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Size-dependent Increase in Prostanoid Levels in Adenomas of Patients with Familial Adenomatous Polyposis[1]

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ABSTRACT

Recent studies indicate that nonsteroidal anti-inflammatory drugs have a chemopreventive effect against colorectal neoplasia. Nonsteroidal anti-inflammatory drugs inhibit cyclooxygenases, principal enzymes that mediate the formation of prostanoids. To determine whether prostanoids are involved in the pathogenesis of colorectal adenomas, we compared the levels of five major stable metabolic products of the cyclooxygenase pathway in the normal-appearing mucosa and in adenomas of patients with familial adenomatosis polyposis. Of 12 patients tested, 6 had elevated levels of at least one prostanoid in the adenomas. More importantly, the relative levels of three prostanoids [prostaglandin (PG)D_2, PGE_2, and 6-keto-$PGF_{1\alpha}$] were elevated in adenomas compared to normal-appearing mucosa from the same patients, and the resulting ratios were correlated with the size of the adenoma. These results suggest a role for prostanoids in progression of colorectal polyposis in familial adenomatosis polyposis patients.

INTRODUCTION

Several lines of evidence suggest that NSAIDs[3] prevent colorectal cancer (1–3). NSAIDs were shown to inhibit chemically induced intestinal tumors in experimental animals (4–6). In human epidemiological studies, NSAIDs decreased the incidence and mortality of colorectal cancer (7, 8) and induced regression of adenomas in patients with FAP (9–11). More recently, in the *Min* mouse, a murine model of human FAP, NSAIDs were shown to suppress formation of intestinal tumors (12–14).

The major known effect of NSAIDs is the inhibition of cyclooxygenase (prostaglandin H synthase), the principal enzyme that mediates the formation of prostanoids, which is a collective term for prostaglandins, prostacyclins, and thromboxanes (15, 16). Although the mechanism by which NSAIDs prevent colorectal cancer is unclear, previous studies suggest that prostanoids may be involved in tumor formation. *In vitro,* prostaglandins modulated cell proliferation and tumor growth (17, 18). In carcinogen-treated rats, levels of PGE_2 in the normal-appearing colonic mucosa in cancer-bearing animals were significantly higher than those of control, non-cancer-bearing animals (19). In addition, PGE_2 levels in the tumor tissue were increased when compared with the surrounding normal-appearing colonic mucosa of carcinogen-treated animals (20). Similarly, PGE_2 levels in adenomas and cancers were higher than in normal colonic mucosa of patients with sporadic colorectal neoplasms (21, 22). Lastly, in FAP patients treated with the NSAID sulindac, colonic mucosal PGE_2 and $PGF_{2\alpha}$ levels were significantly decreased when compared with levels either before the initiation of treatment or in patients treated with placebo (23–25). Thus, these studies provide strong correlative evidence for the association between tissue prostaglandins and colorectal cancer.

Despite several published reports on the effect of sulindac in causing regression of adenomas in FAP patients (9–11), limited data are available on whether mucosal prostanoids are involved in adenoma formation. Recently, we showed that levels of five major prostanoids (PGD_2, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TXB_2) in the normal-appearing mucosa of FAP patients treated with sulindac were lower than those before treatment (25). However, there were no statistical differences in the levels of these prostanoids in the normal-appearing mucosa in FAP patients before treatment when compared with control unaffected individuals. Because the inhibition of prostanoid synthesis correlates with the regression of adenomas, it is reasonable to implicate a role for prostanoids in colorectal tumorigenesis. The present study therefore addresses the question of whether there are differences in prostanoid levels between the adenomas and the normal-appearing mucosa in FAP patients. Our results indicate that the levels of three prostanoids in adenomas are significantly elevated in a size-dependent manner, suggesting that prostanoids may be important mediators of tumor formation.

MATERIALS AND METHODS

Study Subjects. Twelve Caucasian FAP patients (5 males and 7 females; mean age, 20 ± 9 years) and five otherwise normal, healthy Caucasian individuals without FAP (3 males and 2 females; mean age, 28 ± 7 years) in whom colonoscopic examinations were performed for screening purposes were studied. Informed consents were obtained from all subjects who participated in the study. None of the study subjects took NSAIDs within at least 1 week prior to the procedure.

Colonoscopy was performed after routine oral cathartic solution. In FAP patients, the total number of polyps in the rectum between 20 cm and the anal verge was counted, and photography was obtained in this area for documentation purposes. The diameter of the first five polyps distal to 20 cm was measured with a graduated scale passed through the biopsy channel of the colonoscope (9). The diameters of the measured polyps ranged between 2.4 and 10 mm (mean, 5.3 ± 3.3 mm). The number of polyps in the 20 cm of rectum surveyed ranged between 6 and 100 (mean, 36 ± 28). Mucosal specimens were obtained using standard biopsy forceps from the normal-appearing tissue and from a polyp in which the size was first determined. Specimens were similarly obtained from the rectal mucosa 20 cm from the anal verge in control individuals. Two pieces were snap-frozen in liquid nitrogen for prostanoid determination, and two were placed in formalin for histopathological examination. All normal-appearing mucosal samples showed no histological evidence of adenomatous epithelial proliferation.

Processing of Specimens. Specimens were thawed on ice and rinsed with HBSS (containing 138 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5.6 mM D-glucose, 0.3 mM Na_2HPO_4, and 0.3 mM KH_2PO_4). Each sample was manually homogenized in a glass micro-homogenizer in 50 μl of HBSS containing 1 mM $CaCl_2$ and then transferred to a microcentrifuge tube. An additional 60 μl of the same solution were used to rinse the homogenizer and combined with the initial homogenate. The combined solution was then sonicated for 20 s with a Fisher Scientific model 550 Sonic Dismembrator equipped with a microtip

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[3] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatosis polyposis; PG, prostaglandin; TX, thromboxane; APC, adenomatous polyposis coli; COX, cyclooxygenase.

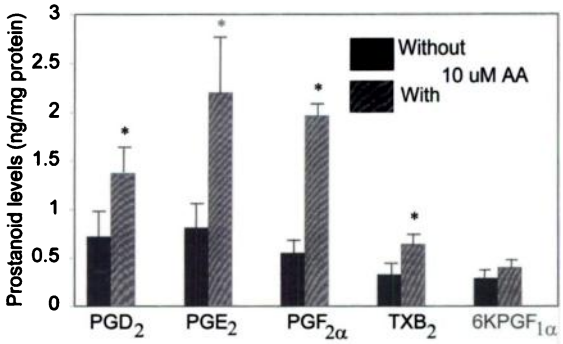

Fig. 1. The effect of arachidonic acid (*AA*) on tissue prostanoid levels. Homogenates of mucosal biopsy specimens from the rectum of normal control individuals were incubated in the absence (■) or presence (▨) of 10 μM arachidonic acid at 37°C for 30 min prior to being processed for quantification by gas chromatography-mass spectrometry. Shown are the means of five individuals; *bars*, SD. *, the difference was statistically significant ($P < 0.05$). *6KPGF*$_{1\alpha}$, 6-keto-PGF$_{1\alpha}$.

probe. Tissue debris was removed by centrifugation at 12,000 × g in a microcentrifuge for 15 s. Ten μl of the supernatant were removed for determination of protein concentration by the Bradford protein assay method (Bio-Rad). Fifty μl of the remaining homogenate were then incubated at 37°C for 30 min in the absence or presence of 10 μM arachidonic acid (Sigma). This concentration of arachidonic acid is above the K_m for both cyclooxygenases 1 and 2 (26) and should enhance the formation of prostanoids above baseline levels if one or both cyclooxygenases were present in the tissues at the time of the biopsy. Following incubation, 50 μl of deuterated prostanoid standards and 250 μl of acetone were added, and the solution was vortexed and centrifuged for 5 min. The supernatant was then divided equally between two vials and dried under a steady stream of nitrogen gas. At the point of complete dryness, 25 μl of 2% *O*-methoxylamine HCl in pyridine were added to each sample. The samples were stored at −20°C until they were delivered to the gas chromatography-mass spectrometry laboratory for quantification of prostanoid levels.

Determination of Prostanoid Levels. Samples were brought to room temperature, and the pyridine solvent evaporated under a nitrogen stream. Following evaporation, the residue in each vial was treated with reagents to synthesize the pentafluorobenzyl ester-trimethylsilyl ether derivatives of the prostanoids for gas chromatography-mass spectrometric analysis as described previously (27). Levels of prostanoids were normalized to the amount of protein in each specimen.

As shown in Fig. 1, the level of each prostanoid was increased when homogenates of the biopsied rectal mucosa were incubated in the presence of 10 μM arachidonic acid. With the exception of 6-keto-PGF$_{1\alpha}$, the increase in levels for each prostanoid was statistically significant. This result is consistent with a recent report that showed an increase in PGE$_2$ levels in intestinal tissues of several species when stimulated with arachidonic acid (28). Thus, this methodology increases the sensitivity of detection of the relatively small amounts of prostanoids in the biopsied tissues. Furthermore, the levels of prostanoids detected in the presence of arachidonic acid represented the maximal synthetic capacity of the tissue at the time of biopsy, compared with the detection of preexisting prostanoids when assays were performed in the absence of arachidonic acid. All subsequent measurements in patients were therefore performed under arachidonic acid-stimulated conditions.

Statistical Analysis. Statistical analysis was performed using True Epistat statistical software.

Comparisons of prostanoid levels (Fig. 1 and Table 1) were performed by two-tailed Student's *t* test. Correlation (Table 2) was evaluated by two-tailed Spearman rank correlation coefficients. Significance was defined as $P < 0.05$.

RESULTS

The levels of five prostanoids in the normal-appearing mucosa and in the adenomatous polyps of 12 phenotype-positive FAP patients were examined. Table 1 shows the mean concentration and SD for each prostanoid. Although the mean concentrations of PGD$_2$ and PGE$_2$ were slightly elevated in the adenomas when compared to the normal-appearing tissues, the increase did not reach a statistical significance. Similarly, the decrease in PGF$_{2\alpha}$ levels in the adenomas as compared with normal mucosa was also not statistically significant. When prostanoid levels in the normal-appearing mucosa were compared with those in the adenoma within individual patients, six patients were noted to have elevated levels of at least one metabolite in the adenoma. Of these six patients, three had elevated levels for all five metabolites and two had elevated levels for four metabolites. The elevated ratios of three prostanoids (PGD$_2$, PGE$_2$, and 6-keto-PGF$_{1\alpha}$) in adenomas compared with normal-appearing mucosa were significantly correlated with the size of the adenoma (Table 2, fourth row). A correlation was also noted between increased ratio of TXB$_2$ in adenomas over normal-appearing mucosa and size, although it failed to reach statistical significance (Table 2, fourth row; $P = 0.12$). In addition, the absolute levels of two prostaglandins, PGD$_2$ and PGE$_2$, in the adenomas were also correlated with size (Table 2, third row). In contrast, none of the absolute prostanoid levels in the normal-appearing mucosa were correlated with adenoma size (Table 2, second row). No correlation was observed between the ratios of prostanoid level or the absolute prostanoid levels and patient gender, age, or number of adenomas (results not shown). When the ratios of individual prostanoids in adenomas over normal-appearing tissues were plotted against the size of the adenoma (Fig. 2), only in adenomas above a size of 6–7 mm were the elevations evident.

DISCUSSION

A large body of evidence indicates that the development of neoplasia is the result of cumulative genetic changes. Germ-line mutation in a single gene called *APC*, for example, leads to a marked familial predisposition to colon cancer, resulting in FAP (29). Somatic mutations of additional genes eventually lead to colon cancer. Evidence from recent studies suggests that cyclooxygenases, especially COX-2, may be associated with colorectal neoplasia. Expression of *COX-2*,

Table 1 *Comparison of prostanoid levels between normal-appearing mucosa and adenomas in FAP patients*

	PGD$_2$	PGE$_2$	PGF$_{2\alpha}$	TXB$_2$	6-keto-PGF$_{1\alpha}$
Normal mucosa	0.964 ± 0.437[a]	1.874 ± 0.891	2.815 ± 1.493	0.704 ± 0.551	0.409 ± 0.314
Adenomas	1.146 ± 1.023	2.073 ± 1.267	1.878 ± 0.889	0.739 ± 0.280	0.302 ± 0.236

[a] Mean concentration and SD of each prostanoid in ng/mg protein. $n = 12$.

Table 2 *Spearman rank correlation coefficients between prostanoid levels and adenoma size*

	PGD$_2$	PGE$_2$	PGF$_{2\alpha}$	TXB$_2$	6-Keto-PGF$_{1\alpha}$
PG level in normal mucosa	−0.477	−0.379	−0.347	−0.337	−0.589
PG level in adenomas	0.624[a]	0.659[a]	0.147	0.414	0.449
Ratio of PG[b] (Adenoma/Normal)	0.849[a]	0.758[a]	0.249	0.453	0.646[a]

[a] $P < 0.05$ by two-tailed analysis.
[b] The ratio of prostanoid in the adenoma over that in the normal-appearing mucosa in the same patient.

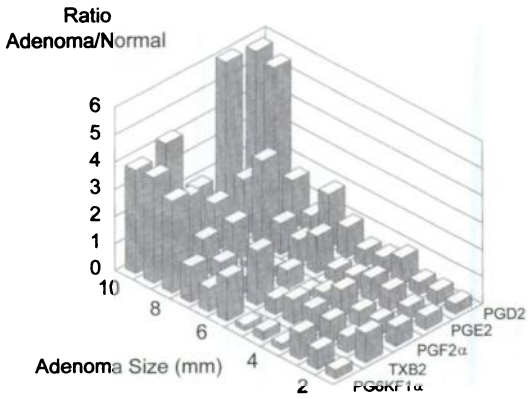

Fig. 2. Size-dependent elevation of prostanoid levels in adenomas. The ratios of prostanoids between the adenoma and normal-appearing mucosa in each of the 12 patients are plotted against the size of the adenoma in mm. *PG6KF1α*, 6-keto-$PGF_{1\alpha}$.

ordinarily at very low levels in normal colonic epithelium, was increased in colorectal adenomas and cancers in both humans (30, 31) and experimental animals (14, 32, 33). Moreover, NSAIDs, compounds that inhibit cyclooxygenase activity, decreased the formation of colon cancer in experimental animals as well as in humans (1–11). Additional evidence implicating COX-2 in colonic neoplasia comes from a recent report demonstrating that intestinal polyposis in mice with targeted ablation of *APC* was markedly attenuated when such mice were mated to mice with targeted ablation of *COX-2* (34).

Although the aforementioned studies suggest that an increased expression of *COX-2* may promote intestinal tumorigenesis, the mechanism by which COX-2 exerts this effect is by no means clear. A number of nonprostanoids may result from the metabolic activity of cyclooxygenases, some of which may contribute to carcinogenesis (35). However, because prostanoids are the major products of cyclooxygenases, it is difficult to ignore the potential role of these compounds in neoplasia. Presently, the role of prostanoids in the formation of colon cancer is controversial. The elevation of certain prostaglandins in colonic adenomas and cancers in both humans and animals (19–22) supports a function for prostanoids in tumor formation. Moreover, the chemopreventive effects of NSAIDs and the ability of these drugs to lower mucosal prostanoid levels (23–25) also suggest that prostanoids may be involved in tumor formation. On the other hand, sulindac sulfone, a metabolite of sulindac that does not inhibit cyclooxygenases, also exhibits a chemopreventive effect (36, 37), suggesting that prostanoids may not be essential for neoplasia.

The results of the present study established a relationship between elevated prostanoid levels and the size of adenomas in FAP patients. This elevation, however, was not observed until an adenoma reached an approximate size of 6–7 mm in diameter. Elevated prostaglandin E_2 levels in human colonic adenomas (21) and carcinomas (22) have been reported previously, but neither study correlated the elevated levels with clinical parameters. Similarly, increased expression of *COX-2* has been noted in adenomas of a subset of FAP patients (30), but the effect of adenoma size was not analyzed. One study has analyzed the relationship between elevated cyclooxygenase expression and the size of adenomas by measuring the levels of COX-1 and COX-2 proteins in adenomas from mice with targeted ablation of *APC* (34). Although COX-1 levels remained relatively constant in normal epithelium as well as intestinal adenomas of different sizes, COX-2 was not present in normal epithelium and was detectable only when adenomas were >2 mm (34). Although the study did not directly measure prostanoid levels in the tumor tissues, these results are consistent with our present findings. Taken together, these observations support the hypothesis that increased COX-2 expression with the resultant elevation of prostanoid levels in adenomas provides a potential mechanism for tumor promotion (35).

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