Chemoprevention of Colon Cancer by Specific Cyclooxygenase-2 Inhibitor, Celecoxib, Administered during Different Stages of Carcinogenesis


INTRODUCTION

Colorectal cancer, one of the leading causes of cancer deaths in both men and women in Western countries, accounts for about 56,000 deaths annually in the United States (1) and is therefore a major public health problem. Several epidemiological studies have shown a significant inverse association between the intake of aspirin and the risk of colorectal cancer in the general population (2, 3). This may be a general feature of NSAIDs3 because clinical studies have shown that the NSAID sulindac also suppresses polyph development in patients with FAP (4). Model studies in laboratory animals have provided convincing evidence that administration of the NSAIDs aspirin, piroxicam, sulindac, ibuprofen, and others inhibited chemically induced colon carcinogenesis (5–8). In addition, piroxicam, aspirin, and sulindac reduced the number of intestinal tumors in mice with inherited defects in the Apc gene (9–11).

The mechanism by which traditional NSAIDs act to reduce the risk of colon carcinogenesis is not clearly understood. Accumulating evidence points to inhibition of arachidonic acid metabolism via COX enzymes, which, in turn, modulates the synthesis of PGs that affect cell proliferation, tumor growth, and immune responsiveness (12, 13). Two mammalian isoforms encoded by different genes, COX-1 and COX-2, are known to be present in colon tumors of humans and rodents (13–18) and to catalyze the conversion of arachidonic acid to PGs. We have previously shown increased levels of COX-2 mRNA and protein in chemically induced colon tumors (17). Intestinal adenomas from Apcmin mice and Apc3716 mice were also found to have elevated COX-2 levels (10, 18). Several studies have revealed that although both isoforms carry out essentially the same catalytic reaction, many of the inflammatory, inducible effects of COX appear to be mediated by COX-2, whereas the normal physiological functions of COX are carried out by COX-1 (19–21). The expression of COX-1 does not fluctuate due to stimuli, whereas cytokines, mitogens, growth factors, and tumor promoters induce COX-2 expression (19–21). Prolonged administration of traditional NSAIDs also causes unwanted side effects, such as gastrointestinal bleeding, ulceration, and renal toxicity, which are manifested mainly by the blocking of COX-1

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2 To whom requests for reprints should be addressed, at the American Health Foundation, One Dana Road, Valhalla, NY 10595.

3 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; AOM, azoxymethane; FAP, familial adenomatous polyposis; PG, prostaglandin.
effects as well as their adverse side effects, it is likely that inhibitors of COX-2, which do not inhibit COX-1 at therapeutic doses in humans, can serve as effective chemopreventive agents without causing side effects (23–25). In this context, it is noteworthy that the development of intestinal adenomas was strikingly (more than 6-fold) reduced in the COX-2 null mice compared to their occurrence in COX-2 wild-type mice; this suggests that COX-2 plays a key role in polyplformation (18). Additional evidence in support of a role for COX-2 comes from studies showing that administration of the COX-2 inhibitor MF Tricycl inhibited the number and size of intestinal tumors in ApcΔ716 mice, a model in which a targeted truncation deletion in the tumor suppressor gene APC causes intestinal polyposis (18). We reported earlier that celecoxib, a COX-2 inhibitor with significant anti-inflammatory and analgesic properties (26, 27), significantly inhibited colonic preneoplastic lesions in rats (28). We had also observed that continuous administration of 1500 ppm celecoxib throughout the initiation and postinitiation phases significantly diminished the incidence and multiplicity of AOM-induced colonic adenocarcinomas in F344 rats (29).

The studies cited above clearly demonstrate the potential chemopreventive activity of celecoxib against colon carcinogenesis when this COX-2 inhibitor was administered during the initiation and postinitiation stages of carcinogenesis. However, the multistep nature of carcinogenesis provides opportunities for intervention with agents targeted at specific mechanisms involved in the initiation, promotion, and progression stages of cancers. Because there were no studies on the efficacy of celecoxib during the promotion/progression stage, at which point premalignant lesions are known to have developed, it was important to verify whether celecoxib treatment can still be effective long after the carcigen administration in experimental carcinogenesis. Determining this in model assays is important with regard to the eventual clinical use of celecoxib in secondary colon cancer prevention among patients with colonic polyps. Because no dose-related study on the inhibition of colon carcinogenesis by celecoxib has been reported in any previous publications, we deemed it important to conduct this dose-response assay and to determine the efficacy of different levels of celecoxib to identify the lowest dose with optimum efficacy. Furthermore, we analyzed the steady-state plasma levels of celecoxib in rats after chronic administration of different doses of this agent in the diet.

**MATERIALS AND METHODS**

**Materials.** AOM (CAS: 25843-45-2) was purchased from Ash Stevens (Detroit, MI). Celecoxib (Fig. 1) was kindly supplied by Searle Research and Development (St. Louis, MO). Weanling male F344 rats were received from the Charles River Breeding Laboratories (Kingston, NY). All ingredients of the semipurified diet were purchased from Dyets Inc. (Bethlehem, PA) and stored at 4°C before preparation of experimental diets. Celecoxib was added to modified AIN-76A diet at 500, 1000, or 1500 ppm dose levels for the dose-response study and at the 1500 ppm level for the tumor promotion/progression study. The modified AIN-76A diet consisted of 20% casein, 0.3% DL-methionine, 52% corn starch, 13% dextrose, 5% corn oil, 5% alphacel, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, and 0.2% choline bitartrate (6). All control diets and the experimental diets containing celecoxib were prepared weekly in our laboratory and were stored in a cold room.

**Efficacy Study.** The experiments were designed to evaluate the dose-response effect of 500, 1000, and 1500 ppm of celecoxib administered continuously beginning 2 weeks before, during, and after carcinogen treatment and then throughout the study (initiation and postinitiation stages) and to determine the efficacy of 1500 ppm celecoxib first given 14 weeks after carcinogen treatment (promotion/progression stage) and continued throughout the study (Fig. 2). Our studies in AOM-induced colon carcinogenesis indicate that colonic neoplasms have developed by week 14 after carcinogen administration (6, 30). The highest dose for the dose-response study and for the promotion/progression protocol was selected on the basis of our previous assay, which had used 1500 ppm of celecoxib (29). The experimental methods were as described previously (29, 30). A total of 180 male F344 rats were received at weaning, quarantined for 1 week, and had free access to the AIN-76A diet. After quarantine, all rats were randomly distributed so that the body weights in each group were evenly distributed (36 animals for each AOM-treated group and 6 animals for each saline-treated group). Beginning at 5 weeks of age, the rats in the initiation and postinitiation study had access to their respective control and experimental diets containing 500, 1000, or 1500 ppm celecoxib, whereas the rats in the assay for the efficacy of celecoxib in the promotion/progression stage were fed the control diet at this stage. At 7 weeks of age, the rats scheduled to receive carcinogen treatment were injected s.c. with a solution of AOM at a dose rate of 15 mg/kg body weight, once weekly for 2 successive weeks. Rats intended for vehicle treatment received an equal volume of normal saline. Starting 14 weeks after the second AOM treatment, the groups of rats designated for the intervention during the promotion and progression stage and maintained thus far on the control diet began to receive the experimental diet supplemented with 1500 ppm celecoxib. All dietary regimens were continued until termination of the experiment 50 weeks after the second AOM treatment. Body weights were recorded every week for the first 10 weeks and then every 4–6 weeks. Dying or moribund rats were killed and necropsied. As scheduled, all rats were killed by asphyxiation with CO2 and necropsied. At the time of sacrifice, blood was drawn by cardiac puncture from animals fed the control and the experimental diets to study the effects of different levels of celecoxib in the diet on its levels in blood plasma. Blood plasma was quickly frozen in liquid nitrogen and stored at −80°C until analysis. All organs were examined grossly for tumors under the dissection microscope. Colon tumors were noted grossly for their location, number, and size, and the length, width, and depth of each tumor were measured with calipers. Estimates of tumor volume were determined using the formula $V = \frac{4}{3}\pi \frac{W \times D}{6}$, where $L$ is length, $W$ is width, and $D$ is depth of colon tumor (6).

For histopathological evaluation, colon tumors were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by routine histological methods with H&E staining. The sections were examined for tumor types according to the classification of Pozharrisski, which is routinely used in our laboratory (6). Adenocarcinomas of the colon were malignant tumors that have the tendency to form adenomatous structures. Adenomas were benign tumors that formed abnormal glandular structures with less atypism.

**Analysis of Blood Plasma for Celecoxib.** Plasma samples (0.3 ml) containing celecoxib and an internal standard were treated with 100 μl of 1.0 N phosphoric acid and extracted in a cation exchange/hydrophobic mixed mode solid-phase extraction column (Jomes Chromatography, Lakewood, CO) preconditioned with 2×1 ml of acetonitrile followed by 2×1 ml of water. The sample was eluted from the extraction column with 1.0 ml of 1% ammonium hydroxide in methanol. The extract was evaporated under nitrogen, and the sample was taken up in 200 μl of high-performance liquid chromatography mobile phase acetonitrile:0.01 M sodium phosphate buffer (pH 9; 50:50, v/v).

An aliquot of the sample extract was injected onto a reverse-phase high-performance liquid chromatography, C18 NOVA PAKTM column (15 cm × 3.9 mm, 4 μm; Waters Associates, Milford, MA) using a 15 × 3.2-mm, 7 μm RP-18 New Guard Cartridge (Brownlee Labs, Inc., Santa Clara, CA). The
 mobile phase acetonitrile:0.01 m sodium phosphate buffer (pH 9; 50:50, v/v) was run at 1.0 ml/min. The analyte was quantified by peak height ratio to that of the internal standard using a fluorescence detector with excitation at 240 nm and emission at 380 nm. The analyte was compared against a standard curve (0.01–10 μg celecoxib/ml) prepared as described above.

Statistical Analysis. Body weights, colon tumor incidence (percentage of animals with tumors), multiplicity (mean number of tumors/animal), and tumor volumes were determined for the animals fed the control diet and for those given experimental diets containing celecoxib. Body weights, tumor multiplicity, and tumor volume were analyzed and compared by Welch’s t test; tumor incidence was analyzed by Fisher’s exact probability test.

RESULTS

General Observations. As summarized in Table 1, the body weights of rats treated with AOM and fed the control diet or the experimental diets containing different levels of celecoxib were comparable throughout the study. In saline-treated animals, chronic administration of various levels of celecoxib did not produce any gross changes in several organs that would indicate toxicity.

Plasma Celecoxib Levels. Steady-state plasma levels of celecoxib (± SD) administered during the initiation and postinitiation stages were as follows: (a) 500 ppm celecoxib, 2.21 ± 0.84 μg/ml; (b) 1000 ppm celecoxib, 2.61 ± 0.69 μg/ml; and (c) 1500 ppm celecoxib, 4.29 ± 0.28 μg/ml. Rats receiving 1500 ppm celecoxib supplements during the promotion/progression stage had plasma levels of 4.13 ± 0.94 μg/ml.

Efficacy of Various Levels of Celecoxib Administered during Initiation and Postinitiation Stages. The results summarized in Table 2 indicate that in rats treated with AOM and fed the control diet, more than 95% of the colon tumors were adenocarcinomas, and the rest were adenomas. None of the saline-treated animals fed the control diet or experimental diets with celecoxib developed colon tumors (data not shown in the table). Administration of celecoxib at 500, 1000, and 1500 ppm dose levels during the initiation and postinitiation stages significantly inhibited the incidence of total colon tumors (adenomas plus adenocarcinomas) and adenocarcinomas (P < 0.01 and P < 0.001; tumor inhibition ranged from 53–78%) when compared with the incidence in rats fed the control diet. These results were analyzed by the linear correlation method, and a correlation coefficient of r = −0.93, which is significant at P < 0.05, was obtained, indicating a dose-dependent inhibition of colon tumor incidence with increasing levels of celecoxib in the diet. Multiplicities of colon adenocarcinomas and total colon tumors were also significantly suppressed in the rats receiving celecoxib at 500, 1000, or 1500 ppm dose levels (P < 0.001 and P < 0.0001; 67–84% inhibition). These results were also analyzed using a linear correlation method for a dose-dependent effect. This analysis yielded the correlation coefficient of r = −0.96 for multiplicity of adenocarcinomas with increasing levels of celecoxib from 0–1500 ppm, suggesting a dose-related inhibition (P < 0.05). The incidences and multiplicities of adenomas could not be compared among different groups because of the low yield of this lesion. Data summarized in Table 3 demonstrate that colon tumor volume was significantly reduced in the rats that were given celecoxib during the initiation and postinitiation stages (P < 0.0001).

Efficacy of Celecoxib Administered during the Promotion/Progression Stage. Administration of 1500 ppm celecoxib during the promotion/progression stages (beginning 14 weeks after the carcinogen treatment) significantly inhibited the incidence of colonic adenocarcinomas and total colon tumors (P < 0.01 and P < 0.001; 45–47% inhibition). The suppression of multiplicities of adenocarcinomas and total tumors (P < 0.01; 57–60% inhibition) was also significant in rats given celecoxib when compared with the results for rats fed the control diet. Colon tumor volume was significantly suppressed when celecoxib was administered during the promotion/progression stage of colon carcinogenesis (P < 0.0001; 72% inhibition).

DISCUSSION

The overall objective of our program is to identify chemopreventive agents with minimal or no side effects but increased

<table>
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<tr>
<th>Experimental group</th>
<th>No. of rats/group</th>
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<th>4</th>
<th>7</th>
<th>15</th>
<th>23</th>
<th>39</th>
<th>47</th>
<th>52</th>
</tr>
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<tbody>
<tr>
<td>Control diet (AOM-76A)</td>
<td>36</td>
<td>119 ± 10</td>
<td>225 ± 11</td>
<td>267 ± 12</td>
<td>345 ± 15</td>
<td>385 ± 22</td>
<td>447 ± 23</td>
<td>460 ± 34</td>
<td>462 ± 30</td>
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<td>Celecoxib, 500 ppm</td>
<td>36</td>
<td>113 ± 10</td>
<td>224 ± 11</td>
<td>270 ± 13</td>
<td>345 ± 18</td>
<td>386 ± 19</td>
<td>443 ± 29</td>
<td>463 ± 27</td>
<td>468 ± 24</td>
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<td>Celecoxib, 1000 ppm</td>
<td>36</td>
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<td>227 ± 11</td>
<td>274 ± 13</td>
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<td>391 ± 19</td>
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<td>114 ± 11</td>
<td>227 ± 11</td>
<td>274 ± 13</td>
<td>347 ± 17</td>
<td>384 ± 19</td>
<td>444 ± 24</td>
<td>461 ± 23</td>
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<td>384 ± 19</td>
<td>444 ± 24</td>
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<td>464 ± 28</td>
</tr>
<tr>
<td>Celecoxib, 5000 ppm</td>
<td>36</td>
<td>114 ± 11</td>
<td>227 ± 11</td>
<td>274 ± 13</td>
<td>347 ± 17</td>
<td>384 ± 19</td>
<td>444 ± 24</td>
<td>461 ± 23</td>
<td>464 ± 28</td>
</tr>
<tr>
<td>Control diet</td>
<td>6</td>
<td>117 ± 4</td>
<td>237 ± 10</td>
<td>283 ± 13</td>
<td>364 ± 22</td>
<td>439 ± 28</td>
<td>466 ± 32</td>
<td>478 ± 35</td>
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<tr>
<td>Celecoxib, 1500 ppm</td>
<td>6</td>
<td>116 ± 5</td>
<td>237 ± 14</td>
<td>284 ± 19</td>
<td>361 ± 23</td>
<td>435 ± 30</td>
<td>471 ± 34</td>
<td>487 ± 36</td>
<td>489 ± 32</td>
</tr>
</tbody>
</table>

a Values represent the mean ± SD.

b Weeks after the last AOM or saline injection.

c Celecoxib was administered in diet beginning 2 weeks before and during carcinogen treatment and until termination of the study at week 52.

d Celecoxib was administered in the diet starting 14 weeks after the second AOM treatment and until termination of the study at 52 weeks.
efficacy against colon carcinogenesis in a preclinical model that will facilitate the application of such agents in a clinical setting. Specifically, the present study, which is a part of a large-scale preclinical investigations of NSAIDs as chemopreventive agents against colon cancer, evaluated the dose-related efficacy of celecoxib, a specific COX-2 inhibitor. Importantly, our study also examined the chemopreventive efficacy of celecoxib when administered as an inhibitor during the promotion/promotion stage of chemically induced colon carcinogenesis. The strong inhibitory effect of celecoxib at the 1500 ppm dose level when administered continuously during the initiation and postinitiation phases of colon carcinogenesis (29) provided the rationale for evaluating the efficacy of this agent during the promotion/promotion stage. This preclinical model provides baseline information for eventual evaluation of the efficacy of celecoxib in late intervention/prevention protocols of colonic tumors in high-risk individuals, such as patients with sporadic colonic polyps or FAP.

The results of this study are in agreement with our earlier investigations, which had shown that administration of 1500 ppm celecoxib during the initiation and postinitiation periods profoundly inhibited colon carcinogenesis (29). This study shows that the suppression of colon carcinogenesis by celecoxib is dose dependent. To our knowledge, this study is the first one to demonstrate a dose-response effect of celecoxib in a preclinical model. It is noteworthy that the difference in the incidence and multiplicity of adenocarcinomas between the lowest (500 ppm) and highest (1500 ppm) doses of celecoxib is limited (55% versus 77% and 69% versus 83% compared to control), suggesting that celecoxib is very effective, even at the lower dose level. The present study also demonstrates for the first time that celecoxib given in the diet during the promotion/promotion period is still an effective inhibitor of colon carcinogenesis. This suggests that the administration of celecoxib may retard the growth and/or development of existing neoplastic lesions in the colon. This study extends our earlier findings that NSAIDs such as piroxicam, sulindac, and the naturally occurring antiinflammatory agent curcumin given to rats during the promotion/promotion stage inhibit colon tumorigenesis (6, 30, 32). Importantly, celecoxib did not induce any gastrointestinal and renal toxicity, unlike nonselective NSAIDs; thus, it provides an advantage over NSAIDs that are not selectively targeted at COX-2 inhibition. This underscores the potential usefulness of celecoxib as a chemopreventive agent for individuals at high risk for colon cancer development.

With regard to the mode of action of celecoxib against colon carcinogenesis, several reports indicate that PGs produced through COX activity have a role in the pathogenesis of colon cancer because they modulate signal transduction pathways (14, 20, 24) and affect cell proliferation, tumor growth, and immune responsiveness (12). COX also appears to play a role in the regulation of angiogenesis as it relates to neoplastic tumor cells (33) so that COX inhibitors may possibly block the growth of blood vessels in and around the developing tumors. The available data support the hypothesis that COX-2 may have a key role in colon tumor growth and progression. Several studies have shown that COX-2 but not COX-1 gene expression and protein expression are markedly elevated in most human colon tumors and also in chemically induced colon tumors in rats as compared with accompanying normal mucosa (15–17). In *in vitro* studies demonstrate that COX-2 expression contributes significantly to the tumorigenic potential of epithelial cells by increasing adhesion to the extracellular matrix and making these cells resistant to apoptosis (25). In addition, a highly selective COX-2 inhibitor decreased cell growth in both *in vitro* and *in vivo* assays only in the COX-2-expressing cell line (34). Thus, the suppression of colon carcinogenesis by celecoxib is mediated by the inhibition of COX-2 activity.

In summary, administration of celecoxib during the initiation and postinitiation stages significantly inhibits colon tumor incidence and multiplicity in a dose-dependent manner. Importantly, administration of celecoxib at the lower dose level significantly suppressed adenocarcinomas in the colon. The study described here demonstrates for the first time that administration of celecoxib during the promotion/promotion stage still significantly inhibits colon tumor development and tumor burden, suggesting indirectly that the chemopreventive efficacy of this agent is achieved even during the later phases of colon tumor development in this model. Although our understanding of the exact mechanism of the chemopreventive action of celecoxib is still evolving, the development of preventive strategies on the basis of experimental studies will serve as a practical approach to design chemoprevention trials in humans. The results described here make a strong case for the use of celecoxib as a chemopreventive agent for the secondary prevention of colon cancer in high-risk individuals, such as patients with sporadic polyps and FAP.

### Table 2 Chemopreventive efficacy of celecoxib on the incidence and multiplicity of AOM-induced colon carcinogenesis in male F344 rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of tumors</th>
<th>Tumor volume (mm³)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet (AIN-76A)</td>
<td>49</td>
<td>228 ± 310f</td>
<td>64</td>
</tr>
<tr>
<td>500 ppm Celecoxib</td>
<td>16</td>
<td>81 ± 39c</td>
<td>74</td>
</tr>
<tr>
<td>1000 ppm Celecoxib</td>
<td>10</td>
<td>38 ± 26d</td>
<td>83</td>
</tr>
<tr>
<td>1500 ppm Celecoxib</td>
<td>21</td>
<td>64 ± 36d</td>
<td>72</td>
</tr>
</tbody>
</table>

* Values represent the mean ± SD.

### Table 3 Effect of celecoxib on colon tumor volume in male F344 rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of tumors examined</th>
<th>Tumor volume (mm³)</th>
<th>% inhibition</th>
</tr>
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<td>49</td>
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</tbody>
</table>

* Values represent the mean ± SD.

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**Termination of the study.**

Continued during carcinogen treatment until termination of the study at week 52.

In the incidence and multiplicity of adenocarcinomas between the administration of celecoxib in a preclinical model. It is noteworthy that the difference of celecoxib in late intervention/prevention protocols of colonic tumors in high-risk individuals, such as patients with sporadic colonic polyps or FAP.**
ACKNOWLEDGMENTS

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