Size- and Invasion-dependent Increase in Cyclooxygenase 2 Levels in Human Colorectal Carcinomas

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Abstract

Nonsteroidal anti-inflammatory drugs reduce the incidence and mortality of colorectal carcinoma. Their chemopreventive effects appear to be due to inhibition of cyclooxygenase (COX)-2. Here, we have studied the relationship between the COX-2 mRNA levels and pathological characteristics in 43 primary colorectal carcinomas. COX-2 levels were significantly higher in tumors with larger sizes and in those with deeper invasions but were not correlated with whether the patients had metastasis or not. These results suggest that larger carcinomas produce more COX-2 to support their own growth and that COX-2 inhibitors may be effective agents of carcinoma growth suppression.

Introduction

Accumulating evidence indicates that aspirin-like drugs or NSAIDs can reduce the incidence of colorectal carcinomas (1-5). First, NSAIDs were shown to decrease the incidence, multiplicity, and/or size of colorectal carcinomas in rodents caused by chemical carcinogens (1). Second, both noncontrolled and randomized trials of sulindac on FAP patients demonstrated that the NSAID can reduce the number and size of colorectal polyps, which are benign adenomas and precursors of malignant carcinomas (2, 3). Third, epidemiological studies indicated that the relative risk of developing colon carcinoma was significantly lower in patients who took aspirin or other NSAIDs (4, 5). The major target of NSAIDs is cyclooxygenase, which catalyzes the conversion of arachidonic acid to prostaglandin H2, the common precursor for all prostanooids, such as prostaglandins, prosta-acyclin, and thromboxanes (6). Two COX isozymes have been identified: the constitutive COX-1 and the inducible COX-2 (7-9). In addition to the involvement of COX-2 in many inflammatory processes, it has been reported that COX-2 is induced also in various carcinomas, including those in the colorectal axis as well as in the intestinal polyps of the Apc gene knockout mouse, a model for FAP (10-13). Moreover, the knockout mice with compound mutations of both the Apc and the COX-2 gene (Pigs2) demonstrated a dramatic decrease in the number and size of the intestinal polyps, indicating that COX-2 plays a key role in tumorigenesis (13). Inhibition of COX-2 by selective inhibitors also reduced the growth of the intestinal polyps in the knockout mice (13) and colon carcinoma incidence in chemical carcinogen-treated rats (1). Recently, Yang et al. (14) reported that the amounts of prostanoids showed a size-dependent increase in FAP adenomas. Here, we have determined COX-2 mRNA levels in primary colorectal carcinoma tissues and investigated the relationship between COX-2 expression and clinicopathological characteristics of the tumors.

Materials and Methods

We studied a total of 43 surgical specimens of primary colorectal carcinoma from randomized patients who underwent surgery between January 1997 and January 1998. They were all sporadic colorectal carcinomas; samples of FAP or hereditary nonpolyposis colorectal cancer were excluded. Materials were obtained from nonneoplastic areas of carcinomas and from the normal mucosa at the resection margin of the surgical specimens. All materials were frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated according to the method of Chomczynski and Sacchi (15) and quantitated by reading the absorbance at 260 nm. cDNA was amplified from the total RNA (1 µg) using the RNaseasy kit (Qiagen, Chatsworth, CA). PCRs were performed concomitantly for both COX-1 and COX-2 in the same tube on each sample. Primers were designed so that the antisense primer was shared for the amplifications of both COX-1 and COX-2 mRNAs. The primers used were: 5'-TGC CCA GCT CCT CGC CCG CCG CTT-3' (COX-1 sense; bases 522-545), 5'-AGT CAA AGA TAC TCA GGC AGA-3' (COX-2 sense; bases 851-872), and 5'-GGA GTT CTG GTT CAA ATT TCA G-3' (common antisense; bases 1080-1101 for COX-1 and 1133-1154 for COX-2; Y indicates an equimolar mixture of T and C). The base numbers are as appear in the GenBank database under accession no. M59979 (COX-1) and M90100 (COX-2). To choose the optimal PCR amplification conditions in the linear range, three cDNA amounts (10-200 ng) were tested for each sample, as reported previously (16). The optimized amount of cDNA was amplified by PCR in a 20-µl reaction mixture that contained: 2 µl of 10× Ex Taq buffer (TaKaRa), 2 µl of 2.5 mm dNTP mix, 0.1 µl of Ex Taq polymerase (TaKaRa), 1 µl (4 µM) of each sense primer, and 2 µl (4 µM) of common antisense primer. Samples were amplified for 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were electrophoresed in 1.5% agarose gels and visualized by ethidium bromide. Their band densities were quantified with the NIH Image software (Version 1.54; provided by Wayne Rasband, NIH, Bethesda, MD) using gel-scanning macros. To estimate COX-2 expression levels, a “COX-2 index” was designated by the band density ratio of COX-2/COX-1. The COX-2 index was analyzed based on clinicopathological findings of the tumors: sex, age, tumor location, tumor size in maximum diameter, tumor surface area, depth of tumor invasion, lymph node metastasis, hematoxylin metastasis, lymphatic permeation, vascular permeation, and stage. The TNM classification was used for pathological classification and staging (18). Tumor sizes were determined by the maximum diameters of fresh specimens and divided into three classes: ≤3 cm, ≤6 cm, and >6 cm. The tumor surface areas, which were calculated by multiplying the maximum diameter by the diameter crossing at the right angle, were classified as: ≤15 cm², ≥30 cm², and >30 cm². Statistical significance was determined by Wilcoxon signed-ranks test, one-factor ANOVA, Student’s t test, Mann-Whitney test, Kruskal-Wallis test, simple regression, and Spearman’s correlation coefficient by rank.
Table 1  Clinical and pathological characteristics of patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of samples/no. of cases</td>
<td>86/43</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>64.3 ± 1.7 (44–84)a</td>
</tr>
<tr>
<td>Sex (no. of males:no. of females)</td>
<td>22:21</td>
</tr>
<tr>
<td>Tumor location (no. in colon:no. in rectum)</td>
<td>25:18</td>
</tr>
<tr>
<td>Tumor size in maximum diameter (cm)</td>
<td>54.2 ± 3.9 (14–120)a</td>
</tr>
<tr>
<td>Tumor surface area (cm²)</td>
<td>26.9 ± 3.5 (14–120)a</td>
</tr>
<tr>
<td>Histological differentiation (well:moderately:poorly)</td>
<td>22:19:2</td>
</tr>
<tr>
<td>Depth of invasion (pT1:pT2:pT3:pT4)</td>
<td>2.6:19:16</td>
</tr>
<tr>
<td>Lymph node metastasis (pN0:pN1:pN2)</td>
<td>16:18:9</td>
</tr>
<tr>
<td>Distant metastasis (pM0:pM1)</td>
<td>34:9</td>
</tr>
<tr>
<td>Lymphatic permeation (−:+:;++:+++)</td>
<td>6:21:12:4</td>
</tr>
<tr>
<td>Stage (I:II:II:III:IV)</td>
<td>4:10:20:9</td>
</tr>
</tbody>
</table>

a Mean ± SE (range).

Results

Clinical and pathological characteristics of the patients are listed in Table 1. Of 43 patients with colorectal carcinomas, 27 had metastasis to lymph nodes. Of nine patients with distant metastasis, seven had liver tumors, one had lung and bone tumors, and another had ovarian tumors. Tumor sizes of the fresh specimens varied from 1.4 to 12 cm in the maximum diameter, with a mean value of 5.4 ± 0.4 cm. Their mean tumor surface area was 26.9 ± 3.5 cm².

To estimate the COX-2 level, we amplified a COX-2 mRNA fragment by reverse transcription-PCR and determined its ratio to that of COX-1 (i.e., COX-2 index = COX-2/COX-1 ratio; see “Materials and Methods”). Both COX-1 and COX-2 mRNAs were detected in all carcinoma tissues. Although COX-2 was detected in the normal colorectal mucosa in 15 patients (35%), their levels were very low (see “Discussion”). In contrast, colorectal carcinomas showed significantly higher COX-2 index values than did normal colorectal mucosae (1.7 ± 0.2 compared with 0.2 ± 0.7, P < 0.001, by Wilcoxon signed-ranks test). Interestingly, the COX-2 indices were significantly higher in larger tumors. Namely, there was a significant increase with tumors of larger maximum diameter (P = 0.036, by Kruskal-Wallis test; Fig. 1A) and of larger surface area (R = 0.48, P = 0.001, by simple regression and Spearman’s correlation coefficient by rank; Fig. 1B). In addition, the COX-2 indices were higher in carcinomas with deeper invasions, with a marginal statistical significance (P = 0.061, by one-factor ANOVA; Fig. 2A). On the other hand, the COX-2 index showed no correlation with any of the following parameters: existence of lymph node metastasis (P = 0.61, by Student’s t test; data not shown), grades of lymph node metastasis (P = 0.84, by one-factor ANOVA; Fig. 2B), distant metastasis (P = 0.37, by Mann-Whitney test; Fig. 2C), or clinical stages (P = 0.32, by one-factor ANOVA; Fig. 2D). The COX-2 index was not affected by sex, tumor location, histopathological type, lymphatic permeation, or vascular permeation (P = 0.69, 0.97, 0.70, 0.33, and 0.12, respectively).

Discussion

We have demonstrated that COX-2 was expressed in all primary colorectal carcinoma samples at significant levels. Moreover, the COX-2 index values were significantly higher in tumors with larger diameters and greater surface areas. The COX-2 indices were also higher in tumors with deeper invasions. On the other hand, COX-2 levels were not correlated whether the patients had metastatic foci or not. Recently, Yang et al. (14) reported that the prostanoid levels in adenomas of FAP patients were significantly elevated with increased tumor sizes. Interestingly, however, the elevation of prostanoid levels was not observed until an adenoma reached a size of 6–7 mm in diameter (14). Although we did not have carcinoma samples that were <1 cm in diameter, the fact that the least squares line intercepted the ordinate around 1.0 in Fig. 1B suggests that COX-2 was expressed from very early stages of the carcinoma growth. The least squares line intercepted the ordinate at a positive value when COX-2 indices were plotted against the diameter as well (data not shown). Different from tumors that grow in such tissues as liver and lung, primary colorectal carcinomas are not usually globular in shape but rather grow flat along the colonic lumen. Accordingly, it is difficult to determine their precise volumes. Practically, however, their maximum diameters and surface areas can be used to estimate their volumes (19). Because most colorectal carcinomas are derived from adenomas (20), these results, taken together, support a hypothesis that the COX-2 level increases significantly upon progression of adenomas to carcinomas. Although this hypothesis is based on the assumption that the adenomas in FAP are of the same nature as the precursors of sporadic colorectal carcinomas, it appears reasonable because suppressive effects of NSAIDs have been reported in sporadic colorectal adenomas as well (21, 22).

In an immunoblot analysis, Kargman et al. (12) reported expression of COX-2 in 19 of 25 human colon carcinoma tissues. Although their report does not contain the size information on the tumors examined, there was no association between the stage and the COX-2 level. However, they had only one sample in Dukes’ stage A, which indicates that the tumor was within the intestinal mucosa. This tumor...
showed no increase in the COX-2 level. On the other hand, they examined 10 samples in Dukes’ stage B, which had invasions through the muscularis propria, and these tumors ranged from no COX-2 to the highest COX-2 expression in their results. Because the Dukes’ classification does not separate the tumor and invasion parameters as does the TNM classification, their Dukes’ B samples could contain tumors of various sizes. Accordingly, the results of Kargman et al. (12) do not necessarily contradict our results. In both their study and our study, no associations were found between metastasis and the COX-2 level. It is also worth noting that they reported reduced COX-1 protein levels in colorectal carcinoma tissues, as compared with those in normal colon. Some studies, in contrast, revealed similar amounts of COX-1 mRNA between carcinoma and normal mucosal tissues (10, 17). The COX-1 mRNA levels we determined by reverse transcription-PCR remained in a similar range in 17 carcinoma samples compared with the normal colonic mucosa (data not shown). We also estimated the protein levels of COX-1 and COX-2 by Western analysis in seven specimens and found similar amounts of COX-1 (data not shown). These results justify that the COX-2 index (i.e., the ratio of COX-2/COX-1) can be used as a convenient estimate for the actual COX-2 level. Although 15 samples (35%) we examined were positive for COX-2 expression in normal mucosal tissues, their COX-2 indices were significantly lower (0.2 ± 0.7) than those of carcinoma tissues (1.7 ± 0.2). It is conceivable that the low levels of COX-2 were derived from macrophages, vascular endothelial cells, and neuroendocrine cells in the normal colorectal tissues (11, 23).

Although evidence has been presented that COX-2 plays a key role in colorectal carcinogenesis, the mechanism by which COX-2 supports the tumor growth remains to be investigated (24). We have demonstrated that COX-2 levels are significantly higher in tumors with larger sizes (i.e., volumes) and those with deeper invasions, suggesting that tumors with more COX-2 grow larger and in a more invasive manner. Although it is expected that development of carcinomas in FAP patients can be prevented by NSAIDs, unwanted adverse effects exemplified by gastrointestinal bleeding often hamper their long-term administrations. Our results, taken together with those of others, suggest a possibility that COX-2 inhibitors that should be devoid of such side effects can be effectively used not only in reducing benign polyps but also in suppressing malignant carcinoma growth.

Fig. 2. Relationships between the COX-2 index and clinicopathological characteristics. A, correlation with the depth of invasion (a marginal statistical significance: P = 0.061, by one-factor ANOVA). pT1, pT2, pT3, and pT4 indicate invasions into submucosa, muscularis propria, subserosa or nonperitonealized pericolic/perirectal tissues, and other organs or structures/visceral peritoneum, respectively. B, no correlation with grades of lymph node metastasis (P = 0.84, by one-factor ANOVA). pN0 indicates no regional lymph node metastasis. pN1 and pN2 indicate metastasis in one to three and four or more regional lymph nodes, respectively. C, no correlation with existence of distant metastatic foci (P = 0.37, by Mann-Whitney test). Patients in the pM0 group had. D, no correlation with the clinical stage (P = 0.32, by one-factor ANOVA). Stages I, II, III, and IV indicate invasion within muscularis propria without metastasis, invasion beyond muscularis propria without metastasis, existence of lymph node metastasis without distant metastasis, and existence of distant metastasis, respectively. Stages I, II, and III coincide with Dukes Stages A, B, and C, respectively. The number and vertical bar to the right of each group indicate the mean and SD of COX-2 indices, respectively. Each factor was determined according to the TNM classification (18).

References

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