A Dose-finding Study of Aspirin for Chemoprevention Utilizing Rectal Mucosal Prostaglandin E\textsubscript{2} Levels as a Biomarker\textsuperscript{1}

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Abstract

Epidemiological and experimental evidence indicates that aspirin can protect against colorectal cancer. Aspirin inhibits cyclooxygenase enzymes and blocks prostaglandin (PG) biosynthesis. Using rectal PGE\textsubscript{2} levels as a mucosal biomarker, we sought to determine the optimal aspirin dose that would significantly suppress PGE\textsubscript{2} levels for chemoprevention trials. We conducted a randomized, double-blinded study in 810 subjects with prior sporadic colorectal adenomas and evaluated three aspirin doses (81, 325, and 650 mg) or placebo taken daily for 4 weeks. PGE\textsubscript{2} levels in rectal biopsies performed at baseline and week 4 were analyzed by competitive immunoassay. Plasma salicylate levels, pill counts, and subject calendars were used to assess compliance. The 81-mg aspirin dose significantly suppressed PGE\textsubscript{2} levels relative to placebo (\(P = 0.005\)) and did so to an equivalent extent as did higher doses (\(P > 0.4\)) in evaluable subjects (\(n = 55\)) over a 4-week treatment period. Serum salicylate levels were associated with aspirin dose (\(P = 0.0002\)). Pill counts and calendars indicated that \(>98\%\) of doses were taken by all subjects. No adverse events occurred in this short-term study. The 81-mg daily aspirin dose suppressed PGE\textsubscript{2} levels to an equivalent extent as did the 650-mg dose and supports the use of this dose for chemoprevention trials.

Introduction

With an estimated 130,200 new cases and 56,300 deaths in 2000, CRC\textsuperscript{3} is the fourth most common cancer among men and women in the United States and is the second leading cause of cancer-related death (1). Epidemiological studies indicate that frequent and prolonged intake of aspirin is associated with a marked reduction in CRC incidence and mortality (2–7). Furthermore, aspirin and other NSAIDs are effective chemopreventive agents in carcinogen-treated and genetically manipulated rodent models of colon carcinogenesis (8–10). The best-defined target of NSAIDs is COX, which catalyzes PG production from arachidonic acid (11). COX exists in both constitutive (COX-1) and inducible (COX-2) isoforms (11). COX-2 expression is induced by growth factors, tumor promoters, and cytokines and is up-regulated at sites of inflammation and in colorectal neoplasms (12–14). The most abundant PG in human colorectal tumors is PGE\textsubscript{2} (15). PGE\textsubscript{2} has been shown to block apoptosis induced by a selective COX-2 inhibitor and to induce the antiapoptotic Bcl-2 protein in colon carcinoma cell lines (15). PGE\textsubscript{2} levels are suppressed by NSAIDs (10, 15, 16) and may serve as a surrogate end point biomarker in chemoprevention studies. Whereas the mechanisms underlying the antitumor effects of NSAIDs in the colorectum are incompletely understood, they appear to involve both COX-dependent and -independent effects (16–18). NSAIDs exert antiproliferative and proapoptotic effects and may also influence angiogenesis (17) as well as the activity of mitogen-activated protein kinases and nuclear factor \(\kappa\)B (16). Recent evidence suggests that peroxisomal proliferator-activated receptor \(\delta\) is a target for NSAIDs (18).

The purpose of this study was to determine the optimal aspirin dose that would reproducibly and significantly suppress PGE\textsubscript{2} levels in rectal mucosa from patients with prior sporadic colorectal adenomas who are at risk of recurrent adenomas and cancer. Optimal doses of chemopreventive agents including aspirin remain undetermined, largely due to the lack of an established surrogate end point biomarker. Determining optimal dosages is of utmost importance for achieving efficacy while avoiding significant toxicity. GI and other toxic effects of NSAIDs are believed to occur secondary to inhibition of the constitutive COX-1 enzyme (11). Aspirin predominantly suppresses the COX-1 isof orm, and therefore determination of the lowest effective aspirin dose is important when considering the risk:benefit ratio for chemoprevention (11, 19). Aspirin is an attractive agent for chemoprevention given its widespread usage, known toxicity profile, established benefit for cardiovascular prophylaxis in men (20), and its low cost. Selective COX-2 inhibitors are commercially available and demonstrate reduced GI mucosal toxicity, at least in short-term studies (19, 21). However, their efficacy as chemopreventive agents in patients with sporadic colorectal adenomas is unknown, and their cardiovascular benefits remain in question (21). Furthermore, selective COX-2 inhibitors are significantly more expensive than aspirin, which is an important issue when considering the large number of subjects at increased risk for colorectal adenomas and cancers.
Materials and Methods

Study Design and Patient Population. We conducted a Phase II randomized, double-blinded, and placebo-controlled trial in patients with prior sporadic colorectal adenomas to determine the minimal daily dose of aspirin that would significantly and reproducibly lower PGE₂ levels in rectal mucosal biopsy specimens. Sixty subjects (34 males and 26 females; age range, age 40–80 years) with a history of colorectal adenoma(s) within the past 5 years that was ≥2 mm in size and a colonoscopy within the previous 3 years were eligible and randomized. The subject population was recruited from a health maintenance organization in Houston, Texas over a 2-year period. Dosage levels of aspirin examined included 81, 325, and 650 mg and placebo taken once daily for 4 weeks (last day of drug administration). Rectal biopsies were obtained at endoscopy at baseline and at 4 weeks. Informed consent was obtained from each subject. Patients were regarded as evaluable if they completed both baseline and week 4 endoscopic exams.

Patient demographics were sought and included age, gender, ethnicity, education, occupation, and income level. Eligible participants could not have taken aspirin or other NSAIDs at a frequency of more than four 325-mg doses per month for the 4 months prior to enrollment. Patients were ineligible if they had a history of invasive cancer in the previous 5 years, right hemicolectomy, a personal or family history of familial adenomatous polyposis or hereditary nonpolyposis CRC, or prior pelvic radiation. Participants were paid $200.00 upon completion of the enrollment period and provided with parking vouchers and a souvenir coffee mug encrypted with the name and slogan of the study.

Study Drug and Procedure. Aspirin (81, 325, and 650 mg) and matching placebo tablets were supplied by Bayer Corp. (Consumer Care Division, Bayer Corp., Morristown, NJ). The drug was given as a single daily dose of one tablet, and subjects were instructed to swallow and not chew the tablets. Subjects in the placebo arm took a tablet containing dextrose. Subjects were randomized in blocks of four using The University of Texas M. D. Anderson Cancer Center Protocol Data Management System. Fifteen subjects were assigned to each of the following doses: placebo or aspirin at 81, 325, or 650 mg p.o. daily. Study participants were required to undergo a history and physical examination at baseline and an unprepared flexible sigmoidoscopy both at baseline and at 4 weeks. Pretreatment evaluation included blood studies (complete blood count, platelet count, and SMAC profile) within 8 weeks of first study drug dose and on the day of the second endoscopy. Before and after endoscopies, blood was obtained for measurement of plasma salicylate levels. Before entering the trial, all study procedures were reviewed with the patient in detail to help ensure compliance, including instruction to avoid other medications containing aspirin or other NSAIDs for the duration of the study. Subjects were supplied with acetaminophen for use as an analgesic if required during the trial. Dietary assessment was obtained from each subject at baseline and at the conclusion of the study using the Harvard Food Frequency Questionnaire to monitor total calories, fat, fiber, calcium, and other major dietary components. Such data were collected for subsequent analysis in conjunction with additional biomarker assays not included in this report. Subjects were instructed not to change their diet or consume new dietary supplements during the trial.

An unprepared flexible sigmoidoscopy was performed on the first day of the trial. Study drug was initiated after this procedure and assurance of acceptable laboratory results. A second unprepared flexible sigmoidoscopy was performed on the last day of study medication intake. Rectal mucosal biopsies were obtained at each procedure at a distance of 8–10 cm from the anal verge using standard biopsy forceps. The rectum was chosen for sampling, given that patients underwent unprepared endoscopic exams. The left bowel preparation was omitted to avoid potential interference with biomarker assays by the preparation solution. Three mucosal biopsy specimens were obtained for PGE₂ measurements at baseline (pretreatment) and week 4 (posttreatment) exams and immediately placed into an indomethacin (5 µg/ml)-containing mixture and then snap-frozen in liquid nitrogen. Other specimens were individually placed on tin foil in Nunc tubes and flash-frozen in liquid nitrogen within 10 s of biopsy. Specimens were stored at −80°C until the time of analysis. Specimens intended for proliferation assays were placed on bilbous paper and immediately placed into a tube containing normal saline for transfer to the laboratory, where each was oriented and then fixed in alcohol within 3–5 min of biopsy procurement.

Toxicity was evaluated by weekly telephone contact, and subjects were encouraged to contact the investigator or study nurse if toxicity occurred between these calls. Compliance with the study drug was monitored through weekly telephone calls, completion of a drug calendar, pill counts, and plasma salicylate measurements.

PGE₂ Content in Mucosal Biopsies. Analysis of PGE₂ in rectal mucosa was performed using a competitive immunonasay, as described previously (22). Snap-frozen biopsies were placed in 2.0 ml of ice-cold 0.05 M Tris–HCl buffer (pH 7.4) containing 5 µg/ml indomethacin and homogenized for 1 min on ice using four 15-s bursts. A 100-ml aliquot was removed for crude protein determination using the BCA Protein Assay (Pierce, Rockford, IL). The homogenate was then transferred to 30-ml siliconized Corex tubes, and methanol: H₂O (acidified to pH 3.0) was added to yield a 20% final concentration. After centrifugation at 10,000 × g, the supernatant was applied to a preconditioned C18 silica gel column (Alltech, Deerfield, IL). The column was washed with petroleum ether:ethyl ether (9:1), and PGE₂ was eluted with methanol into siliconized tubes. The methanol was dried under nitrogen, and the sample was frozen at −80°C until the time of the RIA. The RIA was performed according to manufacturer’s instructions (NEN Life Sciences, Boston, MA). Data are expressed as pg PGE₂/µg protein.

Salicylic Acid in Plasma. The aspirin metabolite, salicylic acid, was assayed from 1 ml of plasma to which 10 µl of m-hydroxybenzoic acid (70 µg/liter) were added as an internal standard. One ml of 1.0 M oxalic acid and 10 ml of hexane-ether (1:1 volume) were added to this solution, and the solution was then shaken vigorously for 1 min and centrifuged at 514 × g for 5 min at 4°C. The organic layer was then removed, and to it was added 300 µl of 0.5 M phosphate buffer (pH 7.0), and the solution was then shaken and centrifuged as described above. From the aqueous phase, 200 µl was removed, and 200 µl of 1.0 M phosphate buffer (pH 2.0) were added and vortexed for 10 s. The sample was then subjected to HPLC assay using methods described previously (23, 24). Sample analysis was performed in triplicate, and the mean values and SDs were determined.

Statistical Analysis. Analysis of PGE₂ levels in relation to aspirin dose was performed by fitting a linear mixed model that took into account three separate measurements at two time points for each subject. The three PGE₂ measurements obtained pretreatment and posttreatment were averaged for each individual. The results of comparing pretreatment and posttreatment PGE₂ values for each aspirin dose level are presented in...
a box plot (Fig. 1). The CV for PGE₂ at pretreatment and posttreatment time points for all four dose levels was calculated as the SD/mean × 100 and expressed as a percentage. Median CV values of <50% were interpreted as showing moderate reproducibility. Mean differences in plasma salicylate levels were analyzed using the (two-sided) Kruskal-Wallis rank-sum test, and pairwise comparisons were performed using Tukey’s method with 95% confidence intervals for specified linear combinations. Statistical significance was defined as \( P < 0.05 \).

**Results**

**Subject Demographics.** Fifty-five of 60 (92%) registered subjects were evaluable (placebo, \( n = 14 \); 81 mg, \( n = 13 \); 325 mg, \( n = 14 \); 650 mg, \( n = 14 \)). Inevaluable subjects included those with abnormal baseline laboratory tests (\( n = 2 \)), study dropouts (\( n = 2 \)), and those admitting to regular NSAID use after study registration (\( n = 1 \)). Demographic variables on the 55 evaluable subjects are shown in Table 1. There were 31 men and 24 women with an overall mean age of 58.2 years. All but one patient reported completing high school, and 53% reported completing 16 or more years of education. Eighty-one percent of the subjects were Caucasian, 79% were married, and 74% reported a professional occupation. Fifty-three percent of subjects worked full-time, and 27% were retired. Fifty-eight percent reported a family income of at least $50,000/year.

**Rectal Mucosal PGE₂ Levels.** All aspirin doses (81, 325, and 650 mg), but not placebo, were found to significantly suppress PGE₂ (pg/μg protein) levels in rectal mucosal biopsy specimens obtained at 4 weeks compared with baseline (Fig. 1). We found that the 81-mg dose significantly suppressed PGE₂ levels relative to placebo (\( P = 0.005 \)), as did the higher doses (325 mg, \( P = 0.0004 \); 650 mg, \( P = 0.0001 \)). The extent of PGE₂ suppression (posttreatment minus pretreatment) at week 4 (mean suppression: 77%, 81 mg; 70%, 325 mg; and 79%, 650 mg) of continuous drug administration did not differ among aspirin doses (\( P > 0.40 \)). Therefore, the lowest aspirin dose evaluated, i.e., 81 mg, was as effective as the highest dose, 650 mg, in suppressing PGE₂ levels in rectal mucosa. Reproducibility of PGE₂ measurements was determined using the CV. Median CV values (percentages) were calculated from three repeat measurement both pretreatment and posttreatment for each study arm (Table 1). PGE₂ results were moderately reproducible as indicated by median CVs of <50%. For the 81-mg dose, the higher CV found for the baseline PGE₂ was due to marked variability among three subjects.

**Plasma Salicylic Acid Levels.** Levels of the salicylate metabolite of acetylsalicylic acid (aspirin) were determined in plasma stored at −80°C using HPLC. Blood was drawn within 6 h of the last aspirin dose on the day of endoscopy for measurement of salicylates. Salicylate levels were available for analysis from 50 of 55 evaluable subjects (91%) at baseline and at week 4 (data not shown). Mean differences (posttreatment minus pretreatment) in salicylate levels were positively associated with aspirin dosage (\( P = 0.0002 \)). Pairwise comparisons were performed of salicylate levels among the placebo and the three aspirin doses. The mean increases in salicylate levels were statistically significant only when comparing the 650 mg aspirin dose with lower doses.

**Compliance and Toxicity.** Of the 55 evaluable participants, pill counts for all study arms indicated 99% compliance with the prescribed dosing. Subject self reports, as recorded in patient calendars, were concordant with pill count data. Plasma salicylate levels, as determined by HPLC, demonstrated that 93% of subjects taking 81 mg aspirin/day, 100% of those taking 325 mg aspirin/day, and 79% of those taking 650 mg aspirin/
day had an end of study salicylate level that significantly exceeded the baseline value. No difference in reported or elicited symptoms or significant adverse effects were found for participants during the course of this study for any aspirin dosage compared with placebo.

Discussion
We conducted this study to determine the optimal dose of aspirin for a planned Phase II chemoprevention trial in subjects at increased risk of CRC. We evaluated PGE$_2$ levels in rectal mucosa as a surrogate end point biomarker, given that aspirin inhibits PG synthesis (10) and the fact that PGE$_2$ is increased in colorectal neoplasms and appears to play a role in colon carcinogenesis (15, 16). PG inhibition by NSAIDs may contribute to the chemopreventive effects of these drugs against CRC; however, this may not be the primary mechanism. In this regard, studies to evaluate PGE$_2$ levels in relation to risk of adenomas and cancer and as a predictor of polyph prevention and/or regression in clinical trials are awaited. We found that the 81 mg/day aspirin dose significantly suppressed PGE$_2$ levels relative to placebo ($P = 0.005$), and suppressed PGE$_2$ levels to an equivalent extent as did aspirin doses of up to 650 mg/day ($P > 0.14$). Plasma salicylate levels were positively associated with prescribed aspirin dosing and indicated compliance with the study medication. Salicylate levels were detected in all evaluable subjects within approximately 6 h of the last aspirin dose, in contrast to a report in healthy subjects wherein salicylate levels were undetectable in plasma 24–30 h after ingestion on day 14 of daily aspirin intake (25). In this study, no toxicity was observed for any aspirin dose taken daily for 4 weeks. GI toxic effects from aspirin and other NSAIDs are dosage dependent and are most frequent during the first month of regular intake (26). Accordingly, identification of the lowest effective dose for chemoprevention is of critical importance. Our data demonstrate the utility of PGE$_2$ as a biomarker of aspirin intake in patients with prior colorectal adenomas and support the use of the 81-mg daily dose for chemoprevention studies in subjects at increased risk of CRC. Based upon this result, we initiated a Phase II randomized trial evaluating aspirin (81 mg daily) alone and in combination with calcium carbonate in this same patient population.

Given that GI mucosal toxicity secondary to aspirin and other NSAIDs is dependent upon dosage and duration (26), our finding for aspirin is important for chemoprevention studies in which such agents are taken regularly and over a protracted time period. Epidemiological studies demonstrating a reduction in CRC incidence and mortality have largely evaluated aspirin (2–7). Aspirin is an attractive chemopreventive agent, given that it is widely used and efficacious for cardiovascular prophylaxis (20) and is well tolerated at low doses. Aspirin inhibits the COX-1 isofrom and, to a lesser extent, the COX-2 isofrom, and it is unclear at this time whether inhibition of both isofroms is required for maximal chemopreventive efficacy (10, 11, 16). It will be difficult to address this issue in clinical trials, given the frequent use of aspirin for cardiovascular prophylaxis in candidates for polyp prevention studies. As a result, many ongoing trials permit the use of low-dose aspirin out of necessity to accrue study patients. Additionally, current data suggest that the selective COX-2 inhibitor rofecoxib may not provide protection from myocardial infarction, as do aspirin and other NSAIDs (21). Selective COX-2 inhibitors produce less GI mucosal toxicity than do other NSAIDs in short-term studies (19, 21). However, these drugs are considerably more expensive than aspirin and other conventional NSAIDs, which may limit their cost effectiveness for chemoprevention.

Our study participants had prior premalignant colorectal adenomas and were motivated, as indicated by their high rate of compliance with study drug intake, maintenance of a written record of compliance, and completion of two nonsedated sigmoidoscopies at our cancer center. Other likely contributors to this outcome were the study’s short duration and research nurse encouragement. Furthermore, our study population demonstrated high rates of compliance with colonoscopic surveillance. In a clinical trial among women at high risk of breast cancer, educational level was found to be the key determinant in the decision to participate (27). The majority of participants in our study reported at least some college education, classified their employment as professional, and reported a household income in excess of $50,000/year. These factors tend to select patients who are motivated toward preserving and improving their personal health and, in general, characterize prevention study candidates. An analysis of factors motivating participation in this study and the influence of study participation on post-study aspirin intake are reported separately (28).

In conclusion, the 81-mg daily aspirin dose significantly suppressed PGE$_2$ levels in rectal mucosa and suppressed PGE$_2$ levels to an equivalent extent as did higher doses. Therefore, 81 mg is the desired aspirin dose for evaluation in Phase II-III chemoprevention trials in sporadic adenoma patients.

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References


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