RAPID COMMUNICATIONS

Elevated Cyclooxygenase-2 Levels in *Min* Mouse Adenomas

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Background & Aims: **Mutations in the *APC* gene result in an increased propensity to develop intestinal neoplasia; however, a complete understanding of the mechanisms resulting in tumor formation has remained elusive. *Min* mice possess a mutation in the *APC* gene and display a neoplastic phenotype similar to that observed in familial adenomatous polyposis coli in humans. Cyclooxygenase (COX) inhibitors decrease tumor multiplicity in the *Min* mouse intestine. The present study was designed to determine if there was an increase in COX-2 in adenomas harvested from *Min* mouse intestine. *Methods:* COX-2 messenger RNA levels were determined by Northern blots and reverse-transcription polymerase chain reactions of B6^{Min} × 129 mouse-derived tumors. Protein levels and localization were determined by Western blots and immunohistochemical staining. *Results:* The Northern blots revealed an approximately threefold increase in the level of COX-2 messenger RNA in *Min* mouse adenoma compared with normal mucosa. COX-2 protein levels in adenomatous tissues were also approximately threefold higher compared with normal mucosa from the same mouse. Immunohistochemical staining with a monospecific COX-2 antibody confirmed that increases in COX-2 immunoreactivity were restricted to dysplastic and neoplastic foci within intestinal mucosa. *Conclusions:* These data show that COX-2 levels may be increased at an early stage in colorectal neoplasia during polyp formation and before invasion.**

Familial adenomatous polyposis (FAP) is an autosomal-dominant disease that has been linked to germline mutations in the *APC* gene.[1,2] Individuals possessing these mutations develop numerous intestinal polyps at an early age. Additionally, *APC* mutations occur in at least 60% of spontaneous human colon cancers and are also found in adenomatous polyps. Currently, the precise mechanisms by which inactivation of the *APC* gene results in tumor formation are unknown; however, additional mutations are necessary for progression to a transformed state.

C57BL/6J mice were treated with ethylnitrosourea and bred for transmission of germline mutations. A line was developed that had multiple intestinal neoplasms (*Min*).[3] These mice exhibited a phenotype similar to FAP in humans. Specifically, they developed numerous intestinal polyps, with a subpopulation exhibiting a neoplastic phenotype that was inherited in a fully penetrant autosomal-dominant fashion. Linkage studies revealed that germline transmission of a mutant allele of the *APC* gene was tightly linked with the multiple intestinal neoplasia phenotype.[4] Subsequently, it was shown that a germline nonsense mutation in the *APC* gene was strongly correlated with this phenotype.[5] Additional studies have revealed that a second inactivating mutation in the normal allele of the *APC* gene results in adenoma formation.[6,7] This is consistent with Knudson's hypothesis, which states that at least two separate events are required for tumor formation.

Two cyclooxygenase (COX) isoforms have been identified, which will be referred to as COX-1 and COX-2 in this report. COX-1 is expressed constitutively in a number of cell types, whereas COX-2 is inducible by a variety of factors including cytokines, growth factors, and tumor promoters.[8,9] COX-2 was identified by many groups as a member of a class of genes referred to as "immediate early" or "early growth response" genes.[10–14] These genes are rapidly and transiently induced after growth factor or phorbol ester stimulation of quiescent cells.[15–17] Members of the immediate early gene family are quite diverse, ranging from nuclear transcription factors to cytokines. The exact role many of these play in regulation of cellular responses to growth stimuli and tumor promoters has not been clearly defined.

Abbreviations used in this paper: bp, base pairs; COX, cyclooxygenase; FAP, familial adenomatous polyposis; *Min,* multiple intestinal neoplasia; PCR, polymerase chain reaction; RT, reverse transcription; SDS, sodium dodecyl sulfate.

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Both isoforms of COX convert arachidonic acid to prostaglandin endoperoxide H_2, which is a substrate for a number of cell- and tissue-specific prostaglandin synthases. Recently COX-2 has been implicated in intestinal neoplasia, because increased levels of COX-2 messenger RNA (mRNA) and protein have been observed in colonic adenomas and carcinoma samples from humans.[18–20] Additionally, patients with FAP receiving sulindac (a COX inhibitor) have a significant reduction in the size and number of adenomatous polyps.[21–24] Furthermore, recent work in our laboratory has revealed that cultured rat intestinal epithelial cells constitutively expressing COX-2 are resistant to sodium butyrate–induced apoptosis and exhibit increased adhesion to extracellular matrix proteins, which are two characteristics likely to affect the tumorigenic potential of intestinal epithelial cells.[25] Finally, a recent report has indicated a reduction in tumor multiplicity in $B6^{Min}$ mice treated with piroxicam, a potent COX inhibitor.[26] These data strongly suggest a possible role for eicosanoids in tumor formation in the *Min* mouse.

The present study was designed to determine whether COX-2 levels were altered in adenoma tissue harvested from $B6^{Min} \times 129$ mice (*Min* mice). We found increased COX-2 mRNA and protein levels in adenomas obtained from *Min* mice.

Materials and Methods

Extraction of Total RNA From Tissue Samples

Intestinal tissue samples were obtained from C57BL6/J-*Min*/+ × 129 +/+ mice after death. Samples were harvested and immediately snap frozen in liquid nitrogen. Tissue samples were divided and weighed, and an appropriate amount of reagent was added before homogenization. Total RNA extraction was performed as previously described.[18]

Northern Blotting

Forty micrograms of total RNA from each sample was electrophoresed in denaturing agarose gels and transferred to nitrocellulose. Nitrocellulose blots were hybridized using standard conditions followed by 0.1% standard saline citrate/0.1% sodium dodecyl sulfate (SDS) posthybridization washes at 42°C.[18] Blots were exposed for various lengths of time before they were developed. Neoplastic intestinal mucosa samples were evaluated and compared with results from adjacent normal mucosa. Blots were stripped and rehybridized with a ^{32}P-labeled 18S ribosomal probe to ensure loading consistency. mRNA levels were quantified by scanning the autoradiographs and analyzing band density via NIH Image 1.59 software (National Institutes of Health, Bethesda, MD).

Reverse-Transcription Multiplex Polymerase Chain Reaction

First-strand complementary DNA (cDNA) was generated using 10 μg of total RNA as template. Random hexamer (3 μmol/L) was used to prime a standard reverse-transcription (RT) reaction. Polymerase chain reaction (PCR) cocktail consisted of 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, gelatin 0.01% (wt/vol), 0.2 mmol/L each deoxynucleoside triphosphate, 2.0 U AmpliTaq Polymerase (Perkin-Elmer Corp., Norwalk, CT), and 0.4 μmol/L each of the following primers: COX-2 5′-GTCTGATGATGTATGCCACAATCTG, 3′-GATGCCAGTGATAGAGGGTGTTAAA, rat β-actin (Clontech #5506-3) 5′-TTGTAACCAACTGGGACGATATGG, and 3′-GATCTTGATCTTCATGGTGCTAGG in a total volume of 50 μL. Two microliters of cDNA template was added and finger-vortexed. β-Actin cDNA (positive control) and double-distilled water (negative control) were analyzed in parallel to ensure the integrity of the reaction cocktail. Thermocycling was performed according to the following profile: 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes, repeated 35 times followed by a final extension at 72°C for 7 minutes. Analysis of amplicons was performed on a 1.8% Tris acetate ethylenediaminetetraacetic acid agarose gel. Eight microliters of each sample was mixed with 2 μL of DNA loading buffer and loaded onto the gel, and electrophoresis was performed at 125 V for 1.5 hours. A 100–base pair (bp) ladder (Promega, Madison, WI) was used as a size standard. The gel was stained with ethidium bromide and then photographed. The amplicons generated by COX-2 and control β-actin primers were 276 and 764 bp in length, respectively.

Western Blotting

The tissues were homogenized at 4°C in radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 1% Nonidet P40, and 50 mmol/L Tris, pH 8.0) containing 10 μg/mL aprotinin, 1 mmol/L sodium orthovanadate, and 100 μg/mL phenylmethylsulfonyl fluoride. Centrifuged homogenates (100 μg) were denatured and fractionated on 7.5% polyacrylamide gels containing SDS and then transferred to nitrocellulose membrane using a semidry cell (Bio-Rad Laboratories, Richmond, CA). Filters were incubated overnight at room temperature in blocking solution (Tris-buffered saline containing 5% nonfat dried milk and 0.05% Tween 20), followed by a 4-hour incubation period with primary rabbit antisera[25] in blocking solution. Filters were washed three times and incubated with a horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin as a secondary antibody (1:2000 dilution) for 1 hour. After three additional washes, filters were treated by the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL) and exposed to X-omat AR film (Kodak, New Haven, CT). Protein levels were quantified by scanning the autoradiographs and analyzing band density via NIH Image 1.59 software.

Histopathology and Immunohistochemistry

Serial sections cut from each paraffin-embedded block were stained with H&E for morphological examination by a pathologist who was unaware of the results of the immunoperoxidase, Northern blot, or Western blot results. Each specimen was examined for the presence of adenomatous polyps, which

were classified as tubular, villous, or tubulovillous.[27] The degree of cytological dysplasia within each adenomatous polyp was further classified as low grade or high grade according to the World Health Organization criteria.[28]

Paraffin-embedded sections (5 μm) were adhered to glass slides, dewaxed, deparaffinized, and rehydrated in xylene, graded alcohol, and phosphate-buffered saline (PBS), respectively. The sections were then incubated in 1% hydrogen peroxide for 15 minutes at room temperature to quench endogenous peroxidase and then digested in 0.1% trypsin for 5 minutes at 37°C. After the sections were blocked in 1.5% normal horse serum for 30 minutes at room temperature, the excess serum was removed and sections were incubated with 1:25 diluted mouse anti–COX-2 monospecific antibody for 18 hours at 4°C. Then, the biotinylated horse anti-mouse immunoglobulin and horseradish peroxidase–conjugated antibiotin antibody were respectively applied to the sections for 30 minutes at RT. The sections were washed in PBS three times between each step. Peroxidase activity was shown by applying 3,3′-diaminobenzidine tetrahydrochloride containing 0.05% hydrogen peroxide for 5–10 minutes at RT. The sections were then thoroughly rinsed in tap water and counterstained with hematoxylin. Finally, the sections were dehydrated, cleared, and mounted with coverslips. The specificity of the immunohistochemical staining with the COX-2 antibody has been established previously. Intestinal epithelial cell lines engineered to express COX-2 or COX-1 have been probed with the COX-2 antibody, and specificity was shown via specific staining of the COX-2 cell line with the absence of a signal in the COX-1 cell line (data not shown).

Results

Northern Hybridization

Forty micrograms of total RNA from *Min* mice neoplastic and normal intestinal mucosa was electrophoresed on a denaturing agarose gel and transferred to a nitrocellulose filter. Loading consistency was determined by direct comparison of 18S ribosomal RNA (present at high levels in eukaryotic cells) visualized by ethidium bromide staining and through a separate hybridization with a ^{32}P-labeled 18S specific probe. The murine COX-2 cDNA was radiolabeled with [^{32}P] deoxycytidine triphosphate and hybridized to the nitrocellulose filter where the RNAs were transferred. We observed an approximately threefold increase in COX-2 RNA levels in the neoplastic mucosal samples compared with adjacent normal tissue (Figure 1*A*).

RT-PCR

Ten micrograms of *Min* mouse total RNA from each sample was used to generate cDNA, which was subsequently used as template in a standard RT-PCR reaction using both COX-2 and β-actin control-specific primer pairs. The predicted size of the PCR-generated amplicon for COX-2 primer amplification was 276 bp. The contamination control (water blank) indicated that no amplification occurred in the absence of bona fide template. Comparison of the COX-2 amplicons with β-actin control amplicons indicated the high quality of the cDNA template. Direct comparison of the COX-2 tumor and normal PCR products showed an increased COX-2 in tumor, corroborating the previous Northern blot observations (see Figure 1*A* and *B*).

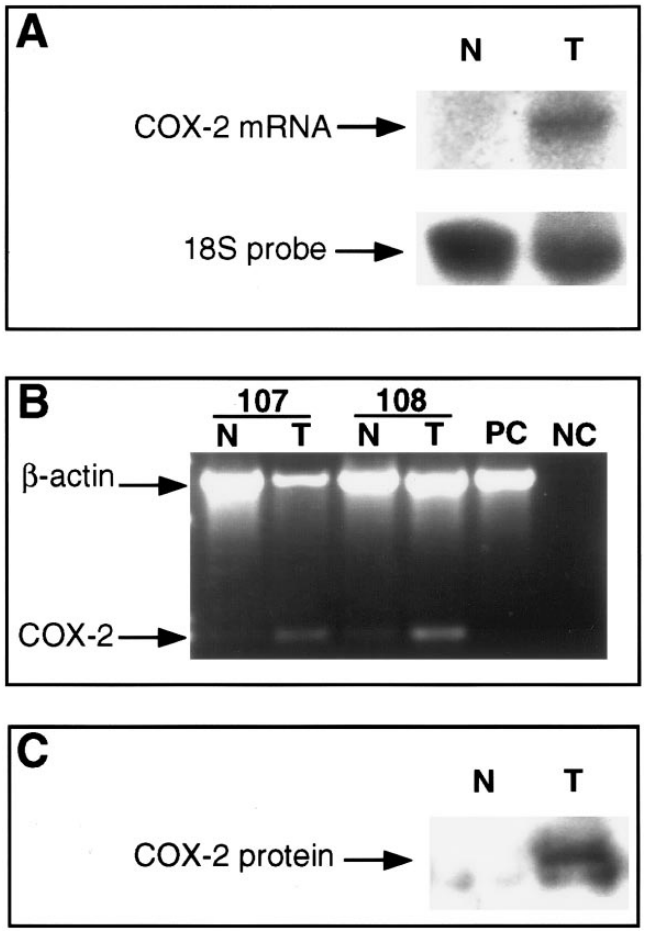

Figure 1. (*A*) Analysis of COX-2 mRNA levels in total RNA extracted from both adenomas and normal mucosa from selected C57BL6/J-*Min*/+ × 129 +/+ mice. Northern blotting was performed using 40 μg of total RNA per sample. The blot was hybridized with a radiolabeled COX-2 cDNA coding region fragment and was then stripped and rehybridized with a radiolabeled 18S ribosomal RNA probe to ensure loading consistency between samples. N, normal mucosa; T, tumor. (*B*) Analysis of COX-2 mRNA levels with RT multiplex PCR. Ten micrograms of total RNA extracted from matched tumor and normal mucosal samples was used as template to generate cDNA. Two microliters of cDNA was used as template in a 50 μL multiplex RT-PCR reaction primed with COX-2 and β-actin control primers. Eight microliters of sample reaction was mixed with 2 μL of DNA loading buffer followed by agarose gel electrophoresis. 107 and 108, animal identification numbers; N, normal mucosa; T, tumor; PC, positive control; β-actin, cDNA control; NC, negative control. (*C*) Immunoblotting of COX-2 protein from both adenomas and normal mucosa from *Min* mice. N, normal mucosa; T, tumor.

Western Blotting

After determining that the COX-2 RNA level was increased, we sought to determine if there was a similar increase in the amount of COX-2 protein in the adenoma. We electrophoresed 100 μg of protein from each sample on a SDS-polyacrylamide gel, transferred the separated proteins onto a nitrocellulose membrane, and probed the filter with monospecific rabbit antisera to COX-2. Immunoblot analysis indicated an approximately threefold increase in the amount of protein present in the adenomatous tissue compared with samples obtained from normal mucosa (Figure 1*C*).

Histopathology

Four adenomatous polyps were identified in specimens from three *Min* mice: two tubular, one villous, and one tubulovillous. Three adenomas contained low-grade dysplasia, and the tubulovillous adenoma contained high-grade dysplasia throughout the lesion. No invasive carcinoma or carcinoma in situ were found in these samples. Sections of adjacent grossly normal intestinal epithelium were histologically unremarkable.

Immunohistochemical Staining

Representative tissue sections from adenomatous lesions and normal epithelium were prepared and probed with a monospecific COX-2 antibody. COX-2 protein staining was not observed in either wild-type mouse intestinal tissue sections (Figure 2*A*) or sections derived from *Min* mice—normal intestinal mucosa (Figure 2*B*). However, in accordance with the previous data, high levels of COX-2 protein were observed in subpopulations of adenomatous and dysplastic intestinal epithelium (Figure 2*C*). Some of the sections of polypoid tumors contained normal (white arrow) and neoplastic cells (black arrow) and therefore afforded internal positive and negative controls (Figure 2*D*). We have also included some representative immunostaining results from a human colorectal adenocarcinoma for comparison. The most striking result from the human studies is that the bulk of COX-2 protein is expressed predominantly in the neoplastic epithelial cells (arrows). This observation confirms a previous report.[20]

Discussion

The precise cause of colorectal cancer is currently under intense investigation. Complex genetic and environmental factors contribute to the predisposition for and subsequent development of neoplastic transformation of colonic epithelial cells. Animal models are extremely useful for investigating components contributing to tumor progression because both genotypic and environmental factors can be rigidly controlled. Important work with mouse models of colorectal cancer has recently shown some of the key genetic components involved in certain types of neoplastic transformation in the human gastrointestinal tract.

The *Min* mice line was originally derived from a founding male C57BL/6J mouse that had been treated with the carcinogen ethylnitrosourea and bred for transmission of germline mutations.[3] On close examination, some of the progeny possessed numerous intestinal polyps, a phenotype similar to FAP in humans. Later it was discovered that these mice shared an additional feature in common with FAP; they also harbored a mutation in the *APC* gene, and thus they are justifiably a model of FAP. However, tumor distribution differs in the *Min* mice because most of the tumors are localized in the small intestine and not in the colon, as in FAP. This murine line presents a unique opportunity to dissect the genetic components responsible for neoplastic progression in tumors occurring as a result of mutations in the *APC* gene.

We report an increased level of both COX-2 mRNA and protein in tumors harvested from intestinal mucosa of a particular strain of *Min* mice ($B6^{Min} \times 129$). COX-2 mRNA or protein in normal mucosal samples were barely detectable; however, COX-2 was present in the tumor samples. Levels of both COX-2 mRNA and protein in the tumor samples were increased approximately threefold over adjacent normal intestinal mucosa. These assays probably underestimated the relative expression of COX-2 in dysplastic cells because processing involved the homogenization of grossly dissected adenomatous tissue, which is likely to contain a variety of epithelial phenotypes ranging from normal to neoplastic.

RT-PCR was performed to confirm the differential expression of COX-2 in tumor samples when compared with normal mucosa. After standardization to a β-actin control, a definitive increase in the level of COX-2 mRNA in the tumor samples was observed. The presence of a faint COX-2 amplicon in the normal mucosal samples is not surprising, because the sensitivity of the RT-PCR reaction and the harvesting technique we used could not distinguish microscopic lesions from normal mucosa. Alternatively, the up-regulation of COX-2 may occur at a very early stage in neoplastic transformation, but such a point would be difficult to distinguish at the gross level. However, the RT-PCR experiment confirms the initial Northern blotting results that indicate an increase in COX-2 mRNA levels in neoplastic tissue.

The immunohistochemical localization patterns from the *Min* mouse intestinal mucosal samples support the Northern and Western blotting data. Within these sec-

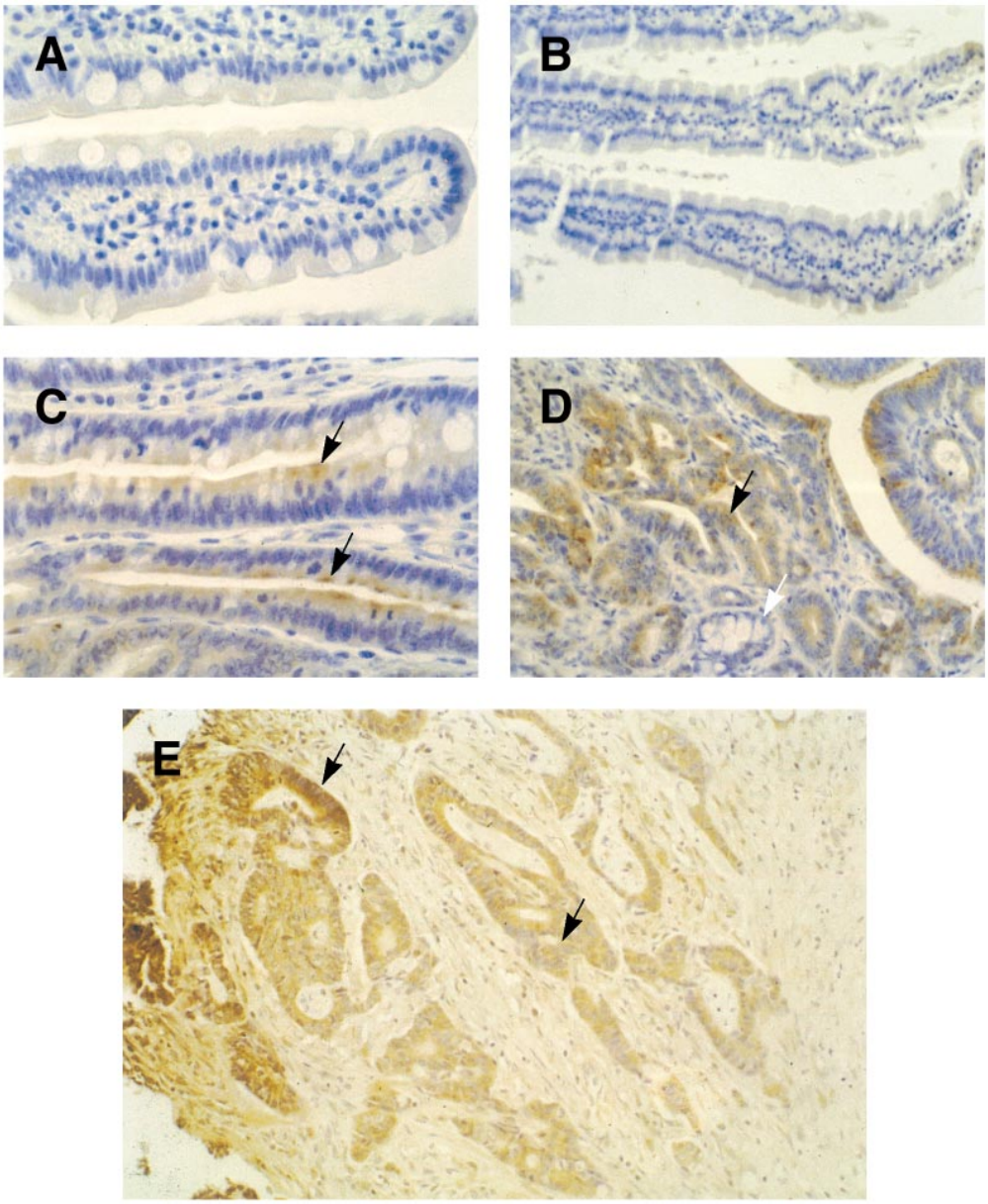

Figure 2. Immunohistochemical staining with a monospecific COX-2 antibody. (*A*) C57BL6/J Apc (+/+) wild-type mouse normal intestinal mucosa sample (original magnification 300×). (*B*) C57BL6/J-*Min*/+ × 129 +/+ normal mucosa sample (original magnification 130×). (*C*) C57BL6/J-*Min*/+ × 129 +/+ neoplastic mucosa sample (original magnification 320×). (*D*) C57BL6/J-*Min*/+ × 129 +/+ mixed normal and neoplastic section (original magnification 200×). (*E*) Human adenocarcinoma sample indicating increased COX-2 immunohistochemical localization in neoplastic regions (original magnification 130×).

tions, multiple cell types such as lymphocytes and connective tissue components were present, which showed some immunoreactivity to COX-2 antiserum. Sections also included normal mucosal cells with no immunoreactivity for COX-2 and dysplastic or neoplastic intestinal epithelial cells, which were predominantly stained positive with COX-2 antibody. Therefore, the low level of expression observed in the Northern and Western blotting data is understandable considering the ratio of dysplastic cells to normal cells observed after immunohistochemical staining.

It has long been known that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit COX enzymatic activity. Several large studies have shown a positive correlation between continuous NSAID use and decreased risk of colorectal cancer in humans. This suggests a possible role for COX-2 in tumor formation, although NSAIDs have other effects in addition to their ability to inhibit COXs. Interestingly, COX-2 up-regulation has been observed in tumor samples harvested from patients with colorectal cancer. Additionally, our laboratory has recently reported increased levels of COX-2 mRNA and

protein in rat colonic tumors that developed after carcinogen treatment.[29] Most interesting, perhaps, is the recent report that showed a decrease in tumor load in $B6^{Min}$ mice treated with piroxicam, which is a COX inhibitor.[26] To the best of our knowledge, the findings of Jacoby et al.[26] represent the first report demonstrating a role for NSAIDs in tumor prevention in the *Min* mouse. Confirmation of these results has been obtained from a study in which *Min* mice were treated with sulindac, which is another potent NSAID. These mice also had a decrease in tumor multiplicity as well as an increase in apoptosis when compared with control *Min* mice.[30] Further research will be necessary to determine mechanisms whereby NSAID treatment results in decreased tumor multiplicity in these mice.

COX-2 protein is undetectable in normal intestinal mucosa; however, at some step in the transformation of normal intestinal epithelial cells to malignant cells an increase in COX-2 expression occurs. The data presented in this report indicate that COX-2 expression is upregulated at an early, preinvasive stage in *Min* mouse adenomas. It is highly likely that additional genetic alterations such as the loss of *p53* or *DCC* mutations are required for progression to a malignant state.

Whereas the precise role that COX-2 plays in tumor formation remains unclear, an enlarging body of evidence suggests that COX-2 expression is altered during the development of colorectal cancer in rodent models and in humans. Our work in cultured epithelial models suggests that the increase in COX-2 may enable the cell to resist apoptosis and enhance the ability of the cell to bind extracellular matrix. This report shows an increase in COX-2 mRNA and a subsequent increase in COX-2 protein in adenomas harvested from *Min* mice. The specificity of COX-2 protein expression in transformed epithelial cells is shown by immunohistochemical staining.

Detailed studies are underway that will more carefully evaluate the temporal pattern of COX-2 expression in *Min* mice and in selected backcrosses that present with lower tumor multiplicity and, consequently, have an increased life span enabling long-term studies. These future studies will include a more detailed, long-term investigation to fully analyze the role of COX-2 in tumorigenesis in the *Min* mouse.

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