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Cyclooxygenase-2 Overexpression and Tumor Formation Are Blocked by Sulindac in a Murine Model of Familial Adenomatous Polyposis[1]

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ABSTRACT

Inducible cyclooxygenase (Cox-2), also known as prostaglandin H synthase 2 (PGH-2) is a key enzyme in the formation of prostaglandins and thromboxanes. Cox-2 is the product of an immediate-early gene that is expressed in response to growth factors, tumor promoters, or cytokines. Overexpression of Cox-2 is associated with both human colon cancers and suppression of apoptosis in cultured epithelial cells, an activity that is reversed by the nonsteroidal anti-inflammatory drug, sulindac sulfide. To address the relationship between Cox-2, apoptosis, and tumor development *in vivo*, we studied C57BL/6J-*Min*/+(Min) mice, a strain containing a fully penetrant dominant mutation in the *Apc* gene, leading to the development of gastrointestinal adenomas by 110 days of age. Min mice were fed AIN-76A chow diet and given sulindac (0.5 ± 0.1 mg/day) in drinking water. Control Min mice and homozygous C57BL/6J-+/+ normal littermates lacking the *Apc* mutation (+/+) were fed AIN-76A diet and given tap water to drink. At 110 days of age, all mice were sacrificed, and their intestinal tracts were examined. Control Min mice had 11.9 ± 7.8 tumors per mouse compared to 0.1 ± 0.1 tumors for sulindac-treated Min mice. As expected, +/+ littermates had no macroscopic tumors.

Examination of histologically normal-appearing small bowel from Min animals revealed increased amounts of Cox-2 and prostaglandin E_2 compared to +/+ littermates. Using two different *in situ* techniques, terminal transferase-mediated dUTP nick end labeling and a direct immunoperoxidase method, Min animals also demonstrated a 27–47% decrease in enterocyte apoptosis compared to +/+ animals. Treatment with sulindac not only inhibited tumor formation but decreased small bowel Cox-2 and prostaglandin E_2 to baseline and restored normal levels of apoptosis. These data suggest that overexpression of Cox-2 is associated with tumorigenesis in the gastrointestinal epithelium, and that both are inhibited by sulindac administration.

INTRODUCTION

An inducible isoform of Cox,[3] designated Cox-2, has been characterized as an immediate-early response gene that, like c-*fos* and c-*jun*, is rapidly induced following stimulation of quiescent cells by mitogens (1, 2). In contrast to Cox-1, which is constitutively expressed, Cox-2 is inducible in a variety of cell types including squamous cells, endothelial cells, macrophages, and intestinal epithelial cells (3–5). The biosynthesis of Cox-2, which has approximately 61% amino acid homology with Cox-1, is stimulated by serum, growth factors, cytokines, and phorbol esters, whereas Cox-1 is unaffected by these agents (6). Recently, overexpression of Cox-2 has been associated with epithelial malignancy. In studies of human colorectal cancer, Cox-2 is increased in about 90% of cancers and 40% of premalignant colorectal adenomas, but it is not expressed in nontumor colon tissue (7). NSAIDs, agents that block the activity of Cox-2, are associated with a decreased incidence of colon cancer in human studies (7, 8). In patients with FAP, the NSAID sulindac is also effective in mediating regression of colorectal adenomas (9–11). Treatment with NSAIDs is associated with a decrease in Cox-2 in colonic tumors (8).

Although a relationship between overexpression of Cox-2 and cancer is suggested by these observations, no mechanism for this process has been established. A recent report by Tsujii and DuBois (12) suggests a possible link between Cox-2 and epithelial cell growth. In this study, rat intestinal epithelial cells expressing high levels of Cox-2 were resistant to apoptosis. Treatment with sulindac sulfide blocked Cox-2 activity and restored the apoptotic response in these cells. This relationship between Cox-2 and apoptosis might also be valid *in vivo*, because sulindac has been found to induce apoptosis of intestinal epithelial cells of humans with FAP (13). In this study, we determined the relationship between Cox-2, apoptosis, and tumor formation in an *in vivo* model. The Min mouse carries a fully penetrant dominant mutation at codon 850 of the murine *Apc* gene that converts a leucine into a stop codon and results in a truncated APC protein (14). *Min/Min* homozygous mice are not viable, whereas 100% of heterozygous female *Min*/+ mice raised on a high-fat diet develop adenomas throughout the intestinal tract. The majority of these adenomas are located in the small intestine, and most *Min*/+ mice die of tumor-related anemia or bowel obstruction by 120 days of age (15). The adenomas that develop in Min mice contain multiple epithelial cell lineages, suggesting that the defect arises in a multipotent epithelial stem cell. In addition, these adenomas show loss of the wild-type *Apc* locus, consistent with the tumor suppressor gene designation of *Apc* (16). For unknown reasons, phenotypic expression of an *Apc* mutation in the Min mouse is quite different from that of humans with FAP, where adenomas are found exclusively in the colon and duodenum. Nevertheless, the use of this model allows us to study carcinogenesis related to *Apc* while controlling for both genetic and environmental factors in the development of intestinal neoplasia.

In the Min mouse, we found that Cox-2 was overexpressed in histologically normal-appearing small bowel tissue. This was associated with increased levels of PGE_2 and decreased apoptosis. All of these abnormalities were reversed with sulindac, which also prevented the formation of tumors in these animals. These data suggest that overexpression of Cox-2 is associated with both modulation of apoptosis and tumorigenesis of the gastrointestinal epithelium.

MATERIALS AND METHODS

Treatment of Min Mice with Sulindac. 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 The Jackson Laboratory (Bar Harbor, ME). Beginning at 5–6 weeks of age, 10 Min mice were fed a low-fat AIN-76A chow diet modified with 0.001% ethoxyquin (Research Diets, New Brunswick, NJ; Ref. 17) and sulindac (Sigma Chemical Co., St. Louis, MO), 0.5 ± 0.1 mg/day (0.05 mg/kcal/day or approximately 160 ppm) in drinking water. This dose was determined using preliminary information provided by

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[3] The abbreviations used are: Cox, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; FAP, familial adenomatous polyposis; PGE_2, prostaglandin E_2; APC, adenomatous polyposis coli; TUNEL, terminal transferase-mediated dUTP nick end labeling; TNF, tumor necrosis factor; IL, interleukin.

Dr. Alex Wood (Hoffman-LaRoche, Nutley, NJ). As controls, 9 Min mice and 5 C57BL/6J-+/+ non-affected littermates (+/+) were fed AIN-76A diet without sulindac. Animals were checked daily for signs of distress or anemia. Animals and their food were weighed twice weekly. During the course of the experiment, there was no difference in body weight or food consumption among the various study groups. No toxicity was observed in the Min/sulindac group. At 110 days of age, all mice were euthanized by CO_2 inhalation, and their intestinal tracts were removed from esophagus to distal rectum, opened, flushed with saline, and examined under ×3 magnification to obtain tumor counts. Tumors were counted by an individual blinded to the animal's genetic status and treatment. Multiple samples of grossly normal, full-thickness bowel were harvested from the mid small intestine and either frozen in liquid nitrogen or fixed in 10% formalin for histological examination. All samples used for the analyses in this study were taken from mid small intestine.

Tissue Histology. For each animal, 10–15 sections of small bowel mucosa were examined. Specimens of small bowel of approximately 5 mm in length were formalin fixed, embedded in paraffin, and sectioned at 3 μm. Sections were stained with hematoxylin and eosin for evaluation of mucosal histology.

For studies of nuclear density, Fuelgen stain was performed. Small bowel specimens were immersed in a 5 N hydrochloric acid solution and hydrolyzed for 1 h, producing sugar aldehyde residues. A stoichiometric Schiff's reaction occurs when placed in the Fuelgen stain, producing a blue color. The specimens were then rinsed and immersed in acid alcohol and Hemo-De. To prepare sections for immunohistochemistry, small bowel sections were deparaffinized and dehydrated by processing the slides with Hemo-De (Fisher Scientific, Pittsburgh, PA) and an alcohol series, followed by washing in PBS (pH 7).

Measurement of PGE_2. Intact small bowel from distal jejunum-proximal ileum was homogenized in 100 μl ice-cold lysis buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin] using a glass-Teflon tissue homogenizer. Intact small bowel, rather than intestinal mucosa only, was used for this study because a relatively large volume of material was required for analysis and difficulty was encountered in stripping mucosa from muscularis in tiny mouse intestine samples. An aliquot of 5 μl from each sample was used to determine protein concentration with a Bicinchoninic Acid Protein Assay Kit (Sigma) according to the manufacturer's protocol. After being homogenized, the sample was transferred to microcentrifuge tubes and vortexed. Homogenates were centrifuged at 4°C for 10 min at 10,000 × *g*, and 5 μl of supernatant from each sample were added to a PGE_2 assay plate. Determination of PGE_2 levels by enzyme immunoassay was accomplished using a Prostaglandin-E_2-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 were performed with InStat 2.00 software.

Measurement of Cox-2. To prepare microsomes for determination of Cox-2 levels, approximately 20 mg of small bowel were added to 200 μl of lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% polysorbate (Tween 20), 50 mM diethyldithiocarbamate, 1 mM EDTA (pH 8.0), and 1 mM phenylmethylsulfonyl fluoride] and homogenized in a glass-Teflon homogenizer. As with PGE_2 measurement, the quantity of tissue necessary for this assay required the use of intact small bowel, rather than intestinal mucosa only. After centrifuging the homogenate at 12,000 × *g* and 4°C for 10 min, the resulting supernatant was centrifuged at 100,000 × *g* in a L8–70 ultracentrifuge (Beckman, Palo Alto, CA) for 1 h. The microsomal pellet was reconstituted in 1 ml of lysis buffer, and the microsomal protein concentration was measured using a modified micro-Lowry assay (Sigma) with BSA as a standard.

Cox-2 protein was identified by Western blotting. Monoclonal antibody specific for Cox-2 was obtained from Dr. Jacques Maclouf (18). Two hundred μg of microsomal protein were prepared and loaded onto a gel for SDS-PAGE (3.5% stacking, 10% resolving gel under reducing conditions with 4% β-mercaptoethanol) by the method of Laemmli (19). Proteins were transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) as described by Towbin *et al.* (20). The membrane was blocked overnight (3% BSA, 0.1% Triton X-100, and 0.01% SDS in PBS), incubated for 3 h with antiserum, then probed with goat antimouse antibody conjugated to alkaline phosphatase (Promega, Madison, WI). Bands were detected using Western Blue Stabilized Substrate for alkaline phosphatase (Promega). Ovine Cox-2 was used as a positive control.

***In Situ* Detection of Apoptosis.** To determine the percentage and distribution of epithelial cells undergoing cell death, we used an immunofluorescence technique, the TUNEL method. Sections of small intestine were treated with 20 μg/ml proteinase K in 10 mM Tris-HCl buffer (pH 8.0) for 30 min at room temperature. The TUNEL reaction mixture (Boehringer-Mannheim, Indianapolis, ID) was applied to the specimens, which were then incubated in a humidified chamber at 37°C for 1 h. Following a wash with PBS and embedding with an anti-fade medium, the specimens were analyzed using a fluorescence microscope. For each assay, 10 separate whole crypt-villus units chosen randomly from serial sections of mucosa were counted by an observer blinded to the treatment group and the genetic status of the tissues. Positive cells were recorded separately for each one-third of the crypt-villus area.

An *in situ* direct immunoperoxidase technique for determining cell death was also employed using the ApopTag Kit (Oncor, Gaithersburg, MD). This method detects digoxigenin-labeled 3′-OH ends of genomic DNA. Small bowel 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, followed by anti-digoxigen-peroxidase for 30 min at room temperature. Color development was achieved by incubation for 5 min with a solution containing 3′-3′ diaminodenzidine with hydrogen peroxide. The specimens were counterstained with ethyl green. To establish a positive control, a section was pretreated with DN buffer [30 mM Trizma base (pH 7.2), 140 mM cacodylate, 4 mM MgCl, and 0.1 mM DTT] for 5 min. The specimen was then incubated in DNase solution for 40 min at room temperature. After rinsing in distilled H_2O, the control was processed as described previously. For each specimen, eight crypt-villus units were chosen randomly from serial sections of small bowel mucosa by an individual blinded to the animal's treatment group and genetic status. The percentage of 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.

RT-PCR Quantitation of Cytokine mRNA. Total RNA was extracted from 20 mg of mouse small bowel from each animal tested using RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. Small bowel specimens were incubated with RNase free-DNase I at 37°C for 20 min to eliminate possible DNA contamination, RNA content was quantitated at 260 nm with a DU-64 spectrophotometer (Beckman, Fullerton, CA), and 0.5 μg of total RNA from each sample was used for RT-PCR. The mRNA were converted to single-strand cDNA using murine leukemia virus reverse transcriptase and 2.5 μM of oligo d(T), according to the manufacturer's protocol (Gene-Amp RNA PCR kit; Perkin-Elmer, Branchburg, NJ). The cDNA samples were divided into equal aliquots for amplification by the specific primers for TNF-α, IL-1, IL-6, and β-actin. PCR reactions were carried out in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, and 5 μM of specific primers using a cycling program of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). Total RNA (0.2–0.5 μg) was used for 25, 30, 35, 40, and 45 cycles to choose PCR cycles with exponential amplification. RT-PCR reactions were also performed with serial two-time dilution of RNA samples to confirm the linear amplification. All PCR primers were gifts from Dr. Steven H. Herrmann of Genetics Institute, Cambridge, MA. The PCR primers used were as follows: TNF-α, 3′-ACAGAAAGCATGATCCGCGA and 5′-ACTTGGGCAGATTGACCTCA; IL-1, 3′-GATCTGGAAGAGACCATCCA and 5′-AGAGACTCAGCACATGCTGT; IL-6, 3′-ACTGATGCTGGTGACAACCA and 5′-TGTGACTCCAGCTTATCTGT; and β-actin, 3′-ACCCTAAGGCCAACCGTGAA and 5′-TAGGAGCCAGAGCAGTAATC.

PCR products were separated by electrophoresis in 1.5% agarose gels, visualized by 0.5 μg/ml ethidium bromide staining for 1 h, and documented by Eagle Eye II Still Video Imaging System (Stratagene, La Jolla, CA). The relative quantitation was determined using NIH Image 1.52 software. Data were normalized to β-actin. Student's *t* test analysis of the data was performed using the InStat 2.00 program.

RESULTS

Min Small Intestine Expresses Increased Amounts of Cox-2 and Exhibits Decreased Epithelial Cell Apoptosis. At 110 days of age, Min mice and +/+ littermates were sacrificed, and specimens of grossly normal small bowel were harvested. 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. The levels of Cox-2 prepared from histologically normal-appearing small bowel of Min and +/+ mice were assessed by Western blot. Cox-2 was not detectable in the tissue from the +/+ mice; however, a faint band indicated the presence of Cox-2 in the small bowel from the untreated Min animals (Fig. 1). To assess the function of this protein, we also examined individual small bowel samples for PGE_2. A quantitative enzyme immunoassay assay for PGE_2 demonstrated a significant increase in PGE_2 in the tissue from the Min mice (Fig. 2).

Overexpression of Cox-2 has been associated with decreased apoptosis in *in vitro* studies of intestinal epithelial cells (12). Apoptosis is decreased in colorectal cancer specimens and has also been reported to be decreased in FAP tissues compared to normal human controls (21). Because apoptosis occurs in specific locations along the crypt-villus axis, we used *in situ* methods to study apoptosis in our model. By the TUNEL and direct immunoperoxidase methods, a comparison between the histologically normal-appearing small bowel mucosa of Min mice and +/+ littermates demonstrated a decrease in apoptotic fraction in Min mice compared to their +/+ littermates (Fig. 3). This decrease was 27% by the TUNEL method and 47% by the direct immunoperoxidase method. All of the apoptotic cells were located in the top (luminal) one-third of the crypt-villus, the normal location of terminally differentiated enterocytes.

These data suggest that, consistent with the results of human FAP studies, apoptosis is decreased in histologically normal-appearing tissue from Min animals. Similar to that observed *in vitro*, increased levels of Cox-2 are associated with decreased epithelial cell apoptosis.

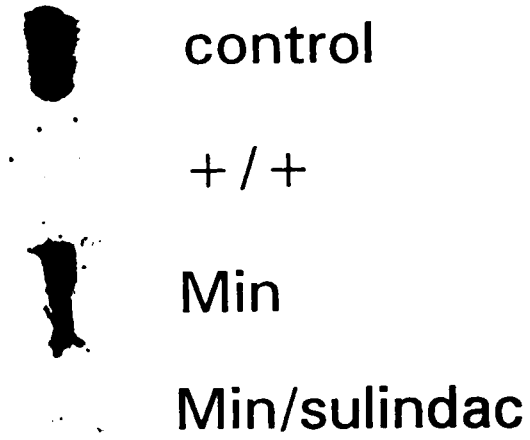

Fig. 1. Cox-2 is elevated in Min small bowel and decreased by sulindac treatment. Microsomes were isolated from small bowel as described in "Materials and Methods." Microsomal protein (200 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and transferred onto nitrocellulose. Cox-2 protein was detected by probing with monoclonal antibody specific for Cox-2. Bands indicating Cox-2 protein were detected at M_r 69,000. By this method, Cox-2 protein was detected in Min (*Lane 2*) but not Min/sulindac or +/+ tissues (*Lanes 1* and *3*).

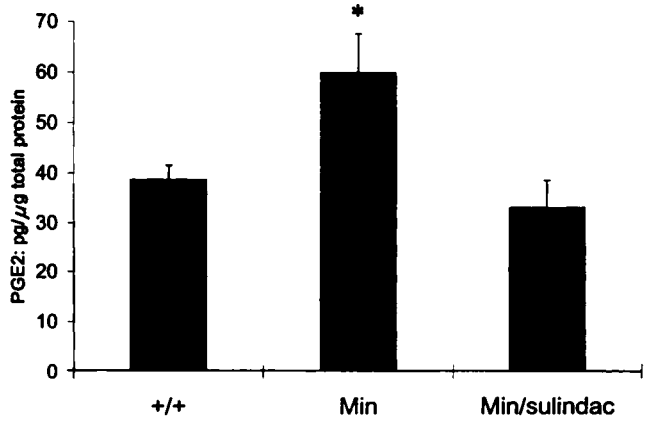

Fig. 2. PGE_2 is elevated in Min small bowel and modulated by sulindac treatment *in vivo*. Levels of PGE_2 were determined in small bowel from +/+, Min, and sulindac-treated Min (*Min/sulindac*) mice. Values represent the means, where $n = 5$ (+/+), $n = 9$ (Min), and $n = 10$ (Min/sulindac); *bars*, SE. *, $P = 0.04$ compared to +/+ and $P = 0.01$ compared to Min/sulindac.

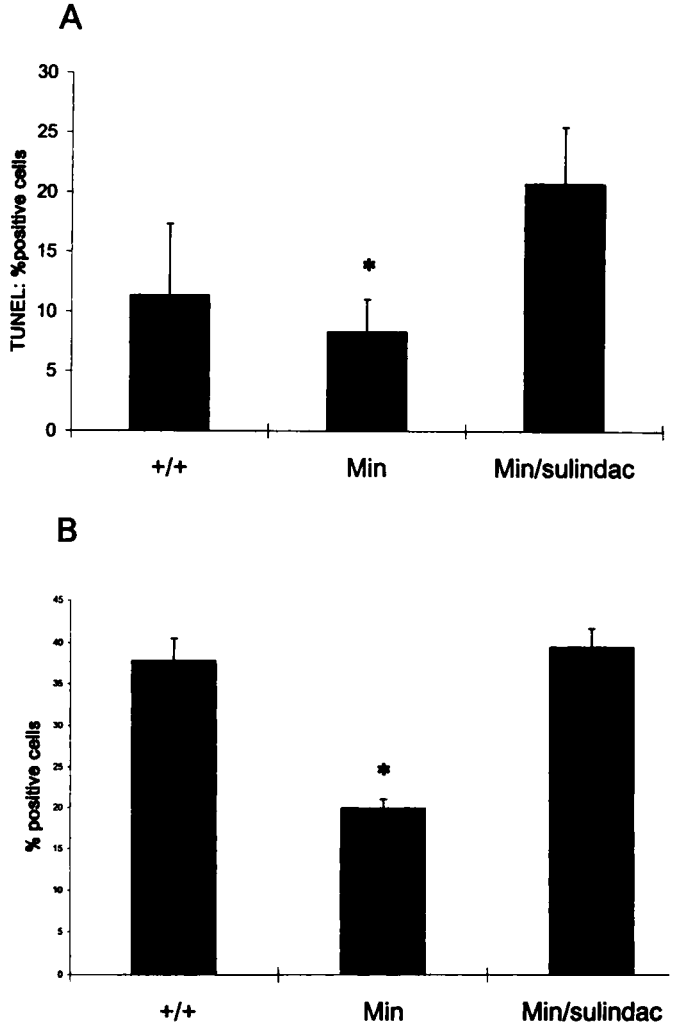

Fig. 3. *A*, enterocyte apoptosis *in situ*: TUNEL. Tissue sections were processed as described in "Materials and Methods" and examined using a microscope that detects fluorescence. All of the positively stained cells were located in the upper one-third of the crypt-villus. Values expressed are the percentage of total cells positive, with 100% equal to the entire crypt-villus population; *bars*, SE. For +/+, $n = 5$; Min, $n = 9$; and Min/sulindac, $n = 10$. *, $P = 0.6086$ compared to +/+; *, $P = 0.0349$ compared to Min/sulindac. *B*, enterocyte apoptosis *in situ*: direct immunoperoxidase. 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 one-third of the crypt-villus. Values expressed are the percentage of total cells positive, with 100% equal to the entire crypt-villus population; *bars*, SE. For +/+, $n = 5$; Min, $n = 9$; and Min/sulindac, $n = 10$. *, $P < 0.0001$ compared to +/+ and $P < 0.0001$ compared to Min/sulindac.

Table 1 *Tumor number and distribution*

Beginning at 6 weeks of age, Min mice were fed AIN-76A chow diet and sulindac, 0.5 ± 0.1 mg/day in drinking water. As controls, 9 Min mice and 5 C57BL/6J-+/+ non-affected littermates (+/+) were fed AIN-76A diet without sulindac. At 110 days of age, all mice were euthanized, and their intestinal tracts were 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 ± SE, where $n = 5$ (+/+), $n = 9$ (Min), and $n = 10$ (Min/sulindac). Values for tumor distribution indicate tumors per mouse per region.

	+/+	Min	Min/sulindac
No. of mice studied	5	9	10
No. of tumors/mouse	0	11.9 ± 2.6[a]	0.1 ± 0.1
Tumor distribution			
Duodenum	0 (0%)	0.8 ± 0.3 (6.5%)	0 (0%)
Jejunum + ileum	0 (0%)	10.7 ± 2.4 (90%)	0 (0%)
Large bowel	0 (0%)	0.4 ± 0.7 (3.5%)	1 (100%)

[a] $p < .0001$ compared to Min/sulindac.

Tumor Formation in the Min Mouse Is Prevented by Sulindac Administration. To investigate the chemopreventive properties of sulindac in Min mice, 10 Min mice were treated for 11.5 weeks with sulindac at a dose of approximately 40% of the maximum tolerated dose in rodents (22). This dose was chosen based upon preliminary work in Min mice treated with sulindac,[4] as well as studies documenting the efficacy of this dose in prevention of azoxymethane-induced carcinogenesis in rats (22). Treatment began with mice at 5–6 weeks of age, an age at which mice should be free of tumor. To confirm this, two Min mice were sacrificed and examined at 6 weeks of age and were found to be free of intestinal adenomas. At 110 days of age, untreated control Min mice had 11.9 ± 2.6 tumors each, 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 1). These adenomas were confirmed by examination of 3-μm sections using H&E stain. No invasive tumors were found. The total polyp count in the untreated Min animals was low compared to some reports published previously (14–16), although comparable to that reported by Jacoby *et al.* (23). This suggests that there is a wide variability in the number of tumors identified in Min mice. One of the reasons for low tumor count in our study may be the use of a low-fat AIN-76A diet. The issue of dietary modification of the Min phenotype warrants further investigation.

In the 10 mice treated with sulindac (Min/sulindac), only one adenoma, located in the colon, was observed. Samples of grossly normal mucosa were harvested from each group of mice. When examined by light microscopy using H&E stain, dysplasias or microadenomas were not found in the Min/sulindac mice. There was no tumor visible in the +/+ mice, either grossly or on microscopic examination of multiple sections from normal-appearing tissue.

Sulindac Modulates Cox-2 and Increases Apoptosis in Small Bowel Intestinal Epithelial Cells. Our results show that sulindac is effective in preventing adenoma formation in the Min mouse. A proposed mechanism of the antitumor activity of sulindac is its inhibitory effect on prostaglandin synthesis. Therefore, we determined the effect of sulindac on Cox-2 and PGE_2 in histologically normal-appearing small bowel samples. By Western blot analysis, sulindac down-regulated Cox-2 levels in the Min mice (Fig. 1). Analysis of PGE_2 in the sulindac-treated Min mice showed that PGE_2 levels in the Min/sulindac mice were reduced to that of the +/+ mice (Fig. 2), a result consistent with the observed decrease in tissue levels of Cox-2 protein.

One important aspect of Cox-2 function is its role in inflammation. Chronic inflammation, with elaboration of reactive oxygen species and subsequent DNA damage, may play a role in gastrointestinal carcinogenesis. Cox-2 is expressed at sites of inflammation and in monocytes and macrophages stimulated with lipopolysaccharide or IL-1. Expression of Cox-2 is inhibited by glucocorticoids, and selective inhibitors of Cox-2 are effective anti-inflammatory agents in animal studies (24). Sulindac, in addition to its effect on prostaglandin synthesis, also blocks the inflammatory response in monocytes and mucosal lymphocytes.

To assess the role of the mucosal inflammatory response in this study, we used reverse transcription-PCR to measure small bowel mRNA levels for the inflammatory cytokines TNF-α, IL-1, and IL-6 (Table 2). Although there was a trend toward increased TNF-α and IL-1 in the sulindac-treated mice, none of the cytokine mRNAs tested by this method showed a statistically significant change, either as a result of the *Min* mutation or in response to sulindac treatment. Our results suggest both that a mucosal inflammatory response in the Min mice is not the cause of increased Cox-2 and that inhibition of monocyte or lymphocyte-mediated inflammation is not an important mechanism of the antitumor activity of sulindac.

In humans with FAP, treatment with sulindac increased apoptosis in the colonic mucosa (13), an effect which may be related to its antitumor activity. When the Min animals in this study were treated with sulindac, small bowel apoptosis increased (Fig. 3). By the TUNEL method, there was no significant difference in apoptosis between the +/+ and Min animals. The direct immunoperoxidase method, however, demonstrated a significant increase in apoptosis in the +/+ animals compared to the Min mice. By both TUNEL and direct immunoperoxidase methods, there was no significant difference in apoptosis between +/+ and Min/sulindac animals. Treatment with sulindac, therefore, returned the level of apoptosis in Min mucosa to that of the normal littermates.

DISCUSSION

Our data demonstrate that sulindac prevents the development of intestinal tumors in a murine model of FAP, suggesting that this agent may be an important chemopreventive as well as therapeutic agent for individuals with FAP. The data also suggest that high levels of Cox-2 may predispose epithelial cells to tumor formation through inhibition of apoptosis, and that this process may be reversed by sulindac.

Sulindac undergoes enterohepatic circulation, with oxidized forms of the drug excreted in the bile. These products are then reduced to sulindac sulfide and sulindac sulfone by the bacterial flora of the colon (10). The sulfide derivative of sulindac is primarily responsible for blockade of prostaglandin synthesis (25, 26). A second metabolite, sulindac sulfone, has been reported to have no anti-prostaglandin activity (27). In FAP patients, the observed reduction of colorectal polyps, but not duodenal polyps, by sulindac has been ascribed to the presence of the sulindac metabolites in the lumen of the colon (9, 10). In the cecum, approximately 75% of the sulindac is transformed to the sulfone derivative, 15% to the sulfide, and 10% remains as unconjugated sulindac (22). All of these compounds are absorbed, however, and serum content in rats is approximately 80% sulfone, 10% sulfide,

Table 2 *Small bowel cytokine mRNA*

Total RNA was isolated from small bowel specimens and the specific primers for TNF-α, IL-1, IL-6, and β-actin were amplified as described in "Materials and Methods." Values for TNF-α, IL-1, and IL-6 were documented by Eagle Eye II Still Video Imaging System. The relative quantitation was normalized to β-actin, using NIH Image 1.52 software. Values expressed indicate the ratio of cytokine/β-actin mRNA ± SE, where $n = 5$ (+/+), $n = 9$ (Min), and $n = 10$ (Min/sulindac).

	+/+	Min	Min/sulindac
TNF-α	0.27 ± 0.08	0.24 ± 0.07	0.39 ± 0.08
IL-1	0.30 ± 0.16	0.22 ± 0.05	0.45 ± 0.15
IL-6	0.46 ± 0.12	0.44 ± 0.10	0.58 ± 0.12

[4] A. Wood, personal communication.

and 10% sulindac (22). In the Min mouse, greater than 95% of tumors are located in the small bowel. This study shows that sulindac prevents tumor formation in spite of the lack of metabolites in the luminal contents of the small bowel and suggests that levels in the serum may be important for preventing tumorigenesis.

The side effects of sulindac administration at doses used for polyp regression in patients with FAP include gastrointestinal upset, peptic ulceration, and bleeding. When calculated based upon percentage of dietary intake, the animals used in this study received 0.05 mg/kcal/day of sulindac, which is less than one-half of the dose normally used in humans with FAP for polyp regression (0.12 mg/kcal/day or 150 mg twice daily; Refs. 9–11). Because colonic adenomas characteristic of FAP usually arise in affected adolescents, the use of sulindac in high doses for long-term prevention would be difficult or impractical. Our results suggest that a lower dose than that normally used for polyp regression may be effective for primary prevention. We did not perform a dose-response in these experiments, and it is possible that even lower doses of sulindac may also be effective. Studies are under way in our laboratory to determine this.

In this study of the Min mouse model of FAP, we observed elevations in small bowel Cox-2, PGE_2, and apoptosis in histologically normal-appearing tissue, suggesting that the *Apc* mutation causes alterations in predysplastic tissue. Our analyses of levels of Cox-2 and PGE_2 required whole-tissue samples. Although these samples were examined histologically for evidence of tumor or dysplasia, it is possible that some microadenomas were included, or that Cox-2 and PGE_2 were produced in the submucosa or muscularis of the small bowel, rather than the mucosa. This reservation cannot be applied to the measures of apoptosis, however, because an *in situ* technique was used. Although several studies suggest that somatic mutation of the wild-type *Apc* allele is necessary for adenoma formation (28–30), recent reports also suggest that mutant APC proteins may exert a dominant-negative effect on the wild-type *Apc* gene product (31, 32). It is unclear from this study whether the observed alterations in histologically normal-appearing mucosa arose because there were somatic mutations present and these alterations represented an early phenotypic expression of FAP, or because the mutant APC protein exerted a dominant-negative effect. Another possibility is that alterations in the histologically normal-appearing tissue resulted from a field effect due to the presence of tumors throughout the bowel.

In summary, sulindac prevents tumor development in Min mice, animals that form intestinal adenomas as a result of a germline mutation in the *Apc* gene. Our results show that the histologically normal-appearing small bowel of these animals contains abnormally high amounts of Cox-2 and PGE_2. By *in situ* methods, intestinal tissue from Min mice also exhibits a decreased fraction of enterocytes undergoing apoptosis. These processes, which are associated with cellular activation, are, therefore, altered before development of clinical tumors, lending support to their role in epithelial carcinogenesis. The observation that sulindac prevents tumor development, as well as down-regulates Cox-2 and restores apoptosis, further supports this hypothesis.

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