Cyclooxygenase-2 Expression and Effect of Celecoxib in Gastric Adenomas of Trefoil Factor 1-deficient Mice

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Abstract

Expression of cyclooxygenase-2 (Cox-2) is elevated in gastric adenocarcinomas and precursor lesions leading to this disease. Mice deficient for trefoil factor 1 (TFF1) develop a pyloric adenoma with full penetrance. Because inhibition of Cox-2 suppresses tumor growth in several animal models, we studied expression of Cox-2 and effect of a selective Cox-2 inhibitor celecoxib in gastrointestinal tissues of the TFF1-deficient mice. Cox-2 mRNA and protein were strongly expressed in the pyloric adenomas of the TFF1−/− mice as detected by in situ hybridization and immunohistochemistry. Nonneoplastic gastrointestinal tissues of wild-type or TFF1−/− mice expressed low or nondetectable levels of Cox-2. Celecoxib (1600 ppm p.o. for 3 months) caused ulceration and inflammation of the adenoma in all treated TFF1−/− mice (n = 7). This effect of the drug was adenoma specific, because no histological alterations were observed in the non-neoplastic gastric or intestinal tissues in the TFF1−/− or wild-type mice receiving the drug treatment. All untreated TFF1−/− mice had an adenoma (n = 7), but none demonstrated the combination of ulceration and inflammation. Our data show that Cox-2 is expressed in gastric adenomas of the TFF1−/− mice and suggest that inhibition of Cox-2 disturbs the integrity of the adenoma by promoting ulceration and inflammation. These findings support the effort to initiate clinical studies to investigate the effect of Cox-2 inhibitors as a chemotherapeutic modality for dysplasias of the stomach.

Introduction

Expression of Cox-2 is elevated in a variety of human carcinomas, and inhibition of Cox-2 suppresses tumor growth in several animal models of carcinogenesis (1). Genetic deletion of Cox-2 leads to a reduced number and size of polyps in a mouse model of familial adenomatous polyposis (2), and a selective Cox-2 inhibitor celecoxib reduced polyp burden in patients who suffer from familial adenomatous polyposis (3). Furthermore, elevated Cox-2 expression associates with poor prognosis in digestive tract carcinomas (4, 5). Thus, Cox-2 seems to be a relevant target in chemoprevention.

Expression of Cox-2 mRNA and protein is elevated in gastric adenocarcinoma and in (dysplastic) precursor lesions of this disease (6–8). In contrast to Cox-2, trefoil peptide TFF1 (named previously pS2) is expressed in normal epithelium of the stomach, where it protects the gastrointestinal mucus membrane from injuries, including those caused by nonsteroid anti-inflammatory drugs (9). Interestingly, expression of TFF1 is lost in ~50% of human gastric adenocarcinomas, and TFF1 knockout mice develop dysplastic gastric adenomas with full penetrance, which can further develop to adenocarcinomas (10). Thus, TFF1 can be considered as a tumor suppressor gene.

Here we describe expression of Cox-2 and the effect of the Cox-2 selective inhibitor celecoxib in gastrointestinal tissues of TFF1 knock-out mice. Our data show that Cox-2 is expressed in the gastric adenomas of the TFF1−/− mice and suggest that inhibition of Cox-2 disturbed the integrity of the adenoma by promoting ulceration and inflammation.

Materials and Methods

Treatment of Mice and Tissue Handling. Expression of Cox-2 was first studied in adenomas derived from TFF1−/− mice (n = 10) and in pyloric tissues derived from wild-type mice (n = 2) as detected by immunohistochemistry. In addition, two TFF1−/− mice adenomas and two wild-type mice pyloric regions were processed for in situ hybridization as described below. Drug treatment trial consisted of four groups of animals: (a) 7 TFF1−/− mice and (b) 7 wild-type mice were fed ad libitum with diet containing 1600 ppm celecoxib starting at the age of 4 weeks, and (c) 7 TFF1−/− mice and (d) 7 wild-type mice were fed with control diet. The weight of the mice was measured weekly. After treatment for 3 months, the mice were sacrificed, and the stomach and proximal part of the small intestine were removed, fixed in formalin, and embedded in paraffin. For histological examination, the tissue sections were stained with H&E. The relative proportion of gastric ulceration in the adenomas was estimated by measuring the length of the adenoma and length of the ulceration under a light microscope using ×400 magnification (0.65 mm) by K. S. and A. R. Heparinized plasma samples were obtained at the time of sacrifice for measurement of celecoxib plasma levels. The study was approved by the local ethical committee.

Histomorphometry. Formalin-fixed and paraffin-embedded specimens were sectioned (5 μm), deparaffinized, and microwaved for 2.5 min in 800 W and for 15 min in 440 W in 0.1 m sodium citrate buffer (pH 6.0). The slides were then immersed in 0.6% hydrogen peroxide in methanol for 30 min and then in blocking solution (0.01 m Tris, 0.1 m MgCl2, 0.5% Tween 20, 1% BSA, and 5% normal goat serum) for 1 h to block endogenous peroxidase activity and unspecific binding sites, respectively. Immunostaining was performed with an affinity-purified antiserum Cox-2 rabbit polyclonal antibody (160126; Cayman Chemical Co., Ann Arbor, MI) in a dilution of 1:300 in the blocking solution at 4°C overnight. The sections were thereafter treated with biotinylated goat antirabbit immunoglobulin (1:200; Vector Laboratories, Inc., Burlingame, CA), and antibody-binding sites were visualized by avidin-biotin peroxidase complex (Vectastain ABCComplex; Vector Laboratories) and 3-amin-9-ethylcarbazole (Lab Vision Co., Fremont, CA). The counterstaining was performed with Mayer’s hemalum (Merck, Darmstadt, Germany). Specificity of the antibodies was determined by preadsorption of the Cox-2 antibody with a mouse Cox-2 control peptide (20 μg/ml; Cayman Chemical) for 1 h at room temperature before the staining procedure.

To measure microvessel density, the paraffin-embedded sections were processed and treated with primary antibodies for antifactor VIII-related antigen/von Willebrand factor (1:800; DAKO Immunoglobulins, Glostrup, Denmark) as de-
scribed previously (11). Trypsin treatment at 37°C for 30 min was used for antigen retrieval, and the detection was performed by using Tyramide Signal Amplification kit (NEN Life Sciences, Boston, MA). Negative controls were done by omitting the primary antibody. The slides were then viewed with a light microscope, and vessels were counted from three \( \times 400 \) fields by K. S. and A. R.

**In Situ Hybridization.** Tissues were fixed in 4% paraformaldehyde at 4°C for 1–2 h, equilibrated in 20% sucrose in PBS at 4°C for 8–10 h, mounted in 7.5% gelatin and 15% sucrose in PBS at 37°C, frozen on dry ice, and cut in 14-μm-thick sections. Thawed sections were hybridized as described previously (12). Sense and antisense mouse Cox-2 riboprobes were generated by transcribing with T3 and T7 RNA polymerases in the presence of \( ^{33} \)P-UTP (1000–3000 Ci/mmol; Amersham, Arlington Height, IL) by using a 453-bp fragment of mouse Cox-2 cDNA (Cayman Chemical). Paraffin-embedded tissue sections were incubated with \( 1 \times 10^{6} \) cpms of \( ^{33} \)P-labeled antisense or sense riboprobe in a total volume of 80 μL.

**Analysis of Blood Plasma for Celecoxib.** Celecoxib was extracted from 25 μL of plasma using Whatman protein precipitation plates. Samples were added to the precipitation plates, precipitated with acetonitrile, and filtered. The filtrates were then collected and evaporated until dry. The dried residues were resuspended in mobile phase and chromatographed onto a liquid chromatography/tandem mass spectrometer system for analysis. The results are shown as micrograms per milliliter.

**Statistical Analysis.** Statistical significance of the effect of celecoxib was calculated with unpaired Fisher’s exact test, and Welch’s t-test, and statistical significance for microvessel density was calculated by using non-parametric unpaired Mann-Whitney test. Statistical significance of the effect of celecoxib was calculated with unpaired Fisher’s exact test, and Welch’s t-test, and statistical significance for microvessel density was calculated by using non-parametric unpaired Mann-Whitney test. Statistical significance of the effect of celecoxib was calculated with unpaired Fisher’s exact test, and Welch’s t-test, and statistical significance for microvessel density was calculated by using non-parametric unpaired Mann-Whitney test.

**Results**

All pretrial TFF1-/- mice developed a dysplastic pyloric adenoma (n = 10), whereas the pylorus of the wild-type mice was of normal histology (n = 2). Expression of Cox-2 mRNA localized to the adenomas as detected by in situ hybridization (Fig. 1, A and B). Most of the signal localized to the stromal cells in the adenomas. No detectable expression of Cox-2 mRNA was evident in the gastrointestinal tissues of the wild-type mice (Fig. 1D). Cox-2 protein was expressed in 90% (9 of 10) of the untreated TFF1-/- mice adenomas. Consistent with the localization of the mRNA signal, Cox-2 protein was expressed in the stromal cells (Fig. 1E). In contrast, non-neoplastic gastric mucosa proximal to the adenoma or intestinal tissues distal to the adenoma did not express detectable levels of Cox-2 protein in these animals. Only low (in Brunner glands) or nondetectable Cox-2 protein expression was evident in the gastrointestinal tissues of the wild-type mice as detected by immunohistochemistry (Fig. 1F).

We next investigated the effect of a Cox-2 selective inhibitor celecoxib in the TFF1-/- and wild-type mice. The treatment was started after weaning at the age of 4 weeks, at which time TFF1-/- mice have been reported to show hyperplasia of the pyloric gland epithelium (10). All untreated TFF1-/- mice (n = 7) had a histologically identified pyloric adenoma (Table 1 and Fig. 2). However, all adenomas in the celecoxib treatment group (n = 7) were ulcerated and contained a heavy infiltration of (mainly mononuclear) inflammatory cells (Table 1 and Fig. 2, C and D). This combination of ulceration and inflammation was exclusively observed in the drug-treated TFF1-/- group, because only one ulceration (without inflammation) was observed in an untreated TFF1-/- mouse (Table 1). Furthermore, none of the pretrial TFF1-/- mice adenomas (n = 10) were ulcerated or inflamed. In the drug-treated knockout mice, the average size of the ulceration was 66 ± 10% (range 50–75%) of the adenoma, which suggests that the majority of the dysplastic lesion was injured. However, no ulcerations were observed in the stomach proximal to the adenoma or in the small intestine distal to the adenoma in these mice.

**Fig. 1.** Expression of Cox-2 mRNA and protein in adenomas of TFF1-deficient mice and pylorus of wild-type mice as detected by in situ hybridization and immunohistochemistry. In A and B, Cox-2 mRNA expression was mainly localized to the stromal cells (arrows) of the adenoma as detected by antisense probe (A, dark field; B, bright field). The sense control probe hybridization is shown in C (dark field). In D, no detectable Cox-2 expression was found in wild-type mice pylorus tissues as detected by the antisense probe (dark field). In E, Cox-2 protein was expressed in stromal cells in the TFF1-/- mice adenomas. Blocking control with the antigenic peptide is shown in the inset. In F, low (Brunner glands) or nondetectable levels of Cox-2 protein expression were evident in the pyloric area of the wild-type mice. Original magnifications are A–D, ×200; E, ×400; and F, ×200.
Celecoxib treatment did not have an effect on Cox-2 expression in the adenomas, because all adenomas in the TFF1−/− group with or without the drug treatment were positive for Cox-2 immunostaining. Wild-type mice pyloric region with or without the drug treatment were virtually negative for Cox-2 immunostaining (data not shown). There was a slight but significant decrease in the weight of the animals in the TFF1−/− group treated with the drug (Table 2). However, no obvious behavioral changes nor gastrointestinal bleeding or perforations were observed in the treatment groups, and none of the mice died during the experiment. Celecoxib plasma concentrations were comparable between the wild-type and TFF1−/− groups (Table 2). Microvessel density was measured from TFF1−/− groups with or without celecoxib treatment by staining for von Willebrand factor (11). The vessel density was 10.4 ± 2.6 in nontreated adenomas (n = 7) and 11.2 ± 2.9 in the dysplastic region of the drug treated (and ulcerated adenomas; n = 7). This difference was not statistically significant. However, microvessel density was more intense in the ulcer bed of the treated mice (39.2 ± 17.2; n = 7) when compared with either adenoma group (P < 0.01).

Table 1. Effect of celecoxib treatment on wild-type (WT) mice gastrointestinal tissues and on TFF1 knockout (KO) mice adenoma.

<table>
<thead>
<tr>
<th>TFF1 status</th>
<th>Intact adenoma</th>
<th>Ulceration + inflammation</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>No adenoma (n = 7)</td>
<td>0% (0/7)</td>
</tr>
<tr>
<td>WT + celecoxib</td>
<td>86% (6/7)</td>
<td>0% (0/7)</td>
</tr>
<tr>
<td>KO</td>
<td>0% (0/7)</td>
<td>100% (7/7)</td>
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*Statistical significance was calculated with unpaired Fisher’s exact test.

*One noninflamed ulceration was observed in this group.

*P = 0.052 as compared with KO group without treatment.

*P = 0.047 as compared with any other group.

Table 2. Effect of celecoxib treatment on wild-type (WT) and TFF1 knockout (KO) mice weight and drug plasma concentrations.

<table>
<thead>
<tr>
<th>TFF1 status</th>
<th>No. of mice</th>
<th>Initial weight (grams)</th>
<th>Final weight (grams)</th>
<th>Celecoxib (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7</td>
<td>14.14 ± 1.26</td>
<td>20.97 ± 1.48</td>
<td>ND</td>
</tr>
<tr>
<td>WT + celecoxib</td>
<td>7</td>
<td>14.76 ± 1.01</td>
<td>21.36 ± 0.83</td>
<td>2.18 ± 0.59</td>
</tr>
<tr>
<td>KO</td>
<td>7</td>
<td>15.16 ± 1.33</td>
<td>21.66 ± 0.84</td>
<td>ND</td>
</tr>
<tr>
<td>KO + celecoxib</td>
<td>7</td>
<td>13.60 ± 2.10</td>
<td>19.64 ± 1.72</td>
<td>2.96 ± 0.76</td>
</tr>
</tbody>
</table>

*Statistical significance was calculated with unpaired Welch’s t test, and data are shown as mean ± SD.

*ND = nondetectable (<5 ng/ml).

*P = 0.562 as compared with WT group without treatment.

*dP > 0.128 as compared with any other group.

*P = 0.024 as compared with KO group without treatment.

*P = 0.045 as compared with WT group treated with celecoxib.

*P = 0.149 as compared with WT group without treatment.

*P = 0.053 as compared with WT group treated with celecoxib.

Fig. 2. Histology of pyloric region in TFF1-deficient and wild-type mice. A, pylorus of a wild-type mouse showing normal histology. B, pyloric adenoma of an untreated TFF1−/− mouse. C, ulcerated and inflamed pyloric adenoma of a celecoxib-treated TFF1−/− mouse. Arrows, the infiltrating mononuclear inflammatory cells in the submucosa. Note the ulceration on the left, dysplastic epithelium in the center, and normal glands of the antrum on the right. D, higher magnification of the area under the square shown in C. Note the ulcer material on the luminal side (+). Original magnification for A–C, ×40 and D, ×200.
Discussion

TFF1-deficient mice develop a dysplastic pyloric adenoma with full penetrance, and thus, it is a well-characterized genetic model for gastric neoplasias (10). Treatment of TFF1−/− mice with the selective Cox-2 inhibitor celecoxib caused ulceration and infiltration of chronic inflammatory cells of the adenoma. Only 1 of 17 (10 in pretrial and 7 in the trial) untreated TFF1−/− mice showed an ulceration of the adenoma that was not inflamed. Because celecoxib caused no injuries in non-neoplastic gastric mucosa or small intestinal mucosa in either wild-type or TFF1−/− mice, our data suggest that the effect of the drug is adenoma specific.

High expression of Cox-2 mRNA and protein were exclusively detected in the adenomas of the TFF1−/− mice. This is consistent with our finding that the effect of the Cox-2 selective inhibitor celecoxib was adenoma specific. Expression of Cox-2 mRNA and protein localized primarily to the stromal cells of the adenomas. Similarly to our data, it has been reported previously that Cox-2 is expressed in stromal cells in mouse models for intestinal neoplasms (13, 14). Furthermore, Cox-2 promoter-driven LacZ gene was expressed in stromal cells in adenomatous polyps of Apc knockout mice (2). We have detected previously Cox-2 expression primarily in neoplastic epithelial cells in human gastric adenocarcinomas and noninvasive dysplasias (8). The significance of stromal versus epithelial Cox-2 expression is not known, but it is possible that Cox-2 expression is first induced in stromal cells and later in neoplastic epithelial cells. At least in mice, this stromal Cox-2 expression may promote growth of carcinoma cells (15).

Gastrointestinal ulceration is the major side effect of conventional nonsteroid anti-inflammatory drugs (1). Administration of Cox-2 selective inhibitors impairs healing of experimentally induced gastric ulcers in rodents, and inhibition of angiogenesis was suggested as one mechanism of action (16, 17). Celecoxib has been shown to inhibit basic fibroblast growth factor-induced corneal neovascularization in vivo (18), which suggests that Cox-2 may also participate in tumor angiogenesis. Indeed, colon cancer cells that overexpress Cox-2 produce angiogenic factors and stimulate endothelial cell migration and tube formation in vitro that was inhibited by a selective Cox-2 inhibitor NS-398 (19). It is thus possible that the adenoma-specific ulcerogenic effect of celecoxib in the TFF1 knockout model is, at least partially, attributable to inhibition of angiogenesis. We also made an attempt to measure microvessel density in the adenomas and at the site of the ulcer bed. Our data show that the vessel density in the dysplastic mucosa in the treated versus nontreated mice did not differ. However, we cannot rule out the possibility that the adenoma tissue that had been destroyed (ulcerated) would also have had the same microvessel density. Interestingly, in the ulcer bed, we found a high density of microvessels, which may suggest that the angiogenesis process itself was in fact intact at these sites of injury. These points need to be more carefully determined in the future to determine the role of angiogenesis at the early phase of the treatment and, on the other hand, the end result of the healing process.

Tumor growth can be modulated by host immune response, and prostanoids have been shown to induce immunosuppression (1). Inhibition of Cox-2 has been implicated to exacerbate dietary antigen-induced injuries in the small intestine of mice and intestinal damage in a rat model of inflammatory bowel disease (20, 21). Thus, Cox-2-derived prostanoids protect gastrointestinal tract from immunological damage in certain experimental models. Mechanistically, this can depend on cytokine microenvironment, because Cox-2-dependent synthesis of prostanoids by lung cancer cells altered release of interleukin-10 and interleukin-12 from lymphocytes and macrophages, resulting in repression of host immunity (22). In our model, a heavy infiltration of chronic inflammatory cells was observed in all celecoxib-treated adenomas. We cannot exclude the possibility that the inflammation is simply associated with the ulceration. However, because one untreated knockout mouse had an ulceration that was not inflamed, it is possible that the exacerbated inflammatory reaction at sites of the ulcerated adenomas in the drug-treated TFF1−/− mice was promoted by the therapy. In contrast to the inflammatory bowel disease model in the rat (21), we did not observe any perforations or deaths of the mice in our model. However, the weight of the drug-treated TFF1−/− mice was slightly reduced. This could be caused by the chronic inflammation, but it is unlikely that the weight loss was caused by reduced food intake, because the plasma levels of the drug were comparable between the drug-treated TFF1−/− and wild-type mice.

In conclusion, our results show that Cox-2 is expressed in gastric adenomas of TFF1−/− mice and that inhibition of Cox-2 disturbed the integrity of the adenoma by inducing ulceration and inflammation. These findings support the effort to initiate clinical studies to investigate the effect of Cox-2 inhibitors as a chemotherapeutic modality for dysplasias of the stomach.

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References

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