Regional response leading to tumorigenesis after sulindac in small and large intestine of mice with Apc mutations

Kan Yang1, Kanhu Fan1, Naoto Kurihara1, Hiroharu Shinozaki1, Basil Rigas2, Leonard Augenlicht3, Levy Kopelowich3, Winfried Edelmann3, Raju Kucherlapati2 and Martin Lipkin1

1Strang Cancer Research Laboratory at The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA, 2American Health Foundation, Valhalla, NY 10595, USA, 3Albert Einstein College of Medicine, Bronx, NY 10461, USA, 4Division of Cancer Prevention, National Cancer Institute, Bethesda, MD 20892, USA and 5Division of Genetics, Brigham and Women Hospital, Boston, MA 02115, USA

Sulindac and other NSAIDs have been widely studied as potential chemopreventive agents for colon cancer. Short-term studies have shown adenomatous polyps to regress in patients with familial adenomatous polyposis (FAP). In this study the effect of sulindac on cancer as an endpoint was evaluated in ApcMin mice, a preclinical model of FAP with an Apc mutation in codon 850 that leads to gastrointestinal adenomas and carcinomas. Three groups of mice were studied all of which were fed AIN-76A diet: one group was fed AIN-76A diet alone, a second group received sulindac 200 p.p.m. premixed in the diet and a third group received sulindac 180 p.p.m. added in drinking water. ApcMin mice were killed 9 weeks after feeding was initiated. Mice receiving sulindac developed fewer tumors in the intestine overall; the major decrease in tumor development after sulindac treatment was demonstrated in the cecum and colon. Regional responses of apoptosis, and Bax/Bcl-xL expression in tumors developing after sulindac treatment were demonstrated in the ApcMin mice.

Materials and methods

Animals and diets
Thirty ApcMin mice (Jackson Laboratories, Harbor, ME), ~4 weeks old, were randomly divided into three groups and fed one of three diets: AIN-76A diet alone, AIN-76A diet with 200 p.p.m. sulindac premixed, and AIN-76A diet with 180 p.p.m. sulindac added to drinking water. The AIN-76A diet provides 3.9 kcal/g and consists of corn oil 5% (12% of calories), calcium 0.52 mg/g (1.3 mg/kcal), phosphorus (as PO4) 0.4 mg/g (1.1 mg/kcal) and vitamin D3 1.0 IU/g (0.26 IU/kcal). ApcMin mice were maintained on their respective AIN-76A or treatment diets for 9 weeks. The duration of treatment was dictated by the lifespan of 4-5 months of the mouse strain (18,19). The average consumption of sulindac was 0.6 mg/day/mouse, an amount equivalent to the dose that inhibits tumor growth reported in ApcMin mice (23). Mice were weighed and feces were tested for gastrointestinal bleeding (hem occult) at baseline, and weekly thereafter, until animals were killed.

Evaluation of tumor development
Mice were killed by cervical dislocation, the gastrointestinal tract was removed, opened longitudinally and the contents were washed in cold phosphate-buffered saline (PBS) pH 7.4. The mucosa was flattened on filter paper and fixed in 10% neutral-buffered formalin. Each specimen was examined under a dissecting microscope. Tumors were numbered and their location was recorded. Tumor volume, measured using a grid in the eyepiece of the microscope, was expressed in units of mm3. The flat mucosa of the stomach, duodenum, cecum, proximal and distal colon was processed for further study, Representative tumors from the small intestine and all the tumors in the cecum and colon were sampled and processed. All other internal organs and lymph nodes were examined for tumors. Sections of paraffin-embedded tumor
Table I. Incidence, number and volume of intestinal tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Overall</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Incidence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIN-76A</td>
<td>10</td>
<td>10 (100%)</td>
<td>10 (100%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Sulindac in AIN-76A</td>
<td>10</td>
<td>10 (100%)</td>
<td>7 (70%)</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Sulindac in drinking water, mice fed AIN-76A</td>
<td>10</td>
<td>10 (100%)</td>
<td>10 (100%)</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>B. Tumor number per mouse, mean ± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIN-76A</td>
<td>10</td>
<td>37.1 ± 5.3</td>
<td>36.7 ± 5.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Sulindac in AIN-76A</td>
<td>10</td>
<td>5.5 ± 2.4</td>
<td>3.4 ± 2.2</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>Sulindac in drinking water, mice fed AIN-76A</td>
<td>10</td>
<td>14.9 ± 3.9</td>
<td>11.1 ± 3.7</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>C. Tumor volume (mm³), mean ± SEM (median)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIN-76A</td>
<td>10</td>
<td>43.2 ± 51.4 (42.3)</td>
<td>41.9 ± 51.4 (41.6)</td>
<td>1.3 ± 0.9 (0.06)</td>
</tr>
<tr>
<td>Sulindac in AIN-76A</td>
<td>10</td>
<td>3.6 ± 0.6 (3.5)</td>
<td>0.6 ± 0.3 (0.02)</td>
<td>3.0 ± 0.5 (3.0)</td>
</tr>
<tr>
<td>Sulindac in drinking water, mice fed AIN-76A</td>
<td>10</td>
<td>6.0 ± 1.4 (4.5)</td>
<td>1.7 ± 1.0 (0.6)</td>
<td>4.3 ± 0.7 (3.9)</td>
</tr>
</tbody>
</table>

n, number of mice studied.

Comparisons are made to corresponding control (AIN-76A diet, no treatment) using two-tailed Fisher exact probabilities test for tumor incidence; Mann-Whitney and exact binomial calculation for tumor number and volume: *P < 0.01; **P < 0.001. Comparisons between tumor volume in the large intestine and small intestine: †P < 0.001; ‡P < 0.01.

The proteins were made in tumors and corresponding flat mucosa in those regions of the ApcMin mice.

**Apoptosis**

Apoptosis originated as a morphological phenomenon, and measurements in the present study were based on morphologic features. Characteristic features required for identification of apoptotic bodies included: a single, condensed small cell with or without cytoplasm (apoptotic body) or a cluster of apoptotic bodies inside another healthy cell (host cell), with or without a halo around the apoptotic body. Measurements were carried out on well-oriented intestinal crypts of flat mucosa. Cells on the mucosal surface of the proximal and distal colon were also included. Twenty-five crypts (50 crypt columns) were scored for each mouse. The apoptotic cells per crypt column were computed. Apoptotic cells in the villi of small intestine were measured separately. The number of apoptotic cells in each villous column was scored. Twenty five villi (50 villous columns) were studied for each mouse. The number of villous columns with apoptotic cells and number of apoptotic cells per villous column were calculated. In tumors, 1000 tumor cells or more were scored for apoptotic bodies.

**Evaluation of Bax and Bcl-XL expression**

Expression of Bax and Bcl-XL was evaluated on immunohistochemical preparations of tumors taken from small and large intestine and flat mucosa of duodenum, cecum and distal colon of ApcMin mice in sulindac and control groups. The antibodies to Bax (N-20) and Bcl-XL (A-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Six slides with five serial tissue sections per slide were cut from a paraffin block and labeled. To avoid variation of staining intensity, all tissue-slides were stained at the same time, and all tissue sections were cut by one technologist with the same microtome. Three slides with an odd number (nos 1, 3 and 5) were used for immunohistochemical staining for Bax and Bcl-XL, and the first two slides with an even number (nos 2 and 4) were stained for H&E for apoptosis study. On the slides with odd numbers the specific antibody to Bax and Bcl-XL was applied to every other section in each slide. This parallel staining for two different antibodies facilitated the data being more comparable. The last slide (no. 6) was used for negative control on which neither antibody to Bax nor to Bcl-XL was added to tissue sections as immunohistochemical staining. Measurements were carried out with a computer-assisted cell image analyzer (Samba 4000). This system uses a color CCD camera to separate red (R), green (G) and blue (B) colors and convert these colors to digital form. The size of the resulting pixels was 0.25 µm under a magnification of 40×. By using a software program, immuno 4.04 per Area Program for cytoplasmic labeling measurement, it was possible to assay the size of positive areas (Labeling Index) and staining intensity (Mean Optical Density) of a given area in an immunohistochemical preparation. Through segregation the assay was confined only to epithelial cells, i.e. in a crypt column of flat mucosa, and to neoplastic glands of a tumor. Ten crypts (20 crypt columns), well oriented, were measured from each intestinal segment of flat mucosa for each mouse. For tumors, 1000 cells from different areas were randomly selected for measurement. Bax and Bcl-XL protein expression was assayed separately in two differently staining tissue sections nearby on one slide for each mouse or tumor. The data were automatically generated with an arbitrary unit (a.u.) including percentage of positive area, staining intensity and overall expression of a combination of

![Fig. 1. Tumorigenesis in the gastrointestinal tract of ApcMin mice after sulindac. (A) tumor incidence, (B) tumor multiplicity, (C) tumor volume (*P < 0.01; **P < 0.001, sulindac group compared with controls).](https://academic.oup.com/carcin/article-abstract/24/3/605/2608455)
positive area and staining intensity (percentage of positive area × staining intensity). The overall expression was used for the comparisons. The ratio of overall expression of Bax and Bcl-xL are reported for each group, i.e. ratio of Bax and Bcl-xL expression = mean overall expression of Bax/mean overall expression of Bcl-xL. Comparisons of protein expression between treatment and control groups were based on the ratio.

Statistical tests
Comparisons between the study and control groups were carried out using Fisher exact probabilities, Mann–Whitney test, and binomial calculations. Significant difference was considered as \( P < 0.05 \).

Results

All tumors found in the ApcMin mice were in the intestinal tract. There were no tumors in the stomach and none were detected in the lungs, liver, kidneys or lymph nodes of these mice.

Tumorigenesis

Tumor incidence. We determined the tumor incidence in the small and large intestine as well as the overall tumor incidence, i.e. tumor incidence in a group of mice regardless of anatomical location of the tumor in the intestinal tract (Table I and Figure 1A). In the small intestine the tumor incidence in the group given sulindac premixed in the solid diet decreased 30\% compared with the control group (\( P > 0.05 \)). However, in the large intestine, the tumor incidence was 2.3-fold increased in both sulindac-treated groups (100 versus 30\% with \( P < 0.01 \)) compared with controls. The overall tumor incidence was 100\% in each of the three groups having tumors (Table I and Figure 1A).

Number of intestinal tumors per mouse (multiplicity). The overall number of tumors per mouse decreased 85 and 60\% in both sulindac-treated groups compared with controls with \( P \) values \(<0.001\) and \(<0.01\), respectively (5.5 and 14.9 versus 37.1) (Table I and Figure 1B). In the small intestine the number of tumors per mouse decreased 90 and 70\% in both sulindac groups compared with controls with \( P \) values 0.001 and 0.01, respectively (3.4 and 11.1 versus 36.7, representing mean tumor number in mice fed sulindac in solid food and in drinking water versus tumors in control group (same for below if not specified). In the large intestine, however, changes in the tumor number were in the opposite direction from those seen in the small intestine. The tumor number increased 4.3- and 8.5-fold in the two sulindac-treated groups with both \( P < 0.01 \) compared with controls (2.1 and 3.8 versus 0.4).

Tumor volume. The overall tumor volume decreased after sulindac treatment in ApcMin mice. The mean overall volume decreased 92 and 86\% in the two sulindac groups compared with controls with both \( P < 0.001 \) (3.6 and 6.0 versus 43.2 mm\(^3\)). Similar to the overall tumor volume, in the small intestine tumor volume decreased 99 and 96\% in two sulindac groups, respectively, over the controls with \( P < 0.01 \) for both (0.6 and 1.7 versus 41.9 mm\(^3\)). However, in the large intestine, tumor volume increased 1.3- and 2.3-fold in the sulindac-treated groups, \( P < 0.01 \) for both compared with controls (3.0 and 4.3 versus 1.3 mm\(^3\)). In both sulindac-treated groups, tumor volume was greater in the large intestine than in the small intestine with \( P < 0.01 \) for both, but it was opposite in the control group (Table I and Figure 1C).

Distribution and histology of the large intestinal tumors. The large intestinal tumors that were unexpectedly found in the sulindac-treated groups were studied in detail, with respect to their location and their histological features.

Location of tumors in the large intestine. The vast majority of large intestinal tumors were located in the right side of the large intestine, especially in the cecum (Table II and Figure 2A). The mean number of large intestinal tumors in the control group was low (0.4). The preponderance of tumors in the right colon increased in the sulindac-treated groups. The number of tumors in the right side increased 5- and 11-fold in both sulindac-treated groups, \( P < 0.01 \) for both compared with controls (1.8 and 3.6 versus 0.3). The tumor number in the right side was significantly higher than in the left side in both sulindac groups, \( P < 0.01 \), but not in the controls (Table II). Thus, the markedly increased tumor incidence in the large intestine was due to tumors developing in the right colon, especially in the cecum.

Histological types of tumors in the large intestine. All 60 tumors found in the large intestine from all three groups were evaluated histologically (Table III). Control ApcMin mice had four tumors found in the colon with one invasive carcinoma and three benign tubular adenomas. After sulindac treatment, invasive carcinomas increased ~8- to 10-fold in both groups. Invasion of the submucosa was found in all 20 adenocarcinomas in sulindac-treated groups (Figure 2B). The potentially malignant villous adenoma increased in the proximal colon in one sulindac group, and flat adenomas were greatly increased and mostly seen in the cecum of mice treated with sulindac.

These findings demonstrated a complex effect of sulindac on tumorigenesis in this preclinical mouse model: tumors inhibited in the small intestine and tumors increased in the large intestine. Similar effects of sulindac on tumorigenesis were also observed in Apc1638N mice (26), another mouse model of human FAP; the sulindac administration and diets fed were the same as for ApcMin mice, and the feeding duration was 6 months longer than for ApcMin mice because the lifespan is 12–15 months in Apc1638N and 4–5 months in ApcMin mice (26).
Apoptosis

Flat mucosa. To evaluate apoptosis during tumor development, apoptotic bodies were counted with criteria noted in the Materials and methods. Findings are presented in Table IV. Apoptosis was counted in the three groups of mice in intestinal crypt epithelial cells of flat mucosa of duodenum, cecum and distal colon. The number of apoptotic cells per crypt column decreased 44% in the cecum (0.34 versus 0.61, \( P < 0.001 \)) and 33% in the distal colon (0.14 versus 0.21, \( P < 0.01 \)), but did not significantly change in duodenal crypts (0.08 versus 0.10, \( P > 0.05 \)) of mice after sulindac treatment. However, in villi of duodenum the number of villous columns with apoptotic cells increased 30% (19.0 versus 14.6, \( P < 0.05 \)) and the number of apoptotic cells per villous column increased 40% (0.21 versus 0.15, \( P < 0.01 \)) in mice after sulindac. The data thus indicated that in response to sulindac treatment apoptosis differed in flat mucosa of the small and large intestine in ApcMin mice.

Tumors. Apoptosis was measured in tumors located in small and large intestine of sulindac-treated and control groups of mice. After sulindac treatment the percentage of apoptotic cells increased 1.4-fold in small intestinal tumors (0.0156 versus 0.0065, \( P < 0.001 \)), and decreased 34% in the cecal tumors (0.0100 versus 0.0151, \( P > 0.078 \)). The reduction of apoptosis in cecal tumors did not reach statistical significance, which might be due to only two tumors in the control group. Thus, in the cecum of ApcMin mice apoptosis decreased in flat mucosal cells with a trend to decrease in tumor cells, and increased in small intestinal villous epithelial cells and tumor cells during tumorigenesis.

Expression of Bax and Bcl-xL

Flat mucosa. Quantitative measurements by computer-assisted cell image analysis showed Bax expression increased and Bcl-xL decreased in epithelial cells in the duodenum with the opposite result in the cecum after sulindac treatment. The overall expression of proteins related to apoptosis in epithelial cells, presented as a ratio of Bax to Bcl-xL, increased in the small intestine and decreased in the cecum after sulindac. The ratio increased 1.2-fold in the duodenum (1.38 versus 0.62, \( P < 0.05 \)) and decreased 55% in the cecum (0.47 versus 1.04, \( P < 0.001 \)) after sulindac treatment (Figure 3A). However, no significant difference was seen in the distal colon (0.72 versus 0.59, \( P > 0.05 \)) (Figure 3A).

Tumors. Similar to the findings of Bax and Bcl-xL expression in flat mucosa of duodenum, Bax expression increased and Bcl-xL decreased in tumors of the small intestine, and the opposite result was observed in the cecal tumors. Figure 2C is representative of the small intestinal tumors showing Bax with strong expression in neoplastic adenoma cells after sulindac treatment. The ratio of Bax to Bcl-xL in tumors increased 3-fold in the small intestine (1.11 versus 0.28, \( P < 0.01 \)) and decreased 79% in the cecum (0.87 versus 4.18, \( P < 0.05 \)) after sulindac treatment in ApcMin mice (Figure 3B).

Discussion

This study demonstrated that the chemopreventive agent sulindac, when administered to ApcMin mice, caused decreased number and size of tumors in the small intestine, consistent with reports in the literature (23–25,27,28). The reduction of tumors in the small intestine provided an important internal control to findings observed in the large intestine. An overall
Table III. Histologic types of large intestinal tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of tumors found in the large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>AIN-76A</td>
<td>4</td>
</tr>
<tr>
<td>Sulindac in AIN-76A</td>
<td>21</td>
</tr>
<tr>
<td>Sulindac in drinking water mice fed AIN-76A</td>
<td>35</td>
</tr>
</tbody>
</table>

Table IV. Apoptosis of intestinal epithelial cells in flat mucosa and tumors of ApcMin mice after receiving control AIN-76A diet and AIN-76A with sulindac

<table>
<thead>
<tr>
<th></th>
<th>Control AIN-76A</th>
<th>AIN-76A with sulindac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of apoptotic cells/epithelial column</td>
<td>0.10 ± 0.02</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Duodenum</td>
<td>0.61 ± 0.04</td>
<td>0.34 ± 0.01^a</td>
</tr>
<tr>
<td>Cecum</td>
<td>0.21 ± 0.02</td>
<td>0.14 ± 0.01^b</td>
</tr>
<tr>
<td>Number of villous columns with apoptotic cells</td>
<td>14.6 ± 1.1</td>
<td>19.0 ± 1.4^c</td>
</tr>
<tr>
<td>Duodenum</td>
<td>0.15 ± 0.01</td>
<td>0.21 ± 0.02^b</td>
</tr>
<tr>
<td>Tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent of apoptotic cells in 1000 tumor cells</td>
<td>0.65 ± 0.08</td>
<td>1.56 ± 0.22^a</td>
</tr>
<tr>
<td>Small intestine</td>
<td>1.51 ± 0.79</td>
<td>1.00 ± 0.08^d</td>
</tr>
<tr>
<td>Cecum</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

By Mann-Whitney test: compared with corresponding organ with AIN-76A: ^aP < 0.001; ^bP < 0.01; ^cP < 0.05.
Compared with tumors of small intestine after sulindac; ^dP = 0.078.
Mean ± SEM. The number of tumors studied was 17 in control group including 15 in the small intestine and two in the cecum; 27 in the sulindac group including 12 in the small intestine and 15 in the cecum.

Fig. 3. Overall expression of proteins Bax and Bcl-xL, presented as ratio of Bax/Bcl-xL in duodenum, cecum and distal colon. (A) In flat mucosa. (B) In tumors of small intestine and cecum. (*P < 0.05, **P < 0.01, ***P < 0.001, sulindac group compared with controls.)

To evaluate the comparative tumor response after sulindac treatment in colon and small intestine, the tumor development ratio of treatment group/control group was analyzed. The relative benefit/harm (RB/H) was calculated as: parameter studied in small intestine × large intestine in the treatment group/parameter studied in small intestine × large intestine in the control group. Treatment was of benefit with RB/H < 1, and was harmful with RB/H > 1. A 95% confidence interval (95% CI) was also calculated using log transformed means to further evaluate the significance of RB/H. A beneficial effect was suggested in tumor multiplicity after treatment (RB/H = 0.49) but without statistical significance (95% CI = 0.091–2.583). However, a beneficial effect was observed with a reduction of tumor volume after treatment in the group receiving sulindac in AIN-76A (RB/H = 0.03, 95% CI = 0.006–0.185). Mice receiving sulindac supplied in drinking water also had a beneficial effect with the tumor volume decreasing, but not tumor number (data not shown). Findings thus indicated a beneficial relative tumor response in colon.
and small intestine after sulindac treatment with decreased tumor volume.

The mechanisms involved in sulindac effects are complex. Pathways of arachidonic acid metabolism, prostaglandin and COX-2 are believed to be involved. Overexpression of COX-2 and PGE2 has been reported in intestinal tumors of humans and ApCMin mice (39–43). Studies show that sulindac sulfide inhibits COX activity, but not the sulfone metabolite (44), and both sulindac sulfide and sulfone are reported to induce apoptosis in different cell types (44–46). Many COX-2-independent mechanisms have been investigated which mediate inhibition of tumors with NSAIDs (47–52).

Apoptosis induced by NSAIDs is a major mechanism in the inhibition of tumors (53–56). Evidence has indicated that multiple pathways are involved in the control of apoptosis, including death receptors on cellular membranes with apaf-1/caspases via cytochrome c released from mitochondria (57–60). BCL-2 family members, Bax, a pro-apoptosis protein, and Bcl-xL, an anti-apoptosis protein, are believed to mediate increased or decreased release of cytochrome c from mitochondria (59). Apoptosis is completely abolished in response to sulindac and other NSAIDs in colon carcinoma cells with a BAX deficiency, indicating that BAX plays a crucial role in this process (55).

Our studies show sulindac significantly increased apoptosis in the small intestine and decreased apoptosis in the cecum in both the flat mucosa and tumors of mice with this Apo mutation. This regional response of apoptosis to sulindac was consistent with the regional tumor response observed, providing evidence of a role for apoptosis in tumorigenesis in this preclinical FAP model; Bax/Bcl-xL expression further revealed a corresponding regional difference after sulindac. Current information on sulindac metabolism in humans and animals (reviewed in refs 56–59) does not indicate a reason for site-specific effects of sulindac in the intestinal tract.

These findings are the first report of a procarcinogenic effect of sulindac in the large intestine in a preclinical model of human FAP. Mice with an Mlh1 mutation also show increased tumor development in both the small and large intestine after sulindac treatment (61). Therefore, it would appear advisable to carry out further studies of sulindac and other NSAIDs in preclinical models related to colon cancer, to further define regional responses and possible interactions with mutations that might occur. So far there is no systematic follow-up information available regarding patients receiving long-term treatment of sulindac or other NSAIDs, although several individuals have been reported to develop colorectal carcinomas after sulindac treatment.

Acknowledgements

The work was supported by NIH Awards CN65031, R01-CA87559, U01-CA-84301 and the Ann E Woodward Foundation.

References