Changes in Antitumor Response in C57BL/6J-Min/+ Mice during Long-term Administration of a Selective Cyclooxygenase-2 Inhibitor

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Abstract
Selective cyclooxygenase-2 (COX-2) inhibitors are widely prescribed for severe arthritis and are currently under study in human chemoprevention trials. Recently, long-term use of these agents has come under scrutiny due to reports of treatment-associated cardiovascular toxicity. On short-term administration, the selective COX-2 inhibitor celecoxib inhibits adenoma growth in animal tumor models, including the C57BL/6J-Min/+ (Min/+) mouse. With uninterrupted long-term celecoxib administration, intestinal tumors in Min/+ mice initially regressed and then recurred to levels comparable with untreated controls. Celecoxib treatment initially suppressed COX-2 and prostaglandin E2 (PGE2) expression, but long-term use produced significantly higher levels of these molecules and reactivated PGE2-associated growth factor signaling pathways in tumor and normal tissues. These results indicate that COX-2 is an important chemoprevention target and that inhibition of this enzyme alters a paracrine enterocyte regulatory pathway. Chronic uninterrupted celecoxib treatment, however, induces untoward effects that may contribute to treatment toxicity. (Cancer Res 2006; 66(12): 6432-8)

Introduction
Nonsteroidal anti-inflammatory drugs (NSAID) are widely used for treatment of pain and arthritis and, in the case of aspirin, for prevention of cardiovascular disease. NSAIDs target one or both cyclooxygenases (COX-1 and COX-2), enzymes that convert arachidonic acid to prostaglandin G2 (PGG2). By the peroxidase activity of the COX enzyme, PGG2 is reduced to prostaglandin H2 (PGH2) and then converted by isomerases to one of various prostanoids: prostaglandin E2 (PGE2), prostaglandin D2, prostaglandin F2α, prostaglandin I2 (PGI2; prostacyclin), or thromboxane A2 (1). COX activity and PGE2 production are implicated in intestinal tumorigenesis in mice and humans. Case-control and observational studies show that frequent use of NSAIDs is associated with reductions in colorectal adenomas, cancers, and cancer-associated mortality (2). In rodent models, NSAIDs reduced the number, multiplicity, and size of intestinal tumors (3, 4).

Multiple lines of evidence indicate that COX-2, in particular, is an important mediator of tumorigenesis. COX-2 is overexpressed in most epithelial tumors (5), and selective blockade of COX-2 activity by gene deletion or pharmacologic inhibition reduces tumor formation in both animals and humans (3, 4, 6, 7). Knowledge of precisely how COX-dependent pathways promote intestinal tumorigenesis remains incomplete. It is generally thought that COX-2 stimulates tumor growth via complementary pathways affecting interactions between enterocytes and stromal cells. For example, COX-2 promotes tumor cell survival by inhibiting apoptosis and by stimulating angiogenesis (8, 9). Consistent with these findings, selective COX-2 inhibitors induce apoptosis of colon cancer cells in vitro and tumor regression in vivo (6, 10). COX-2 collaborates with growth factor receptors to stimulate tumor cell growth as evidenced by the ability of PGE2 to transactivate the epidermal growth factor receptor (EGFR; refs. 11–14). In the intestinal tract, fibroblasts and endothelial cells of the lamina propria produce PGE2 and PGI2 (15). These prostanoids protect normal enterocytes from apoptosis during episodes of infection, tissue injury, and inflammation. They also protect enterocytes as well as tumor cells from NSAID-induced apoptosis (16, 17). Additional potential mechanisms for the efficacy of NSAIDs in colorectal cancer inhibition include boosting host immune surveillance and inhibiting tumor cell invasion. Although NSAIDs primarily target COX enzyme activities, these drugs may also produce anticancer effects via COX-independent mechanisms (18–20).

Clinical trials are currently under way to assess the efficacy and safety of treatment with selective COX-2 inhibitors, such as celecoxib, for prevention of colorectal adenomas. Results from a study of patients with familial adenomatous polyposis (FAP) treated with celecoxib to induce adenoma regression suggest that some tumors are resistant to suppression by this NSAID (7). Previous studies by Jacoby et al. (6) used the C57BL/6J-Min/+ (Min/+) mouse, an animal model of FAP and sporadic intestinal adenomas, to examine the effect of short-term celecoxib administration on intestinal adenoma formation. A dramatic reduction in tumor number was achieved following use of up to 1,500 ppm celecoxib from ages 30 to 50 days. The effect of long-term drug administration, such as that required for cancer chemoprevention, has not yet been examined. In this study, we treated Min/+ mice with celecoxib over a prolonged interval to investigate the nature of celecoxib-resistant intestinal neoplasia.

Materials and Methods

Mice, antibodies, and reagents. Min/+ mice and their Apc−/− [wild-type (WT)] littermates were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were fed AIN-76A diet with or without celecoxib at 1,500 ppm (Research Diets, Inc., New Brunswick, NJ). Antibodies and reagents were as specified previously (11, 21). Additional reagents included rabbit anti-mouse EP4 (H-160), goat anti-COX-2 (N-20), goat anti-COX-1...
Dietary treatment and tissue harvesting. At age 5 weeks, all animals were placed on AIN-76A diet and provided tap water to drink ad libitum. Fifteen 8-week-old Min/+ mice were started on celecoxib diet (1,500 ppm). Concurrently, age- and gender-matched Min/+ and WT control mice (n = 10 each) were allowed to continue feeding on the AIN-76A diet without added drug. At ages 7 to 8 weeks, adenomas are well established in Min/+ mice, and celecoxib efficacy was similar whether treatment was initiated at weaning or at age 55 days (6). Drug-treated mice were divided into groups of five animals each. Two groups of mice were treated with celecoxib for 4 or 21 days and were then sacrificed along with untreated age-matched controls, and relevant tissues were harvested and preserved as described previously (11, 21). A third group of mice was maintained on celecoxib diet until they showed evidence of ill health, such as lethargy or weight loss. At this time, these animals were euthanized and autopsied. Mice that were provided treatment diet long-term were sacrificed at between 5 to 7 months. The procedures for animal care, euthanasia, tissue dissection, and tumor counting have been described previously (22). StatView was used to perform a two-tailed t test to determine statistical significance of changes in tumor number.

Lyase preparation, immunoblot, and PGE2 analysis. Enterocyte collection, lyase preparation, immunoblot, and PGE2 assays were done as described previously (11, 21). All protein analyses were repeated at least thrice using independently prepared lysates from different animals.

Immunohistochemistry. Serial 4-μm sections of paraffin-embedded tissues were processed to facilitate antigen retrieval in a Biocare Medical Decloaking Chamber (Walnut Creek, CA) for 2 minutes in 10 mmol/L citrate buffer (pH 6.0). Endogenous peroxidases were quenched with DAKO Decloaking Chamber (Walnut Creek, CA) for 2 minutes in 10 mmol/L citrate buffer (pH 6.0). Endogenous peroxidases were quenched with DAKO Peroxidase Block (Carpinteria, CA), slides were washed in PBS, and staining was completed as described previously (11, 21). The COX-2 immunohistochemistry used rabbit anti-COX-2 (murine) antibody at 1:50 for 1 hour at room temperature. Slides were washed in PBS and reacted with DAKO anti-rabbit labeled polymer for 30 minutes. Immunohistochemistry for EP2 and EP4 used rabbit anti-mouse antibodies at 1:500 dilution. The LOX-5 immunohistochemistry used rabbit anti-mouse LOX-5 antibody at 1:1,000 supplemented with biotin block. Immunohistochemistry for β-catenin was done as described previously (11). The chromagen used was 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin, dehydrated, and coverslipped. Images were obtained using an Olympus BX45 microscope (Optical Analysis Corp., Nashua, NH). All immunohistochemistry used celecoxib-treated and untreated Min/+ and WT tissues subjected to the same procedures at the same time and repeated on multiple occasions with specimens from different mice.

Results

Intestinal adenoma formation and COX-2 expression in animals receiving chronic celecoxib treatment. Min/+ mice spontaneously develop multiple intestinal adenomas due to a germ-line mutation in Apc. In agreement with previous reports, administration of celecoxib at 1,500 ppm induced tumor regression or prevented visible tumor formation in the Min/+ mouse that was discernable after 21 days of treatment (P = 0.001; Fig. 1A). The size of residual tumors was reduced and reflected reduction of total tumor load as in a similar study by others (6). When Min/+ mice were continuously maintained on diet containing celecoxib until they were 5 to 7 months old, tumor numbers increased to levels similar to that of the 3-month-old untreated control group (P = 0.80). In one of these chronically treated animals, an invasive adenocarcinoma of the distal colon was identified. No invasive tumors were found in animals treated with celecoxib for <3 months.

Because celecoxib targets the COX-2 enzyme, we evaluated PGE2 production in celecoxib-treated and control Min/+ mice. Figure 1B shows that PGE2 levels in tumors were suppressed during the treatment interval corresponding to a reduction in tumor...
For tumors that either persisted on treatment or developed during treatment, however, PGE\(_2\) expression was 1.5-fold higher than in tumors obtained from untreated Min/+ mice (\(P = 0.0001\)). Immunoblot analyses showed higher expression of COX-2 in Min/+ small bowel whole-cell lysates compared with those of the mucosa of WT mice (Fig. 1C). Following short-term celecoxib treatment, COX-2 expression was suppressed in normal mucosa and tumors. After long-term treatment, COX-2 expression in the nontumor intestinal tissue increased to a level above that observed in untreated mice. In these adenomas, COX-2 expression was also similar to that of untreated tumors. These results correlated the antitumor effect of dietary celecoxib treatment with inhibition of both COX-2 protein levels and PGE\(_2\) synthesis and adenoma recurrence or persistence during chronic celecoxib administration with increased COX-2 and PGE\(_2\) expression.

PGE synthases convert COX-derived PGH\(_2\) into PGE\(_2\). Two PGE synthase isoforms (mPGES-1 and mPGES-2) are glutathione-dependent, microsomal proteins whose expression can be regulated in coordination with COX-2 (23). Immunoblot analysis showed that expression of mPGES-1 was increased in untreated Min/+ mucosa and tumors, with minimal expression of this PGE synthase isoform in WT tissue (Fig. 1D). In comparison, mPGES-2 levels were invariant across the tissue types. Long-term celecoxib treatment did not significantly change mPGES-1 protein expression in the Min/+ adenomas.

Immunohistochemistry was used to determine the origin of COX-2 expression (Fig. 2). Although occasional tumor cells stained positively for COX-2 in untreated tissues, most of the expression was localized in cells of the peritumoral stroma (top left) or in the lamina propria of the normal Min/+ intestine (middle left). The intensity of COX-2 staining was reduced in tumors (top center) and normal lamina propria (middle center) of animals treated with celecoxib for 4 and 21 days, consistent with the reduced expression of PGE\(_2\) in these tissues. In contrast, strong focal COX-2 staining was detected in peritumoral stroma (top right) and normal lamina propria (middle right) of mice treated with celecoxib for >3 months. One animal treated with celecoxib for 5.5 months developed an invasive adenocarcinoma of the colon. Immunohistochemistry of this tumor showed strong COX-2 expression in both stromal cells (dark arrow) and infiltrating tumor cells (white arrow).

Expression of lipooxygenases and prostaglandin receptors are unaffected by chronic celecoxib administration. An alternative pathway of arachidonic acid metabolism uses lipooxygenases to catalyze the oxidation of this lipid to 5(S)-HPETE, the first step in the formation of leukotriene B\(_4\). Immunoblot analyses to examine the expression of 5-LOX, 12-LOX, and 15-LOX in the Min/+ celecoxib-treated and untreated enterocytes and
tumors showed minimal, if any, difference in the expression of these proteins (Fig. 3). 5-LOX is transferred to nuclear membranes on cell activation, and reduced 5-LOX nuclear localization is associated with decreased leukotriene B4 synthesis (24). Because the nuclear localization correlates with activity of this tumor-promoting lipoxygenase, we did immunohistochemistry to examine 5-LOX. Strong nuclear 5-LOX expression, with weaker background cytoplasmic staining, was observed in the histologically normal enterocytes and in stromal fibroblasts. This staining distribution was similar in tumor cells both with and without celecoxib treatment.

Two PGE2 receptors, EP2 and EP4, are implicated in murine intestinal tumorigenesis (25). By immunoblot analysis, EP2 and EP4 expression remained invariant in tumors despite treatment (Fig. 4). Immunohistochemistry to identify EP2 and EP4 expression confirmed these results (data not shown). COX-1 expression remained invariant across all tissue types (Fig. 4). Because COX-2 can increase expression of the multidrug resistance protein, Mdr-1 (26), we determined whether long-term celecoxib use affected this transporter. By immunoblot, levels of Mdr-1 were invariant across all treatment groups, suggesting that this component of multidrug resistance was not responsible for celecoxib resistance (Fig. 4).

Activation of signaling via PGE2 in celecoxib-resistant adenomas. COX-2 and EGFR share a common signaling pathway because of the ability of PGE2 to transactivate EGFR (11, 13). By immunoblot, expression and tyrosine phosphorylation of EGFR protein was increased in Min/+ tumors compared with nontumor enterocytes, with a decrease in activated EGFR following short-term celecoxib treatment of tumors (Fig. 5). Assays conducted in parallel showed that activated p44/p42 mitogen-activated protein kinase (MAPK) was higher in normal Min/+ intestine and in tumors, consistent with the up-regulation of EGFR activity in these tissues relative to the WT mucosa. As predicted, short-term celecoxib treatment also decreased activated p44/p42 MAPK. Long-term celecoxib treatment of Min/+ tumors was associated with increased EGFR expression and tyrosine phosphorylation as well as increased MAPK activity. These results were confirmed by immunohistochemistry (Fig. 6), which showed that activated p44/p42 MAPK was present in adenoma cells and the enterocytes of the intestinal proliferative zone and crypts.

Most tumors of the large intestine are initiated by loss of Apc, a protein that regulates cytosolic levels of β-catenin (27). In intestinal epithelial cells, β-catenin is an adhesion protein that links E-cadherin-mediated junctional complexes to the actin cytoskeleton. This catenin also binds to Tcf-4 in the nucleus to form a transcriptional activator of growth-promoting genes. The transcriptional role of β-catenin is required for stem cell proliferation and continuous tissue renewal (28). Intestinal tumorigenesis also

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Celecoxib treatment did not alter lipoxygenase expression. Immunoblot to detect 5-LOX, 12-LOX, and 15-LOX expression in enterocytes and tumor lysates from untreated and long-term celecoxib-treated Min/+ mice used 60 μg protein/lane and 1:1,000 antibody dilution. Immunohistochemistry using antibody directed against 5-LOX (1:1,000) shows a similar expression pattern for treated and untreated adenomas.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Expression of the PGE2 receptors, COX-1, and Mdr-1 in enterocytes and tumors from Min/+ mice treated with celecoxib. Immunoblots containing 50 μg protein/lane were separately probed with anti-EP2 and anti-EP4 antibodies (A). Blots containing 25 μg protein/lane were separately probed with anti-COX-1 (M-20) and anti-Mdr-1 (C-19) antibodies (B). All antibodies were used at 1:3,000 dilution. Reprobing blots for β-actin (clone AC-40) controlled for sample loading, and a mouse macrophage lysate served as a positive control. Expression of these proteins was invariant across treatment groups.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Activation of EGFR and MAPK in tumors resulting from long-term celecoxib treatment of Min/+ mice. Immunoblot analysis determined the expression of EGFR (clone 13; 1:1,000), phosphotyrosine (4G10; 1:2,000), p44/p42 phosphorylated MAPK, and total MAPK in WT, Min+, treated tumor, and untreated tumor cell lysates. Sample lanes contained 25 and 100 μg protein for the MAPK and EGFR blots, respectively.
depends on this function of β-catenin. Receptor tyrosine kinase transactivation by PGE₂ induces β-catenin-Tcf-4 transcriptional activation in the nucleus of colon cancer cells (29). Because nuclear β-catenin is a minimal requirement for its transactivation potential, we did immunohistochemistry to identify this oncprotein in tumors from untreated and celecoxib-treated Min/+ mice. In untreated nontumor Min/+ mucosa, nuclear staining for β-catenin was present only in the proliferative zone of the crypts (Fig. 7). The staining intensity of β-catenin was increased in both cytoplasm and nucleus of untreated adenoma cells, and nuclear β-catenin expression was decreased considerably by short-term celecoxib treatment. In contrast, prominent nuclear β-catenin expression was detected in adenomas that failed to regress or developed during long-term celecoxib treatment. These data suggest that long-term celecoxib treatment, which was associated with elevated PGE₂ production, may also promote the transcriptional activity of β-catenin-Tcf-4.

**Discussion**

This work confirms the importance of COX-2 and its effects on PGE₂-mediated signaling as causative factors in intestinal tumor formation. Short-term assays determined that the selective COX-2 inhibitor celecoxib effectively blocked COX-2 and PGE₂ expression and that this treatment did not shift arachidonic acid metabolism toward production of tumorigenic lipoygenase metabolites. In these studies, lack of tumor suppression despite high-dose celecoxib treatment was associated with overexpression of COX-2, in concert with the expected downstream consequences in the adenomas, including increased expression of PGE₂, activation of EGFR and MAPK, and nuclear localization of β-catenin. COX-2 overexpression was also found in stromal cells of both nontumor intestine and adenomas of Min/+ mice chronically treated with celecoxib, indicating that this is not a tumor-specific effect.

In agreement with others (4), we found that the main sources of COX-2 in both neoplastic and normal tissues were mononuclear
cells and fibroblasts of the stromal cell compartment. This distribution did not change significantly following long-term celecoxib treatment. Our data support the hypothesis that during early tumorigenesis COX-2 produced by stromal cells mediates growth-promoting changes in adjacent epithelium. This situation may change following malignant transformation. In the invasive adenocarcinoma that developed during chronic drug administration, we observed strong COX-2 staining of a subset of the tumor cells, suggesting a switch for this tumor promoter from paracrine production in benign cases to autocrine production in malignancy.

Nonspecific NSAIDs, such as sulindac and indomethacin, are dual inhibitors of COX-1 and COX-2. Although the active sites of COX-1 and COX-2 are structurally similar, the NSAID-binding region of COX-2 contains a distinct pocket produced by substitution of an isoleucine found in COX-1 with a valine in COX-2. Selective COX-2 inhibitors of COX-1 and COX-2. Although the active sites of COX-1 and COX-2 are structurally similar, the NSAID-binding region of COX-2 contains a distinct pocket produced by substitution of an isoleucine found in COX-1 with a valine in COX-2. Selective COX-2 inhibitors block enzyme activity by binding to this site (30). These agents exhibit minimal effect on COX-1 activity, thereby preserving platelet function and gastric mucosal protection. It remains to be determined whether the development of resistance to COX inhibition is specific to selective COX-2 inhibitors or is a property shared by all chronically administered NSAIDs.

COX-2 is minimally expressed in most normal tissues but is rapidly induced by inflammatory and mitogenic stimuli, including growth factors, cytokines, and other signals capable of stimulating MAPK activity. COX-2 expression is regulated by both transcriptional and post-transcriptional mechanisms. The COX-2 promoter contains consensus sequences for nuclear factor-κB, interleukin-6, activator protein-1, nuclear factor of activated T cells, PEA3, and cyclic AMP–responsive element (31, 32). A Tcf-4-binding element is also present in the COX-2 promoter, and studies in colorectal cancer cells suggest that nuclear β-catenin accumulation up-regulates COX-2 mRNA via β-catenin-Tcf-4 binding to this site (33). Consistent with this hypothesis, transfection of WT Apc into colorectal cancer cells lacking full-length Apc down-regulated COX-2 transcriptional activity (33). Our data extend this important observation to an in vivo system.

Several post-transcriptional modifications regulate COX-2 expression. In colon cancer, COX-2 expression is increased by binding of the mRNA stability factor, HuR, to AU-rich elements (ARE) in the 3′ untranslated region of COX-2 mRNA (34). The ARE-binding protein, TIA-1, is a translational silencer, and colon cancer cells overexpressing COX-2 show deficient TIA-1 mRNA binding (35). It remains to be determined whether any of these mechanisms of COX-2 up-regulation is responsible for resistance to celecoxib during long-term use. It is also possible that a component of celecoxib metabolism or intracellular transport is altered by chronic drug administration, resulting in levels of active drug that are too low to mediate COX-2 inhibition.

Long-term treatment data are not available for patients with FAP treated with celecoxib for adenoma suppression. Prospective cohort studies of FAP patients treated with the nonselective NSAID sulindac show conflicting results. In one prospective study, patients with FAP treated with sulindac maintained an antitumor response at a mean of 63.4 months of treatment (36). In another study, rectal adenomas regressed significantly during the first 6 months of sulindac use, but after a mean of ~48 months of treatment both number and size of adenomas increased, showing no statistical difference with baseline values (37). Case reports also indicate that patients with FAP chronically maintained on sulindac for tumor suppression can develop invasive adenocarcinomas (38), an occurrence also seen in one of the Min/+ mice treated long-term with celecoxib (Fig. 2).

Target enzyme expression was examined in a cohort of FAP patients treated with sulindac for 9 to 54 months, comparing COX-2 expression of pretreatment (baseline) and post-treatment (resistant) adenomas (39). The sulindac-resistant adenomas showed less epithelial COX-2 expression and showed less nuclear accumulation of β-catenin and more localization of β-catenin in the cell membrane. It is interesting that two of the three sulindac-resistant patients in this study were initially in complete tumor remission and only developed resistant tumors after 2 years of continuous sulindac use. One important difference noted between baseline and sulindac-resistant adenomas in this FAP study was the presence of K-ras colon 12 mutations in only the resistant adenomas. In colon cancer tissue culture studies, K-ras transformation produced resistance to NSAID-induced apoptosis (40), and coexpression of mutant β-catenin and mutant K-ras induced COX-2 expression (33). Several other factors may contribute to the difference between this FAP study and the murine study presented here. Although all of the patients examined had FAP, a significant genetic variability existed among the small number (n = 9) of total patients studied. Tissues from these patients were harvested at different times and subjected to variable fixation and storage times. Finally, due to heterogeneity of expression within tumors, it is difficult to accurately quantify COX-2 expression using immunohistochemistry.

The finding of COX-2 overexpression in both histologically normal and tumor tissues has important implications for agent toxicity as well as treatment resistance. Recently, long-term use of selective COX-2 inhibitors was associated with an increase in serious cardiovascular adverse events, such as myocardial infarction and stroke (41, 42). The risk for these events developed after ~18 to
24 months of drug use. One explanation for this toxicity is that selective inhibition of COX-2 blocks the production of endothelial prostacyclin without affecting the synthesis of platelet thromboxane A2, thereby potentially creating a prothrombogenic state (43).

An alternative hypothesis is that these clinical events occurred as a result of supernormal COX-2 activity produced by chronic NSAID administration. Production of COX-2 by mononuclear cells within arterial plaques may lead to generation of PGE2, which can serve as a substrate for thromboxane A2 synthesis. The resulting thromboxane A2 present in the vicinity of an endothelial defect can promote arterial thrombosis. In support of this, in vitro studies showed that production of COX-2 by endothelial cells restored thromboxane A2 synthesis in aspirin-treated platelets (44). It is therefore possible that COX-2 produced within arterial plaques can promote thrombosis even in the setting of concomitant aspirin use (45).

If the results from this animal model are also observed in humans treated with COX-2 inhibitors, the health implications are highly significant. The phenomenon of tachyphylaxis following chronic NSAID administration deserves detailed study in human clinical trials for treatment of arthritis and for prevention of cardiovascular disease and cancer. It is essential to determine the degree to which NSAID resistance occurs in patients treated with NSAIDs, whether this problem is specific to selective NSAIDs, and whether factors defining which individuals are likely to develop resistance can be identified before or during treatment. Overcoming the problem of drug resistance may require combination therapy or may be as simple as providing drug holidays in chemoprevention regimens to allow recovery from resistance in cell populations with high turnover rates, such as the gastrointestinal mucosa.

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