

Short Communication

Effect of Aspirin on Prostaglandin E₂ Formation and Transforming Growth Factor α Expression in Human Rectal Mucosa from Individuals with a History of Adenomatous Polyps of the Colon¹

Christopher J. Barnes, Rhoda L. Hamby-Mason, W. Elaine Hardman, Ivan L. Cameron, K. Vincent Speeg, and Makau Lee²

Departments of Medicine [C. J. B., R. L. H-M., K. V. S., M. L.] and Cellular and Structural Biology [W. E. H., I. L. C.], University of Texas Health Science Center, San Antonio, Texas 78284

Abstract

Colorectal cancer is the second-most frequent cause of cancer mortality in the United States. Human epidemiology and laboratory studies indicate that aspirin may be an effective colorectal cancer chemopreventive agent. This study was designed to determine whether treatment with 81 mg of aspirin per day for 3 months would alter two putative surrogate end point biomarkers of chemoprevention of colorectal cancer [*i.e.*, mucosal prostaglandin E₂ (PGE₂) formation and transforming growth factor α (TGF- α) expression] in normal-appearing rectal mucosa from individuals with a history of adenomatous polyps. Rectal biopsies were obtained by flexible sigmoidoscopy at three sequential time points: (a) after a 1-month placebo run-in period (baseline), (b) after 3 months of ingesting 81 mg of aspirin (as a single tablet) once per day, and (c) after 3 months of ingesting a placebo tablet once per day (washout period). Daily aspirin significantly suppressed PGE₂ formation, but this significant suppression was completely reversed when aspirin was withdrawn. The extent of TGF- α staining in rectal crypts was also reduced significantly ($P = 0.039$) by daily aspirin. After a 3-month placebo-washout period, however, the mean extent of TGF- α staining was not significantly different from either baseline or the aspirin time point. Thus, 81 mg of aspirin daily significantly reduced rectal mucosal PGE₂ formation and TGF- α expression in patients with a history of adenomatous polyps. These putative surrogate end point biomarkers may be useful intermediate end points in future colorectal cancer chemoprevention trials.

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² To whom requests for reprints should be addressed, at Division of Digestive Diseases, Department of Medicine, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505. Phone: (601) 984-4549; Fax: (601) 984-4548.

Introduction

Colorectal cancer is one of the most common malignancies in the United States, representing the second-most frequent cause of cancer mortality (1). Decades of intensive research on colorectal cancer treatment and various screening strategies have failed to have a significant impact on the high mortality rate (2). Research efforts have begun to shift toward identification of potential preventive agents and biomarkers to identify populations at high risk for development of colorectal cancer (2, 3).

One class of compounds of recent interest as chemopreventive agents is the NSAIDs,³ which include aspirin. Indeed, there is ample laboratory and epidemiological evidence supporting an inverse association between the use of aspirin and other NSAIDs and the risk of developing adenomatous polyps and colorectal cancer (2, 4, 5). The precise mechanism(s) by which aspirin and other NSAIDs reduce colorectal cancer incidence and mortality have not been established. However, it is likely that these mechanisms are related to the ability of NSAIDs to inhibit COX, the rate-limiting enzyme for prostaglandin biosynthesis (4, 6). Recent studies have demonstrated the ability of NSAIDs to inhibit prostaglandin synthesis in human rectal mucosa (7–9). Additionally, aspirin and other NSAIDs have been shown to modulate colonic crypt homeostasis through normalization of crypt proliferative and apoptotic changes associated with increased colon cancer risk (10–13).

The long time involved in the development of colon carcinogenesis and the large number of subjects required to test efficacy of various interventions make the direct testing of human colorectal cancer as an end point measure impractical. Therefore, there is a need to identify easily detectable biomarkers of a precancerous state as intermediate end points that will reliably predict cancer risk in chemoprevention trials. Ideal surrogate end point biomarkers fit the following criteria: (a) differential expression in normal and high-risk tissue; (b) alteration early in carcinogenesis; (c) high sensitivity, specificity, and accuracy relative to cancer; (d) ease of measurement; (e) modulation by chemopreventive agents; and (f) correlation of modulation with decreased cancer incidence (3).

Expression of TGF- α in rectal mucosa may serve as a surrogate end point biomarker. TGF- α expression is normally localized to the upper one-third to two-thirds of normal human colorectal crypts (14). The expression of TGF- α is often increased in colonic adenomas and adenocarcinomas (15, 16). We have recently reported that the distribution of TGF- α immunohistochemical staining in rectal crypts of individuals with a

³ The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; TGF- α , transforming growth factor α ; PGE₂, prostaglandin E₂.

history of adenomatous polyps is significantly reduced by a dietary cellulose intervention (17). This study was designed to determine whether treatment with 81 mg of aspirin per day for 3 months would significantly alter mucosal PGE₂ formation and TGF- α expression in normal-appearing rectal mucosa from individuals with a history of adenomatous polyps.

Materials and Methods

Patients and Study Protocol. This was a prospective, placebo-controlled, double-blind study. Patients with a history of adenomatous polyps were recruited from the University Hospital and the Veterans Affairs Hospital in San Antonio, Texas. Informed consent was obtained from patients who met the protocol inclusion criterion of having a history of adenomatous polyps and who were not disqualified based on the exclusion criteria. Exclusion criteria included a history of colorectal cancer and/or resection; familial polyposis coli; inflammatory bowel disease; a history of aspirin allergy, clotting disorder, or bleeding tendency; pregnancy; a history of complication of aspirin therapy (*i.e.*, gastroduodenal ulcer, bleeding, or perforation); a need for continuous aspirin or other NSAID use; abnormal serum levels of liver enzymes; abnormal renal function (*i.e.*, creatinine level of >2 mg/dl); other significant systemic illness; or age of <18 years.

Ten volunteers (4 men and 6 women; mean age, 53.6 years; age range 47–64 years) were enrolled in this study. After informed consent was obtained, patients underwent baseline colonoscopy with polypectomy and an initial screening work-up, consisting of a complete blood count, blood chemistry and liver function, and a history and physical examination. Patients who met the enrollment criteria were then given placebo (one placebo tablet taken *p.o.* per day) for 1 month during a placebo run-in period between the colonoscopy and the first flexible sigmoidoscopy. The placebo run-in period was designed to establish an aspirin-free baseline and to assess adherence and compliance. Compliance during this study was assessed by pill count performed after the placebo run-in, aspirin treatment, and placebo washout period. Pill count suggested that all subjects had taken the study medications as directed.

Rectal biopsies were obtained by flexible sigmoidoscopy after Fleet enema (Lynchburg, VA) preparation of colon at three subsequent time points: (a) after a 1-month placebo run-in period (baseline), (b) after 3 months of ingesting 81 mg of aspirin (as a single tablet) once per day, and (c) after 3 months of ingesting a placebo tablet once per day (placebo-washout period following the aspirin treatment). Volunteers were instructed to take the study medication with breakfast every day (including the day of biopsy). The placebo tablets (each containing 97 mg of cellulose; CMC Corp., Brewster, NY) were identical in appearance to the aspirin tablets (generic 81-mg aspirin tablets; Rugby, Norcross, GA). A research nurse dispensed medications. Volunteers, endoscopists (performing the biopsies), and laboratory personnel were blind to the exact ingredients of the medications and to the sequence of treatment, making the study double-blind. The complete protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

Rectal Biopsies. Biopsy specimens from normal-appearing flat rectal mucosa were obtained ~10 cm above the anal verge using 7-mm pinch forceps. Two biopsies were obtained and immediately frozen in a Tris buffer for later prostaglandin analyses (see below). Two additional biopsies for immunohistochemistry were obtained and fixed in Omnifix (An-Con Genetics, Melville, NY), an alcohol-based fixative that does not

cross link antigen epitopes. We have shown previously that immunolocalization of TGF- α varied significantly with the method of fixation and that consistent, quantifiable results were obtained with Omnifix fixation (17, 18). After receipt in the research laboratory, biopsy specimens were oriented “on edge” with the aid of a stereo dissecting microscope (magnification, $\times 18$) prior to paraffin blocking to obtain complete mid-axial longitudinal crypt sections from the paraffin blocked tissue. Following paraffin embedding, 4- μ m-thick sections were cut and three serial sections from a single block were mounted on coated slides (Fisher Scientific, Pittsburgh, PA).

Measurement of PGE₂ Formation. PGE₂ formation was assessed as previously described by Barnes and Lee (19). Briefly, frozen biopsies were thawed and washed in 10 ml of ice-cold Tris buffer (150 mM, pH 8.4). Biopsy tissue (~10 mg) was placed in a preweighed microfuge tube containing 1 ml of Tris buffer. The specimen was then minced for 60 s with scissors, washed by vortexing, and centrifuged at 10,000 $\times g$ for 15 s. The supernatant was discarded, and an additional 1 ml of Tris buffer was added to the tube before vortex mixing to generate prostaglandins. Each sample was incubated in a vortex mixer at 25°C for 60 s and then centrifuged again at 10,000 $\times g$ for 15 s. The supernatant was then transferred to a second microfuge tube containing 10 μ g of indomethacin dissolved in 10 μ l ethanol (to prevent further prostaglandin formation), and was used on the same day in PGE₂ determination by RIA, as described previously (20). Antibodies against PGE₂ and PGE₂ standards were obtained from Sigma Chemical Co. (St. Louis, MO) and were reconstituted for RIA 30 min prior to the assay. The RIA has been validated by high-performance liquid chromatography (20), and the coefficient of variation for repeat measurement of the same sample was 5%. The capability of the biopsy specimens to generate and release PGE₂ into the supernatant was expressed in picograms per milligram of tissue weight per minute.

Immunohistochemistry. Routine immunohistochemical techniques were used to localize TGF- α epitopes in deparaffinized slide-mounted tissue sections. TGF- α primary antibody (clone 231-4.4, 1:10 dilution; Oncogene Science, Cambridge, MA) was used in TGF- α localization with a routine streptavidin-biotin immunohistochemical technique at room temperature as described previously (17). Briefly, following an endogenous peroxidase block (3% H₂O₂, 5 min) and a protein block (1% goat serum, 20 min), slides were incubated with an anti-TGF- α monoclonal antibody (1:10 dilution, 45 min). Localization of the bound anti-TGF- α antibody was achieved with a biotin-linked antimouse IgG and streptavidin-linked horseradish peroxidase (1:25 dilution, 20 min each) followed by diaminobenzidine staining (1 mg/ml, 5 min) and hematoxylin counterstain. A slide-mounted section of rat colon known to express TGF- α was included with each group of slides stained as a positive control for TGF- α staining. Negative control slides were incubated with normal mouse serum in place of the primary antibody but were otherwise treated the same.

Calculation of TGF- α Parameters. Only complete mid-axial, longitudinally sectioned crypts were selected for determining the extent of TGF- α immunoreactivity. TGF- α staining indices were quantified in terms of the percentage of staining in the entire crypt by two investigators (W. E. H. and I. L. C.) independently, and the interobserver variation was <5%. This method has been validated by our group previously (17). Parameters recorded for TGF- α staining in an average of seven crypts per biopsy were: (a) total crypt height in number of cells and (b) number of cells that exhibited positive stain in a

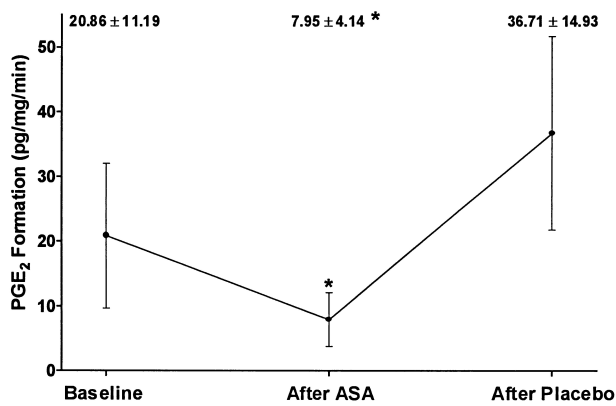


Fig. 1. Data points, mean PGE₂ formation (pg/mg tissue/min) in rectal biopsy tissue from individuals taken after a 1-month placebo run-in period (*Baseline*), after 3 months of consuming 81 mg of aspirin per day (*After ASA*), and after a 3-month placebo washout period (*After Placebo*); bars, SE. The values at each time point are shown at the top. *, $P < 0.05$. *After ASA* values were significantly less than those at the other two time points. There was not a significant difference between the *Baseline* and the *After Placebo* time points.

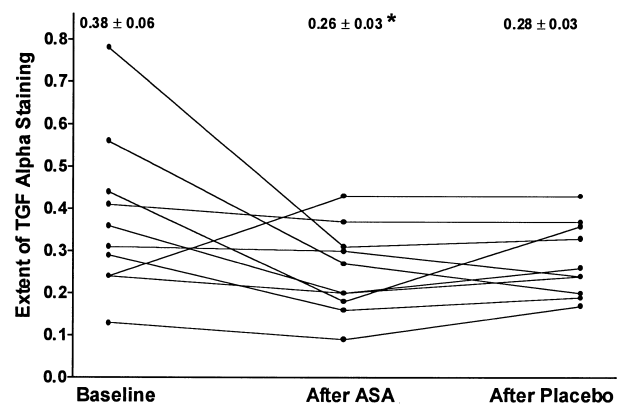


Fig. 2. Extent of TGF- α staining of rectal crypts (*i.e.*, fraction of stained cells) from biopsies of individuals taken after a 1-month placebo run-in period (*Baseline*), after 3 months of consuming 81 mg of aspirin per day (*After ASA*), and after a 3-month placebo washout period (*After Placebo*). Data points, individual means at single time points. The values at each time point are shown at the top. *, $P < 0.05$. Overall TGF- α staining was significantly less in the *After ASA* group as compared to *Baseline*, with no significant differences between the *After ASA* and *After Placebo* time points.

continuous column from the mouth down the side of the crypt. Solitary TGF- α -positive cells and cells at the bottom of the crypt were not included in the count. The positively stained fraction of the crypt was calculated as the number of cells with cytoplasmic and nuclear stain (counted from the top down), divided by the crypt height in number of cells. Because TGF- α is normally localized in the upper one-third to two-thirds of human colon crypts (14, 17), any reduction in the percentage of TGF- α staining was generally a decrease in the extent of TGF- α staining from the luminal surface down the crypt epithelial cell column.

Statistical Analyses. Prism (GraphPad Software, San Diego, CA) statistical software was used for statistical analyses. Due to the small sample size and the biological complexity and intra-subject variability of biomarker measurements, data presented here were analyzed by nonparametric methods. Data on the extent of TGF- α immunoreactivity and prostaglandin data were analyzed for differences between the different biopsy time points for each individual using Wilcoxon signed rank tests. Significance was accepted with a P of <0.05 .

Results

The effect of aspirin on rectal mucosal PGE₂ synthesis is shown in Fig. 1. Three months of 81 mg of aspirin daily significantly suppressed PGE₂ synthesis, but this significant suppression was completely reversed when aspirin was withdrawn (*i.e.*, after the 3-month placebo-washout period).

The extent of TGF- α staining in rectal crypts of individuals taking aspirin for 3 months was also significantly reduced ($P = 0.039$) compared to paired baseline measurements (Fig. 2). Indeed, 8 of 10 study patients showed decreased TGF- α staining. After a 3-month washout period, however, the mean extent of TGF- α staining was not significantly different from that of either baseline or aspirin at the same time point.

Subsequent analysis of the TGF- α data suggested that the study subjects may be divided into two groups: responders ($n = 6$; TGF- α staining reduced by $>25\%$) and nonresponders ($n = 4$; $<25\%$ change in TGF- α staining). As shown in Table 1, subgroup analyses demonstrated that the TGF- α responders accounted for the significant reduction in TGF- α staining ob-

served in the whole group analysis, whereas TGF- α staining was not significantly altered by aspirin or placebo in the non-responders. On the other hand, the reduction in PGE₂ values by aspirin was significant in the responders and nearly significant in the nonresponders ($P = 0.06$). Although the interpatient variability in PGE₂ values resulted in only modest changes in the mean PGE₂ values after aspirin treatment, when each individual served as his/her own control, the mean reductions in PGE₂ synthesis were 60 and 69% for the TGF- α responders and nonresponders, respectively.

Furthermore, none of the subjects reported any dyspeptic symptoms while taking the study medications, and no aspirin-related gastrointestinal complications (such as gastroduodenal ulceration or bleeding) were noted in any subjects during this study. All subjects tolerated the biopsies well, and no complications (*i.e.*, excessive bleeding requiring hemostasis therapy or delayed bleeding) were encountered as a result of sigmoidoscopy and/or biopsies during the entire study.

Discussion

The extended period of time required for the development of colorectal cancer in humans necessitates the use of end points that are intermediate to colorectal cancer development for testing the efficacy of interventions. Indeed, the identification of surrogate end point biomarkers that are altered early in the disease process and that are modified in a predictable direction in short-term studies would reduce the cost of chemoprevention trials and strengthen the rationale for long-term trials with promising chemoprevention agents (3).

Aspirin is a putative colorectal cancer chemopreventive agent that, when used on a regular basis for an extended time, may effectively reduce colorectal cancer morbidity and mortality (2, 4, 5). However, several questions regarding aspirin's mechanism of chemoprevention and a proper dosing regimen remain to be answered prior to recommendation of its use in the general population for preventing colon cancer (21). One putative mechanism of chemopreventive action for aspirin and other NSAIDs is suppression of prostaglandin production. PGE₂ concentrations are significantly increased in colon cancer

Table 1 Subgroup analyses of TGF- α and PGE₂ data according to TGF- α response

	TGF- α staining			PGE ₂ (pg/mg/min)		
	Baseline ^a	After ASA ^a	After placebo ^a	Baseline	After ASA	After placebo
TGF- α responders ^b	0.43 \pm 0.09	0.20 ^c \pm 0.03	0.25 \pm 0.03	30 \pm 18	12 ^d \pm 6.6	42 \pm 22
TGF- α nonresponders	0.30 \pm 0.04	0.33 \pm 0.05	0.33 \pm 0.05	7.2 \pm 2.1	2.6 \pm 1.5	28 \pm 21

^a Data represent values are a 1-month placebo run-in period (baseline), after 3 months of consuming 81 mg of aspirin per day (after ASA), and after a 3-month placebo washout period (after placebo).

^b Responders ($n = 6$), individuals with >25% change in TGF- α immunoreactivity after aspirin treatment; nonresponders ($n = 4$), individuals with <25% change.

^c $P < 0.05$ versus baseline values.

^d $P < 0.05$ versus baseline values and after placebo values.

(4), but aspirin use can significantly decrease colon mucosal PGE₂ concentrations through inhibition of COX, the rate-limiting enzyme in prostaglandin production. Two COX isozymes have been identified to date, a constitutively expressed form (COX-1) and an inducible form (COX-2). Aspirin is a potent inhibitor of COX-1 and a weak inhibitor of COX-2 (22), yet only expression of the inducible COX-2 isozyme has been shown to increase in tumor tissue (5, 23). Thus, recently developed selective COX-2 inhibitors, which have proven efficacious against colon cancer in rodent models (24, 25), may be potent human colorectal cancer chemopreventive agents.

Moreover, an effective low dose of aspirin with chemopreventive benefit and minimal side effects needs to be established. Toward that end, we have shown here that 81-mg aspirin tablets taken once a day were well tolerated and significantly suppressed rectal mucosal PGE₂ formation. Other investigators have shown that short-term use of various aspirin doses (*i.e.*, between 81 and 650 mg of aspirin per day) significantly suppressed rectal mucosal PGE₂ concentrations (8, 9). However, this is the first report of significant suppression of rectal PGE₂ synthesis following long-term daily ingestion of 81 mg of aspirin, the same aspirin dose recommended for prevention of cardiovascular morbidity (26). Moreover, PGE₂ suppression was reversible upon aspirin cessation. Thus measurement of rectal mucosal PGE₂ synthesis may serve as a confirmation of compliance to aspirin therapy as well as a surrogate end point biomarker of aspirin chemoprevention.

The extent of TGF- α stain in human colorectal crypts appears to meet the majority of requirements for a useful surrogate end point biomarker. TGF- α is a mitogenic and differentiation-signaling peptide produced by normal colonic epithelium and is found in the epithelial cells located in the upper one-third to two-thirds of the normal human colon crypt (14, 27). Epithelial cells in the proliferative zone of colon crypts have epidermal growth factor receptors present on their basolateral surface (28, 29), making them capable of interacting with TGF- α . It has been proposed that TGF- α may contribute to the progression of the transformed phenotype independent of growth stimulation (30). Furthermore, TGF- α is overexpressed in 24% of colonic adenomas and 81% of colonic adenocarcinomas (15). Serum levels of TGF- α are also significantly elevated in individuals with colorectal cancer (31, 32). Moreover, we have previously shown that in patients with a history of adenomas, dietary cellulose intervention effectively reduces the extent of TGF- α stain in human rectal crypts (17). Thus, a decrease in TGF- α , an endogenously produced growth factor that is promotional for colorectal cancer, in colorectal mucosa could serve as a biomarker for reduced risk of colon cancer. In this study, significant differences in the extent of TGF- α staining were detected following aspirin treatment in both the whole group and subgroup analyses despite the relatively small number of subjects per group. Although the suppression of TGF- α

staining pattern was not significantly reversed after cessation of aspirin treatment, this long-lasting aspirin effect may fade after 3 months.

In summary, 81 mg of aspirin effectively reduce PGE₂ in rectal mucosa of humans with a history of adenomatous polyps. The reversibility of PGE₂ suppression after aspirin cessation may add to the usefulness of PGE₂ as a surrogate end point biomarker. Also, this second demonstration of TGF- α as an effective surrogate end point biomarker is further evidence that this biomarker may be useful in chemoprevention trials. However, additional work must be performed to understand the physiological implications of reduced TGF- α staining. In addition, it is necessary to validate that alteration of either of these putative surrogate end point biomarkers is predictive of reduced colon cancer risk. Overall, however, rectal mucosal PGE₂ formation and the extent of TGF- α immunoreactivity in rectal biopsies from subjects with a history of adenomatous polyps appear to be useful biomarkers of chemopreventive efficacy.

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