Cyclooxygenase-2 Expression and Prostanoid Biogenesis Reflect Clinical Phenotype in Human Colorectal Fibroblast Strains

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ABSTRACT

Up-regulated cyclooxygenase (COX)-2 expression and prostaglandin E$_2$ (PGE$_2$) synthesis contribute causally to the early stages of colorectal neoplasia and carcinogenesis, yet COX-2 expression is barely detectable in normal and premalignant colorectal epithelium. Rather, COX-2 expression in nonmalignant colonic tissue is probably confined to subepithelial cells, such as fibroblasts. We established a panel of 33 primary subepithelial fibroblast strains from human colorectal biopsies of normal colon (group I), normal segments of colons that harbored synchronous advanced neoplasms in remote segments (group II), advanced neoplasms (group III), and segments of active ulcerative colitis (group IV). In group I strains, mean basal and peak PGE$_2$ levels after 24 h of interleukin (IL)-1$\beta$ stimulation were 5.4 ± 1.1 and 32.8 ± 4.9 ng/mg protein, respectively. Mean IL-1$\beta$-stimulated peak levels in groups II, III, and IV strains were, respectively, 6-, 9-, and 7-fold greater than that in group I ($P < 0.001$ for each comparison), and inductions of COX-2 mRNA and protein were consistent with these findings. IL-1$\beta$-mediated stimulation of PGE$_2$ was fully blocked in the presence of a nonselective COX inhibitor (indomethacin) or a selective COX-2 inhibitor (NS-398). IL-1$\beta$ treatment elicited from group I (normal) and group III (cancer-associated) fibroblasts, respectively, −2- and 3-fold inductions of COX-2 transcriptional activity and −1.4- and 1.7-fold inductions of COX-2 promoter activity. This modestly greater COX-2 transcription rate could not alone account for the dramatically higher levels of COX-2 mRNA and protein and PGE$_2$ in cancer-associated compared with normal fibroblasts. However, incubation of fibroblasts with PGE$_2$ after IL-1$\beta$ stimulation prolonged COX-2 mRNA half-life from −1 to 9 h. Our results strengthen the evidence that fibroblasts and other mesenchymal cells are the source of COX-2 expression in normal and premalignant colorectal tissue. Group II fibroblasts from normal segments of colons that harbored synchronous remote advanced neoplasms behaved like group III fibroblasts from advanced neoplasms and not group I fibroblasts from normal colons. We hypothesize that the effects of modestly greater COX-2 transcription in groups II−IV fibroblasts yield corresponding modest increases in PGE$_2$ synthesis whose effects are progressively amplified through robust stabilization of COX-2 mRNA.

INTRODUCTION

Aspirin ingestion can reduce colorectal cancer mortality by 40–50% (1). Aspirin and other NSAIDs$^3$ inhibit the COX enzymes, COX-1 and -2 (2), which generate PGE$_2$ and other prostanoids from arachidonic acid. PGE$_2$ is the major prostanooid product of arachidonic acid metabolism in colorectal tissue (3). Alterations in colorectal prostaglandin synthesis are thought to contribute to the antineoplastic actions of NSAIDs (4).

Whereas COX-1 is constitutively expressed in a wide range of tissues, COX-2 is induced by various cytokines, growth factors, and tumor promoters (5), and elevated levels occur with colorectal neoplasia (6). A causal role for COX-2 in colorectal carcinogenesis was demonstrated in vivo in a murine gene disruption model (7). The effects of COX-2 up-regulation were mediated predominantly through increased PGE$_2$ production, and the site of COX-2 up-regulation in the earliest microadenomas was in stromal cells of the subepithelial mesenchymal layer. Studies in Min (8) and IL-10$^{-/-}$ knockout mice (9) have provided further evidence of COX-2 expression by stromal cells. Less is known of the source of COX-2 expression in nonneoplastic human colorectal tissue.

Fibroblasts are the dominant cell type in connective tissue and fulfill many functions. They express receptors for cytokines, growth factors, and hormones (10), and a population of specialized subepithelial, intestinal “myofibroblasts” has been described with pleiotropic capabilities (11). We reported that IL-1$\beta$ or tumor necrosis factor $\alpha$ could induce synthesis of PGE$_2$ by as much as 25-fold in a human neonatal colonic fibroblast strain (CCD-18Co) and four human adult primary colorectal fibroblast strains that we initiated from colonscopically pinch biopsies of normal mucosa (12).

DCA, a secondary bile acid and the predominant fecal bile acid, has been implicated in the etiology of colorectal cancer (13). We reported recently that DCA but not cholic acid, a primary bile acid, induced COX-2 expression and PGE$_2$ synthesis in colorectal fibroblast strains initiated from colorectal biopsies (14). Maximal levels of PGE$_2$ synthesis were 5-fold greater in adenocarcinoma-associated strains than in normal fibroblast strains.

In the present study, we examined further the hypothesis that up-regulation of fibroblast COX-2 expression plays an early, causal role in the development of intestinal neoplastic disorders. We investigated basal and inducible PGE$_2$ synthesis and COX expression in a panel of 33 human colorectal fibroblast strains that we initiated from normal, inflamed, and neoplastic bowel. Levels of induction were significantly higher in strains initiated from neoplastic and inflamed tissue or from uninflamed, histologically normal segments of large intestine that harbored a remote, synchronous advanced neoplasms than in strains from a normal large bowel. Differences in prostanoid biogenesis among the different colorectal fibroblast phenotypes reflected levels of COX-2 transcriptional activity and possibly mRNA turnover in these phenotypes.

MATERIALS AND METHODS

Materials. IL-1$\beta$, Klenow enzyme, and random primers were purchased from Boehringer Mannheim (Indianapolis, IN). Indomethacin and NS-398 were purchased from Sigma (St. Louis, MO). Cesium chloride and guanidinium isothiocyanate were purchased from Bethesda Research Laboratories (Bethesda, MD). DMEM and MCDB 153 medium were purchased from Life Technologies, Inc. (Grand Island, NY) and Sigma, respectively. FBS, nonessential amino acids, sodium pyruvate, penicillin, streptomycin, epidermal growth factor, and insulin-transferrin-selenium-X were purchased from Life Technologies, Inc. Kits for measurement of PGE$_2$ levels by radioimmunoassay were purchased from Amersham (Arlington Heights, IL). Goat antihuman COX-1 antibody and rabbit antihuman COX-2 antibody were from Cayman (Ann Arbor, MI). Horseradish peroxidase-conjugated rabbit antigoat IgG and goat antirabbit IgG were obtained from Sigma.

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3 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PGE$_2$, prostaglandin E$_2$; IL, interleukin; DCA, deoxycholic acid; FBS, fetal bovine serum.
Fibroblast Cultures. CCD-18Co, a fibroblast strain derived from normal human fetal colon, was purchased from the American Type Culture Collection (Manassas, VA). CCD-18Co cells were cultured in DMEM supplemented with 10% FBS, 0.1 mm nonessential amino acids, and 1 mm sodium pyruvate. Other fibroblast cultures were initiated from biopsies obtained, with approval of the Institutional Review Board of the Buffalo Veterans Administration Medical Center, at routine colonoscopies. Colorectal mucosal/lesions in the following categories were targeted for biopsy: normal mucosa in patients whose total colonoscopy was normal (group I) or revealed a synchronous advanced neoplasm (adenoma ≥ 1 cm diameter and/or high-grade dysplasia/villous histology, or adenocarcinoma) in a remote segment of that large intestine (group II); advanced neoplasms (group III); and active ulcerative colitis (group IV).

Fibroblast strains were initiated from colonoscopic biopsies as described previously (12, 14). Immunohistochemical stains were performed on representative cells from each putative strain to confirm consistency with a fibroblast phenotype (positive vimentin stain and negative factor VIII, antichymotrypsin, α-antitrypsin, and S-100 stains). Studies were performed on fibroblast strains from the third to the sixth passage in culture.

**PGE\(_2\) Assay.** Fibroblasts were seeded in 24-well plastic culture plates at a density of 5 × 10\(^3\) cells/cm\(^2\) and cultured for 1 week in DMEM supplemented with 10% FBS. Medium was replaced with DMEM supplemented with 1% FBS 24 h before administering cytokine or other treatments to fibroblast cultures. We reported that maximal inductions of PGE\(_2\) synthesis were achieved by treatment of fibroblasts with IL-1β (10 ng/ml) for 24 h (12). This treatment was applied to selected cultures in the current study. Indomethacin or NS-398 at final concentrations of 10 μM (15, 16) was added to selected cultures.

For 30 min before harvesting, medium was replaced with PBS supplemented with 1% gelatin. PGE\(_2\) levels in harvested PBS-gelatin were determined using radioimmunassay kits as instructed. Protein content was determined by the method of Bradford (17), and all cultures were performed in triplicate.

**Isolation of Fibroblast RNA and Northern Analysis.** Isolation of total cellular RNA, gel electrophoresis, Northern blotting, hybridization with cDNA probes, and quantitation of mRNA levels were performed as described previously (12, 18). Fibroblasts were cultivated to confluence in 100-mm plastic culture plates, treated with test compounds, and harvested for isolation of total RNA. Near full-length cDNAs for human COX-1 (19) and -2 (20) were used for hybridizations, the densities of the DNA-RNA hybrids were determined by scanning, and results were normalized by hybridization with β-actin cDNA (Oncor, Gaithersburg, MD).

**Western Analysis of COX-1 and -2 Protein Expression.** Relative levels of the COX proteins were determined as described previously (12, 14). Briefly, confluent cultured fibroblasts were shifted from medium supplemented with 10% FBS to medium containing 1% FBS for 48 h, including the period of treatment with test compounds, before harvesting. Lysates were electrophoresed on polyacrylamide gels in the presence of SDS and electroblotted to an Immobilon-P membrane (Millipore Corp., Bedford, MA). The membrane was incubated sequentially in PBS supplemented with 10% nonfat milk and 0.1% Tween (pH 7.4), with either goat antihuman COX-1 antibody or rabbit anti-human COX-2 antibody diluted 1:1000 in PBS, and, finally, with horseradish peroxidase-conjugated rabbit antigoat IgG or goat antirabbit IgG for detection of COX-1 or -2 protein, respectively. Bound antibodies were detected by chemiluminescence using reagents purchased from KPL (Gaithersburg, MD) and analyzed densitometrically.

**Nuclear Transcriptional Assay.** Nuclei were isolated, and the run-on protocol was carried out as described previously (18, 21). The entire nuclear preparation was used to carry out the run-on protocol. Equal amounts of nascent radiolabeled RNA transcripts (5 × 10\(^3\) cpm/ml) were hybridized for 3 days to 2 μg of nitrocellulose-bound cDNA.

**Transfection and Luciferase Assays.** Normal (group I) or colorectal adenocarcinoma-associated (group III) fibroblasts were seeded at a density of 5 × 10\(^3\) cells/cm\(^2\) in 24-well plates and cultured for 1 week in DMEM supplemented with 10% FBS. Medium supplemented with 1% FBS was substituted 24 h before transfection of cultured fibroblasts. The COX-2 promoter (−1796 to +104)-luciferase plasmid construct that was used to determine the promoter activity of the gene was provided by Dr. Manuel Fresno (Madrid, Spain; Ref. 22). For each culture well, 100 μg of experimental plasmid were mixed with 2 ng of pRL-null plasmid, serving as internal control, in a volume of 450 μl. A DNA transfection mixture of 50 μl of 1 M CaCl\(_2\) and 500 μl of 2× HEPES-buffered saline was incubated at room temperature for 7 min and added to each well. Plates were incubated for 7 h at 37°C in a humidified atmosphere containing 5% CO\(_2\) and shocked by the addition of 10% glycerol for 3 min. After incubation for an additional 24 h, IL-1β (10 ng/ml) was added to selected cultures for 24 h. Cells were washed twice with PBS. Lysates were prepared by incubation in 100-μl volumes of lysis buffer (Promega, Madison, WI) under the conditions specified by the manufacturer. Luciferase assays were performed by using the Promega Dual-Luciferase Reporter Assay System according to the manufacturer’s instructions. Chemiluminescence was measured using a Zylus FB 12 luminesimeter (Promega). Firefly luciferase activities were normalized to internal control Renilla luciferase activities.

**Statistical Analyses.** Mean PGE\(_2\) levels and relative mRNA and protein levels were compared for significance of differences between groups by two-tailed t test.

**RESULTS**

**IL-1β-stimulated PGE\(_2\) Synthesis in Colorectal Fibroblast Strains.** We previously demonstrated maximal increases of PGE\(_2\) synthesis after treatment with IL-1β at a concentration of 10 ng/ml for 24 h in CCD-18Co fibroblasts and four primary fibroblast strains that we initiated from biopsies of normal colorectal mucosa (12). In the present study, experiments were conducted in CCD-18Co fibroblasts or a new panel of 33 fibroblast strains in four categories: (a) group I (8 strains), normal mucosa in 8 patients with a normal colonoscopy; (b) group II (15 strains), uninfamed, normal mucosa in 15 patients, who all harbored elsewhere a synchronous, advanced, colorectal adenoma or adenocarcinoma; (c) group III (5 strains), biopsies in 5 patients directly from an advanced adenoma or carcinoma; and (d) group IV (5 strains), active ulcerative colitis.

Mean basal and IL-1β-stimulated (10 ng/ml for 24 h) PGE\(_2\) levels in CCD-18Co and group I–IV fibroblasts are shown in Fig. 1. Respective levels in CCD-18Co and Groups I–IV fibroblasts increased 25-fold (CCD-18Co), 6-fold (group I), 10-fold (group II), 5-fold (group III), and 6-fold (group IV). Mean basal and peak PGE\(_2\) levels of groups II (basal level, 19.6 ng/mg protein; peak level, 190.9 ng/mg protein), III (basal level, 52.7 ng/mg protein; peak level, 284.3 ng/mg protein), and IV (basal level, 39.8 ng/mg protein; peak level, 245.2 mg/mg protein) fibroblasts were significantly greater than the mean basal (5.4 mg/mg protein) and peak (32.8 mg/mg protein) PGE\(_2\).
levels of group I fibroblasts, which were similar to those of CCD-18Co fibroblasts (basal level, 1.4 ng/mg protein; peak level, 34.6 ng/mg protein).

Indomethacin is a nonselective NSAID that inhibits COX-1 and -2, and NS-398 is a selective COX-2 inhibitor (16). As already described, basal PGE$_2$ levels in group II–IV fibroblasts were significantly greater than those in group I fibroblasts. Treatment with either indomethacin or NS-398 (each at 10 $\mu$g for 24 h) reduced the basal PGE$_2$ levels of group II–IV cultures to less than those of group I fibroblasts; the magnitude of these reductions was $\approx 83\%$ with either NSAID in each of groups II–IV. Both NSAIDs caused nearly complete attenuation of the PGE$_2$ response to IL-1$\beta$ in all fibroblast groups when added simultaneously with the cytokine (data not shown).

**COX mRNA Levels in Primary Colorectal Fibroblasts.** Northern blots were performed to characterize basal COX mRNA levels and their up-regulation after IL-1$\beta$ stimulation. Primary fibroblast strains were initiated, and total RNA samples from unstimulated cultures or cultures treated with IL-1$\beta$ (10 ng/ml) for 24 h were prepared in separate experiments over a period of several months. The following strategy was adopted so that data derived from experiments performed at different times could be combined and analyzed as one set.

Fresh unstimulated CCD-18Co cultures were included with each batch of primary fibroblasts cultures that was processed. Total RNA from a concurrent CCD-18Co culture was electrophoresed on the same gel and blotted on the same filter as material from that batch of primary fibroblast strains. Densitometric measurements were made of bands on the same autoradiograph representing COX-2 mRNA expression in unstimulated and IL-1$\beta$-stimulated primary fibroblast strains and unstimulated CCD-18Co fibroblasts. Thus, values for COX-2 mRNA levels in a given primary fibroblast strain (groups I–IV) were calculated as a ratio to values for concurrent unstimulated CCD-18Co cultures and recorded as relative light units. Levels in Group I fibroblasts before and after stimulation were similar to those for CCD-18Co fibroblasts (data not shown). Poststimulation (peak) COX-2 mRNA levels of groups II, III, and IV strains, respectively, were 21-, 56-, and 21-fold greater than the combined poststimulation level for group I ($P < 0.05$, 0.01, and 0.05, respectively). COX-1 mRNA levels were unaltered by exposure to IL-1$\beta$ in all groups (data not shown).

**COX Protein Levels in Primary Colorectal Fibroblasts.** Western analysis was performed to determine whether variations in stimulated PGE$_2$ level according to fibroblast group (I–IV) reflected similar variations of COX-2 protein level in IL-1$\beta$-stimulated fibroblasts. COX-2 protein levels were normalized, in similar fashion to the representation of COX-2 mRNA levels, as ratios of corresponding levels in concurrent unstimulated CCD-18Co cultures. Levels in Group I fibroblasts before and after stimulation were similar to those of CCD-18Co fibroblasts (data not shown). Relative poststimulation (peak) COX-2 protein levels of groups II, III, and IV fibroblast strains, respectively, were 18-, 22-, and 25-fold greater than the poststimulation level for group I ($P < 0.001$, 0.001, and 0.01, respectively). COX-1 protein levels, which were also assessed by Western analysis, were unaltered by exposure to IL-1$\beta$ in all groups.

**Colonic Fibroblast COX-2 Gene Transcription and Promoter Activity.** Possible mechanisms for IL-1$\beta$-mediated increases in COX-2 mRNA and protein expression include increased COX-2 transcription and reduced rates of mRNA and protein decay. The influence of IL-1$\beta$ on transcription rates of the COX-2 and $\beta$-actin genes was assessed by the nuclear run-on reaction (Fig. 2A). Densitometric analysis indicated that $\beta$-actin transcript levels were unaltered by IL-1$\beta$ treatment in concurrent experiments. IL-1$\beta$ treatment of normal and cancer-associated fibroblasts increased relative COX-2 transcript levels, normalized to corresponding $\beta$-actin levels, by approximately 2- and 3-fold, respectively (Fig. 2B).

We next sought to determine whether differential levels of COX-2 promoter activity could explain increased transcription of the COX-2 gene in cancer-associated colonic fibroblasts. Luciferase activity in fibroblasts transiently transfected with a COX-2 promoter-luciferase plasmid construct is shown in Fig. 3. IL-1$\beta$ treatment of transfected normal and cancer-associated fibroblasts increased luciferase activity approximately 1.4- and 1.7-fold, respectively.

**Stability of COX-2 mRNA in Colonic Fibroblasts.** The amplitudes of enhanced promoter activity and transcription of the COX-2 gene that were elicited by IL-1$\beta$ treatment of colonic fibroblasts were proportionally of much lesser magnitude than inductions of COX-2 mRNA and protein and PGE$_2$ synthesis. To explain this discrepancy, we explored the possibility that, in addition to increasing transcription, IL-1$\beta$ might reduce COX-2 mRNA turnover. To estimate the potential influence of IL-1$\beta$ on the rate of COX-2 mRNA decay, actinomycin D (1 $\mu$g/ml) was added to selected fibroblast cultures after a 24-h exposure to IL-1$\beta$. Four strains each of normal (group I) and cancer-associated (group III) fibroblasts were examined (data not shown). The level of COX-2 mRNA declined rapidly within 1 h of substituting IL-1$\beta$-free medium. The rates of decline were similar in normal and cancer-associated fibroblasts and appeared unimpeded by the presence of actinomycin D. From these findings, there is no evidence that IL-1$\beta$ alone influences COX-2 mRNA decay in either normal or cancer-associated fibroblasts.

Overexpression of COX-2 through inappropriate expression of a factor that stabilizes mRNA was reported in colon cancer cells (23). In human synovial fibroblasts, PGE$_2$-dependent stabilization of COX-2 mRNA made a major contribution to the magnitude and
duration of IL-1β-mediated induction of COX-2 mRNA and protein and PGE2 release (24). We assessed the influence of exogenous PGE2 on COX-2 mRNA stability in IL-1β-stimulated colonic fibroblasts. Four strains each of normal (group I) and cancer-associated (group III) fibroblasts were examined, and results in a representative strain from each group are shown in Fig. 4. Addition of PGE2 (100 nM) to fibroblast cultures that had been exposed to IL-1β for 24 h and then shifted to IL-1β-free medium profoundly delayed COX-2 mRNA decay. In the absence of PGE2, COX-2 mRNA was no longer detectable in cultures of normal or cancer-associated fibroblasts 12 h after substitution of IL-1β-free medium. In the presence of PGE2, COX-2 mRNA remained readily detectable 24 h after cultures were shifted to IL-1β-free medium.

The half-life of COX-2 mRNA in fibroblasts cultured in the absence or presence of exogenous PGE2 after stimulation with IL-1β was ~1 or 9 h, respectively (Fig. 5, A and B). There were no significant differences between the half-lives of COX-2 mRNA in normal and cancer-associated fibroblasts under the conditions tested.

**DISCUSSION**

Recent reports have emphasized the subepithelial/stromal cell origin of COX-2 expression in spontaneous colorectal neoplasms from APC+/- (8) and Min mice (9), carcinogen-induced colorectal carcinomas from IL-10+/- mice (10), and human sporadic colorectal adenomas (25). However, the ability of stromal cells from human nonneoplastic, uninflamed or inflamed colorectal tissue to express COX-2 has received less attention.

We recently identified DCA as a potent inducer of COX-2 expression and PGE2 synthesis in human colorectal fibroblasts (14). In the present study, we compared basal and cytokine-stimulated (peak) prostanooid biogenesis in a previously untested panel of 33 primary fibroblast strains initiated from pinch biopsies of normal and disease-associated colon. Profiles of basal and peak PGE2 production in the CCD-18Co human fetal colonic fibroblast strain and eight human adult normal colorectal fibroblast strains (group I) were similar.

The 7–10-fold and 7–9-fold higher levels, respectively, that we found in mean basal and peak PGE2 synthesis of groups III (advanced adenoma or carcinoma) and IV (active ulcerative colitis) fibroblasts compared with group I strains are compatible with the reported subepithelial immunohistological localization of up-regulated COX-2 expression in murine and human colorectal adenomas (7, 25). More provocative are the ~4- and 6-fold higher mean levels, respectively, of basal and peak PGE2 production in group II (15 strains initiated from normal, uninflamed mucosa in colons harboring remote synchronous advanced adenomas or carcinomas) compared with group I fibroblasts. On this evidence, group II fibroblast strains resemble those of groups III and IV rather than group I with respect to prostanooid biogenesis. Respective levels of COX-2 protein and mRNA reinforce the alignment of group II strains with groups III and IV strains rather than group I strains; peak levels of COX-2 protein
and mRNA in group II strains were ≥18-fold greater than those in group I strains.

We examined the relative contributions of COX-1 and -2 gene expression to increased PGE\(_2\) synthesis in disease-associated fibroblasts. Cytokine-stimulated PGE\(_2\) synthesis was completely blocked by a selective COX-2 inhibitor, NS-398, in all fibroblast groups. Based on the equivalent, near-total abrogation of elevated basal PGE\(_2\) levels in group II–IV fibroblasts by a nonselective and a selective COX-2 inhibitor alike, up-regulated COX-2 gene expression was also largely responsible for enhanced prostanoid biogenesis by disease-associated fibroblasts under basal conditions.

Exposure of fibroblasts to IL-1β caused relatively modest (1.4–3-fold) and mutually consistent inductions of COX-2 gene transcription and promoter activity that were significantly greater in cancer-associated fibroblasts than in normal fibroblasts. But much greater inductions would be required if increased transcription alone were to account for the massive increases in COX-2 expression and PGE\(_2\) synthesis elicited by this agent. The profound, 9-fold prolongation of COX-2 mRNA half-life that was caused by exogenous PGE\(_2\) suggests a plausible positive feedback mechanism that might explain the exaggerated prostaglandin biogenesis of disease-associated (groups II–IV) fibroblasts compared to normal (group I) colonic fibroblasts.

We hypothesize that increased COX-2 transcription in disease-associated fibroblasts on its own yields only a modest increase in PGE\(_2\) production compared with production in normal fibroblasts but that the small differences in levels of PGE\(_2\) synthesis accruing from varying transcriptional activity are progressively multiplied through the differential COX-2 mRNA stabilizing effects of PGE\(_2\) according to fibroblast phenotype.

Our findings suggest a crucial role for fibroblasts in intestinal homeostasis. We propose that subepithelial fibroblasts may be the targets for anti-inflammatory and chemopreventive strategies.

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