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Tissue prostanoids as biomarkers for chemoprevention of colorectal neoplasia:

correlation between prostanoid synthesis and clinical response in familial adenomatous polyposis★

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Abstract

Recent studies indicate that sulindac, a nonsteroidal anti-inflammatory drug (NSAID), lowers mucosal prostanoid levels and regresses colorectal adenomas in patients with familial adenomatous polyposis (FAP). To determine whether they are biomarkers for sulindac-mediated chemoprevention of colorectal adenomas, levels of 5 prostanoids [prostaglandin (PG) D₂, PGE₂, PGF_{2α}, thromboxane B₂, and 6-keto-PGF_{1α}] in the normal-appearing rectal mucosa from 7 FAP patients with a history of subtotal colectomy and ileorectal anastomosis and 4 FAP patients without surgery, were measured in the absence or presence of exogenously added arachidonic acid before the initiation and at the end of 3 months of sulindac treatment. The addition of arachidonic acid resulted in a uniform increase in the levels of all 5 prostanoids although this increase was selectively attenuated in patients with ileorectal anastomosis who took sulindac. In the latter patients, arachidonic acid also augmented the inhibition of prostanoid synthesis by sulindac. In contrast, sulindac failed to attenuate the increase in prostanoid levels resulting from arachidonic acid in patients without previous surgery. Importantly, when measured in the presence of arachidonic acid, the reduction in the levels of all 5 prostanoids due to sulindac was statistically correlated with a reduction in the size and number of adenomas in the two groups of patients combined. These results suggest that tissue prostanoids measured in the

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presence of arachidonic acid may serve as sensitive and reliable biomarkers in monitoring the clinical responsiveness of FAP patients undergoing chemoprevention for colorectal neoplasia with NSAIDs.

Keywords

Prostanoids; Prostaglandins; Arachidonic acid; Familial adenomatous polyposis; Chemoprevention; Sulindac

1. Introduction

Colon cancer is the second leading cause of death from cancer in the United States [1]. Epidemiological, clinical and genetic evidence indicates that most malignant colorectal cancers arise from benign adenomas [2]. Recent research has focused on the ability of nonsteroidal anti-inflammatory drugs (NSAIDs) to prevent colorectal neoplasia including cancers and adenomas (reviewed in Refs. [3-6]). The observation of a reduction in the incidence of colorectal cancer in regular aspirin users was first reported a little over a decade ago [7] and confirmed by many subsequent studies [8-11]. Other NSAIDs including sulindac have been shown to cause regression of adenomas in familial adenomatous polyposis (FAP) [12-16] and in sporadic adenomas [17-19]. Moreover, NSAIDs are effective in reducing both the incidence and multiplicity of tumors in various rodent models of intestinal and colonic neoplasia [20-26]. These studies provide powerful evidence that NSAIDs are effective agents for the chemoprevention of colorectal neoplasia.

Although the mechanism of action of NSAIDs in preventing colorectal neoplasia has not been clearly established, the primary biochemical effect of these agents is to inhibit cyclooxygenase (COX) or prostaglandin H synthase [27]. Recent studies demonstrate the presence of two distinct isoforms of cyclooxygenase, COX-1 and COX-2 [28-30]. Of the two isozymes, synthesis of COX-2 is induced by a variety of extracellular stimuli. Importantly, COX-2 expression is up-regulated in colorectal neoplasia when compared to normal colon in both human and animal models of colorectal tumorigenesis [31-34]. Evidence for a direct role for COX-2 in intestinal tumorigenesis is provided by a recent study that showed a dramatic reduction in polyp number in the offspring of mice lacking the adenomatous polyposis coli (APC) gene, a model of human FAP, that were mated to mice lacking the COX-2 gene [35].

Prostanoids, a collective term that includes prostaglandins, prostacyclins and thromboxanes, are the primary cellular metabolites of cyclooxygenases. Consistent with an elevation of COX-2 in tumor tissues, levels of certain prostanoids such as prostaglandin E₂ (PGE₂) are increased in neoplastic colonic tissues as compared to normal mucosa in both humans and experimental animals [36-39]. Several recent studies also showed that a reduction in mucosal prostanoid levels due to sulindac treatment in FAP patients accompanied regression of colonic adenomas [13,15,40]. These findings suggest that prostanoids may play a potentially important pathophysiological role in the process of colorectal tumorigenesis. Finally, because of the potential involvement of COX-2 in the pathogenesis of colorectal neoplasia, novel COX-2-specific inhibitors are being investigated as agents for chemoprevention [41].

At the present time, there are no clearly established biomarkers to monitor the efficacy of NSAID treatment and patient compliance. Tissue prostanoids, which are the endpoints of cyclooxygenase activity, may serve as potential biomarkers. In addition to the evidence from human investigations [13,15,40], many animal studies also support a correlation between a reduction in tissue prostanoid levels and the clinical efficacy of chemoprevention by NSAIDs [21,24,25]. Our laboratory recently developed a simple and reliable assay using gas chromatography-mass spectrometry to simultaneously quantify 5 major prostanoids in biopsy

specimens of rectal mucosa obtained from FAP patients and control individuals [38,40]. In the current study, we refined this assay to include a stimulation of prostanoid synthesis in vitro when biopsy specimens were incubated in the presence of exogenously added arachidonic acid after tissue homogenization. We show that when measured in the presence of arachidonic acid, the reduction in prostanoid synthesis correlates with the clinical response of FAP patients to sulindac, as judged by changes in the size and number of adenomas. The results suggest that tissue prostanoids, when measured by this refined methodology, may serve as sensitive and reliable biomarkers for monitoring the clinical progression of polyposis in patients undergoing NSAID-mediated chemopreventive treatments.

2. Materials and methods

2.1. Study subjects and specimen collection

Seven FAP patients (3 males and 4 females; mean age, 34 ± 13 years) with a history of subtotal colectomy and ileorectal anastomosis, and 4 FAP patients (2 males and 2 females; mean age, 18 ± 5 years) without prior surgery were included in the study. Each patient was given sulindac 150 mg orally twice a day (mean dosage, 4.2 ± 1.2 mg/kg/day) for 3 months. The inclusion and exclusion criteria, and the methods for evaluating the clinical response to sulindac treatment (changes in the size and number of adenomas), were previously described [16]. Informed consent was obtained from all patients before acceptance into the protocol.

Rectal mucosal specimens were collected before the initiation and at the end of sulindac treatment using standard biopsy forceps through a flexible sigmoidoscope. Tissues were taken from the normal-appearing mucosa at 20 cm from the anal verge, snap-frozen in liquid nitrogen, and stored at -70°C until further analysis. Additional specimens were obtained in the same region for histopathological examination. None of the endoscopically normal-appearing mucosa showed evidence of adenomatous epithelium or aberrant crypt foci formation.

2.2. Specimen processing and prostanoid determination

The processing of biopsy specimens for prostanoid determination was previously described [38,40]. Briefly, specimens were thawed on ice and manually homogenized in a glass micro-homogenizer (Kontes Scientific Glassware, Vineland, NJ, USA) in 50 μl HBSS (138 mM NaCl, 5 mM KCl, 4 mM NaHCO_3 , 5.6 mM D-glucose, 0.3 mM Na_2HP_4 , and 0.3 mM KH_2PO_4) containing 1 mM CaCl_2 and transferred to a microcentrifuge tube. An additional 60 μl of the same solution was used to rinse the homogenizer and added to the initial homogenate. The combined solution was then sonicated for 20 s with a Fisher Scientific Model 550 Sonic Dismembrator with a microtip probe. After sonication, tissue debris was removed by microcentrifugation at $12\,000 \times g$ for 15 s. Ten μl of the supernatant were removed for determination of protein concentrations using the BCA method (Pierce, Rockford, IL, USA). The remaining supernatant was divided into 25- μl aliquots, which were then incubated at 37°C for 30 min in the absence or presence of 10 μM arachidonic acid (Sigma Chemical Co., St. Louis, MO, USA).

At the completion of the reaction, 25 μl of deuterated prostanoid standards and 125 μl of acetone were added to each reaction, and the combined solution was dried under a steady stream of nitrogen gas. As soon as the specimen was dried, 25 μl of 2% *O*-methoxylamine HCl in pyridine were added to each sample. The samples were stored at -20°C until quantification by gas chromatography-mass spectrometry.

Prostanoid levels were determined with a Finnigan MAT SSQ771 gas chromatographmass spectrometer (Finnigan MAT, San Jose, CA, USA) [38,40]. The level of each prostanoid was

determined based on the inclusion of known quantities of deuterated prostanoids as internal standards. All prostanoid levels were normalized to the same amount of protein in the sample.

2.3. Statistical analysis

Comparison of prostanoid levels assayed under different conditions was performed by paired two-tailed Student's *t*-test. Correlation between prostanoid levels and clinical response was evaluated by two-tailed Spearman rank correlation coefficients. Significance was defined as $P < 0.05$.

3. Results

Fig. 1 shows the levels of the 5 prostanoids (PGD₂, PGE₂, PGF_{2α}, TXB₂, and 6-keto-PGF_{1α}), measured in the absence or presence of 10-μM arachidonic acid (AA), in the normal-appearing rectal mucosa obtained from 7 FAP patients with subtotal colectomy and ileorectal anastomosis (IR), and from 4 FAP patients with intact colon (IC). The measurements were performed before the initiation and at the end of 3 months of treatment with sulindac (SUL). Although AA stimulated the synthesis of the 5 prostanoids in almost all patients regardless of sulindac, the stimulation was considerably attenuated in the IR group of patients at the end of sulindac treatment (Fig. 1, second IR column). This difference is clearly illustrated in Fig. 2. Before the IR group of patients received sulindac, AA stimulated the formation of the 5 prostanoids by means ranging between 2.6- and 3-fold (Fig. 2, first column). In contrast, the degree of induction (means 1.4- to 1.5-fold) was significantly lower at the end of treatment (Fig. 2, second column; $P < 0.01$ to < 0.05). The stimulation of prostanoid synthesis by AA in the IC group of patients was not statistically different before and after sulindac treatment (Fig. 2, third and fourth column, respectively; P , 0.4 to 0.9). These results suggest that sulindac effectively suppressed the endogenous cyclooxygenase activity and resulted in a blunted response to AA, but only in the group of patients who underwent previous subtotal colectomy and ileorectal anastomosis.

The effect of including arachidonic acid in the assays on the ability of sulindac to suppress tissue prostanoid levels is shown in Fig. 3. Sulindac reduced the levels of each prostanoid (i.e. $< 100\%$ of pretreatment or baseline levels) when measured in the absence of AA in all but one patient with ileorectal anastomosis (Fig. 3, first column). The addition of AA in the assays produced an additional and statistically significant reduction in prostanoid levels resulting from sulindac intake in the same patients (Fig. 3, second column; $P < 0.01$ to < 0.05). In contrast, the changes in prostanoid levels in the IC group of patients measured in the absence of AA were more variable (Fig. 3, third column). Moreover, the addition of AA to the assays failed to alter the ability of sulindac to significantly reduce prostanoid levels (Fig. 3, fourth column; P , 0.3 to 0.8). Taken together, the results indicate that the ability of sulindac to inhibit tissue prostanoid formation was intensified when assays were performed in the presence of AA but only in those patients with previous subtotal colectomy and ileorectal anastomosis.

To determine whether an alteration in tissue prostanoid levels due to sulindac treatment corresponded to clinical response, we examined the relationship between changes in prostanoid levels and changes in polyp size and number from baseline at the end of 3 months of treatment in all 11 patients (Table 1). As shown, there was a statistically significant correlation between changes in all 5 prostanoid levels measured in the presence of AA and polyp size as well as polyp number. When measured in the absence of AA, only changes in the levels of PGD₂, PGE₂, and TXB₂ correlated ($P < 0.05$) with changes in polyp size and changes in the levels of PGD₂, PGF_{2α}, and TXB₂ correlated ($P < 0.05$) with changes in polyp number. The close correlation between the reduction in prostanoid levels and the clinically observed reduction in polyp size and number suggests that the chemopreventive effect of sulindac is reflected by its ability to inhibit tissue prostanoid formation. These findings also indicate that tissue

prostanoids measured in the presence of AA may be excellent biomarkers for chemopreventive treatments involving sulindac.

4. Discussion

In the past decade significant effort has been devoted toward the identification of chemopreventive drugs for colorectal neoplasia. Numerous studies suggest that NSAIDs may be effective agents. The identification of the potential role of COX-2 in colorectal tumorigenesis increases the opportunity to develop newer and safer inhibitors of cyclooxygenases in the fight against colon cancer. The myriad of recently developed selective COX-2 inhibitors demonstrates the enthusiasm from academia and industries alike (reviewed in Ref. [41]).

We previously evaluated predictive factors for sulindac-induced regression of colorectal adenomas in FAP patients among a multitude of variables including age, sex, body weight, drug dosage, mutation status, and surgical status [16]. By multivariate regression analysis, only one variable, that of previous subtotal colectomy with ileorectal anastomosis, was found to have a positive association with clinical response to sulindac treatment. A potential explanation for this observation may be due to the way by which sulindac exerts its anti-inflammatory effect. Sulindac, administered as a pro-drug, is metabolized to its active sulfide form by bacterial flora in the colon [42]. Consequently, a higher dose of active metabolite may be delivered to a comparatively small segment of colonic mucosa in patients who had surgical resection. This hypothesis is supported by the results of the present study. Treatment with sulindac caused a mean reduction in rectal mucosal prostanoid levels in those patients who had undergone subtotal colectomy (Fig. 3). This suggests that the active sulindac metabolite was present in sufficient concentrations in the retained rectum to exert an influence on tissue prostanoid metabolism.

Comparing to the patients with ileorectal anastomosis, our study indicates that the effect of sulindac on rectal prostanoid metabolism was much less prominent in those patients who never had surgery. Although we cannot rule out a Type II error due to the relatively small number of patients in the latter group, our results nonetheless suggest the possibility of a 'dilutional' effect from the much larger mucosal surface area in these patients compared to those with surgery. This finding contrasts with previous studies in which rectal mucosal PGE₂ levels were found reduced by the administration of aspirin to either normal subjects or patients who had colon adenomas or cancers [43-45]. This variance may be attributed to a difference in the metabolism of the two NSAIDs and by the possibility that the aspirin effect may be systemic rather than local [44]. It remains to be seen whether mucosal prostanoid levels can further be suppressed in patients with intact colons with higher dosages of sulindac.

A central issue in chemopreventive trials is the necessity to employ a sensitive and reliable method to monitor biological response and drug effectiveness. Surrogate end-point biomarkers (SEBs) have been proposed to achieve this purpose [46]. In chemoprevention of colon cancer, several potential biomarkers have been utilized. They include histological criteria such as apoptotic index [47] and aberrant crypt foci [48], and genetic criteria such as ras [49] and p53 [50] mutations. Although each of these biomarkers was shown to correlate with clinical progression of the disease, many are inherently difficult to apply to human trials. For example, the histological evaluations are primarily employed in animal studies and the genetic methods may be difficult to quantify. It is clear that a biomarker that is relatively easy to measure in a quantitative manner is desirable for large-scale chemopreventive trials in humans.

The present study established a positive correlation between the reduction in tissue prostanoid levels and clinical response, as measured by the reduction in size and number of adenomas,

when patients were treated with sulindac. However, this correlation only became consistently significant when prostanoids were assayed after tissue homogenates were first incubated with arachidonic acid (Table 1). The quantity of each prostanoid measured in the presence of exogenously added arachidonic acid represents the total or maximal synthetic capacity of the tissue for that prostanoid at the time of biopsy, as opposed to the pre-existing prostanoid when the assay is performed in the absence of arachidonic acid. The inclusion of arachidonic acid, therefore, increases not only the sensitivity of detection of tissue prostanoids but also the ability to distinguish tissues in which sulindac effectively inhibits the synthesis of prostanoids. Because inhibition of rectal mucosal prostanoids is an end-point of sulindac therapy, the use of arachidonic acid during prostanoid determination seems to be an improved and more reliable method to reflect the efficaciousness of drug treatment.

Because of the increased sensitivity in detecting sulindac-induced changes in prostanoid levels when measured in the presence of arachidonic acid, our study indicates that prostanoids determined in this fashion may qualify as SEBs in NSAID-mediated chemopreventive treatment for colorectal neoplasia. According to Einspahr et al. [46], an ideal SEB should exhibit the following properties: 1) differential expression between normal and neoplastic tissues, 2) early detection in the neoplastic pathway, (3) an association with risk of developing cancer, 4) the potential for modification by a chemoprevention agent, 5) localized to tissues that are easily accessible for multiple biopsies, and 6) the capability of developing adequate quality control procedures. The procedure that we used to sample mucosal prostanoid levels fulfills many if not all of these criteria for SEBs. For example, we and others have previously demonstrated elevated prostaglandin metabolite levels in colonic neoplasm including both adenomas and carcinomas [36-38]. The present and previous studies [43-45] indicated that administration of NSAIDs modified mucosal prostanoid levels. The rectal mucosa are easily within reach of a flexible sigmoidoscope that allows multiple and serial samplings of tissues. The procedures that we described to assay prostanoid levels are straightforward and highly sensitive. Lastly, despite the relatively high degree of inter-subject variability in the levels of mucosal prostanoids (36-38, 43-45, and this study), the assay remains valid as each individual's response is compared to his or her baseline value. Consequently, we propose that tissue prostanoids may serve as excellent SEBs to monitor the clinical response of patients in chemopreventive treatments with NSAIDs for colorectal neoplasia.

In a recent study, we reported that tissue prostanoid levels were elevated in a size-dependent manner in adenomas of FAP patients when compared to the normal-appearing mucosa [38]. Only in adenomas with a diameter of 6 mm or more did the elevation become apparent. These findings suggest that increased prostanoid levels in adenomas may have a relatively late effect during tumor promotion. Although the present study did not examine prostanoid levels within individual adenomas, it is clear that the active suppression of prostanoid formation in normal-appearing mucosa by sulindac correlated with a reduction in the size of adenomas (Table 1). It is therefore possible that larger adenomas are preferentially targeted by sulindac.

A debated issue in NSAID-mediated chemoprevention of colorectal neoplasia is whether cyclooxygenases, with the resultant production of prostanoids, are involved in the mechanism of chemoprevention. Several reports have indicated that inhibition of prostaglandin synthesis may not be necessary for the chemopreventive effect of NSAIDs [51-53]. Most of these studies used sulindac sulfone, a metabolite that does not inhibit cyclooxygenase activity, as a chemopreventive agent [51-53] and all involved animal models of colonic neoplasia. Previous studies in FAP patients, however, suggest that a reduction in tissue prostaglandin levels is associated with clinical response [13,15,40]. In addition, an equal number of chemopreventive studies in animals report a similar finding [21,24,25]. Further-more, a recent investigation in the *Min* mouse model of colorectal neoplasia compared equivalent amounts of sulindac sulfide and sulindac sulfone [54]. Only mice treated with sulindac sulfide, with an associated decrease

in tissue PGE₂ levels, had a reduction in the number of intestinal tumors. Although the discrepancy between studies may be due to a difference in the model systems employed to examine chemoprevention of colon cancer, results of our present study support the concept that prostanoids play an active role in the mechanisms of chemoprevention in FAP patients treated with sulindac.

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Fig. 1.

Prostanoid levels in biopsy specimens of normal-appearing rectal mucosa of FAP patients before and at the end of sulindac treatment. Biopsy specimens were obtained from 7 FAP patients with a history of subtotal colectomy and ileorectal anastomosis (IR) and from 4 patients with intact colon (IC), before the initiation and at the end of 3 months of treatment with sulindac (SUL). Tissues were homogenized and incubated in the absence or presence of 10 μ M arachidonic acid (AA) for 30 min before being processed for prostanoid determination by gas chromatography-mass spectrometry as described in Section 2. All prostanoid levels were normalized to a same amount of protein and shown in logarithmic scales. 6KPGF_{1α} is 6-keto-prostaglandin F_{1α}.

Fig. 2.

The effect of arachidonic acid (AA) on inducing tissue prostanoid levels before and at the end of sulindac treatment. The fold-induction in prostanoid levels due to arachidonic acid (AA) in each patient is expressed as the ratio between the prostanoid levels measured in the presence of AA and those in the absence of AA. The horizontal bar indicates the mean for each group. * $P < 0.05$ and ** $P < 0.01$ in IR group of patients at the end of treatment compared to before treatment.

Fig. 3.

The effect of sulindac on reducing tissue prostanoid levels measured in the absence or presence of arachidonic acid. The level of each prostanoid at the end of 3 months of sulindac treatment is compared to that before treatment (i.e. baseline) and expressed as the percent of the baseline level on a logarithmic scale. A less than 100% value represents a reduction in the prostanoid level caused by sulindac. * $P < 0.05$ and ** $P < 0.01$ samples processed in the presence of AA compared to those without AA in IR group of patients.

Table 1
Correlation between the reduction in mucosal prostanoid levels and the effect of sulindac on reducing polyp size and number^a

AA	PGD ₂	PGF _{2α}	PGF _{2α}	TXB ₂	6KPGF _{1α}
	-	+	-	+	-
Size	0.741 [*]	0.845 ^{**}	0.651 [*]	0.681 [*]	0.586 ^{**}
Number	0.708 [*]	0.796 ^{**}	0.587	0.717 [*]	0.395 ^{**}

^aThe level of each prostanoid measured in the absence (-) or presence (+) of arachidonic acid (AA) at the end of the 3-month therapy was first calculated as a percentage of the baseline value (see Fig. 3) for all 11 patients. This percentage was then correlated with the percentage of baseline value in either the size or number of polyps at the end of treatment. The numbers in the table represent Spearman rank correlation coefficients between each prostanoid and one of the two clinical parameters.

* $P < 0.05$ by two-tailed analysis.
** $P < 0.01$ by two-tailed analysis.