The sulfide metabolite of sulindac prevents tumors and restores enterocyte apoptosis in a murine model of familial adenomatous polyposis

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Sulindac, a non-steroidal anti-inflammatory drug (NSAID), is effective in treating intestinal adenomas in humans with Familial Adenomatous Polyposis (FAP) and in preventing intestinal tumors in the C57Bl6J-Min+ (Min) mouse, an animal model of FAP. Sulindac is a prodrug metabolized by the liver and intestinal flora to a sulfone, which has no anti-inflammatory activity, and a sulfide, which is the active anti-inflammatory metabolite. In this study, we determined which of these metabolites is responsible for the anti-tumor effect of sulindac in Min mice. Min mice were treated with either sulindac sulfone or sulindac sulfide (0.5 ± 0.1 mg/day). Min mice and homozygous C57Bl6J+/+ normal litter-mates lacking the Apc mutation (+/+ ) were used as controls. At 110 days of age, all mice were euthanized and their intestinal tracts examined. Control Min mice had 33.2 ± 6.6 tumors per mouse compared to 0.6 ± 0.3 tumors for sulindac sulfide-treated Min mice (P < 0.001) and 21.9 ± 4.5 tumors per mouse for sulindac sulfone-treated Min mice (P > 0.05). Decreased enterocyte apoptosis was observed in Min control mice and Min mice treated with sulindac sulfone. Sulindac sulfide restored to normal the level of apoptosis in the mucosa of Min animals and decreased levels of PGE2 in the small intestine of treated Min animals by 59% (P < 0.001). These data suggest that the anti-tumor effect of sulindac in Apc-deficient animals is mediated by the sulfide metabolite and correlates with suppression of tissue prostaglandin synthesis.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs*) have been associated with inhibition of colorectal carcinogenesis in both epidemiologic studies and animal tumor models. Regular use of NSAIDs by humans is associated with as much as a 40–50% decrease in colorectal cancer incidence (1,2). In carcinogen-induced rodent colon tumor models, piroxicam (3,4), sulindac (5,6) and to a lesser extent, indomethacin, ketoprofen, ibuprofen, and aspirin (7–12), all inhibit tumor formation. The mechanism by which anti-inflammatory drugs inhibit intestinal carcinogenesis is not fully understood. Most data suggest that the anti-tumor efficacy of these agents resides, at least in part, in their ability to inhibit prostaglandin synthesis. In a previous report by our laboratory, sulindac blocked tumor formation in the multiple intestinal neoplasia (Min) mouse, a murine model of Apc mutation-associated intestinal carcinogenesis (13,14). In the Min mouse, tumor-preventing doses of sulindac inhibited tissue levels of prostaglandin-E2 (PGE2), as well as cyclo-oxygenase-2 (Cox-2), an inducible enzyme involved in prostaglandin synthesis (15).

In humans with Familial Adenomatous Polyposis (FAP), a cancer susceptibility syndrome characterized by an inherited Apc gene mutation, sulindac induces regression of rectal adenomas (16–19) and clinical response to sulindac in humans with FAP also correlates with a decrease in total tissue PGE2 levels (20). In both humans with FAP and Min mice, the intestinal mucosa exhibits a decreased level of epithelial cell apoptosis. In the Min mouse, decreased enterocyte apoptosis precedes development of neoplastic architectural changes in the mucosa (15). Administration of tumor-preventing doses of sulindac to Min mice both inhibits cyclo-oxygenase activity and restores normal levels of apoptosis in the small intestine (15).

Sulindac, a sulfone derivative, does not inhibit prostaglandin synthesis (21). Sulindac is a prodrug metabolized by the liver and the colonic bacteria by irreversible oxidation to sulindac sulfone and reversible reduction to sulindac sulfide. The anti-inflammatory activity of sulindac resides in the sulfide derivative which, following oral administration in animals, is approximately twice as potent as sulindac in suppressing tissue inflammation (21,22). Following oral ingestion of sulindac in rodents, cecal contents contain 77% sulfone, 14% sulfide and 9% sulindac, and serum levels contain 84% sulfone, 9% sulfide and 7% sulindac (6). Although data strongly suggest that anti-inflammatory activity is required for colorectal tumor prevention by NSAIDs, some reports indicate anti-tumor activity by the sulfone derivative. Sulindac sulfone has been reported to prevent tumor formation in a rat breast cancer model (23,24) and to inhibit the in vitro growth of human colorectal cancer cell lines (25,26). In particular, cell line data show that sulindac sulfone induces apoptosis in colonic epithelial cells, an effect which correlates with the anti-tumor effect of sulindac in both in vitro and in vivo studies (24,26).

To determine whether the anti-tumor effect of sulindac correlates with inhibition of prostaglandin activity, we administered the two major metabolites of sulindac independently to Min mice and studied anti-tumor efficacy according to our previous model (15). We found that dietary administration of the sulfide metabolite decreased tissue levels of PGE2 and suppressed tumor formation in the Min mouse. An equivalent dose of the sulfone derivative, however, failed to demonstrate an effect on tissue PGE2 levels or tumor formation. Previous studies of histologically normal-appearing mucosa from the Min mouse demonstrated decreased levels of enterocyte apoptosis. Previous model (15). We found that dietary administration of the sulfide metabolite decreased tissue levels of PGE2 and suppressed tumor formation in the Min mouse. An equivalent dose of the sulfone derivative, however, failed to demonstrate an effect on tissue PGE2 levels or tumor formation. Previous studies of histologically normal-appearing mucosa from the Min mouse demonstrated decreased levels of enterocyte apoptosis.
apoptosis (15), a defect which was corrected upon sulindac administration. In the present study, the decreased level of enterocyte apoptosis in histologically normal-appearing mucosa from Min animals was unaffected by sulindac sulfone administration. The sulfide derivative, however, increased apoptosis to levels observed in mice lacking the Apc mutation. These data suggest that the effect of sulindac upon tumorigenesis and enterocyte apoptosis in the Min mouse is due to the sulfide rather than the sulfone derivative, and correlates with inhibition of tissue prostat gland activity.

Materials and methods

Treatement of Min mice with sulindac sulfide and sulindac sulfone

Female C57Bl/6J-Min/+ (Min) mice, a strain containing a fully penetrant dominant mutation in the Apc gene, were obtained at 5 weeks of age from Jackson Laboratories, Bar Harbor, ME. Beginning at 5–6 weeks of age, Min mice in a control group were fed AIN-76A diet (Research Diets, New Brunswick, NJ) and given tap water to drink. Treatment groups were fed AIN-76A diet and either sulindac sulfide or sulindac sulfone 0.5 ± 0.1 mg/day (0.05 mg/kcal/day; 20 mg/kg body wt/day or ~160 ppm) in drinking water. Because the metabolism of sulindac to sulindac sulfone is irreversible, the orally-administered dose chosen for this study should reflect the activity present in a comparable dose of sulindac. The dose of sulindac sulfone used (20 mg/kg/day) is therefore roughly equivalent to that administered in our previous study of sulindac treatment of Min mice (15). The sulfone preparation used contains ~1.4% sulindac, equivalent to ~0.04 mg/kg/day of sulindac sulfone if metabolized. This degree of impurity is unlikely to be significant in this model. We also chose a dose of 20 mg/kg/day of sulindac sulfide for this study so that the effects could be compared to those obtained with sulindac sulfone treatment. Sulindac sulfide and sulindac sulfone were obtained from Merck & Co., Inc. As additional controls, C57Bl/6J-+/+ non-affected littermates (+/+) were fed AIN-76A diet without drug supplementation. Animals were also fed AIN-76A diet without drug supplementation. Animals were checked daily for signs of wt loss or lethargy that may indicate intestinal obstruction or anemia. Animals and their food were weighed twice weekly.

During the course of the experiment there was no difference in body wt or food consumption among the various study groups and the animals remained healthy. Animals were weighed twice weekly. Multiple samples of grossly-normal full-thickness small intestine were counted by an individual blinded to the animal’s genetic status and examined under 3 magnification to obtain tumor counts. Tumors were counted by an individual blinded to the animal’s genetic status and treatment. Values expressed represent mean number of tumors per mouse ± SEM where n = 11 (Min), and n = 10 (+/+, Min/sulfide, and Min/sulfone). By analysis of variance (ANOVA), values for tumor distribution indicate total number of tumors per mouse

<table>
<thead>
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<th>Treatment group</th>
<th>Tumors per mouse</th>
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<tr>
<td>Min</td>
<td>33.2 ± 6.6</td>
</tr>
<tr>
<td>Min/sulfide</td>
<td>21.9 ± 4.5</td>
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<tr>
<td>Min/sulfone</td>
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*P < 0.001 compared to Min, **P > 0.05 compared to Min.

Microvolumes of supernatant was diluted 1/100 in enzyme immunoassay (EIA) buffer and 50 μl of this dilution was added to a PGE2 assay plate. Determination of PGE2 levels by EIA was accomplished using a Prostaglandin-E2-Monoclonal Enzyme Immunoassay Kit (Caymen Chemical, Ann Arbor, MI), following the manufacturer’s protocol. Plates were read at 410 nm with a UV max Kinetic Plate Reader (Molecular Devices, San Jose, CA). Data were computerized with DeltaSoft 3 and statistics performed with InStat 2.0 software.

In situ detection of apoptosis

To determine the percentage and distribution of epithelial cells undergoing cell death, we employed an in situ direct immunoperoxidase technique for determining cell death using the ApopTagTM Kit (Oncor, Gaithersburg, MD). This method detects digoxigenin-labeled 3'-OH ends of genomic DNA. After deparaffinization in xylene followed by ethanol series, small intestine sections were immersed in 2% hydrogen peroxide for 5 min, followed by a PBS wash. The specimens were then incubated for 1 h at 37°C with TdT working enzyme. A stop-wash buffer was then applied for 30 min, followed by a PBS rinse and incubation in anti-digoxigenin-peroxidase for 30 min at room temperature. Color development was achieved by incubation for 5 min with a solution containing 3,3'-diaminobenzidine with hydrogen peroxide. The specimens were counterstained with methyl green.

Mouse lymphoid tissue with a known 2–3% rate of apoptosis was used as a positive control. For each specimen, 8 crypt-villus units were counted randomly from serial sections of small intestinal mucosa by an individual blinded to the animal’s treatment group and genetic status. The percentage staining of enterocytes in these crypt-villus units was measured using the Cell Analysis System 200 (CAS 200) and CAS 200 Quantitative Nuclear Analysis Software. To confirm that uniform sampling was achieved, nuclear density was measured and confirmed to be equal throughout the three study groups.

Results

Tumor suppression

Previous studies of sulindac’s anti-tumor activity in the Min mouse showed that 0.5 mg/day (20 mg/kg/day) of sulindac inhibited tumor formation in these animals. Based upon studies of the metabolism and intestinal absorption of sulindac in rodents (6,21), this dose corresponds to ~15–16 mg/kg/day of sulindac sulfone, 2–3 mg/kg/day of sulindac sulfide and 1–2 mg/kg/day of non-metabolized sulindac. We therefore chose a dose of 20 mg/kg/day of sulindac sulfone for this study to achieve levels comparable to or above those obtained upon administration of 20 mg/kg/day of sulindac. Beginning at 5–6 weeks of age, Min mice were treated for 10 weeks with either sulindac sulfone or a comparable dose of sulindac sulfide. At 110 days of age, untreated control Min mice had 33.2 ± 6.6 tumors each (Table 1). These adenomas were

Table 1. Sulindac sulfide inhibits tumors in the Min mouse. Beginning at 5–6 weeks of age, Min mice were fed AIN-76A chow diet and sulindac sulfide (Min/sulfide) or sulfone (Min/sulfone), 0.5 ± 0.1 mg/day in drinking water. As controls, Min mice (Min) and C57Bl/6J-+/+ non-affected littermates (+/+) were fed AIN-76A diet without drinking water additives. Each treatment group contained 10 mice, except the Min control group, which contained 11 animals. At 110 days of age (10 weeks of treatment), all mice were killed and their intestinal tracts removed and examined under 3 magnification to obtain tumor counts. Tumors were counted by an individual blinded to the animal’s genetic status and treatment. Values expressed represent mean number of tumors per mouse ± SEM where n = 11 (Min), and n = 10 (+/+, Min/sulfide, and Min/sulfone). By analysis of variance (ANOVA), values for tumor distribution indicate total number of tumors per mouse
distributed throughout the intestinal tract from the duodenum to the rectum, with, as expected, >90% of the tumors located in the jejunum and ileum (Table II). No invasive tumors were found. The total polyp count in the untreated Min animals was high compared to some previously published reports (3,15), although comparable to that reported by others (27–29). This suggests that there is a wide variability in the number of tumors identified in Min mice. To control for this in our studies, littermates were distributed evenly throughout the different treatment groups, and all tumors were counted by the same observer who was blinded to the animal’s genetic and treatment status.

In the 10 animals treated with sulindac sulfone (Min/sulfone), 21.9 ± 4.5 tumors per mouse were identified (Table I). Although this suggested a trend toward reduction in tumor number, a multiple group comparison study (analysis of variance) showed that there was no significant difference in tumor number between the Min/sulfone and the Min animals, nor was there a change in the location of tumors throughout the intestinal tract (Table II). Among the 10 mice treated with sulindac sulfide (Min/sulfide), only three animals developed adenomas. These tumors were also located primarily in the small intestine.

Samples of grossly normal small intestinal mucosa were harvested from each group of mice. When examined by light microscopy using H&E stain, no additional dysplasias, aberrant crypt foci or micro-adenomas were found in the Min/sulfide or Min/sulfone treated mice. There were no tumors visible in the +/- mice, either grossly or on microscopic examination of multiple sections from normal-appearing tissue.

**Inhibition of tissue prostaglandin activity**

Following 10 weeks of treatment with either sulindac sulfide or sulindac sulfone, we determined tissue levels of PGE2 to ensure that the administered metabolites exerted their stated activities. Full-thickness specimens from the mid small intestine of treated and control animals were assayed for PGE2 as described in Materials and methods. We found that oral administration of sulindac sulfide decreased the tissue level of PGE2 in Min animals by 59%, whereas treatment with sulindac sulfone had no effect (Figure 1). These data demonstrate suppression of tissue prostaglandin synthesis by oral administration of the sulfate, but not the sulfone derivative of sulindac. Inhibition of prostaglandin synthesis, therefore, correlates with inhibition of intestinal tumor formation in this experimental model.

**Table II.** Distribution of tumors. Intestinal tracts from each treatment were examined under ×3 magnification and tumors were counted by an individual blinded to the animal’s genetic status and treatment. Values expressed represent mean number of tumors per mouse ± SEM where n = 11 (Min) and n = 10 (Min/sulfide and Min/sulfone). Values for tumor distribution indicate total number of tumors per mouse per intestinal segment. Statistical analysis by Analysis of Variance (ANOVA) demonstrated no significant difference in tumor distribution between the three groups. For tumors located in the small intestine, a significant reduction in tumor number resulted from sulindac sulfide administration.

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<tr>
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<th>Duodenum</th>
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<th>Large intestine</th>
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<tr>
<td>Min</td>
<td>0.3 ± 0.2</td>
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<tr>
<td>Min/sulfide</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.3*</td>
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<tr>
<td>Min/sulfone</td>
<td>0.1 ± 0.1</td>
<td>20.9 ± 4.4**</td>
<td>0.8 ± 0.3</td>
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*P < 0.001 compared to Min; **P > 0.05 compared to Min.

Fig. 1. Decrease in tissue prostaglandin-E2 levels correlates with tumor suppression in the Min mouse. Levels of PGE2 were determined in small intestine from Min, sulindac sulfide (Min/sulfide) and sulindac sulfone (Min/sulfone)-treated Min mice. Values represent mean ± SEM where n = 11 (Min); n = 10 (Min/sulfide) and n = 9 (Min/sulfone). By ANOVA, *P < 0.001 compared to Min; **P > 0.05 compared to Min.

**Reversal of APC-associated changes in enterocyte apoptosis**

A decrease in enterocyte apoptosis has been implicated in intestinal carcinogenesis (15,30–32). In previous studies, administration of tumor-preventing doses of sulindac to Min mice resulted in an increase in enterocyte apoptosis to levels equal to that of unaffected +/- littermates (15). We therefore determined whether this response could be attributed to an equivalent dose of sulindac sulfone.

After 10 weeks of treatment (110 days of age), control Min mice and Min mice treated with sulindac sulfone or sulindac sulfide as described above were euthanized and specimens of grossly normal small intestine were harvested. These tissues were compared to those obtained from unaffected +/- littermates. To confirm that these specimens were free of tumor, sections from each sample were examined by light microscopy using H&E stain. No dysplasias or microadenomas were observed. Because apoptosis occurs in specific locations along the crypt-villus axis, we used an in situ direct immunoperoxidase method to study apoptosis. A comparison between the histologically normal-appearing small intestinal mucosa of the Min and +/- littermates demonstrated an 80% decrease in apoptotic fraction in Min mice compared to their +/- littermates (Figure 2). All of the apoptotic cells were located in the top (luminal) 1/3 of the crypt-villus, the normal location of terminally differentiated enterocytes.

Administration of sulindac sulfide resulted in an increase in enterocyte apoptosis in the Min animals to a level equal to that of the unaffected +/- littermates. Treatment with the sulfone derivative, however, did not alter the level of apoptosis in the small intestinal mucosa.

**Discussion**

Studies in both Min mice (15) and humans (16) demonstrate that oral administration of sulindac induces regression of intestinal adenomas. These studies utilized comparable doses of sulindac, as the effective dose for Min mice was four times higher than that given to humans in keeping with a four-fold less absorption of orally-administered sulindac in rodents as compared to man (21). Due to metabolism of sulindac by intestinal flora, the intraluminal concentration of sulindac sulfide is higher in the distal large intestine than in the small.
intestine. The relatively insoluble sulfide is excreted in only small amounts in the bile (21). It is also interesting that, when administered to humans with FAP, sulindac is successful in treating large intestinal tumors, but not duodenal adenomas. These observations have led researchers to the conclusion that it is the intraluminal drug concentration of sulindac sulfide, rather than serum level, that determines anti-tumor effect. Sulindac-treated Min mice, however, demonstrate near-complete inhibition of small intestinal tumors, a region of the bowel with scant microbial activity where intraluminal sulindac sulfide concentration is relatively low (15). This observation suggests that systemic, rather than intraluminal sulindac sulfide levels may be responsible for tumor suppression.

Sulindac sulfide is a potent inhibitor of cyclo-oxygenase, whereas sulindac sulfone has no anti-prostaglandin activity (21). Numerous studies link cyclo-oxygenase activity to intestinal tumor formation. Cox-2 is up-regulated in ~90% of human colon cancers and 40% of premalignant adenomas, but not duodenal adenomas. Moreover, levels of prostaglandins are increased in benign tumors and cancers of the colon (33). Evidence of a critical link between human colon cancers and 40% of premalignant adenomas, but intestinal tumor formation. Cox-2 is up-regulated in ~90% of these data suggests that there may be two different pathways for achieving cancer prevention in epithelial tissues. Data from mammary tumor models and carcinogen-induced rat intestinal tumor studies suggest that sulindac sulfone prevents cancer by a prostaglandin-independent pathway. Substantial data both in vivo and in vitro suggest the existence of a second prostaglandin-dependent pathway for carcinogenesis that may act through overexpression of Cox-2. Inhibition of prostaglandin synthesis may be sufficient for tumor prevention in this instance. Further support for this pathway awaits additional studies of selective Cox-2 inhibitors in cancer chemoprevention.

Acknowledgements

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References


Fig. 2. Apoptosis in Min intestinal mucosa. Tissue sections were processed as described in Materials and methods, and examined using the CAS system. All of the positively-stained cells were located in the upper 1/3 of the crypt-villus. Values expressed are percentages of total cells positive ± SEM, with 100% equal to the entire crypt-villus population. There were 10 animals studied in all treatment and control groups (n = 10). By ANOVA, *P < 0.001 compared to +/+; **P > 0.05 compared to Min; †P < 0.001 compared to Min.
Tumor prevention by sulindac sulfide


