Suulindac effects on inflammation and tumorigenesis in the intestine of mice with Apc and Mlh1 mutations

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We have previously reported that sulindac, a non-steroidal anti-inflammatory drug, inhibited tumor formation in the small intestine but increased tumors in the colon of ApcMin/+ mouse, a model of human familial adenomatous polyposis. To further explore intestinal regional responses, we studied effects of sulindac on additional gene-targeted mouse models of human intestinal tumorigenesis; these were (i) Apc1638N/+ mouse (chain termination mutation in exon 15 of the Apc gene); (ii) Mlh1−/− mouse (DNA mismatch repair deficiency, a mouse model of human hereditary non-polyposis colorectal cancer) and (iii) double-heterozygous Mlh1+/−/Apc1638N/+ mutant mouse. Mice were fed AIN-76A control diet with or without 0.02% sulindac for 6 months. Intestinal regional tumor incidence, multiplicity, volume and degree of inflammation were used as end points. The results showed the following: (i) sulindac inhibited tumor development in the small intestine of Apc1638N/+ mice; (ii) in contrast, sulindac increased tumors in the small intestine of Mlh1 mutant mice, a neoplastic effect which persisted in heterozygous compound Mlh1+/−/Apc1638N/+ mutant mice; (iii) sulindac increased tumors in the cecum of all mice regardless of genetic background; (iv) sulindac decreased inflammation in the small intestine of Apc1638N/+ mice, but it increased inflammation in the small intestine of Mlh1−/− mice and Mlh1+/−/Apc1638N/+ mice and (v) sulindac enhanced inflammation in the cecum of all mutant mice. Findings indicate that the effects of sulindac in the intestine of these mutant mouse models are probably related to genetic background and appear to be associated with its inflammatory-inducing response.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to possess chemopreventive properties against gastrointestinal neoplasia. For example, sulindac decreased the number and size of colorectal adenomas in familial adenomatous polyposis (FAP) patients (1–8) and in non-FAP cohorts at increased risk of adenoma recurrence (9). Nonetheless, colorectal cancer appears to recur in FAP patients after prolonged sulindac treatment (6,10–13). Sulindac also caused regression of small intestinal tumors in the ApcMin/+ mouse, a mouse model of FAP (14–16), the small intestine being the major site of tumor development in mouse models. We have previously observed (17) regional effects of sulindac on intestinal tumorigenesis in the ApcMin/+ mouse, wherein these mice when given sulindac for 9 weeks showed decreased number and volume of tumors in the small intestine but increased tumor formation in the cecal portion of the colon. A similar regional effect of piroxicam on intestinal neoplasia was reported in double-mutant Mlh1−/−/Apc1638N/+ mice (18). It is noteworthy that sulindac administration induced tumors in the cecum of wild-type C57BL/6J mice, a mouse strain with very low susceptibility to intestinal cancer (19).

Here, we further explored the regional responses of the intestine to dietary sulindac, using additional mouse genetic models that simulate increased sensitivity to human intestinal tumorigenesis, including (i) Apc1638N/+ mice (chain termination mutation in exon 15 of the Apc gene) (20); (ii) Mlh1−/− mice (DNA mismatch repair deficiency, a mouse model of human hereditary non-polyposis colorectal cancer) and (iii) double-heterozygous Mlh1+/−/Apc1638N/+ mutant mice (21). The results indicate that the regional effects of sulindac on tumor development in these mice are associated with chronic inflammation and appears to be related to their genetic background.

Materials and methods

Mice and diets

Genetically modified mice at 5–6 weeks of age were provided by Dr Winfried Edelmann, Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY. These included Apc1638N/+ (n = 31), Mlh1−/− (n = 38) and double-heterozygous Mlh1+/−/Apc1638N/+ mice (n = 22). Mice were fed AIN-76A diet and maintained on a 12 h light–dark cycle in a temperature and humidity-controlled room. After 1 week of diet acclimatization, mice of different genotypes were randomized to two dietary groups and either maintained on AIN-76A diet or AIN-76A diet containing sulindac (200 p.p.m.) (Research Diets, Brunswick, NJ). The average consumption of sulindac was ~0.6 mg/day per mouse, equivalent to the dose that inhibited tumor growth reported in ApcMin/+ mice (14) and to the dose used in our previous study with ApcMin/+ mice (17). Mice were weighed weekly and tested for fecal bleeding to monitor both intestinal inflammation and tumor formation. All animals were euthanized after 6 months of feeding.

Evaluation of tumor and inflammation development

The gastrointestinal tract was removed, opened longitudinally, and the contents were washed in cold phosphate-buffered saline (pH 7.4). Each specimen was examined under a dissecting microscope for tumors after fixation in 10% formalin. Tumor multiplicity (number of mice with tumors), incidence (number of tumors per mouse), location and volume (mm3) were recorded. All tumors found in the gastrointestinal tract were excised for processing; tissue section slides were prepared and stained with hematoxylin and eosin for histological examination. The tumor diagnosis was based on the Histological Typing of Intestinal Tumours and The Pathology of Mouse Models of Intestinal Cancer (22) with modifications. Other organs and lymph nodes were also examined. Two segments of flat mucosa were taken from duodenum, cecum, proximal and distal colon and fixed in 10% neutral-buffered formalin, and tissue sections were stained with hematoxylin and eosin for histological analysis and evaluation of inflammation.

The degree of inflammation was assessed using the following semiquantitative scoring system (23): (i) no inflammatory cells; (ii) few plasma and lymphoid cells; (iii) clusters of plasma and lymphoid cells; (iv) granulomatous tissue formed with acute and chronic inflammatory cells, mononuclear cells, fiber and fibroblastic cells and (v) micro-abscess formation. The depth of inflammatory infiltration was evaluated as follows: (i) confined to the mucosa proper; (ii) extended to the submucosa; (iii) spreading to the muscularis and (iv) spreading to the serosa or beyond. Inflammation severity was calculated as the degree of inflammatory cells × depth of inflammatory infiltration.

Results

Tumor development

Small intestine. Spontaneous tumor incidence in the small intestine of Apc1638N/+ mice, Mlh1−/− mice and double-mutant Mlh1+/−/Apc1638N/+ mice fed AIN-76A for 6 months was 68, 6 and 73%, respectively.

Abbreviations: FAP, familial adenomatous polyposis; NSAID, non-steroidal anti-inflammatory drug.

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Sulindac treatment for the same period resulted in marked changes in tumor development in each of the genetic groups (Figure 1 and Table I). Sulindac completely eliminated tumor formation, as measured by tumor incidence (0 versus 68%, \( P < 0.001 \)), multiplicity (0.0 versus 1.5, \( P < 0.001 \)) and volume (0.0 versus 18.0 mm\(^3\), \( P < 0.05 \)) in the small intestine of Apc\(^{1638N/þ}\) mice, compared with mice fed unmodified AIN-76A control diet (Figure 1A, D and G). In contrast, dietary sulindac increased tumor incidence 5.8-fold (41 versus 6%, \( P < 0.05 \)) and tumor number 8-fold (0.9 versus 0.1, \( P < 0.05 \)) in the small intestine of Mlh1\(^{þ/þ}\) mice (Figure 1B, E and H).

Sulindac was ineffective in suppressing tumor incidence (91 versus 73% control) or multiplicity (3.0 versus 3.3) in the small intestine of compound Mlh1\(^{þ/þ}\)/Apc\(^{1638N/þ}\) mice, but there appeared to be a decrease in tumor size (4.1 versus 14.5 mm\(^3\)), which was not statistically significant (Figure 1C, F and I). Importantly, the increased susceptibility to spontaneous tumors in the small intestine appears to be related to alterations in the Apc gene in both the Apc\(^{1638N/þ}\) and the compound Mlh1\(^{þ/þ}\)/Apc\(^{1638N/þ}\) mice.

Cecum. Tumors were mainly located in the cecum and upper part of the colon near the junction with the ileum. Tumor incidence was 0, 6 and 9%, respectively, in single-mutant Apc\(^{1638N/þ}\) mice, Mlh1\(^{1/þ}\) mice and double-mutant mice fed AIN-76A diet, respectively (Figure 1). In mice treated with sulindac, tumor incidence, multiplicity and volume were greatly increased regardless of genetic status. Thus, in Apc\(^{1638N/þ}\) mice, tumor incidence increased to 92%, multiplicity to 1.8 tumors per mouse and volume to 4.7 mm\(^3\) per mouse, with all increases significant at \( P < 0.001 \) (Figure 1A, D and G, Table I). In Mlh1\(^{1/þ}\) mice, dietary sulindac increased tumor incidence in cecum by 5.8-fold (41 versus 6%, \( P < 0.05 \)) and tumor number 8-fold (0.9 versus 0.1, \( P < 0.05 \)) in the small intestine of Mlh1\(^{þ/þ}\) mice (Figure 1B, E and H).

### Table I. Summary of intestinal tumor development in mutant mice after feeding sulindac

<table>
<thead>
<tr>
<th></th>
<th>Apc(^{1638N/þ})</th>
<th>Mlh1(^{1/þ})</th>
<th>Mlh1(^{1/þ})/Apc(^{1638N/þ})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulindac added</td>
<td>AIN-76A diet</td>
<td>AIN-76A diet</td>
<td>AIN-76A diet</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor incidence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Colon</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tumor multiplicity</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Small intestine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Colon</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tumor volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Colon</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Decreased (–) or increased (+) tumor incidence, multiplicity or volume compared with mice fed unmodified control AIN-76A diet.
and proximal colon 14-fold (91 versus 6%), tumor multiplicity (1.5 versus 0.1 tumors per mouse) and increased tumor volume by 17.5-fold (3.7 versus 0.2 mm³), all increases significant at P < 0.001 (Figure 1B, E and H). In double-heterozygous Mlh¹⁻⁻/Apc¹⁶⁸⁸⁹¹⁻⁻ mice, dietary sulindac led to a 10-fold increase of tumor incidence (100 versus 9%), 14-fold increase of tumor multiplicity (1.5 versus 0.1) and increased tumor volume 50-fold (5.1 versus 0.1 mm³), all values significant at the P < 0.001 (Figure 1C, F and I).

Most of these tumors in control group mice fed AIN-76A were adenomas. After sulindac treatment, however, the number of invasive adenomas increased 15-fold (5.7 versus 0.4%) in cecum, 5-fold (2.2 versus 0.4%) in the proximal colon and 10-fold (0.8 versus 0.08%) in the distal colon (Table II). Significantly, after sulindac treatment, the inflammatory severity score decreased in small intestine (0.00 versus 2.68, P < 0.001) and greatly increased in cecum (6.00 versus 0.29, P < 0.001) compared with mice fed unmodified AIN-76A control diet; no inflammation was detected in the remainder of the colon after sulindac treatment (Table II).

Slight inflammation occurring spontaneously was observed in the cecum of Mlh¹⁻⁻ mutant mice maintained on AIN-76A control diet. However, following sulindac treatment, the inflammatory severity score in these mice increased significantly in both small intestine (3.64 versus 0.00, P < 0.001) and cecum (4.73 versus 0.33, P < 0.001) compared with mice fed AIN-76A control diet (Table II).

In all instances, inflammatory cells after sulindac administration were located mainly in and around the area of tumor formation and included neutrophils, lymphoid cells, plasma cells and mononuclear cells extending from the upper part of intestinal wall to the muscularis and serosa. Increased inflammation was evident in the proximal colon after sulindac treatment, although not statistically significant.

**Discussion**

It is mandatory that the effects of sulindac as a potential chemopreventive agent are taken in context. Present results using mice with different genetic backgrounds, all with an increased propensity to develop colorectal cancer, provide further insight on the potential effects of the drug. Sulindac inhibited tumorigenesis in small intestine of Apc¹⁶⁸⁸⁹¹⁻⁻ mice but significantly increased the incidence, multiplicity and volume of tumors in the cecum of these mice. These results are in agreement with previous findings in Apc⁰⁰⁰⁰⁰⁰⁻⁻ mice (17). We extended these studies to Mlh¹⁻⁻⁻⁻ and Mlh¹⁻⁻⁻⁻/Apc¹⁶⁸⁸⁹¹⁻⁻ mice wherein sulindac treatment resulted in a marked increase in tumor incidence, multiplicity and volume in both small intestine and cecum. Thus, in contrast to the single-mutant Apc¹⁶⁸⁸⁹¹⁻⁻ mice in which sulindac protected against tumor formation in the small intestine, we saw no such protection by sulindac in the small intestine of Mlh¹⁻⁻⁻⁻ or Mlh¹⁻⁻⁻⁻/Apc¹⁶⁸⁸⁹¹⁻⁻ mice which could be due to loss of wild-type Apc allele. These genetic alterations would presumably affect the histopathology of the intestine in these mice, accounting for their increased propensity to develop intestinal cancer and in turn differentially alter their response to sulindac in the small intestine and cecum.

In the present study, we have examined these findings as related to the inflammatory process and its potential association with the development of intestinal tumorigenesis in Apc¹⁶⁸⁸⁹¹⁻⁻ and Mlh¹⁻⁻⁻⁻ mice fed either unmodified AIN-76A diet or AIN-76A supplemented with sulindac. Surprisingly, moderate to severe inflammation was present in the duodenum of Apc¹⁶⁸⁸⁹¹⁻⁻ mice fed AIN-76A but was absent in mice with an Mlh¹⁻⁻⁻⁻ mutation maintained on the same diet. In Apc¹⁶⁸⁸⁹¹⁻⁻ mice, inflammation was decreased after sulindac. Whether the presence or absence of inflammation in the same region of the intestine reflects the different genetic lesions in mice or an interaction of diet with a specific genotype remains to be determined. It is also noteworthy that sulindac-enhanced inflammation developed predominantly in the cecum of these mice where most tumors were also found.

Using quantitative computer-assisted image analysis, we previously showed (17) that intestinal regional response of Apc⁰⁰⁰⁰⁰⁰⁻⁻ mice to sulindac may be accounted for, at least in part, by the increased expression of Bax, a pro-apoptotic protein, and decreased expression of Bcl-xl, an anti-apoptotic protein of the Bcl-2 family. An opposite Bax/Bcl(-xl) pattern was observed in cecal mucosa and in tumors therein; these changes were also associated with widespread ulceration and limited perforation in cecal mucosal surface and tumors after sulindac treatment, which are histopathologic signs of severe inflammation.

These observations are consistent with reports showing that sustained use of NSAIDs, including sulindac, induces damage not only to the upper gastrointestinal tract but also to the small and large intestine, resulting in the activation of an inflammatory cascade leading to mucosal ulceration (24–26). While this proinflammatory process has been attributed to NSAIDs’ induction of changes in mucosal permeability and the consequent influx of toxic luminal factors into the mucosa (24,26,27), an important compounding effect might be the intrinsic ability of NSAIDs, including sulindac, to generate reactive oxygen species (28–31).

While sulindac-induced reactive oxygen species was shown to enhance apoptosis in cancer cells (29), we surmise that sustained stimulation by sulindac of reactive oxygen species formation would ultimately lead to oxidative stress, a key stimulus of the inflammatory response and carcinogenesis (32,33). Thus, sulindac-induced oxidative

### Table II. Inflammation measurements in the intestine of mice with Apc¹⁶⁸⁹¹ and Mlh¹⁻⁻ mutations

<table>
<thead>
<tr>
<th></th>
<th>Degree</th>
<th>Depth</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apc¹⁶⁸⁹¹⁻⁻ mice</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AIN-76A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.32 ± 0.27</td>
<td>1.21 ± 0.26</td>
<td>2.68 ± 0.78</td>
</tr>
<tr>
<td>Cecum</td>
<td>0.29 ± 0.16</td>
<td>0.21 ± 0.11</td>
<td>0.29 ± 0.16</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Distal colon</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Sulindac</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Cecum</td>
<td>2.42 ± 0.29</td>
<td>2.25 ± 0.28</td>
<td>6.00 ± 0.91</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Distal colon</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td><strong>Mlh¹⁻⁻⁻⁻ mice</strong></td>
<td></td>
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<tr>
<td>AIN-76A</td>
<td></td>
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<td></td>
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<tr>
<td>Duodenum</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Cecum</td>
<td>0.33 ± 0.17</td>
<td>0.33 ± 0.17</td>
<td>0.33 ± 0.17</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Distal colon</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Sulindac</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.18 ± 0.32</td>
<td>1.23 ± 0.34</td>
<td>3.64 ± 1.26</td>
</tr>
<tr>
<td>Cecum</td>
<td>2.18 ± 0.24</td>
<td>1.82 ± 0.18</td>
<td>4.73 ± 0.60</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>0.09 ± 0.09</td>
<td>0.09 ± 0.09</td>
<td>0.18 ± 0.18</td>
</tr>
<tr>
<td>Distal colon</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

Mean ± SEM.

*Inflammatory scores included degree of inflammatory cells, depth of inflammatory cell infiltration and inflammatory severity (degree × depth). By Mann–Whitney test and binomial exact calculation compared with corresponding AIN-76A: *P < 0.001; compared with corresponding diet group of Apc¹⁶⁸⁹¹⁻⁻ mice: *P < 0.001. 

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stress was found to induce oncogenic COX-2 overexpression in various cell lines (31) and to activate p38 (29,34), a mitogen-activated protein kinase that participates in the generation of key proinflammatory cytokines and chemokines and in cancer development (35–38).

There is a large body of evidence showing that chronic inflammatory conditions increase the risk of cancer: the causative link between inflammation in inflammatory bowel disease and colon cancer is a salient case in point (39–41). One may therefore propose that sulindac-induced inflammation contributes to the regional effects of sulindac on tumor formation in the small intestine and cecum of mice. This view is supported by the observation that chemically induced inflammation accelerated colonic adenoma formation in ApcM<sup>Min/+</sup> mice (42,43) and enhanced colon carcinogenesis in Mlh1<sup>±/−</sup> mice (44).

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Conflict of Interest Statement: None declared.

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14. Boolbol,S.K. et al. (1996) Cytochrome<sub>x</sub>-overexpression and tumor formation are blocked by sulindac in a murine model of familial adenoma


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