Chemoprevention of Intestinal Polyposis in the Apc\textsuperscript{&716} Mouse by Rofecoxib, a Specific Cyclooxygenase-2 Inhibitor

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ABSTRACT

Mutations in the human adenomatous polyposis (APC) gene are causative for familial adenomatous polyposis (FAP), a rare condition in which numerous colonic polyps arise during puberty and, if left untreated, lead to colon cancer. The APC gene is a tumor suppressor that has been termed the “gatekeeper gene” for colon cancer. In addition to the 100\% mutation rate in FAP patients, the APC gene is mutated in >80\% of sporadic colon and intestinal cancers. The APC gene in mice has been mutated either by chemical carcinogenesis, resulting in the Min mouse Apc\textsuperscript{&716}, or by heterologous recombination, resulting in the Apc\textsuperscript{&716} or Apc\textsuperscript{-1366} mice (M. Oshima et al., Proc. Natl. Acad. Sci. USA, 92: 4482–4486, 1995). Although homozygote Apc\textsuperscript{-176} mice are embryonally lethal, the heterozygotes are viable but develop numerous intestinal polyps with loss of APC heterozygosity within the polyps (M. Oshima et al., Proc. Natl. Acad. Sci. USA, 92: 4482–4486, 1995). The proinflammatory, prooncogenic protein cyclooxygenase (COX)-2 has been shown to be markedly induced in the Apc\textsuperscript{&716} polyps at an early stage of polyp development (M. Oshima et al., Cell, 87: 803–809, 1996). We demonstrate here that treatment with the specific COX-2 inhibitor rofecoxib results in a dose-dependent reduction in the number and size of intestinal and colonic polyps in the Apc\textsuperscript{&716} mouse. The plasma concentration of rofecoxib that resulted in a 55\% inhibition of polyp number and an 80\% inhibition of polyps >1 mm in size is comparable with the human clinical steady-state concentration of 25 mg rofecoxib (Vioxx) taken once daily (A. Porras et al., Clin. Pharmac. Ther., 67: 137, 2000). Polyps from both untreated and rofecoxib- or sulindac-treated Apc\textsuperscript{&716} mice expressed COX-1 and -2, whereas normal epithelium from all mice expressed COX-1 but minimal amounts of COX-2. Polyps from either rofecoxib- or sulindac-treated mice had lower rates of DNA replication, expressed less proangiogenic vascular endothelial-derived growth factor and more membrane-bound \(\beta\)-catenin, but showed unchanged nuclear localization of this transcription factor. This study shows the inhibition of polyps in the Apc\textsuperscript{&716} mouse suggests that the specific COX-2 inhibitor rofecoxib (Vioxx) has potential as a chemopreventive agent in human intestinal and colon cancer.

INTRODUCTION

The majority of epidemiological studies that included the use of NSAI\textdagger\textsuperscript{D}s\textsuperscript{2} as a risk factor have demonstrated that constant use of NSAI\textdagger\textsuperscript{D}s is associated with a significantly reduced risk of colon cancer (1). Although each NSAI\textdagger\textsuperscript{D} has unique physical properties and pharmacokinetics, the mechanism of action common to all at clinically achievable drug concentrations is the inhibition of COX enzymatic conversion of the polyunsaturated fatty acid arachidonic acid to PGG\textsubscript{2} (2). PGG\textsubscript{2} is converted to prostaglandin H\textsubscript{2} by the peroxidase activity of the COX enzyme, and then PGG\textsubscript{2} may be converted to one of several of the five biologically active prostanooids, PGE\textsubscript{2}, prostaglandin D\textsubscript{2}, prostaglandin F\textsubscript{2a}, prostacystin, or thromboxane (3). Elevated PGE\textsubscript{2} has been measured in rodent and human colonic tumors, and the inhibition of prostaglandin synthesis by NSAID treatment has been shown to inhibit tumor growth in animal models (4–6). On the basis of such observations, the NSAID sulindac was studied in FAP patients for prevention of polyp growth (7). This clinical trial showed that treatment with sulindac decreased polyp number and size, and that when sulindac treatment was stopped, polyp growth recurred (7).

In the early 1990s, a second form of COX, termed prostaglandin G/H synthase-2 or, more commonly, COX-2, was identified that was 60\% identical to the original COX-1 (8–10). COX-2 mRNA and protein were highly inducible by inflammatory and growth factors, whereas COX-1 expression was constitutive in most tissues, including the GI tract (8–11). The discovery of the second COX isof orm led to the hypothesis that COX-2-specific inhibitors would be as efficacious as nonspecific COX-1/COX-2 inhibitor NSAI\textdagger\textsuperscript{D}s with respect to prostaglandin-mediated pain and inflammation in arthritis, but with a much-improved GI safety margin (12). Two specific COX-2 inhibitors, i.e., rofecoxib (Vioxx) and celecoxib (Celebrex), have been shown preclinically and clinically to have comparable efficacy to NSAI\textdagger\textsuperscript{D}s for relief of pain and inflammation in osteoarthritis, but to have decreased risk of GI damage (13–18).

Given the epidemiology of NSAI\textdagger\textsuperscript{D} protection for colon cancer, we and others investigated whether this chemopreventive effect might be specifically through the inhibition of COX-2-produced prostaglandins. COX-2 mRNA and protein were shown to be markedly elevated in human colon tumor tissue, whereas COX-1 expression remained the same or decreased (19, 20). COX-2 is also overexpressed in human colonic polyps and in macrophages in intimate contact with these sporadic polyps (21, 22). The growth of human colon tumor cells expressing COX-2 can be inhibited in vitro and in vivo by treatment with COX-2 inhibitors (23, 24). Mechanistic studies have revealed that this growth inhibition results from antiproliferative, proapoptotic, and antiangiogenic effects (23–27). Elevated concentrations of COX-2 mRNA and protein have now been associated with esophageal, head and neck, breast, lung, prostate, and other cancers, and it has been suggested that COX-2 inhibitors may have benefit in malignancies other than colon cancer (28).

A relevant animal model in which to test COX-2 inhibitors for prevention of the polyp precursors of adenocarcinomas is the Apc\textsuperscript{&716} mouse, which develops hundreds of intestinal polyps from birth through the first 3 months of development (29). Both the genetic deletion of COX-2 expression and pharmacological inhibition with the specific COX-2 inhibitor, MF-tricyclic, have been shown to markedly attenuate the number and size of polyps in the Apc\textsuperscript{&716} mouse (30). The specific COX-2 inhibitor celecoxib (Celebrex) has been shown to decrease polyp number and size in the chemically induced Apc mutant Min mouse (31). In clinical trials in FAP patients, celecoxib has also shown moderate efficacy, at twice the approved ar-

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\textsuperscript{2}The abbreviations used are: NSAI\textdagger\textsuperscript{D}, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PGG\textsubscript{2}, prostaglandin G\textsubscript{2}; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; FAP, familial adenomatous polyposis; GI, gastrointestinal; VEGF, vascular endothelial growth factor; HPLC, high-performance liquid chromatography; BioTdx, bromodextrin.
thritic dose, for the inhibition of colonic polyps (32). In the study described here, we carefully investigated the efficacy of the specific COX-2 inhibitor rofecoxib (Vioxx) for chemoprevention of intestinal polyposis in the ApcΔ716 mouse. To profile the potential for long-term prophylactic use as a chemopreventive agent, we chose doses of rofecoxib at or below the steady-state concentrations achieved at the clinical doses of Vioxx for arthritis. In addition, we monitored parameters of COX expression, angiogenesis, and polypl proliferation to further our understanding of the potential mechanism of chemoprevention by rofecoxib.

MATERIALS AND METHODS

Reagents and Chemicals. Mouse chow containing compounds, synthesized at Merck Research Laboratories, was prepared at Purina as described previously (30). Full-length sheep seminal vesicle COX-1- and sheep placental COX-2-purified proteins were purchased from Cayman (Ann Arbor, MI) and were used to generate rabbit polyclonal antibodies, as described previously (20). Under the immunoblotting conditions used in this study, the anti-COX antibodies demonstrated no significant cross-reactivity with the alternate ovine COX isoform. VEGF antiserum (SC-507), purchased from Santa Cruz Biotechnology (Santa Cruz, CA), was an affinity-purified rabbit polyclonal antibody raised against an epitope corresponding to amino acids 1–140 of human origin. The antiserum recognizes all splice variants of VEGF and cross-reacts with mouse VEGF. β-catenin antisera was purchased from Sigma (St. Louis, MO). All other reagents, if not specifically noted, were of highest reagent grade.

HPLC Quantitation of Rofecoxib and Sulindac and Its Metabolites and in ApcΔ716 Mouse Plasma. Drug concentrations were measured in terminal bleed plasma samples taken from Cayman (Ann Arbor, MI) and were used to generate rabbit polyclonal antibodies, as described previously (20). Under the immunoblotting conditions used in this study, the anti-COX antibodies demonstrated no significant cross-reactivity with the alternate ovine COX isoform. VEGF antiserum (SC-507), purchased from Santa Cruz Biotechnology (Santa Cruz, CA), was an affinity-purified rabbit polyclonal antibody raised against an epitope corresponding to amino acids 1–140 of human origin. The antiserum recognizes all splice variants of VEGF and cross-reacts with mouse VEGF.

Preparation of Microsomal Membranes from Intestinal Tissues. Normal mouse intestinal mucosa and autologous polyp tissue were excised, frozen immediately in liquid N2, and stored at −70°C. Frozen tissues were thawed in ice-cold homogenization buffer [50 mm potassium phosphate (pH 7.1) containing 0.1 mM NaCl, 2 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 60 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin; all from Sigma Chemical Co., St. Louis, MO]. Tissues were disrupted twice, for 15 s each, on ice using a tissue-tearer (IKA Labortechnik, Germany). Samples were homogenized by sonication at 4°C using a Cole Parmer 4710 series ultrasonic homogenizer (Cole Parmer Instrument Co., Chicago, IL). Debris was removed by centrifugation at 1,000 × g for 15 min at 4°C, and the resultant supernatants were subjected to centrifugation at 100,000 × g for 45 min at 4°C. Membrane fractions were resuspended in homogenization buffer, and then sonicated to obtain a homogenous membrane suspension. Protein concentrations were determined for each sample using a protein assay kit (Bio-Rad, Mississauga, Ontario, Canada).

SDS-PAGE and Immunoblot Analysis. Membrane fractions were mixed with 0.5 volume of SDS sample buffer [20 mM Tris-HCl (pH 6.8) containing 0.4% (w/v) SDS, 4% glycerol, 0.24 M β-mercaptoethanol, and 0.5% bromphenol blue], boiled for 5 min and analyzed by SDS-PAGE on 9 × 10-cm precast 4–20% Tris-glycine acrylamide gels (NOVEX, San Diego, CA) according to the method of Laemmli (18). Proteins were electrophoretically transferred to nitrocellulose membranes as described previously (19). Primary antisera to COX-1 and COX-2 were used at a final dilution of 1:3,000 and 1:5,000–10,000, respectively. Primary antisera to VEGF (Santa Cruz) and β-catenin (Sigma) were used at final dilutions of 1:500 and 1:5,000 according to the manufacturer’s instructions. The secondary horseradish peroxidase-linked goat antirabbit IgG antibody (Santa Cruz Biotechnology) was used at dilutions of 1:3,000–1:6,000 for COX-1 and 1:2,000–1:3,000 for VEGF and 1:5,000 for β-catenin. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham). Protein bands were visualized using a FUJI LAS-1000-plus Luminescent Image Analyzer.
Rofecoxib Is a Specific COX-2 Inhibitor in in Vitro Human COX-2 Recombinant Whole Cell and ex Vivo Human Whole Blood Assays. We have shown previously, in both human recombinant COX-2-expressing cell lines (33) and in human whole-blood assays (14, 34, 35), that rofecoxib is a highly selective inhibitor of COX-2 with minimal activity against COX-1 (Table 1). Sulindac is a prodrug in vivo that is inactive as a COX inhibitor until it is converted to the metabolite sulindac sulfide, which is a potent inhibitor of both COX-1 and COX-2, which is derived prostaglandin production in in vitro human recombinant COX-1 and COX-2 whole-cell assays and also is active in human whole blood assays (Table 1). The oxidative metabolite of sulindac, i.e., sulindac sulfone, does not inhibit COX-1 or COX-2 enzyme activity in human whole-blood assays (Table 1). Mouse whole-blood assays have not been able to be developed, therefore exact activity of the COX inhibitors in mouse blood is not available.

**RESULTS**

**Rofecoxib and Sulindac Treatment Resulted in a Marked Diminution of Mouse by Rofecoxib and Sulindac.** As depicted in Fig. 3, both rofecoxib and sulindac-treated mice intestines had many fewer polyps, and those present were much smaller than those in the untreated mouse intestines (Fig. 2, B and C).

**In Vivo Inhibition of Polyp Number and Size in the Apc<sup>Δ716</sup> Mouse by Rofecoxib and Sulindac.** As depicted in Fig. 3, both rofecoxib and sulindac treatment resulted in a marked diminution of polyp number in the Apc<sup>Δ716</sup> mice. The average inhibition of number of polyps for rofecoxib at 0.0025% w/w or 4.7 mg/kg/day was 36%, for rofecoxib at 0.0075% w/w or 14.7 mg/kg/day was 55%, and for sulindac 0.015% w/w or 32.6 mg/kg/day was 38%. In addition, the rofecoxib- and sulindac-treated mice had the greatest decreases in numbers of the larger-sized polyps, as shown in Fig. 4 and in Table 4.

**Effect of Rofecoxib and Sulindac Treatment on the Expression of COX-1, COX-2, VEGF, and β-Catenin Expression.** Samples of both normal intestine and polyps from three mice from each treatment group were assessed for expression of COX-1, COX-2, VEGF, and β-catenin proteins by specific immunoblot analyses, as shown by a representative set of immunoblots in Fig. 5 and Fig. 7. COX-1 and COX-2 protein are microsomal membrane-bound proteins, and expression was investigated in 100,000 × g pellet fractions only. COX-1 was expressed in both normal Apc<sup>Δ716</sup> intestine and in polyps from all samples, with no significant changes with rofecoxib or sulindac treatment (Fig. 5 and Fig. 6). COX-2 was expressed at low or undetectable levels in normal intestine samples, but the COX-2 protein was markedly up-regulated in polyp samples (Figs. 5 and 6). In some mice, treatment with rofecoxib or sulindac slightly enhanced the protein amount of COX-1 and COX-2 in polyps, possibly through stabilization of the protein by decreased proteolysis as has been reported by others for COX-1 in the presence of indomethacin (39).

VEGF has been shown to be associated with membranes and cytoplasm and also has been shown to be secreted from cells. Using an antisera that recognizes all forms of VEGF, we investigated the

### Table 2 Plasma concentrations of rofecoxib and sulindac in Apc<sup>Δ716</sup> mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample no.</th>
<th>Inhibitor (µg/ml)</th>
<th>Average inhibitor (µg/ml)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vioxx</td>
<td>2-1</td>
<td>0.198</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.7 mg/kg/day</td>
<td>2-2</td>
<td>0.217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.7 mg/kg/day</td>
<td>2-3</td>
<td>0.222</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulindac</td>
<td>2-4</td>
<td>0.169</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015%</td>
<td>2-5</td>
<td>0.229</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.26 mg/kg/day</td>
<td>2-6</td>
<td>0.328</td>
<td>0.23</td>
<td>0.05</td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>3-1</td>
<td>0.024</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.7 mg/kg/day</td>
<td>3-2</td>
<td>0.059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>3-3</td>
<td>0.073</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015%</td>
<td>3-4</td>
<td>0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-5</td>
<td>0.017</td>
<td>0.04</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>4-1</td>
<td>2.485</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015%</td>
<td>4-2</td>
<td>0.726</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>4-3</td>
<td>0.266</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015%</td>
<td>4-4</td>
<td>2.476</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.26 mg/kg/day</td>
<td>4-5</td>
<td>2.057</td>
<td>1.6</td>
<td>1.04</td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>4-4</td>
<td>1.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015%</td>
<td>4-5</td>
<td>1.57</td>
<td>1.78</td>
<td>0.69</td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>4-5</td>
<td>1.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015%</td>
<td>4-6</td>
<td>15.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>4-6</td>
<td>6.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015%</td>
<td>4-7</td>
<td>4.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>4-7</td>
<td>12.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015%</td>
<td>4-8</td>
<td>12.56</td>
<td>10.4</td>
<td>4.67</td>
</tr>
</tbody>
</table>

### Table 3 Drug concentrations in Apc<sup>Δ716</sup> mice or in humans at clinical steady-state conditions

<table>
<thead>
<tr>
<th>Apc&lt;sup&gt;Δ716&lt;/sup&gt; mice</th>
<th>Plasma concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rofecoxib</td>
<td>0.05–0.22</td>
</tr>
<tr>
<td>Rofecoxib 0.0025%</td>
<td>0.5–1.0</td>
</tr>
<tr>
<td>Sulindac 0.015%</td>
<td>0.8–7.5</td>
</tr>
<tr>
<td>Human steady-state</td>
<td>4–9</td>
</tr>
<tr>
<td>Vioxx 25 mg q.d.</td>
<td>15–47</td>
</tr>
<tr>
<td>Clinoril 200 mg b.i.d.</td>
<td>0.3–1.0</td>
</tr>
</tbody>
</table>

* Ref. 7 (trough-peak). q.d., every day.
* Ref. 41. b.i.d., twice a day.
expression of cellular VEGF in both 100,000 g pellets (membranes) and supernatants (cytosol and nucleoplasm) from both normal intestine and polyp samples (Figs. 5 and 6). Under our SDS-PAGE conditions, VEGF remained a dimer at approximately Mr 42,000, although this appeared as a doublet in the membrane fractions (Fig. 5). VEGF was markedly up-regulated in polyps in comparison with the matching normal epithelium in both membrane and cytosolic fractions (Figs. 5 and 6). Normal intestinal concentrations of VEGF were subtracted from polyp amounts in each mouse (for three mice from each group), and the mean cytosolic or membrane VEGF was calculated for each group. Then the VEGF concentrations in each group were compared, and although there was high variability in the increases in control polyps there was 68% decrease in membrane-bound VEGF (P = 0.053; t test) for the rofecoxib-treated (0.0075% w/w) group versus control. The mice treated with the lower rofecoxib concentration (0.0025% w/w) and the sulindac-treated animals showed a ~35% and ~45% decrease, respectively, in membrane-bound VEGF, but with the high control variation, this was not statistically significant. For cytosolic VEGF concentrations, there was a nonstatistically significant trend for decreases in polyps of all treatment groups compared with control polyps.

A representative set of β-catenin gels and the quantitation of data from three mice from each group is shown in Fig. 7. There was an increase in cytosolic β-catenin expression in the polyp as compared with normal intestine in all samples: control and rofecoxib- and sulindac-treated. However, in agreement with the nuclear localization seen by immunohistochemical analysis, treatment did not alter significantly the amount of β-catenin in the cytoplasmic/nucleoplasmic fractions (Fig. 8). The total amount of supernatant β-catenin in the normal tissues was at least 100-fold less than the total amount of β-catenin in the membrane/microsomal 100,000 g-pelleted fraction (Fig. 7). In comparison to normal intestine from the same animal, control polyps all had greatly reduced membrane-bound β-catenin.

Table 4  
Inhibition of polyp size in Apc<sup>D716</sup> mice treated with rofecoxib or sulindac

<table>
<thead>
<tr>
<th>Polyp Size (mm)</th>
<th>Rofecoxib (0.0075% w/w)</th>
<th>Rofecoxib (0.0025% w/w)</th>
<th>Sulindac (0.015% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>39</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>1–2</td>
<td>80</td>
<td>57</td>
<td>76</td>
</tr>
<tr>
<td>2–3</td>
<td>97</td>
<td>91</td>
<td>80</td>
</tr>
<tr>
<td>&gt;3</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
However, the concentration of membrane-bound β-catenin in control polyps was still 10-fold higher than the cytosolic concentration. Treatment with rofecoxib and sulindac tended to increase the membrane-bound β-catenin in polyps, with a P value of 0.059 for the 0.0075% rofecoxib dose (Fig. 8).  

Unchanged Nuclear Localization of Polyp β-Catenin in the ApcD716 Mouse after Treatment with Rofecoxib or Sulindac. As shown in Fig. 8, immunohistochemical staining for β-catenin was observed in both the cytoplasm and the nucleus of all treatment groups with no apparent inhibition in nuclear localization by any of the drug treatments.  

Decreased DNA Replication in ApcD716 Mouse Polyps after Treatment with Rofecoxib or Sulindac. We investigated an index of cellular proliferation, i.e., BrdUrd-labeling in polyps from control and rofecoxib- and sulindac-treated mice. In comparison to control BrdUrd-labeling, the rofecoxib (0.0075% w/w and 0.0025% w/w) and sulindac (0.015% w/w) showed a 35%, 50%, and 35% decrease in BrdUrd labeling, respectively (Fig. 9).  

DISCUSSION

We show here that treatment with either rofecoxib or sulindac results in a diminution in the number and size of intestinal polyps in the ApcD716 mouse. Rofecoxib at 0.0075% w/w in chow, a dose that gave mouse plasma concentrations comparable with those achieved in humans at clinical steady-state with a 25-mg tablet taken once daily, decreases ApcD716 polyp number by 55% and inhibits 80% of polyps >1 mm in size. Rofecoxib at 0.0025% w/w in chow resulted in a 35% inhibition of polyp number and a 57% inhibition of polyps >1 mm in size. We used sulindac as our positive COX-1/COX-2 nonselective inhibitor control because this compound had been shown previously to inhibit polyp number in the Min mouse model (6). There was greater variability in the plasma concentrations of sulindac and its metabolites in our study in comparison with rofecoxib, but an average 38% inhibition of polyp number and >75% inhibition of polyps >1 mm in size was observed after treatment with sulindac at 0.015% w/w.
in chow. Rofecoxib and sulindac both resulted in a marked size reduction and a flattened morphology of the remaining polyps. Our sulindac dosing achieved concentrations of the active COX-1/COX-2 inhibitor sulindac sulfide of 4–9 μM, which should have resulted in significant inhibition of both COX-1 and COX-2 (Tables 1 and 3).
our mouse study, in comparison with humans, there is a greater conversion of sulindac to sulindac sulfone, and this metabolite is approximately six times the concentration of sulindac itself or of sulindac sulfide (Table 3). In our study, the terminal bleed-plasma concentration of sulindac sulfone was between 15–50 μM, equivalent to about four times that achieved in humans given sulindac at a 200-mg twice-daily dosing (37). It has been shown previously that sulindac sulfone at equivalent concentrations to sulindac sulfide (0.5 mg/day in drinking water) was relatively ineffective at inhibiting polypl number in the Min mouse (40). However, no terminal drug blood concentrations were determined in the latter study. In a Phase I trial of sulindac sulfone (Exsulind) as a chemopreventive in patients with FAP, doses of 200 mg and 300 mg twice daily were tolerated and achieved peak plasma concentrations of ~15–30 μM sulindac sulfone (41). However, in the latter study, even at these relatively high drug concentrations, there was a very modest clinical effect with a nonsignificant trend toward an increase in apoptosis but with no decrease in polypl number or cellular proliferation (41). Therefore, we assume that the majority of polypl-number and -size inhibition seen with sulindac and its metabolites in our mouse study is attributable to the inhibition of COX-2 by sulindac sulfide; but there may be a minor contribution of sulindac sulfone at another target. With regard to sulindac itself, one study has shown an effect of the parent compound at ≥100–μM concentrations in vitro on peroxisome proliferator activated receptor δ transcription (42). Given the blood levels of <10–20 μM at steady state achieved in our mouse study or for clinical doses of sulindac (Table 3), it is unlikely that this mechanism would be relevant either in our mouse study or with sulindac in clinical use in humans.

COX-1 protein was observed in all samples of normal epithelium and polypl, with a weak trend for increase in concentration in the sulindac-treated mice. It seems that COX-1 activation is not linked with the process of polypl formation inasmuch as COX-1 protein concentration is equivalent in the normal intestine and in the polypl, and rofecoxib shows marked inhibitory growth effects at concentrations far below any possible COX-1 inhibition (Table 1). COX-2 concentrations were either unchanged or slightly increased in normal intestine or polypl from the rofecoxib- and sulindac-treated mice, possibly because of the inhibitor stabilization of protein, as has been reported previously (38). We see no evidence for a transcriptional decrease in the concentration of COX-2 by rofecoxib.

VEGF protein was markedly elevated in all polypl from each group in comparison with the normal intestinal epithelium control tissue. VEGF concentrations in 100,000 X g pellet and 100,000 X g supernatant fractions of rofecoxib- or sulindac-treated polypl showed a decreasing trend, with less membrane-bound VEGF in the higher-dose rofecoxib-treated polypl. This probably reflects both the decreased vasculature of smaller-sized drug-treated polypl and also the downregulation of VEGF production within these polypl. Previous studies have shown that the overexpression of COX-2 causes increased cellular VEGF, and that in vivo tumors are less vascularized and grow more slowly in a COX-2-negative host (26, 43). Membrane-bound β-catenin was reduced ~5-fold in the polypl of control mice in comparison with normal intestinal tissue. This is a novel finding and the first quantitative measurement of β-catenin and its intracellular distribution in mouse polypl. Although there was a small increase in the cytoplasmic/nucleoplasmic β-catenin in polypl, this was <5% of the concentration of β-catenin lost from the membrane fraction. Therefore the dramatic reduction in membrane-bound β-catenin in control polypl must reflect a decrease in transcription, translation, or stabilization of the protein. A quantitatively small amount of β-catenin, which possibly is qualitatively important as a transcriptional activator, is localized to the soluble fraction in the control polypl but not in normal intestine. Rofecoxib treatment partially restored to normal intestinal levels the concentration of membrane-bound β-catenin in polypl. The rofecoxib-treated polypl may be more differentiated, and hence, may maintain a more normal complement of β-catenin-E-cadherin complexes. Free and bound intracellular pools of catenins have been shown previously to be in dynamic equilibrium (44). A trend for loss of β-catenin complexes at cell-cell junctions as has been reported in primary colorectal tumors and in the corresponding liver metastases (45). It is possible that the loss of membrane β-catenin expression may be important in early polypl growth, and subsequent up-regulation of cytoplasmic/nucleoplasmic β-catenin may be important in a later adenoma stage. Progression to adenocarcinoma may involve both the loss of β-catenin from membranes and the nuclear localization of transcriptionally active β-catenin.

We showed that both rofecoxib and sulindac treatment of mice decreased DNA replication within polypl, as demonstrated by decreased BrdUrd incorporation (although this was not dose-dependent for rofecoxib). We did not investigate apoptosis in this study, although others have shown that sulindac sulfide increases enterocyte apoptosis in Min mice (40). We assume that rofecoxib- or sulindac sulfide-inhibition of COX-2 produced PGE2 results in decreased proliferation and perhaps increased apoptosis through EP prostanoid receptor-mediated changes in second-messenger signaling.

The potential effects of COX-2 inhibition on immune surveillance were not investigated in the present study, but COX-2 has been localized to macrophages in Apc716 and Min mouse polypl and in human sporadic polypl (22, 30, 46). In addition, enhanced secretion of PGE2 has been shown by tissue-fixed macrophages in colon carcinomas (47). Specific inhibition of COX-2 in a murine Lewis lung carcinoma model restores host antitumor reactivity by decreasing the immune suppressor cytokine interleukin 10 and increasing the antitumor cytokine interleukin 12 (48). Given the potential for inhibition of COX-2 in tumor, stromal, and immune cells, it is not surprising that combination therapy of COX-2 inhibitors with antiproliferative agents and radiation therapy result in synergistic benefits in tumor regression (49–51).

In conclusion, we present here the first demonstration of the chemopreventive efficacy of the specific COX-2 inhibitor rofecoxib in the Apc716 mouse model at blood levels comparable with those achieved in humans with a clinical anti-inflammatory dose. We demonstrate the reduction in both number and size of polypl by rofecoxib treatment, and that this is associated with a decrease in membrane-bound VEGF. In addition, we make the novel observation of a marked decrease in membrane-bound β-catenin in control polypl versus normal intestine, and that rofecoxib treatment partially restores this membrane localization. On the basis of the data presented here, we suggest that the specific COX-2 inhibitor rofecoxib (Vioxx) may have a therapeutic benefit in colorectal cancer.

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This paper is dedicated to Lison Bastien and her ongoing battle with cancer.

REFERENCES


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