Colonic Mucosal Prostaglandin E₂ and Cyclooxygenase Expression before and after Low Aspirin Doses in Subjects at High Risk or at Normal Risk for Colorectal Cancer₁,₂

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Abstract

Development of potential cancer chemopreventive drugs involves the systematic evaluation of these drugs in preliminary Phase I and II studies in human beings to identify the optimal drug dose, drug toxicity, and surrogate end point biomarker modulation.

Objectives: We tested the hypothesis that aspirin, at a single, once-daily 81-mg dose, will reduce colonic mucosal concentration of prostaglandin estradiol (E₂) in individuals at high risk for colorectal cancer development similar to our prior observations in a young normal-risk population.

Methods: Aspirin was administered at a dose of 81 mg once daily for 28 days in a cohort of 92 matched high-risk and normal-risk colorectal cancer subjects. Prostaglandin E₂ and cyclooxygenase expression were assayed from distal sigmoid biopsies from all of the subjects before and after treatment.

Results: The mean prostaglandin E₂ for normal-risk subjects before aspirin treatment was 11.3 ± 1.7 pg/µg (mean ± SE) tissue protein and after aspirin treatment was 4.9 ± 0.91 pg/µg tissue protein (P < 0.0001). In high-risk subjects, mean pretreatment prostaglandin E₂ was 14.4 ± 1.7 pg/µg tissue protein and after aspirin treatment was 4.7 ± 0.70 pg/µg tissue protein (P < 0.0001). Aspirin treatment did not alter cyclooxygenase-1 protein expression.

Conclusions: Aspirin treatment at a dose of 81 mg reduces colorectal mucosal prostaglandin E₂ concentration after 28 daily doses. Risk for colorectal carcinoma did not modify colorectal mucosal baseline or post-aspirin prostaglandin E₂, or cyclooxygenase expression. Colorectal mucosal prostaglandin concentration may be used as a “drug-effect surrogate biomarker,” that is, a surrogate to assess sufficient delivery and tissue effect of a chemopreventive agent.

Introduction

The chemopreventive potential of NSAIDs is derived from several lines of evidence. Numerous published epidemiological studies indicate that NSAIDs reduce the incidence of colorectal cancer by 30–40% (1–8) in humans. NSAIDs have anticarcinogenesis effects in chemically induced and in genetic mutational rodent carcinogenesis models (9–14).

The mechanisms underlying the anticarcinogenic effects of NSAIDs remain unknown. Hypothesized mechanisms of action have included the effects of suppression by Coxs on immune function (15), cellular oxidative stress (15–17), and apoptosis regulation (18–21). Non-Cox-mediated mechanisms have also been postulated including suppression of NF-κB activation (22), inhibition of activator protein-1 activity (23) and Cox-independent enhancement of apoptosis (13, 24–27). Inhibition of angiogenesis may also be a critical factor attributable to suppression of Cox-2 as demonstrated in colon cancer cell lines by Tsuji et al. (28).

Aspirin and other NSAIDs have been extensively studied, and the complexities regarding dose variations for different clinical indications are well recognized. Before the development of large clinical trials to assess colorectal cancer risk reduction with NSAIDs, it is necessary to estimate optimal doses and schedules of any agent proposed for such trials. Most NSAIDs are approved by the United States Food and Drug Administration and similar regulatory bodies for over-the-counter or prescription treatment of inflammation or pain. For this indication, high doses and frequent administration schedules are recommended. Assumptions of equivalent clinical dose and schedule response for preventive intent as compared with

other therapeutic intentions (e.g., anti-inflammatory) are un-founded. For a cancer chemopreventive indication as opposed to an anti-inflammatory indication, dose response is likely to differ. Whereas many centers have studied differing NSAID doses for the purpose of colon cancer chemoprevention (6, 7, 29–32), few have performed systematic dose response and validation studies (6, 7, 33). Such studies may identify potentially effective chemopreventive doses that are much lower and schedules that are less frequent that those used for other clinical indications. Reduced doses and schedules have the potential of enhancing adherence and reducing drug-induced toxicity.

An ideal chemopreventive dose will be one that alters a validated biomarker that has a strong correlation with colorectal carcinogenesis. For colorectal chemopreventives, a biomarker other than reduction of adenomatous polypl occurrence or recurrence (34) with these characteristics has not yet been identified. Specifically, a biomarker that might be detected biochemically or genetically in morphologically normal mucosa has yet to be validated. However, there is an urgent need to establish potential NSAID chemopreventive doses. To address this need, we (33), as well as others (30–32), have come to rely on tissue PGs as surrogates of drug delivery to colorectal epithelial mucosa as a “drug effect” surrogate end point biomarker.

To test these assertions, we have studied the dose response of aspirin given once daily on distal sigmoid mucosal PGE2 and F2, concentration (33). Our data suggested that doses as low as 40 mg once daily were sufficient to inhibit distal sigmoid mucosal PG content in 50% or more of normal human subjects studied. We found that a once-daily dose of 81 mg inhibited distal sigmoid PG concentration in >90% of 10 subjects who took the dose for a 14-day period. In this trial, we studied healthy subjects without known risk for colorectal cancer. At the completion of the Phase I trial, we were concerned that baseline PG production in the colorectal mucosa might differ with age and with risk for development of colorectal cancer. Therefore, we hypothesized that aspirin at a single, once-daily 81-mg dose, reduced colorectal mucosal concentration of PGE2, in individuals at high risk for colorectal cancer development similar to our prior observations in a young normal-risk population. We hypothesized that colorectal cancer risk did not alter aspirin effects on colorectal mucosal PGE2 concentration. To address these hypotheses, we designed a Phase Ila chemoprevention trial of aspirin at 81 mg a day on PGE2 and Cox-1 and -2 concentrations in age-, sex-, and colorectal-cancer-risk-matched individuals.

Materials and Methods

Clinical Protocol

Human Subjects. A total of 92 subjects were recruited between June 1995 and January 1997. To be eligible for the study, participants had to be 18 years or older; able to provide written, informed consent; and be in normal health as demonstrated by medical history, physical examination, and normal hematology and biochemical laboratory values (WBC, >4000 mm3; hemoglobin, >12 g/dl; platelet count, >120,000/mm3; normal urinalysis; and negative fecal occult blood tests). Exclusion criteria included pregnancy, lactation, peptic ulcer disease (active or inactive), history of gastrointestinal bleeding from gastric or duodenal ulcers or gastrin-secreting tumors, history of medication intake in the preceding week, history of cancer except basal cell and squamous cell skin cancer or Duke’s A, B, or C colon cancer, or unwillingness to stop taking aspirin or other NSAIDs. All of the participants were reimbursed for their time.

The protocol was reviewed and approved by the University of Michigan Institutional Review Board (IRB MED) and the Human Use Committee of the Ann Arbor VA Medical Center.

Subjects were assigned as high-risk for colorectal cancer in the study if they had a history of familial colorectal cancer syndromes; two or more first-degree relatives with colon cancer; personal history of Duke’s A, B, or C colorectal carcinoma without disease recurrence and at least 8 weeks since completion of adjuvant chemotherapy; or resection of a villous adenoma >1 cm in size or any adenoma containing carcinoma in situ. Normal-risk subjects had no history of either familial colorectal cancer syndromes nor two or more first-degree relatives with colon cancer. Normal-risk individuals did not have a personal history of Duke’s A, B, or C colorectal cancer or resection of a villous adenoma >1 cm in size or any adenoma containing carcinoma in situ. High-risk and normal-risk subjects were matched for age (± 5 years) and gender.

Drug. Eighty-one-mg aspirin tablets, formulated with a dextrose excipient, Yellow #6, saccharin sodium, and starch, were provided as Bayer’s Children’s Formulation (Bayer Corporation, Consumer Care Division, Morristown, NJ) as a single, recently manufactured lot (No. KC326). The drug was given as a single, daily dose for 28 days. The subjects were instructed to swallow the tablet and not to chew the tablet.

Diet. Two days before distal sigmoid biopsy specimens were obtained, the dietary intake of the participants was controlled to reduce potentially confounding variables. Specifically, a meal plan was developed to distribute energy and micronutrients equally in each of three meals per day, so that variable meal size and composition would not interfere with variations attributable to diurnal variations. Meal plans were individualized to accommodate different energy requirements for weight maintenance. These energy requirements ranged from 7,531 to 12,552 KJ/day (1800 to 3000 kcal/day). All of the subjects were required to eliminate caffeine-containing beverages from their diet during this diet control period.

Adherence. Subjects were assessed for adherence by a research coordinator through weekly telephone calls, self-report, and pill counts at the end of the study. Subjects were asked to keep a calendar of the time and date when they took the medications. Reasons for violation of prescribed time and dose were solicited. Adherence was defined as taking the tablets within 4 h of the agreed on time, once daily. Adherence was monitored by weekly self-report and by pill counts at the end of the study. Subjects were classified as adherent if the adherence monitoring suggested that 80% or more of the doses were taken as prescribed.

Toxicity Assessment. Subjects were assessed for toxic effects by direct questioning in person or by telephone at weekly intervals. Symptoms germane to aspirin toxicity were solicited by the research coordinator. The National Cancer Institute (NCI) Common Toxicity Scale (Regulatory Affairs Branch, Cancer Therapy Evaluation program, Division of Cancer Treatment, Diagnosis, and Centers, NCI, Bethesda, MD) and the Costa Toxicity Frequency Scale (35) were used to quantify toxicity. Subjects experiencing Common Toxicity Criteria grade 2 or grade 1 and Costa Toxicity Frequency Scale 2 were considered to have unacceptable toxic effects and were removed from the study.

Flexible Sigmoidoscopy and Tissue Collection. Subjects underwent two flexible sigmoidoscopies, one before drug treatment and the second, 29 days after drug treatment commenced. The second procedure was performed at a time as close as possible to 24 h after the subject took the final aspirin dose. The
subjects were not prepared for the procedure with any enemas. Subjects were asked to evacuate their rectum within 12 h of the procedure but to not take any laxatives to enhance evacuation. Subjects were placed in a left lateral decubitus position and a flexible sigmoidoscope was passed to 15 cm from the anal sphincter. Ten tissue samples were taken by opening and pressing the biopsy forceps perpendicular to the mucosal surface with mild pressure. To reduce artifactual PG activation by the biopsy forceps were taken at least 2 cm proximal to the endoscope. Each biopsy specimen was taken ~2 cm or more from other biopsy sites in distal sigmoid colonic mucosa that had no visual appearance of trauma or recent biopsy.

**Tissue Handling and Disposition.** Of the 10 biopsy samples, 5 were placed immediately into a sterile 1.5-ml Eppendorf tube and frozen in liquid nitrogen within 20 s of the time the biopsy forceps were closed. The specimens were stored at −70°C until immediately before analysis. The remaining tissue samples were fixed in 10% buffered formalin for paraffinization and future study.

Frozen biopsy samples weighed ~5 mg and yielded between 400 and 600 μg protein. Triplet assays for PGE₂ and Coxs required 10–20 μg each. Two frozen samples were used for these assays because of the different buffers used for tissue homogenization. The remaining frozen tissue samples were stored at −70°C for future use.

**Analytical Methods**

PGE₂. An ELISA (EIA; Cayman Chemicals, Ann Arbor, MI) was used for quantitation of PGE₂. A single colonic biopsy specimen was removed from the freezer, placed in 0.5 ml of PBS at room temperature, immediately homogenized for 30 s, vortex-mixed for 2 min, and centrifuged at 2100 × g for 30 s at room temperature. The supernatant was removed and three 50-μl aliquots were assayed for PGE₂. If the PG value obtained did not fit within the linear portion of the assay standard curve, then an aliquot of the supernatant was diluted 1:10 or 1:100 with PBS and reassayed for PGE₂. This process was repeated until the PGE₂ value measured was within assay limits. Validity of the data was further confirmed by the assay in triplicate of low, intermediate, and high PGE₂ concentrations of 250 pg/ml, 500 pg/ml, and 1000 pg/ml, respectively, in PBS. Batch assays in which the known standards were greater than or less than 15% of the known concentrations were reassayed until within tolerance limits. Crude protein was assayed from 6 μl of the supernatant by Coomassie Blue microtiter Plate (Pierce Chemical Co., Rockford, IL) protein assay. The PGE₂ concentration (in picograms) was normalized per μg of protein. The PGE₂ assays were performed in 10 batches. All of the biopsy samples from a given individual were assayed in the same batch to eliminate any batch effect in within-subject responses.

Cox Isoenzymes. The Cox isoenzymes, Cox-1 and Cox-2, were identified and quantified by Western immunoblotting, chemiluminescence, and densitometric image analysis. Frozen biopsies were placed in ice-cold antiprotease buffer and homogenized on iced ethanol using a homogenizer followed by sonication for 1.5 min. Total protein content was determined by the Coomassie Blue microtiter plate protein assay. Equal amounts of protein (10 μg) and Cox-1 and Cox-2 standards (Cayman Chemicals) were separated on 10% SDS PAGE. High-molecular and low-molecular weight rainbow markers (Amersham corp., Arlington Heights, IL) were also loaded on each gel. After overnight transfer to nitrocellulose membrane, blots were blocked by incubation for 1 h with 10% nonfat dried milk in Tris-buffered saline (TBS), and washed in TBS containing 0.1% Tween 20. The membranes were then incubated with a 1:1500 dilution of human Cox-1 antibody (Cayman Chemicals) for 1 h followed by washing and incubation for 1 h with the horseradish peroxidase-labeled secondary antibody and detection using enhanced chemiluminescence detection system (ECL; Amersham Corp., Arlington Heights, IL). The blots were then stripped of Cox-1 antibody and the process was repeated with primary Cox-2 antibody (polyclonal ovine antibody provided as a gift from Dr. D. L. DeWitt, Michigan State University, East Lansing, MI). All of the samples from an individual subject were run on the same gel for reliable comparisons. Cox expression was quantified by digitizing the antibody-hybridized protein blot with a Javelin camera and quantified densitometrically using NIH Image Freeware (version 1.47).

**Statistical Analysis**

All of the statistical analyses were produced on a Sun Microsystems SPARC Ultra 10 computer using SAS software (SAS Institute, Cary, NC). Measures of statistical significance were obtained by repeated measures ANOVA (36) as implemented in SAS PROC MIXED. Repeated measures ANOVA is a generalization of the paired-comparison t test that takes into consideration the correlation between assays of multiple biopsies from the same subject. Cox-1 and PGE₂ concentrations were log-transformed as required for variance stabilization.

**Results**

**Study Population.** A total of 92 subjects (61% males, 39% females) were enrolled. The distribution of age by risk group, sex, and race are presented in Table 1. The distribution of ages was similar in the normal-risk and control groups.

**Adherence.** Twenty-three of the 92 subjects either missed a single dose or took the drug at a different time during the 28 days of the study. Eight of the 92 subjects forgot or missed more than one dose during the study period. All of the subjects took the drug as prescribed at least 80% of the time, and, hence, data from all of the treated subjects were included in the final analysis.

**Toxicity.** None of the subjects experienced any drug-related toxicities. One consented subject withdrew before receiving drug, because of discomfort from the flexible sigmoidoscopy procedure, and was replaced.

**Colorectal Mucosal PGs.** At baseline, the normal-risk subjects tended to have lower PGE₂ colorectal mucosal concentration than the high-risk subjects (mean ± SE: PGE₂, 11.3 ± 1.7 pg/μg protein versus 14.4 ± 1.7 pg/μg protein, respectively; Fig. 1), but the difference was not statistically significant.

<table>
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*Mean ± SD.
Aspirin and PGE\(_2\) in Human Subjects

In a repeated-measures ANOVA that tested for the effects of aspirin on Cox-1 expression, a significant decrease in PGE\(_2\) concentration over 28 days was observed in both high-risk and normal-risk subjects (Fig. 3). This decrease in PGE\(_2\) concentration was consistent with the administered aspirin treatment. In contrast, the average decrease of 70% in high-risk subjects was not statistically significant compared to that of normal-risk subjects (Fig. 2). The decrease in PGE\(_2\) concentration over 28 days in both high-risk and normal-risk subjects was not significantly different from the average decrease of 65% in normal-risk subjects (all, \(P > 0.45\)).

There were a few subjects in each group whose concentration was much higher than the mean. The sex, race, and age of the subjects were not significant in predicting baseline PGE\(_2\) colorectal mucosal concentration (all, \(P > 0.45\)). By day 28, the PGE\(_2\) concentrations of 32 (70%) of the normal-risk subjects and 36 (78%) of the high-risk subjects had decreased (mean ± SE: PGE\(_2\) after treatment, 4.9 ± 0.91 pg/μg protein versus 4.9 ± 0.70 pg/μg protein, respectively; Fig. 1). The decrease in PGE\(_2\) concentration over 28 days in both normal- and high-risk subjects was statistically significant (\(P < 0.0001\)), and there was no significant difference between the decrease in normal-risk versus high-risk subjects (\(P = 0.22\)). The average decrease of 70% in the high-risk subjects was not significantly different from the average decrease of 65% in the normal-risk subjects.

**Colorectal Mucosal Cox.** At baseline, the difference in the distributions of Cox-1 values between normal-risk and high-risk subjects was negligible (Fig. 2). The sex, race, and age of the subjects were not significant in predicting baseline Cox-1 protein expression (all, \(P > 0.40\)). Posttreatment, the Cox-1 expression of 25 (54%) of the high-risk subjects and 23 (50%) of the normal-risk subjects had decreased (Fig. 2). The decrease in Cox-1 expression over 28 days was not statistically significant (\(P = 0.40\)).

The expression of Cox-1 was unrelated to the concentration of PGE\(_2\). In a repeated-measures ANOVA that considered the risk status of the subject and whether the biopsy was collected before or after treatment, Cox-1 added no significant predictive power to the statistical model (\(P = 0.82\)).

**Discussion**

Data published over the last 3 years has established that p.o. administration of NSAIDs inhibits colorectal mucosal PG synthesis in morphologically normal human colorectal mucosa (30–33). The dose response of this effect with aspirin, using a once-daily dosing schedule, is steep, with doses as low as 40 mg inhibiting 50% of subjects' PGE\(_2\) and F\(_{2α}\) concentration and 81 mg inhibiting 80% (33).

Most published trials consist of small cohorts (<20 human subjects) considered at higher-than-normal risk for the development of colorectal cancer through their diagnoses of adenomatous polyps or previously resected cancers (30–32). Frommel et al. (32) established that a high dose of aspirin (325 mg, once daily) effectively inhibits rectal mucosal PGE\(_2\) synthesis while not shifting arachidonic acid metabolism to the 5-lipoxygenase pathway. Giardiello et al. (31) confirmed the inhibition of PGE\(_2\) by another NSAID in four familial adenomatous polyposis patients and four healthy matched controls and extended these findings by assaying a full profile of PGs using mass spectroscopy. Of interest are the findings by Giardiello et al. that PGE\(_2\) is the most abundant PG detected in human colorectal mucosa and the PG most profoundly inhibited by an NSAID (sulindac) intervention (31). Giardiello et al. found no differences in baseline or treated PG mucosal concentration between small cohorts (four subjects each) of polyposis and normal risk human subjects (31). The data of both Frommel et al. (32) and Barnes et al. (30) suggest that the magnitude of aspirin-induced reduction in PGE\(_2\) formation in human mucosa...
lines demonstrate the difference in PGE2 colorectal mucosal content before and after aspirin treatment, but there was no relationship between PGE2 mucosal content and Cox-1 expression in the same sample.

The regression lines were determined by the mixed effects model described in "Results." The difference in baseline PGE2 values was large, the difference in baseline PGE2 values seen an average difference with significant spread. Although differences between normal-risk and high-risk colorectal cancer subjects, either at baseline or after NSAID administration. These data might be explained in a number of ways. Although there is considerable debate regarding their mechanistic role in the carcinogenesis process, PGs, particularly PGE2, remain consistently elevated in the later stages of colorectal carcinogenesis, such as adenoma, in animal chemoprevention models (12, 19). Some laboratories have reported the lack of NSAID inhibition of PG synthesis in animal models with enhanced cellular apoptosis (13, 26). These results could be associated with variability of technical aspects of eicosanoid assays from tissue or dose-response differences of weaker metabolites of more potent NSAIDs (e.g., sulindac sulfone versus sulindac sulfide; Ref. 38). However, if this were true, we would have seen an average difference with significant spread. Although the spread was large, the difference in baseline PGE2 values between the normal- and high-risk groups is large enough to be of consequence. In fact, the medians were nearly identical. Thus, we do not feel that technical variability plays a role in these results.

In vitro data support the relationship of PGE2 production, up-regulation of Cox-2 (39), and associated mitogenic cellular phenotype (40). Whether cellular PGE2 represents a downstream product of carcinogenesis associated with Cox-2 induction, plays a role in the cellular transformation process itself, or has no role whatsoever in colorectal epithelial carcinogenesis, is unclear. Whereas its role as a surrogate biomarker of chemopreventive drug efficacy remains to be defined, recent data in a familial adenomatous polyposis cohort support its use as a drug efficacy biomarker (37). However, its use as a surrogate end point biomarker for drug effect, evidence of drug delivery to target and cause of some biological change at a target, is justifiable based on our data and data from many other groups (6, 7, 29–33).

In addition to studying product formation of Cox in human colorectal mucosa, we assessed expression of Cox-1 and Cox-2 before and after aspirin treatment. We found that aspirin did not alter Cox-1 expression in our subject samples. Although aspirin-induced acetylation inactivates the dioxygenase activity of the enzyme, the peroxidase activity remains intact (41–44). The long (>24 h) suppression of PG synthesis in the setting of short aspirin and salicylic acid plasma half-lives (33) in nucleated cells capable of enzyme synthesis, may reflect the continued intracellular concentration of structurally intact enzymes capable of inhibiting Cox synthesis. This effect is of importance because this type of inhibition is of a relatively long period with no apparent resistance to its effects for 28 days.

Although Sinicrope et al. recently reported low concentrations of Cox-2 expression in paired normal tissue from three subjects with sporadic colon cancer (45), we did not detect Cox-2 protein expression by Western blot in normal mucosal biopsies, similar to the normal mucosa studied as paired samples with carcinomas or adenomas (46, 47). Whereas the Cox-2 gene is expressed in morphologically normal colon tissue (48), it does not appear to be translated to functional Cox-2 protein in most normal mucosa.

Whereas one mechanism by which NSAIDs promote anticarcinogenesis through inhibition of COX-2, recent data suggest a multiplicity of Cox and PG-dependent and -independent carcinogenesis mechanisms. For example, NSAID down-regulation of nuclear transcription factors like NF-xB or inhibition of binding of PPAR-6 to DNA have been recently demonstrated (49). Sulindac sulfone enhanced apoptosis and reduces neoplas-
tic events in in vivo rodent models of colorectal carcinogenesis; yet does not inhibit Cox or reduce tissue PGs (25–27). One possible explanation for the diversity of chemopreventive efficacy mechanisms might be explained by upstream events being mediated through the PPAR-δ pathway with PGs serving as ligands that activate this pathway. With the rapid increase in potential chemopreventive agents that target some aspect of arachidonate metabolism (50–55), new data regarding the mechanisms of NSAID anticarcinogenesis activity will provide more useful biomarkers of both drug effect and drug efficacy in the future.

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References


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