Expression of Prostaglandin G/H Synthase-1 and -2 Protein in Human Colon Cancer

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ABSTRACT

Prostaglandin G/H synthase (PGHS), a key enzyme leading to the formation of prostaglandins, is the target of nonsteroidal antiinflammatory drugs. Two forms of the enzyme have been identified, PGHS-1 and PGHS-2. Epidemiological evidence has suggested that aspirin and other nonsteroidal antiinflammatory drugs may reduce the risk of colorectal cancer. We examined by immunoblot analyses the expression of human PGHS-1 and PGHS-2 protein in 25 matched colon cancer and nontumor tissues, 4 premalignant polyps, 5 control colon tissues from noncancer patients, and 3 matched normal and cancerous breast tissue samples. PGHS-1 was detected in all normal and tumor tissue. In contrast, PGHS-2 was not detected in 23 of 25 normal colon tissues but was detected in 19 of 25 colon tumors. PGHS-2 protein was not observed in four human premalignant polyp samples, control colon from noncancer patients, or matched normal or cancerous breast tissues. These results suggest that the beneficial effects of nonsteroidal antiinflammatory drugs in colon cancer may be mediated by inhibition of PGHS-2.

INTRODUCTION

The enzyme PGHS[2] is a key enzyme in the biosynthetic pathway leading to the formation of prostaglandins (1, 2). These prostanoids are potent biological mediators with diverse normal physiological effects and are also implicated in a variety of pathological conditions including inflammation and neoplastic transformation (1–3). Two isoforms of PGHS have been identified (4). PGHS-1 is constitutively expressed in most tissues and has been proposed to generate prostaglandins for normal physiological functions. The second isoform, PGHS-2, is characterized by a rapid induction by a variety of stimuli, including mitogens, hormones, cytokines, and growth factors (4, 5). In conditions such as inflammation, PGHS-2-derived prostaglandins may be the predominant effectors (6). Both PGHS-1 and PGHS-2 have been shown to be the target of NSAIDs (5, 7, 8).

Elevated levels of prostaglandins have been demonstrated in various cancers including lung and colon carcinomas (9, 10). In particular, prostaglandin levels have been shown to be elevated in benign adenomatous polyps and further increased in cancerous colon tissue, as compared to histologically normal mucosa (11). Since prostanoids have been shown to be immunosuppressive, it has been suggested they play a role in tumor development by decreasing immunosurveillance (11).

In this study, we examined the expression of PGHS protein in colorectal cancers in view of the data showing the benefit of NSAIDs for prevention of colorectal neoplasms (12). We have analyzed the expression of human PGHS-1 and PGHS-2 protein in 25 paired normal and autologous colon tumors, 4 premalignant colon polyps, 5 control colon tissues (from noncancer patients), and 3 matched normal and cancerous human breast tissues. The observations from this study

are that PGHS-1 immunoreactive protein was reduced in colon tumor tissue as compared to histologically normal colonic mucosa and that PGHS-2 was detected in the majority of colon tumor samples, while being virtually undetectable in normal tissues, polyps, and breast cancer samples. These results suggest that the increased levels of prostaglandins in tumor tissue of the colon may be derived from the inducible PGHS-2 isoform.

MATERIALS AND METHODS

Patient Samples. Colon cancer and matched normal mucosal tissues, adenomatous polyps, normal colonic mucosa from noncancer patients, and matched normal and cancerous breast tissues were examined. The mean age and range of matched normal and colon cancer samples were 63.3 and 41–93 years, respectively. Colon tissue specimens were obtained from a nonnecrotic area of the tumor and from autologous normal mucosa from the same patient at a resection margin located at more than 5 cm from the tumor. Histologically, all colon tumors were adenocarcinomas, and all patients had sporadic colon cancer. One patient had a focus of adenocarcinoma within an adenomatous polyp. Colonic adenomas were from patients with familial adenomatous polyposis.

Preparation of Microsomal Membranes from Colon Tissues. Frozen tissues were thawed in ice-cold homogenization buffer [50 mM potassium phosphate (pH 7.1) containing 0.1 M NaCl, 2 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 60 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin], all from Sigma Chemical Co. (St. Louis, MO). Tissues were disrupted twice on ice using a tissue tearer (Biospec Products, Bartlesville, OK) and homogenized by sonication at 4°C using a Cole Parmer 4710 series ultrasonic homogenizer (Cole Parmer Instrument Co., Chicago, IL). Cellular debris was removed by centrifugation at 1,000 × g for 15 min at 4°C, and the resultant supernatants were subjected to centrifugation at 100,000 × g for 60 min at 4°C. Membrane fractions were resuspended in homogenization buffer and sonicated in order to obtain a homogenous membrane suspension. Protein concentrations were determined for each sample using a protein assay kit (Bio-Rad, Mississauga, Ontario, Canada).

Antisera. Full-length sheep seminal vesicle PGHS-1 and placental PGHS-2 purified proteins were purchased from Cayman (Ann Arbor, MI) and used to generate rabbit polyclonal antibodies. New Zealand White female rabbits were injected with 1 ml of Freund's complete adjuvant containing 200 μg of purified PGHS-1 or PGHS-2. Two weeks after the primary injection, rabbits were boosted with 100 μg of purified PGHS-1 or PGHS-2 in 0.5 ml of Freund's incomplete adjuvant. The anti-PGHS antisera recognize the homologous human PGHS isoforms with approximately 1000-fold selectivity for the appropriate isoform. Under the conditions used in this study, the anti-PGHS antibodies demonstrated no significant cross-reactivity with the alternate PGHS isoform. For each experiment, two concentrations of both PGHS-1 and PGHS-2 protein standards were loaded on each gel to assess selectivity of the antibodies.

SDS-PAGE and Immunoblot Analysis. Membrane fractions were mixed with SDS sample buffer [20 mM Tris-HCl (pH 6.8) containing 0.4% (w/v) SDS, 4% glycerol, 0.24 M β-mercaptoethanol, and 0.5% bromophenol blue], boiled for 5 min, and analyzed by SDS-PAGE according to the method of Laemmli (13). Proteins were electrophoretically transferred to nitrocellulose membranes as described previously (14). Primary antibodies to PGHS-1 and PGHS-2 were used at a final dilution of 1:5000 and 1:7500, respectively. The secondary horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Life Sciences, Oakville, Ontario, Canada) was used at a dilution of 1:3000. Immunodetection was performed using enhanced chemiluminescence

2556

Fig. 1. Representative immunoblot analysis of PGHS-1 and PGHS-2 protein expression in normal human colonic mucosa and autologous tumor tissue. Immunoblot analysis using an anti-PGHS-1 antiserum (*a*) and immunoblot analysis of the same samples as those shown in *a* using anti-PGHS-2 antiserum (*b*). Purified PGHS standards and microsomal protein samples (50 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-PGHS antisera with detection by enhanced chemiluminescence. Numbers *1–4*, samples from 4 representative patients of 25 patients examined. The densitometric values for patients 1–4 in this figure correspond to patients 17–20 shown in Fig. 2. *N* and *T*, matched normal colonic mucosa and tumor tissue for each patient, respectively. Purified PGHS-1 and PGHS-2 standards are shown at the *right*. The positions of molecular weight markers are indicated on the *left*.
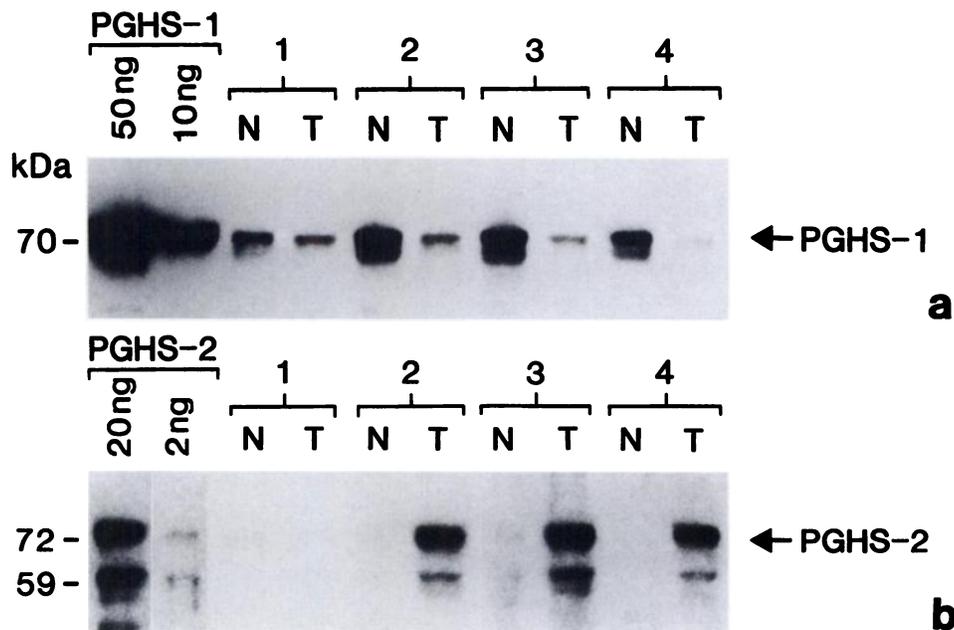
according to the manufacturer's instructions (Amersham). Autoradiographs were scanned using a computing densitometer (Molecular Dynamics, Sunnyvale, CA), and the volume of absorbance corresponding to the purified PGHS isoform was used to calculate quantity (ng) of PGHS protein in histologically normal colonic and tumor tissue.

Statistical Analysis. The results of this study were analyzed by a Wilcoxon signed-rank nonparametric test to determine significant differences between normal and tumor PGHS-1 and PGHS-2 levels (15).

RESULTS

Expression of PGHS-1 in Human Colon: Normal and Tumor Tissue. Fig. 1 is a representative immunoblot demonstrating PGHS expression in 4 of the 25 patients examined in this study. The M_r 72,000 band, which is the reported molecular weight for PGHS-1 (16), comigrated with purified sheep seminal vesicle PGHS-1 standard (Fig. 1*a*) and with recombinant human PGHS-1 expressed in COS-7 cells (data not shown; Ref. 7). The immunoblot results from all 25 patients were quantitated by densitometric analysis and are shown in the bar graph in Fig. 2. In 21 of 25 patient, PGHS-1 levels were

reduced in tumor tissue as compared to normal colon. The mean decrease of PGHS-1 in tumor as compared to normal tissue for all 25 patients examined was 170 ng/mg microsomal protein. In comparison to the purified PGHS-1 standard, the range of concentrations of PGHS-1 in normal and tumor tissue were 0–760 ng (median, 199.8) and 4–540 ng (median, 51.1) per mg microsomal protein, respectively. The difference in PGHS-1 expression in normal *versus* tumor tissue was highly statistically significant as determined by nonparametric analyses (Wilcoxon signed-rank test, $P < .0001$).

Expression of PGHS-2 in Human Colon: Normal and Tumor Tissue. A representative immunoblot analysis of 4 of the 25 matched colon samples using a specific anti-PGHS-2 antibody is shown in Fig. 1*b*. Duplicate immunoblots were performed in order to assess the expression of PGHS-1 (Fig. 1*a*) and PGHS-2 (Fig. 1*b*) in samples derived from the same patient. PGHS-2 immunoreactivity was not detected in any of the four normal colon tissue samples. In contrast, immunoreactive bands of M_r 70,000–72,000, which comigrated with purified sheep placental PGHS-2 (Fig. 1*b*) and human recombinant PGHS-2 expressed in COS-7 cells (data not shown; Ref. 7), were

Fig. 2. Quantitation of change in PGHS-1 and PGHS-2 protein expression in matched tumor tissue as compared to autologous normal mucosa. Aliquots of PGHS standards and of microsomal proteins from 25 patients (50 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-PGHS-1 or anti-PGHS-2 antisera, with detection by enhanced chemiluminescence. The amounts of PGHS protein were determined by densitometry. The absorbance reading from the scanned autoradiograph for known quantities of purified PGHS-1 and PGHS-2 standards were used to quantitate approximate amounts of PGHS-1 and PGHS-2 protein in microsomal samples from normal mucosa and tumor samples. Values represent the change in expression in nanograms from normal mucosa to tumor tissue for each patient.
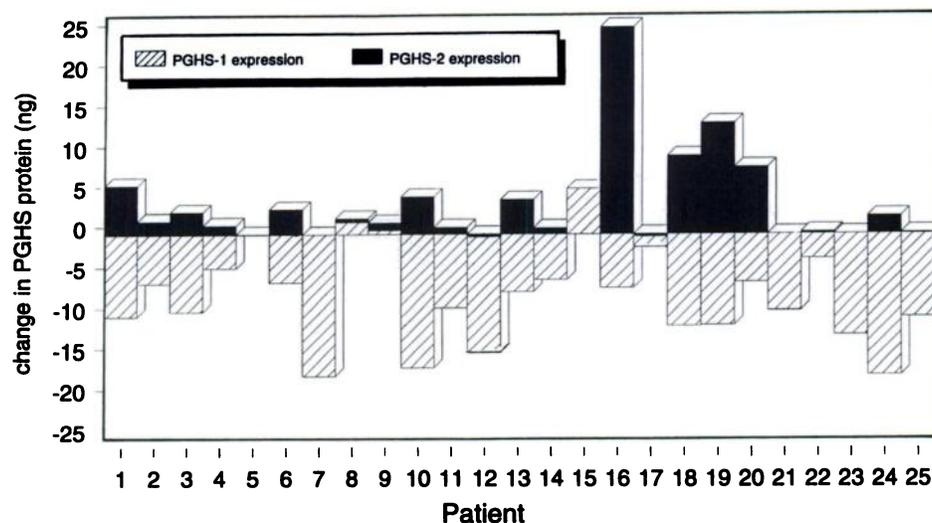
2557

Table 1 *Patient data*

Information for each patient examined in this study is shown. When available, medications taken by patients are indicated. Dukes' stage of disease is as follows: A, tumor within mucosa; B, tumor into muscularis mucosa; C, metastasis to lymph nodes; and D, metastasis to other tissues. Amount of PGHS immunoreactive protein is expressed as relative change in expression from normal colonic mucosa to tumor tissue.

Patient #	Medication	Dukes' stage	Change in protein expression (ng) PGHS-2	PGHS-1
1	NA[a]	NA	6.20	-10.00
2	NA	NA	1.70	-6.00
3	NA	NA	2.80	-9.40
4	NA	NA	1.20	-4.10
5	None	B	0.10	ND
6	Chlordiazepoxide hydrochloride/clidinium bromide, magaldrate	C	3.20	-5.90
7	NSAID	A	0.00	-17.30
8	Levothyroxine sodium, lorazepam, omeprazole	C	0.50	1.50
9	None	C	0.90	0.50
10	NA	C	4.70	-16.30
11	None	B	0.90	-9.00
12	NA	D	-0.20	-14.40
13	NA	B	4.30	-7.10
14	Aspirin	C	0.80	-5.60
15	Deltacortisone, azathioprine, lovastatin	B	0.00	5.70
16	Enalapril maleate	B	25.60	-6.60
17	None	B	-0.30	-1.50
18	None	B	9.70	-11.20
19	Aspirin	C	13.70	-11.10
20	None	B	8.30	-5.90
21	None	C	-0.10	-9.30
22	Aspirin	C	0.20	-3.00
23	None	C	0.00	-12.30
24	Diltiazem hydrochloride, omeprazole	B	2.20	-17.00
25	Flurbiprofen, aspirin, nifedipine, metoprolol tartrate	B	0.10	-10.00

[a] NA, data not available; ND, not detectable.

detected in tumor tissue of three of the four patients shown here. In total, PGHS-2 immunoreactive protein was detected in tumor tissue from 19 of 25 patients examined (Fig. 2). Overall, the mean PGHS-2 increase in colonic tumors for all 25 patients examined was approximately 73 ng/mg microsomal protein. In comparison to the PGHS-2 standard, the range of concentrations of PGHS-2 immunoreactive protein in normal and tumor tissue was 0–49 ng (median, 3.8 ng) and 1.6–580 ng (median, 37.7 ng) per mg microsomal protein, respectively. The difference in PGHS-2 expression in normal *versus* tumor tissue was highly statistically significant as determined by nonparametric analyses (Wilcoxon signed-rank test, $P < 0.0001$).

Colonic tumors are classified by four Dukes' stages: A, tumor within the intestinal mucosa; B, tumor into muscularis mucosa; C, metastasis to lymph nodes; and D, metastasis to other tissues. In this study, colonic tumors were from patients with all stages of Dukes' classification (Table 1). There was no association between stage and change in expression of PGHS-1 or PGHS-2 protein. This lack of association is consistent with the observation that prostaglandin E_2 levels in human colon tumors do not appear to correlate with Dukes' stage (10). Interestingly, PGHS-2 expression was either low or undetectable in five of six patients currently using antiinflammatory medications (Table 1).

Expression of PGHS-1 and PGHS-2 Protein in Normal Colon, Premalignant Polyps, and Matched Normal and Breast Cancer Tissue. The expression of PGHS-1 and PGHS-2 protein was analyzed in a variety of other human tissues including five colon tissues from noncancer patients, four premalignant polyps, and three matched normal and cancerous breast tissues. Although PGHS-1 protein was observed in normal and cancer tissues, PGHS-2 protein was not detected in any of these samples (data not shown).

DISCUSSION

In this study, 19 of 25 colon tumors examined expressed PGHS-2 protein, whereas only 2 of 25 normal colon tissue samples expressed this protein. Concomitant with the induced expression of PGHS-2, PGHS-1 expression was reduced in 21 of 25 of the tumor samples in comparison with normal adjacent colonic mucosa; however, PGHS-1 protein concentrations were similar in control colon and polyp samples.

To our knowledge, this is the first study examining the expression of PGHS-1 and PGHS-2 protein in human colon cancer. Previous research has shown increased eicosanoid levels, in particular, prostaglandin E_2, in human colon cancer (10, 17, 18). The increase in PGHS-2 enzyme that we observe in colonic tumor tissue may result in elevated prostaglandin levels in these tumors. Since prostaglandins may have multiple effects in the biology of cancer, including growth promotion and modulation of immune surveillance, elevated prostanoid levels within tumors may aid in tumor growth or development (11). Interestingly, PGHS-2 protein was not observed in a limited examination of breast cancer samples, suggesting that PGHS-2 protein expression may not be a common feature of malignant transformation.

Eberhart *et al.* (19) demonstrated up-regulation of PGHS-2 mRNA in human colorectal adenomas and adenocarcinomas. This is an interesting finding but may not be indicative of actual enzyme expression due to the complex posttranscriptional and posttranslational regulation of PGHS-2 mRNA. For example, Hoff *et al.* (20), Lee *et al.* (21), and our laboratories have shown substantial expression of PGHS-2 mRNA without concomitant expression of PGHS-2 protein.[3] Therefore, examination of PGHS protein expression is critical to estimate the concentration of PGHS-2 enzyme.

It is surprising the PGHS-2 protein was not detected in polyps from four patients with familial adenomatous polyposis, especially in light of evidence by several groups that sulindac (inhibiting both PGHS-1 and PGHS-2) results in polyp regression in patients with familial polyposis. The human polyp samples were pools of small colon polyps ranging in size from 0.4 to <5 mm in diameter. It is possible that PGHS-2 protein is expressed at a later stage in the polyp-cancer sequence, when polyps are larger in diameter. A recent study by Ladenheim *et al.* (22) reports that sulindac did not result in a regression of sporadic colonic polyps; however, they emphasized that their study addressed early sporadic polyps (67% of the polyps were ≤5 mm) and suggested that response to NSAIDs may be more favorable in polyps at a "particular stage along the adenoma-carcinoma sequence" (22).

The prolonged use of NSAIDs is associated with side effects including renal toxicity, gastrointestinal ulceration, and increased bleeding. Current NSAIDs such as aspirin, sulindac, and indomethacin have little selectivity for inhibition of either PGHS-1 or PGHS-2 (5, 7, 8). It has been suggested that selective inhibitors of PGHS-2 would have useful therapeutic effect with a decreased capacity to induce mechanism-based side effects. Recently, a selective PGHS-2 inhibitor, NS-398, has been shown in the rat to have antiinflammatory, antipyretic, and analgesic effects without being ulcerogenic (23, 24). The present study examining the expression of PGHS-1 and PGHS-2 in the colon demonstrates that both PGHS isoforms are present in colon tumors. We cannot rule out the possibility that the beneficial effects of NSAIDs on polyp regression in familial polyposis patients or in reducing the risk of colon cancer is related to inhibition of PGHS-1. Nevertheless, it is interesting that PGHS-2 is significantly induced in many of the tumor samples. The investigation of a selective PGHS-2 inhibitor in an animal model

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[3] S. Kargman and B. Kennedy, unpublished data.

of colon cancer is strongly warranted by the intriguing induction of PGHS-2 protein in colon tumors. A selective PGHS-2 inhibitor may be beneficial in preventing the development and/or progression of the neoplastic process and at the same time have reduced potential for gastric toxicity.

ACKNOWLEDGMENTS

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