Cell Proliferation and Apoptotic Indices Predict Adenoma Regression in a Placebo-Controlled Trial of Celecoxib in Familial Adenomatous Polyposis Patients

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Abstract

Background: Celecoxib was shown to regress colorectal adenomas in familial adenomatous polyposis (FAP) patients relative to placebo. To address the mechanism of polyp regression, we determined whether celecoxib can modulate cell proliferation, apoptosis, and prostaglandin E2 (PGE2) levels in colorectal epithelia from FAP trial participants and whether such alterations correlate with observed reductions in polyp number. Materials and Methods: Colorectal mucosal biopsies were obtained at baseline and on last day of celecoxib (100 or 400 mg twice daily) or placebo administration (6 months). Residual paraffin-embedded adenomas and normal mucosa from the same patients (n = 17) or normal tissue alone (n = 15) were analyzed. Immunoperoxidase staining for Ki-67 was performed and apoptotic cells were identified by their morphology. Ki-67 and apoptotic labeling indices and their ratios were calculated in superficial (s) and nonsuperficial (ns) regions of adenomas and normal mucosa, and baseline to 6-month differences were calculated. PGE2 levels were analyzed by mass spectroscopy (normal, n = 64; adenoma, n = 56). Biomarkers were analyzed by treatment arm and correlated with previously determined mean percentage reductions in colorectal polyp number. Results: In adenomas, a reduction in the superficial proliferative activity i.e., Ki-67s labeling index, accompanied polyp regression (r = 0.75, P = 0.006). An increase in the apoptotic ratio [i.e., superficial apoptotic index (AI)/nonsuperficial apoptotic index (AIs)] was found to correlate with reduced polyp counts in that higher apoptotic ratios correlated with better response to celecoxib (r = 0.71, P = 0.004). Furthermore, the AI/Ki-67s ratio (r = 0.58, P = 0.026) accompanied polyp regression. Changes in biomarker levels (baseline to 6 months) were correlated in adenomas and normal mucosa (AI, r = 0.33, P = 0.053) and polyp regression was found. PGE2 levels did not significantly correlate with polyp regression. Changes in biomarker levels (baseline to 6 months) were correlated in adenomas and normal mucosa (AI, r = 0.34, P = 0.009; PGE2, r = 0.50, P = 0.059) within individual patients. Conclusion: Suppression of cell proliferation and an increased apoptotic ratio, as well as the ratio of apoptosis to cell proliferation, accompany polyp regression in a chemoprevention trial in FAP patients. These findings suggest potential mechanisms for the efficacy of celecoxib and warrant further study of these biomarkers as intermediate endpoints in FAP patients. (Cancer Epidemiol Biomarkers Prev 2004;13(6):920–7)

Introduction

Epidemiologic studies have consistently shown an inverse association between long-term intake of nonsteroidal anti-inflammatory drugs (NSAIDs), especially aspirin, and incidence of colorectal adenomas and carcinomas relative to nonusers (reviewed in ref. 1). NSAIDs, including selective cyclooxygenase (COX)-2 inhibitors, are potent inhibitors of intestinal tumor incidence and multiplicity in carcinogen-induced and genetically manipulated animal models of colon cancer (reviewed in ref. 2). In patients with familial adenomatous polyposis (FAP), the NSAID sulindac has been shown to regress rectal polyps in uncontrolled studies (3-5) as well as in placebo-controlled trials (6, 7). FAP is an autosomal dominant disorder that is due to a germ-line mutation in the adenomatous polyposis (APC) gene on human chromosome 5 (8). The FAP phenotype consists of at least hundreds of colorectal adenomas and the eventual development of colorectal cancer (3-7). Recently, the selective COX-2 inhibitor celecoxib given continuously to FAP patients for 6 months was found to significantly regress the number of colorectal adenomas compared with placebo (9). Specifically, high-dose (400 mg twice daily) but not lower-dose (100 mg twice daily) celecoxib produced significant polyp regression in comparison with placebo. In an effort to gain insights into the mechanism of celecoxib-induced polyp...
COX enzymes regulate prostaglandin (PG) synthesis from arachidonic acid and are the best defined molecular targets of NSAIDs (2). Two COX isozymes have been identified and include constitutive COX-1 and inducible COX-2. COX-2 is induced by cytokines, growth factors, and tumor promoters (10) and its expression is up-regulated at sites of inflammation and in certain neoplastic epithelia, including colorectal adenomas and carcinomas (11-13). The best evidence for the role of COX-2 in intestinal tumorigenesis derives from mice with a mutation in the APC gene (APC716), which, when mated to COX-2 knockout mice, resulted in double-knockout progeny with a dramatic reduction in intestinal polyp burden (14). While the exact mechanism(s) by which COX-2 contributes to intestinal tumorigenesis remains unknown, studies indicate that COX-2 may inhibit apoptosis, promote angiogenesis, and increase tumor cell invasiveness (2). Selective COX-2 inhibitors (i.e., coxibs) were developed which exert an anti-inflammatory effect but are associated with significantly less gastrointestinal mucosal injury compared with nonselective NSAIDs (15, 16).

In animal models of colon cancer, the coxibs appear at least as efficacious as traditional NSAIDs in suppressing intestinal tumor development (3). While PG inhibition is the best studied antitumor mechanism of NSAIDs, COX-independent mechanisms also exist and experimental studies have consistently shown their importance (2).

Epithelial homeostasis is maintained by a balance between cell proliferation and apoptosis (17). A disruption in cellular kinetics in intestinal epithelia may cause tumors (18). In normal colorectal mucosa, proliferating cells are in the lower one-third of crypts, and apoptotic cells are detected near or at the luminal surface (17). In normal-appearing colonic epithelium from FAP patients, a significant shift in the proliferative compartment toward the luminal surface is seen, as is a reduction in apoptosis at the cell surface (19, 20). These changes appear to accompany adenoma development. Furthermore, Bedi et al. (20) reported a progressive decrease in apoptotic rates during colorectal tumorigenesis in FAP. We (21) and Moss et al. (22) reported that adenomas demonstrate an inverted apoptotic gradient relative to normal colorectal mucosa. Taken together, these findings suggest that suppression of apoptosis and expansion of the proliferative compartment are key events during colorectal neoplastic development and progression.

We sought to test the hypothesis that celecoxib induces polyp regression in FAP by modulating in vitro rates of cell proliferation and apoptosis. In support of this hypothesis are abundant data demonstrating that NSAIDs and coxibs inhibit cell proliferation and induce apoptosis in cultured colon cancer cell lines (23-26). However, the doses of NSAIDs found to exert these effects in vitro have generally been in excess of doses that can be achieved in vivo, thereby casting suspicion as to the clinical relevance of these findings. Yet, in intestinal tissues from animal models of colon cancer treated with NSAIDs, modulation of rates of apoptosis and cell proliferation were found and were associated with tumor inhibition and/or regression (27-29).

We analyzed and compared mucosal biomarkers including apoptotic and proliferative indices and PGE2 levels in biopsies of normal-appearing mucosa and adenomas obtained at baseline and at 6 months in FAP patients treated in a randomized, placebo-controlled trial of celecoxib (high and low dose) versus placebo (9). Correlations were sought between biomarker levels and treatment arm as well as the primary study endpoint (i.e., percentage change in colorectal polyp number at 6 months relative to baseline).

Materials and Methods

Patient Population and Tissue Resource. FAP patients were enrolled in a randomized, double-blinded, placebo-controlled trial to determine the effect of two doses of celecoxib on colorectal polyps as reported previously (9). Eligible patients had a genotype and phenotype consistent with FAP, were 18 to 65 years of age, and had five or more polyps 2 mm or greater in diameter that could be assessed endoscopically. Patients with intact colons or ileorectal anastomoses were eligible. Patients (n = 77) received either of two celecoxib doses (100 or 400 mg twice daily) or placebo given continuously for 6 months. The primary study endpoint was the percentage reduction in the number of colorectal polyps at 6 months compared with the baseline colonoscopic examinations. Informed consent was obtained from all patients, and the protocol was approved by the Institutional Review Board of the University of Texas M.D. Anderson Cancer Center. Pretreatment and posttreatment biopsies were obtained at baseline and on the last day of drug administration. A rigorous biopsy protocol was used to obtain tissue specimens for biomarker studies, thus ensuring standardized collection. For collection of normal-appearing mucosa, the endoscopist biopsied areas where no polypoid tissue or mucosal elevations were identified at endoscopy. Specimens of normal-appearing mucosa (n = 64) and adenomatous polyps (n = 56) were snap-frozen for PGE2 analysis. Residual, paired paraffin-embedded sections of normal colorectal mucosa (n = 15) and adenomas (n = 17) were available for analysis. These specimens had been fixed in 10% neutral-buffered formalin and embedded in paraffin, and four to six micron sections were subsequently cut for immunohistochemistry. A section from each tissue specimen was stained with H&E for analysis of histology.

Cell Proliferation and Apoptosis. Endoscopic biopsy specimens were examined for cell proliferation by measuring Ki-67 protein expression. Immunoperoxidase staining was performed using an anti-Ki-67 polyclonal antibody (DAKO Corp., Carpenteria, CA), as described below. Apoptotic cells and bodies were identified in H&E-stained tissue sections using established morphologic criteria that were uniformly applied to all specimens examined (17, 18, 21, 30). The analysis of apoptotic and proliferative indices was restricted to epithelial cells. Calculation of labeling indices (LIs) is described below.

Immunohistochemistry for Ki-67. Slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% H2O2 in methanol for 20 minutes at room temperature. Sections were microwaved in PBS...
cells examined in five high-power fields (400×) determined independently and calculated by counting
the number of apoptotic or Ki-67-positive nuclei per 500
also determined in both superficial and nonsuperficial
regions of adenomas and normal mucosa. Superficial
regions included intact and nondetached normal or neo-
plastic cells at or near the lumenal surface. Nonsuperficial
regions included all other regions, except for basal colonic
crypts identified by the presence of the mucularis
mucosae in normal tissue sections. Apoptotic cells and
crypts identified by the presence of the mucularis
colon carcinoma sections by morphol-
phases in the negative ion chemical ionization mass spectrum
were assayed with each batch as quality control
standard is m/z 528. Endogenous PGE2 levels are cal-
culated from the ratio of intensities of the ions m/z 524
and 30 minutes at 37°C. Reagents were dried under nitrogen,
and the residue was reconstituted in 30 μL chloroform
water. Slides were counterstained with hematoxylin
and mounted. A human colorectal cancer known to
intensely stain for Ki-67 was included with all slide
runs (positive control). A negative control was included
with each slide run and omitted the primary antibody
but included all other procedural steps.
Labeling Indices. Lls were determined in adenomas
and normal mucosal biopsy specimens at light micros-
copy. Apoptotic or proliferative (Ki-67 staining) Lls were
determined independently and calculated by counting
the number of apoptotic or Ki-67-positive nuclei per 500
cells examined in five high-power fields (400×) per slide,
with the result expressed as a percentage (21, 31). Lls were determined in both superficial and nonsuperficial
regions of adenomas and normal mucosa. Superficial
regions included intact and nondetached normal or neo-
plastic cells in the top two epithelial cell layers including
cells at or near the luminal surface. Nonsuperficial re-
gions included all other regions, except for basal colonic
crypts identified by the presence of the mucularis
mucosae in normal tissue sections. Apoptotic cells and
bodies detected in colon carcinoma sections by morphol-
ogy have been shown to contain DNA strand breaks
using terminal deoxynucleotidyl transferase-mediated nick end labeling assay (21). Quantification of Lls was
performed by a single observer who has considerable experience in quantifying apoptosis in histologic sec-
tions and who was blinded to all clinical data. Furth-
enermore, biomarker ratios of superficial proliferative activity
(Ki-67s)/nonsuperficial proliferative activity (Ki-67ns),
superficial apoptotic index (AI)/nonsuperficial apoptotic
index (Alns), and AI/Ki-67s and AI ns/Ki-67 ns were
also computed. Biomarker levels were determined at
both baseline and 6-month time points, and the absolute
change between these time points was calculated.
PGE2 Content in Mucosal Biopsies. PGE2 levels were
quantified in two snap-frozen biopsy specimens at
baseline and 6-month examinations and were stored at
−70°C as determined previously (32). To extract PGE2,
rozen samples were submerged in a pestle containing
liquid nitrogen and pulverized with a mortar. After the
niter evaporated, the sample was immediately mixed
with 0.5 mL methanol containing 10 μmol/L indometh-
acin that had been cooled to −20°C. The mixture was
allowed to warm to 4°C and was diluted with water
(pH 3). [3H]PGE2 (1.5 ng) was added to the aqueous
tissue homogenate as an internal standard and the solu-
tion was adjusted to pH 3. The sample was applied to a
C-18 Sep-Pak cartridge that had been prewashed with
5 mL methanol and 5 mL H2O (pH 3). The cartridge was
washed with 10 mL H2O (pH 3) followed by 10 mL
heptane, and compounds were eluted with 10 mL ethyl
acetate. The eluate was applied to a silica Sep-Pak car-
tridge and rinsed with 5 mL ethyl acetate, and comp-
pounds were eluted with 5 mL ethyl acetate/methanol
(50:50 v/v) and dried under nitrogen. Compounds were
methoximated by treatment with 2% solution of aqueous
methoxyamine-HCl (250 μL) for 30 min at room tem-
perature and extracted with 1 mL ethyl acetate. The or-
ganic layer evaporated under nitrogen. Conversion to a
pentafluorobenzyl ester was performed by the addition of
40 μL pentafluorobenzyl bromide (10% solution) in
acetonitrile and 20 μL diisopropylethylamine in acetoni-
trile (10% solution) and allowed to incubate for
30 minutes at 37°C. Reagents were dried under nitrogen,
and the residue was reconstituted in 30 μL chloroform
and 20 μL methanol and chromatographed on a silica
TLC plate to 13 cm in a solvent system of ethyl acetate/
methanol (98:2 v/v). The methyl ester of PGE2 and the
O-methyloxime, pentafluorobenzyl ester derivative PGD2 (−5 μg each) were chromatographed on a separate
lane and visualized with 10% phosphomolybdic acid in
ethanol by heating. Compounds migrating in the region
1 cm above the PGF2α standard to 1.5 cm below the
PGD2 standard were scraped from the TLC plate, extracted
with 1 mL ethyl acetate, and dried under nitrogen.
Following TLC purification, compounds are converted to
trimethylsilyl ether derivatives by addition of 20 μL
N,O-bis(trimethylsilyl)trifluoroacetamide and 10 μL
dimethylformamide. The sample is incubated at 37°C
for 10 minutes and dried under nitrogen. The residue is
redissolved for gas chromatographic/mass spectrometric
analysis in 10 μL undecane.
Mass Spectroscopy. Gas chromatography/negative ion chemical ionization/mass spectrometry was carried out on a Hewlett-Packard 5982A mass spectrometer (Palo Alto, CA), calibrated daily, and interfaced with an IBM Pentium computer. Gas chromatography is performed using a 15 m, 0.25 μm film thickness DB-1701 fused silica capillary column (J&W Scientific, Folsom, CA). The column temperature is programmed from
190°C to 300°C at 20°C/min. The major ion generated in the negative ion chemical ionization mass spectrum of the pentafluorobenzyl ester, O-methyloxime, tetrame-
ethylsilane or tetramethylysilyl ether derivative of PGE2 is
the m/z 524 carboxylic anion [M-181 (N-CH2C8F5)]. The
corresponding ion generated by the [H3]PGE2 internal
standard is m/z 528. Endogenous PGE2 levels are cal-
culated from the ratio of intensities of the ions m/z 524
to m/z 528. As part of the assay procedure, a blank and a
control sample containing a known amount of eicosani-
oid were assayed with each batch as quality control
measures.
Statistical Considerations. The major statistical end-
point in this study was the correlation between mucosal
biomarkers and response to treatment (i.e., percentage
reduction in number of colorectal polyps at 6 months
from baseline). Additionally, biomarker levels were ana-
yzed in relation to study treatment arm. The biomarkers
assessed included apoptotic and proliferative Lls and

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PGE₂ levels. Biomarkers of interest included the apoptotic or proliferative (Ki-67) LI$s in the superficial and nonsuperficial compartments and their respective ratios. The relationship between biomarker levels and clinical response were assessed using Spearman correlations between the absolute change in biomarker levels from baseline to 6 months and the percentage reduction in polyp number from baseline to 6 months. The primary analysis was done using Spearman correlation coefficients, which are based on the ranks of the data. Because they are computed on the ranks, they are robust to outliers and do not require linearity in the scale of the data. Scatter plots were also generated to show the actual data on which the Spearman correlations were computed. The relationship between biomarker levels and study arm was assessed using nonparametric rank sum tests. The primary focus was placed on the placebo versus 400 mg twice daily celecoxib comparison, because this was the comparison that demonstrated the chemopreventive efficacy of celecoxib. Additionally, we examined the correlation between biomarker values within individual patients using Spearman correlations. All statistical tests were conducted with a significance level of 0.05.

Results

The results of this clinical trial in FAP patients have been published (9) and are summarized below. At baseline, the mean (± SD) number of polyps in focal areas where polyps were counted was 15.5±13.4 in the 15 patients assigned to placebo, 11.5±8.5 in the 32 patients assigned to 100 mg of celecoxib twice daily, and 12.3±8.2 in the 30 patients assigned to 400 mg of celecoxib twice daily (P = 0.66) for the comparison among groups. After 6 months of continuous drug treatment, the patients receiving 400 mg celecoxib twice daily had a 28.0% reduction in the mean number of colorectal polyps (P = 0.003 for the comparison with placebo) as compared with a reduction of 4.5% in the placebo group. The reduction in the group receiving 100 mg celecoxib twice daily was 11.9% (P = 0.33 for the comparison with placebo). The improvement in the extent of colorectal polyposis in the treatment groups was confirmed by a panel of endoscopists who reviewed procedure videotapes in a blinded manner. The incidence of adverse events was similar among the groups. More than 90% of patients completing the study took 80% of the study drug.

We retrospectively examined a panel of biomarkers in normal mucosa and in adenoma tissue from FAP clinical trial participants. Our aim was to determine whether modulation of the selected biomarkers correlated with the primary clinical study endpoint (i.e., the percentage change in colorectal polyp number at 6 months relative to baseline). To this aim, we determined the absolute change in a given biomarker from baseline to 6 months and sought its correlation with treatment arm and with polyp response data. We regarded this correlation as most important for providing mechanistic insights into the antitumor effects of celecoxib in vivo.

Apoptotic and Proliferative Indices. Apoptotic and proliferative indices were determined in superficial (AIs and Ki-67s) and nonsuperficial (AI ns and Ki-67 ns) regions of normal-appearing colorectal mucosa and in adenoma specimens from baseline and 6-month (end of study) examinations (Table 1). The change in each biomarker (baseline to 6 months) was determined and ratios between Al, or Al ns, and Ki-67 s or Ki-67 ns were computed. A significant reduction in Ki-67 s in adenomas was seen at 6 months relative to baseline (P = 0.016; Table 1). No significant differences were found for apoptotic indices in adenomas between these time points, nor were any significant differences found for these biomarkers in normal mucosa.

Correlation between Apoptotic and Proliferative Indices and Polyp Regression. We investigated whether biomarker values are related to clinical response, defined as the percentage change in polyp number from baseline to 6 months. In this study, the mean reduction in colorectal polyp number was −4.5% for placebo, −11.9% for

### Table 1. Summary of median values and ranges for apoptotic and proliferative indices and PGE₂ expression

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Histology</th>
<th>Study Evaluation (mo)</th>
<th>Mean</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
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<tr>
<td>AIs</td>
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<td>Baseline</td>
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<td></td>
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<td>0.00</td>
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<td>23</td>
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<td></td>
<td></td>
<td>6 months</td>
<td>1.38</td>
<td>1.40</td>
<td>0.00</td>
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<td>0.40</td>
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<td>0.00</td>
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<tr>
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<td>PGE₂ (pg/μg protein)</td>
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<td>415.30</td>
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<tr>
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<td>6 months</td>
<td>112.01</td>
<td>75.43</td>
<td>3.11</td>
<td>454.45</td>
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low-dose celecoxib, and -28% for high-dose celecoxib (9). We regarded the correlation between biomarker levels and clinical response to suggest modulation by celecoxib and to indicate a mechanism of polyp regression. For each biomarker and tissue type, we calculated the correlation between polyp response and (1) biomarker value at baseline and 6 months and (2) absolute change in biomarker from baseline to 6 months. Scatter plots of change in biomarker values versus polyp regression in adenoma and normal tissue versus polyp regression are shown in Fig. 1A-D and are discussed below.

**Adenomas.** We found that the change in the Ki-67s LI in adenomas from baseline to 6 months was significantly correlated with polyp regression ($r = 0.72$, $P = 0.0055$; Fig. 1A). Patients whose adenomas showed the greatest reduction in Ki-67s LIs over the study period had the best clinical response. The change in the apoptotic ratio of $A_I/Al$ ($r = 0.71$, $P = 0.004$) was significantly correlated with polyp regression (Fig. 1B). This change represented an increase in apoptosis at or near the luminal surface and a concomitant decrease in the nonsuperficial region. Furthermore, the change in the $A_I/Ki-67s$ ratio also correlated with clinical response ($r = 0.58$, $P = 0.026$; Fig. 1C). These data suggest that the extent of superficial apoptosis and cell proliferation are modulated in association with celecoxib-induced polyp regression. No other significant relationships were found either for individual biomarkers or for their ratios in adenomas. We also analyzed the relationship between treatment arm and absolute change in the biomarker level from baseline to 6 months. Emphasis was placed on the comparison between high-dose celecoxib and placebo, given that significant polyp regression was seen for this comparison. An association between celecoxib dose and Ki-67s LI in adenomas was suggested when comparing the higher dose (400 mg twice daily) to the placebo group from baseline to 6 months ($-1.34$ for placebo and $-16.28$ for 400 mg), but this difference did not reach statistical significance ($P = 0.077$) given the small sample size. Correlations between biomarker levels in normal colorectal mucosa and adenomas were sought. We found a positive

Figure 1. Scatter plots of the change in biomarker levels (baseline to 6 months; $x$ axis) versus percentage reduction in polyp number ($y$ axis) for adenomas (A, B, and C) and normal-appearing colorectal mucosa (D). Treatment arms include 400 mg ($\times$) and 100 mg (+) celecoxib twice daily or placebo (○). A. A reduction in the Ki-67s LI correlates with polyp regression in FAP patients. B. An increase in $A_I/Al$ ratio of adenomas is positively correlated with polyp regression. C. An increase in $A_I/Ki-67s$ ratio accompanies polyp regression. D. In normal mucosa, the $Al$ is associated with polyp regression.
correlation between normal and adenoma tissue for measurements of $A_{L}$ ($r = 0.29, P = 0.02$) and $A_{L_{0}}$ ($r = 0.34, P = 0.01$) within individual patients, suggesting that similar modulation is occurring in both normal and neoplastic tissues.

**Normal Mucosa.** In normal-appearing colorectal epithelia, the change in the $A_{L}$ (baseline to 6 months) correlated with polyp regression ($r = 0.33, P = 0.053$). In this regard, the largest increases in $A_{L}$ occurred in patients with greater percentage reductions in polyt number. No association was found between Ki-67, LI and polyp regression over the treatment period ($r = −0.04, P = 0.86$), nor for any of the ratios examined.

**PGE2 Levels.** PGE2 levels were determined both in normal-appearing colorectal mucosa and in adenomas (Table 1). No significant differences were found in median PGE2 levels comparing normal and adenoma tissue at baseline ($P = 0.17$), 6 months ($P = 0.45$), or the change in PGE2 over this interval ($P = 0.86$). Specifically, PGE2 levels were not reduced in biopsy specimens at 6 months relative to baseline in either normal tissue or adenomas, indicating that celecoxib treatment failed to suppress PGE2 production. PGE2 levels did not differ significantly among treatment arms. Importantly, more than 90% of the patients who completed the study took at least 80% of the study drug, suggesting that non-compliance is unlikely to account for these negative results. Furthermore, the changes in PGE2 levels from baseline to 6 months for normal epithelia ($r = 0.02, P = 0.84$) and adenomas ($r = 0.05, P = 0.71$) were not significantly associated with polyp regression. Within individual patients, PGE2 levels were positively correlated between normal and polyp tissues ($r = 0.50, P = 0.06$).

**Discussion**

Celecoxib was shown in a placebo-controlled trial study to significantly regress colorectal polyps in FAP patients administered this drug at a high dose (400 mg twice daily) for a continuous period of 6 months (9). Specimens from patients treated in this trial provided a unique tissue resource in which to analyze biomarkers that may be mechanistically related to the antitumor efficacy of celecoxib and may therefore be associated with the clinical response to this agent. In this study, we analyzed biomarkers, including cell proliferation and apoptosis, shown to be modulated experimentally by celecoxib and other NSAIDs (23-29) in adenomas and in normal-appearing mucosa. We also analyzed the biosynthetic product (i.e., PGE2) of the COX-2 enzyme in tissues from FAP study participants. We found that significant reductions in cell proliferation, as measured by the Ki-67 LI, in the superficial compartment of adenomas accompanied their regression in FAP patients. Alteration in the ratio of apoptosis at or near the mucosa surface ($A_{L}$) compared with the nonsuperficial region ($A_{L_{0}}$) was also found to significantly correlate with polyt regression. Specifically, an increase in the apoptotic ratio in adenomas was associated with a greater reduction in polyt number. Additionally, the ratio of apoptosis to cell proliferation in the superficial ($A_{L}/A_{L_{0}}$) compartment was predictive of clinical response. In normal colorectal epithelia, the correlation between the $A_{L}$ and polyp regression was of borderline statistical significance. Of note, apoptotic events in normal mucosa occur with a reduced frequency relative to adenomas and our sample size was relatively small. Similar to our findings in adenomas, Keller et al. (33) found that sulindac treatment modulated the ratio of superficial to nonsuperficial apoptosis in normal rectal mucosa of FAP patients, which accompanied polyp regression. However, this same group found that the apoptotic ratio did not predict response to sulindac treatment nor adenoma development in genotype-positive, phenotype-negative FAP patients (34). While cell proliferation was not examined in those reports (33, 34), sulindac was shown to modulate cell proliferation in normal-appearing epithelia from FAP patients in one report (7) but not in others (35, 36). In our study, we clearly demonstrate that reduced cell proliferation in adenomas accompanies their regression. We emphasize that the correlations between biomarker modulation in adenomas and clinical response were highly statistically significant despite the relatively small sample size. While we were unable to establish any statistically significant correlations between biomarkers and study treatment arm, this does not mean that there is no such correlation. An association between celecoxib dose and Ki-67, LI in adenomas was suggested when comparing the higher dose (400 mg twice daily) to the placebo group, where a large difference was suggested with respect to mean change in Ki-67, from baseline to 6 months (−1.34 for placebo and −16.28 for 400 mg; $P = 0.077$). The small study sample size limited the statistical power to detect significant associations among treatment arm and tissue biomarkers unless they were of extremely large magnitude. Taken together, our data suggest that celecoxib is modulating cell proliferation as well as the frequency and spatial distribution of apoptosis in colorectal mucosa from FAP patients. Alterations in cell kinetic parameters (i.e., hyperproliferation and attenuated apoptosis; refs. 20, 35) found in the colorectal mucosa of FAP patients may therefore be potentially reverted by celecoxib, suggesting that their modulation may be mechanistically related to the chemopreventive and antitumor efficacy of celecoxib in FAP.

Effective treatment of an established tumor requires that the tumor regress by a reduction in cell growth and/or an induction of apoptosis (18). A chemopreventive effect requires that an intervention interrupt or revert the cellular changes associated with tumorigenesis back to their normal physiological state. In normal colorectal mucosa, proliferating cells are restricted to the lower two-thirds of the crypts and apoptosis occurs predominantly in colonocytes at or near the luminal surface (17). These processes are dysregulated in FAP as indicated by an upward shift of the proliferative compartment and reduced superficial apoptosis in normal-appearing colorectal mucosa (19, 20). The distribution of proliferating and apoptotic cells have been shown to be topographically reversed in adenomatous polyps compared with normal colorectal mucosa. Specifically, colorectal adenomas have increased numbers of proliferating cells at or near the luminal surface and more frequent apoptotic cells at or near the crypt base (21, 22). Our results suggest that celecoxib may regress adenomas by reverting abnormal cellular kinetics toward the pattern found in
normal mucosa. Our findings are consistent with in vitro data for celecoxib where this drug and other NSAIDs inhibit cultured colon cancer cell growth, arrest cells in the G1 phase of the cell cycle, and induce apoptosis (23-26). Furthermore, Mahmoud et al. (29) found that sulindac sulfide reverted the reduction in enterocyte apoptosis found in the intestinal mucosa of ApoMin mice, compared with normal littermates lacking the APC mutation, in association with its chemopreventive effect. These data suggest that NSAIDs may reverse the suppression of apoptosis resulting from a loss of APC function (37). In this regard, reexpression of APC in human colorectal cancer cells containing endogenous inactive APC alleles resulted in a substantial reduction in cell growth shown to be due to the induction of apoptosis (37). Other potential mechanisms by which NSAIDs induce apoptosis include nuclear factor-κB inhibition (38), ceramide production (39), induction of membrane receptor DR5 expression (40, 41), and mitochondrial cytochrome c release (42, 43).

We did not find evidence for suppression of PGE2 levels in colorectal epithelia by celecoxib treatment although a high level of patient compliance was achieved. Our findings are consistent with a study in ApoMin mice where sulindac markedly reduced intestinal tumor number but did not alter the level of PGE2 in intestinal tissues (44). Celecoxib is a selective inhibitor of the COX-2 enzyme and studies indicate that PGE2 is regulated to a greater extent by the COX-1 isoform (10). We found previously that low-dose aspirin (81 mg per day) markedly suppressed rectal mucosal PGE2 levels and to an equivalent extent as did higher doses (up to 650 mg per day; ref. 45). Furthermore, the nonselective COX inhibitor sulindac reduced PGE2 levels in 11 FAP patients and such suppression correlated with adenoma regression (46). Marked interpatient heterogeneity in sulindac-induced PGE2 suppression was observed. While methodological issues may have impacted on our findings, it is highly plausible that the observed biomarker modulation and polyP reduction by celecoxib are independent of COX-2 inhibition. Considerable evidence indicates that NSAIDs exert their antitumor effects through both COX-dependent and COX-independent mechanisms (2). Interestingly, recent data by Gao et al. (47) demonstrate a second pathway for PG production that is independent of COX and would therefore not be inhibited by NSAIDs.

In summary, we found that cell proliferation and apoptosis are modulated in colorectal epithelia during a 6-month trial of continuous celecoxib versus placebo treatment in FAP patients. Such modulation was found to significantly correlate with the percentage reduction in colorectal polyp number. These findings suggest that the mechanism of celecoxib’s chemopreventive and tumor-regressing effects are related to alterations in these cell kinetic parameters. Cell proliferation and apoptotic ratios in colorectal epithelia may therefore represent intermediate biomarkers for the efficacy of celecoxib in FAP patients and studies are warranted to further address this issue.

References


Cell Proliferation and Apoptotic Indices Predict Adenoma Regression in a Placebo-Controlled Trial of Celecoxib in Familial Adenomatous Polyposis Patients

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