation Assay (Active Motif, Carlsbad, CA) was performed according to the manufacturer’s instructions. Whole-cell extracts from frozen colon tissues were collected using Nuclear Extract Kit (Active Motif) and protein concentrations were determined by using Coomassie Plus Protein Assay Reagent (PIERCE, Rockford, IL). Lysates (5 µg of total protein) were incubated at room temperature for 1 h in 96-well dishes containing immobilized oligonucleotides that comprise the NF-κB consensus DNA-binding site (5’-GGGACTTTCC-3’). Consecutively, the primary antibody against activated p65 and the horseradish peroxidase-conjugated secondary antibody were incubated in the same manner, separated by washing steps. The reaction was developed for 5 min at room temperature and its intensity measured immediately at 450 nm using a microplate reader (Vidort 1420, Perkin Elmer, Waltham, MA).

Immunohistochemistry
Fresh colon specimen of inflamed tissues, tumors and of untreated WT control colons were fixed in 10% neutral-buffered formalin overnight followed by automated processing and embedding in paraffin. For staining, rabbit anti-nitrotyrosine polyclonal antibody (Chemicon, Temecula, CA, 1:500) was used as primary antibody. The secondary antibody was BGAR (biotinylated goat anti-rabbit, Vector Labs, Burlingame, CA) at a concentration of 1:250. Antigen retrieval was performed with Na Citrate (pH 6) for 10 min in a microwave. Lysates (5 µg of total protein) were incubated at room temperature for 1 h in 96-well dishes containing immobilized oligonucleotides that comprise the NF-κB consensus DNA-binding site (5’-GGGACTTTCC-3’). Consecutively, the primary antibody against activated p65 and the horseradish peroxidase-conjugated secondary antibody were incubated in the same manner, separated by washing steps. The reaction was developed for 5 min at room temperature and its intensity measured immediately at 450 nm using a microplate reader (Vidort 1420, Perkin Elmer, Waltham, MA).

Statistical analysis
All results are presented as mean ± standard deviation, except where stated otherwise. Student’s T-test was used to evaluate the difference between two groups. RT–PCR was analysed by using the 2A Ct method. For comparison of tumor incidences in the distal colon, Chi Square test was used. Statistical significance was accepted at the level of P < 0.05 and Prism 4 for Windows Software (Graph Pad) was used for all calculations.

Results
Tumor induction in fat-1 versus WT mice
Transgenic fat-1 mice used in this study had a ratio of long-chain n-6 PUFA to n-3 PUFA of 6.6 in the colon, whereas the WT littersmates used as control group had a ratio of 49.8 (Table I). Treatment with AOM and DSS (Figure 1a) resulted in a tumor incidence of 56.3% (9 out of 16) in the distal part of colon (where the DSS-induced inflammation is most severe) in fat-1 mice as compared with 90.9% (10 of 11) in their WT littersmates (P = 0.053, Figure 1b, c, d and e). In the intermediate part of the colon, total tumor incidence was similar between the two groups of animals (75% fat-1 versus 72.7% in WT animals, Figure 1e). Interestingly, there were significantly fewer tumors in the fat-1 animals (2.4 ± 0.47) than in WT animals (4.2 ± 0.71, P < 0.05, Figure 1f). Tumors in the intermediate part of fat-1 colons were smaller (7.8 ± 1.9 mm3 in fat-1 versus 25.6 ± 7.0 mm3 in WT animals, P < 0.01, Figure 1g). Overall tumor incidence was 87.5% in fat-1 mice as compared with 100% in WT animals. No tumors were found in the proximal part of the colon in either group of animals.

Inflammatory status in the colons of fat-1 versus WT mice
DSS administration leads to inflammation in the distal and intermediate colon (16,19,20). Fat-1 mice showed significantly less colon shortening than WT mice (Figure 2a), indicating less severe inflammation and swelling of the large bowel. Colon length was inversely correlated with inflammatory activity. While both groups of animals (fat-1 and WT animals) lost weight during DSS administration, they quickly recovered and gained weight again after DSS was stopped. Body weight was higher in fat-1 mice throughout the course of the experiment, confirming the results of our previous study on acute DSS colitis in fat-1 mice (10). In addition to colon length, the microscopic evaluation of the distal part of the colon documented significantly decreased inflammation severity and mucosal thickness in colons of fat-1 animals (Figure 2b).

Table I. Differential fatty acid profiles in WT control and fat-1 transgenic mice

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>WT (n = 11)</th>
<th>Fat-1 (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-6 PUFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>18.70 ± 5.86</td>
<td>16.65 ± 2.98</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>12.17 ± 2.90</td>
<td>10.69 ± 1.28</td>
</tr>
<tr>
<td>22:4 n-6</td>
<td>2.59 ± 0.62</td>
<td>1.70 ± 0.24*</td>
</tr>
<tr>
<td>22:5 n-6</td>
<td>2.30 ± 0.53</td>
<td>0.60 ± 0.12*</td>
</tr>
<tr>
<td>Total n-6</td>
<td>36.11 ± 3.73</td>
<td>29.64 ± 1.82*</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:3 n-3</td>
<td>0.17 ± 0.04</td>
<td>0.17 ± 0.09</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>n.d.</td>
<td>0.79 ± 0.25*</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>n.d.</td>
<td>1.01 ± 0.12*</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>0.55 ± 0.21</td>
<td>2.53 ± 1.12*</td>
</tr>
<tr>
<td>Ratio n-6/n-3</td>
<td>0.72 ± 0.22</td>
<td>4.50 ± 1.27*</td>
</tr>
</tbody>
</table>

*LA, linolenic acid; AA, arachidonic acid; DTA, docosatetraenoic acid; DPA, docosapentaenoic acid; ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n.d., fatty acids not detected in the sample. P < 0.01.

Inflammation and proliferation markers in fat-1 versus WT mice
NF-κB activity was assessed by measuring activated p65 in cell extracts from the colons of fat-1 and WT animals. There was significantly lower activity of NF-κB in the fat-1 mice, as compared with the WT group (Figure 3a). These results are consistent with the findings in acute DSS colitis in fat-1 mice (Figure 4A in ref. (10)).

Nitrotyrosine as a marker for nitrosative stress was detectable in colon tumors (Figure 3b), with increased expression of inducible nitric oxide synthase (iNOS) in the colon tumors of WT animals (Figure 3c). Expression of iNOS in the tumors of fat-1 animals was significantly lower. These results were consistent with the results in acute DSS colitis, where iNOS expression was lower in the fat-1 mice that were protected from colitis (10). In addition, however, the non-tumorous colon, there was no significant difference of iNOS mRNA expression between the fat-1 and WT animals in this study (Figure 3c). Furthermore, we could not document significant tumor necrosis factor alpha activity in the plasma of the animals tested here (data not shown).

mRNA expression of the anti-proliferative transforming growth factor beta (TGF-β) was increased in the colons of fat-1 mice (Figure 3d), which could contribute to the suppression of tumorigenesis in the fat-1 animals.

Discussion
The results presented here show that an increased n-3 PUFA tissue status in the colon decreases inflammation-triggered colon tumorigenesis. The suppressed tumorigenesis in the fat-1 mouse was evidenced by a lower incidence of colon neoplasia in the fat-1 mice as well as a smaller size of the tumors formed. Inflammation was less severe in the distal colon of fat-1 mice with high endogenous n-3 PUFA tissue levels, confirming the findings of protection from acute colitis in fat-1 mice (10). The lower degree of inflammatory changes was associated
with a decreased tumor incidence in the distal colon. These changes were, on a molecular level, accompanied by a lower NF-$\kappa$B activity in the fat-1 mice together with a significantly lower mRNA expression of iNOS in the tumors and an increased expression of anti-proliferative TGF-$\beta$ in colonic tissue.

Together with the suppressed NF-$\kappa$B activity observed in this study, the results showing a decrease of tumor incidence in the distal part of the colons of fat-1 animals (56.3 % in the fat-1 mice as compared with 90.9 % in the WT mice), and a decreased tumor size in the fat-1 mice, support the link between inflammation and tumorigenesis through increased NF-$\kappa$B activity in the inflamed colon (6). In the study by Greten et al. (6), deletion of the NF-$\kappa$B-activator inhibitor kappa B kinase beta in intestinal epithelial cells decreased tumor incidence by increasing apoptosis of transformed cells; deletion of inhibitor kappa B kinase beta in myeloid cells decreased tumor growth by decreasing the expression of pro-inflammatory cytokines. This correlates well with previous findings of inflammatory cytokine suppression by $n$-3 PUFA (7,10,21).

The observed inhibition of colon tumor formation and growth rate by the increased $n$-3 PUFA tissue status is probably due to the inhibitory effect of these fatty acids on NF-$\kappa$B (22), and thus suppression of NF-$\kappa$B-mediated pro-inflammatory, pro-proliferative and anti-apoptotic activities. The increased levels of $n$-3 PUFA-derived lipid mediators (resolvins and protectins) in the colons of fat-1 mice (10), and particularly resolvin E1, are known to suppress NF-$\kappa$B activity (9,11).

In addition, the lower iNOS expression in tumors from fat-1 transgenic animals with high $n$-3 PUFA tissue content probably further contributes to the decreased tumor growth in these animals. Recent data have implicated pro-inflammatory iNOS expression and nitrosative stress in the growth of colon carcinomas by nitrosylation of caspases and subsequent inhibition of apoptosis. Strategies to lower nitric oxide...
have been tested experimentally for the prevention of colon tumorigenesis (23–26). The data presented here are consistent with our previous findings (10) and indicate that n-3 PUFAs can inhibit iNOS expression and suppress nitrosative stress in colon tumors, leading to an increased apoptosis of tumor cells.

The data presented here also suggest that the observed antitumor effect of increased n-3 PUFAs tissue content might be mediated partially by an increased presence of TGF-β. Reduction of TGF-β expression has been shown to increase chemical-induced colon carcinogenesis significantly (27). On a molecular level, TGF-β was shown to inhibit transformation through inhibition of the Akt pathway in intestinal epithelial cells (28).

The findings that increased tissue status of n-3 PUFAs reduced colon inflammation and tumorigenesis in the fat-1 mice will lead to future studies to elucidate the molecular interactions and pathways underlying these effects. More studies to address the relationship between the formation of lipid mediators and carcinogenesis using approaches of lipidomics and proteomics are warranted.

The results presented here support a protective role of n-3 PUFAs in the prevention of CRC. In this context, dietary supplementation with n-3 PUFAs may be an effective and safe means of CRC prevention and it may be an alternative to the use of anti-inflammatory cyclooxygenase inhibitors, particularly cyclooxygenase-2 inhibitors, which exhibit side effects when used for a long term. Because n-3 PUFAs has many other beneficial effects, such as cardioprotective effect (29), supplementation with n-3 PUFAs to prevent colon cancer is a strategy worth pursuing now (30).

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References


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