Increased Expression of Cyclooxygenase-2 to -1 in Human Colorectal Cancers and Adenomas, but not in Hyperplastic Polyps

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Background: Non-steroidal anti-inflammatory drugs can reduce the risk of colorectal cancer. Reportedly, mRNA expression of cyclooxygenase-2 (COX-2) is elevated in human colorectal cancers compared with accompanying normal mucosa. The present study was undertaken to establish a simple analytical procedure to quantify COX-2 expression levels and to characterize COX-2 expression levels in human colorectal cancers, adenomas and hyperplastic polyps.

Methods: The combination of PCR using common primers designed in the highly conserved regions and fluorescence-based single-strand conformation polymorphism (F-SSCP) analysis of the products is used for quantitative determination of the proportions of COX-2 mRNA in human colorectal cancers, adenomas, hyperplastic polyps and accompanying normal mucosa.

Results: The present F-SSCP analysis was a simple and powerful method for quantitative determination of the proportions of COX-2 mRNA. The proportion of COX-2 mRNA was higher in cancer tissues than in accompanying normal mucosa in 46 of the 50 cancers. There was no significant correlation between the increase of the COX-2 proportion and tumor location or stages. The enhanced COX-2 expression was also observed in colorectal adenomas. On the other hand, the proportion of COX-2 mRNA in hyperplastic polyps was not significantly different from that in normal mucosa.

Conclusions: The proportion of COX-2 to COX-1 expression was elevated in most human colorectal cancers and adenomas, but not in hyperplastic polyps. Therefore, the increased proportion of COX-2 expression might be an early event in the carcinogenesis of colorectal cancer.

Key words: cyclooxygenase, colorectal cancer, adenoma, hyperplastic polyp, reverse transcribed polymerase chain reaction, single-strand conformation polymorphism

INTRODUCTION

Recent studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenase (COX: prostaglandin endoperoxide synthase; EC 1.14.99.1) and the synthesis of prostaglandins (PGs), can reduce the formation of colon cancers in experimental animals given carcinogens (1). Epidemiological analyses have indicated that administration of NSAIDs can reduce the incidence of colorectal cancer in humans (2-4). Mammalian cells contain two related, but unique, isoforms of COX, referred to as COX-1 and COX-2. These two proteins are encoded by separate genes (5,6). The most dramatic difference between these two isozymes is observed in their patterns of expression. COX-1 is present in most tissues and is involved in the physiological production of PGs for maintaining normal homeostasis, whereas COX-2, which is induced by mitogens, cytokines and growth factors, is primarily responsible for PGs produced in inflammatory sites (7-9). Eberhart et al. (10) have shown that COX-2, but not COX-1, mRNA expression is markedly elevated in the tissues of most human colorectal cancers and some colorectal adenomas compared with accompanying normal mucosa.
normal mucosa. More recent studies suggest that COX-2 is related to colon carcinogenesis and may be the target for the chemopreventive effect of NSAIDs. Elevated levels of COX-2 protein and mRNA, but not those of COX-1, are found in chemically introduced rat colon carcinoma tissues (11,12) and in human colon carcinoma (12-15). Furthermore, genetic disruption of the COX-2 gene or treatment with a COX-2 specific drug suppresses the polyp formation in mice for familial adenomatous polyposis and marked increases in COX-2 enzyme concentrations are found in early polyps in these animals (16).

Previously, we reported the combined use of an automated fluorescence-based DNA sequencer and a data processing computer, allowing the exact quantification of polymorphic DNA sequences and the combination of reverse transcribed polymerase chain reaction (RT-PCR) and single-strand conformation polymorphism (SSCP) analysis, for the quantitative detection of expression levels of genes encoding homologous sequences (17,18). The purpose of the present study was to establish a simple procedure to analyze proportions of COX-2 and COX-1 mRNAs using RT-PCR–SSCP, thus permitting application to small amounts of biopsied specimens or surgical resected specimens (colonrectal cancer, adenoma and polyps). The principle of the present investigation is based on the condition that COX-1 is constantly expressed in most tissues and that the proportion of COX-2 to total COX mRNA could reflect the amount of COX-2 mRNA.

METHODS

SUBJECTS

Colorectal tissues were obtained from the National Cancer Center Hospital, cancer and normal accompanying mucosa resected at surgery and hyperplastic polyps, adenoma and corresponding normal mucosa at endoscopic biopsy. The tissues were stored in liquid nitrogen or at -80°C until use.

DNA EXTRACTION, RNA PREPARATION AND CDNA SYNTHESIS

DNA was extracted from tissue specimens by a method described previously (19). Total RNA was prepared from various cancer cell lines and colorectal tissues using a standard guanidium thiocyanate–phenol–chloroform–isoamyl alcohol extraction method (20). Reverse transcriptase reactions (20 µl) were carried out with -0.5–1.0 µg of total RNA as templates and random hexamer as primers, using SuperScript RNase free reverse transcriptase (GIBCO BRL, Gaithersburg, MD).

DESIGN AND SYNTHESIS OF FLUORESCENCE-LABELED PRIMERS

Oligonucleotide primers were prepared using an Oligo 1000 DNA synthesizer (Beckman Instruments, Fullerton, CA). In order to coamplify both COX-1 and COX-2 cDNAs, we designed a set of PCR primer pairs that anneal to the conserved region of COX cDNA sequences, i.e. in exons 5, 6 and 7 (Fig. 1) (21–23). Both primers contained several mismatches to the target sequences of either COX-2 or COX-1 cDNA. The nucleotide sequences of the primers were 5'-AAG-TTC-ATA-CCT-GAT-

Figure 1. Homology alignment of the nucleotide and amino acid sequences highly conserved in human COX-1 and COX-2 and PCR primer sequences for COX cDNA. Part of the nucleotide and amino acid sequences of COX-1 and those of COX-2 that differ from those of COX-1 are indicated. Identical nucleotides are indicated by hyphens and primer sequences are denoted by bold characters.

CCC-CA-3′ (forward) and 5′-GGA-GGA-TAC-ATT-TCT-CCA-TC-3′ (reverse). The primers yielded a product of 236 bp from both COX-2 and COX-1 cDNAs. The 5′-terminus of the forward primer was labeled with indodicarbocyanine (Cy5 amridite) fluorescent dye, using ALFred (Cy5 amidite) reagent (Pharmacia, Uppsala, Sweden).

We also designed specific primers for COX-2 and COX-1 to make cDNA fragments containing sequences of either COX-2 or COX-1. The nucleotide sequences of the primers were 5′-CCA-GTA-TAA-GTG-GGA-TAC-ATT-TCT-CCA-TC-3′ (forward; 211–232) and 5′-CCG-TAG-ATG-CTC-AGG-GAC-TTG-A-3′ (reverse; 907–886) for COX-2 and 5′-TCC-ATT-CTG-GGA-CCA-GCA-GGA-CAT-C-3′ (forward; 114–135) and 5′-TGG-CTC-TGA-CTT-CCA-CAT-C-3′ (reverse; 848–827) for COX-1.

PCR PROCEDURES

The cDNAs were amplified by PCR using a Cy5 labeled primer. The reaction mixture (25 µl) contained 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl2, 1 g/l gelatin and 0.2 mmol/l each of four dNTPs, oligonucleotide primers (6 pmol each), 0.6 units of AmpliTaq polymerase (Perkin-Elmer-Cetus, Branchburg, NJ) and 0.5 µl of the cDNA prepared above. After the first denaturation step at 94°C for 3 min, 45 cycles of reaction were performed at 94°C for 30 s, at 53°C for 30 s and at 72°C for 1 min, with a final extension at 72°C for 7 min. The yields of amplified DNA fragments were determined by electrophoresis in 8% polyacrylamide gels and visualized by ethidium bromide staining. A 5 µl volume of the reaction mixture was mixed with 0.5 units of Klenow fragment (Takara Shuzo, Shiga, Japan) and incubated at 37°C for 1 h in order to generate blunt-end (16,17).

SSCP ANALYSIS

An ALFred DNA sequencer (Pharmacia) equipped with a short gel plate (200 mm height × 345 mm width × 0.35 mm thickness) was used for SSCP analysis. A 1 µl volume of the amplified
products after treatment with Klenow fragment was mixed with 10 µl of loading solution containing 90% deionized formamide, 20 mmol/l EDTA and 0.05% bromophenol blue. After denaturation at 80°C for 5 min, 1 µl of the mixture was applied to a 10% polyacrylamide gel (the ratio of acrylamide and bisacrylamide was 30:1) containing Tris–glycine buffer (25 mmol/l Tris, 192 mmol/l glycine). Electrophoresis was performed at 20 W for 5 h at 20°C, using a running buffer consisting of 25 mmol/l Tris and 192 mmol/l glycine. Electrophoretic profiles were analyzed by the software Fragment Manager (Pharmacia). The proportion of COX-2 eDNA to total COX eDNA was calculated as COX-2 versus COX-2 plus COX-1, from the area of the signals for COX-2 and COX-1.

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed with the Vectorstain–biotin peroxidase complex kit (Vector, Burlingame, CA) following previous reports (14,21,24). The antisera used were raised against human COX-1 and COX-2 polypeptide (14,24). Colon tissues were preserved in 10% formalin and the specimens were embedded in paraffin, serially sectioned onto microscope slides at a thickness of 4 µm. The slides were stained with antibodies against COX-2 and COX-1 and normal rabbit serum as a negative control at dilution of 1:100. In the final step, color was developed with a solution containing 0.02% peroxide, tetrahydrochloride, 0.04% nickel chloride and 0.01% hydrogen peroxide in 0.05 M Tris–HCl (pH 7.2) for 2–5 min. The sections were counterstained with 0.5% light green (Sigma, St Louis, MO). Specificity was determined by preadsorption of anti-COX-1 or -2 antibody with the COX-1 or -2 synthetic polypeptide (1 mg/ml) before staining. For each tissue specimen, the extent and intensity of staining with COX-1 and -2 antibodies were graded on a scale of 0 to 4+, as described previously (24).

RESULTS

EXPERIMENTAL CONDITIONS FOR SSCP ANALYSIS

The forward primer anneals COX-I and COX-2 sequences with one mismatch and the reverse primer anneals them with COX-1 and COX-2 sequences with two and one mismatches, respectively. The COX-1 or COX-2 could be amplified by the primers described above from solutions containing COX-1 or COX-2 cDNA, respectively. In PCR amplification, a combination of a Cy5 labeled forward primer and a non-labeled reverse primer provided a good separation of DNA fragments from COX-2 and COX-1 cDNAs by the SSCP analysis as used in the present experiments (Fig. 2). The peaks of COX-1 and COX-2 were electrophoresed with similar migrations in both normal and cancer tissues. The relative proportion of COX-2 to COX-2 plus COX-1 was calculated from the densitometric curves.

COX-2 and COX-1 cDNAs (each ~10 pg/µl) amplified by using the specific primers for them were mixed in different ratios (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1). The mixtures were subjected to PCR followed by SSCP analysis. Determination of the proportion of COX-2 in the mixture confirmed good linearity, as in previous reports using lactate dehydrogenase isozymes (Fig. 3).
Table 1. COX-1 and COX-2 expression in normal and cancer tissues of colon

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Location</th>
<th>Histological differentiation</th>
<th>Normal COX-1*</th>
<th>Normal COX-2*</th>
<th>Normal COX-2 (%)†</th>
<th>Cancer COX-1*</th>
<th>Cancer COX-2*</th>
<th>Cancer COX-2 (%)†</th>
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<tr>
<td>7</td>
<td>53</td>
<td>M</td>
<td>Sigmoid colon</td>
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<td>0</td>
<td>0</td>
<td>36.7</td>
<td>0</td>
<td>1.5+</td>
<td>99.9</td>
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<tr>
<td>10</td>
<td>50</td>
<td>F</td>
<td>Rectum</td>
<td>Well</td>
<td>0</td>
<td>0</td>
<td>39.0</td>
<td>0</td>
<td>1.5-2+</td>
<td>90.5</td>
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<tr>
<td>21</td>
<td>73</td>
<td>F</td>
<td>Ascending colon</td>
<td>Poorly</td>
<td>0</td>
<td>0</td>
<td>35.9</td>
<td>0</td>
<td>0.5-1+</td>
<td>97.9</td>
</tr>
<tr>
<td>23</td>
<td>51</td>
<td>M</td>
<td>Rectum</td>
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<td>0</td>
<td>0</td>
<td>27.5</td>
<td>0</td>
<td>0.5-1+</td>
<td>99.9</td>
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<tr>
<td>26</td>
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<td>M</td>
<td>Sigmoid colon</td>
<td>Well</td>
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<td>0</td>
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<td>Rectum</td>
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<td>3.5-4+</td>
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<td>0</td>
<td>8.3</td>
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<tr>
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<td>1.0</td>
<td>0.5+</td>
<td>2.5-3+</td>
<td>96.2</td>
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</table>

*The intensity of immunostaining was graded on a scale of 0-4+ by a blind observer: 0, no staining; 4+, maximum intensity. †COX-2 (%) means the proportion of COX-2 mRNA to total COX (COX-2 plus COX-1), which was calculated from the RT-PCR-SSCP.

**The proportion of COX-2 mRNA in colorectal tissues, cancer, adenoma and hyperplastic polyps**

A total of 50 cases of paired adenocarcinoma and normal mucosa obtained from surgical materials were evaluated (Fig. 4). The proportion of COX-2 mRNA to total COX mRNA was higher in cancer tissues than in normal mucosa in 46 of the 50 cases. Ten pairs of colorectal adenoma, nine pairs of colorectal hyperplastic polyps and corresponding normal mucosa were also evaluated. The proportion of COX-2 mRNA was higher in adenoma than in normal mucosa in nine of the 10 cases, whereas in hyperplastic polyps only three of the nine cases showed slightly higher proportions of COX-2 mRNA than normal mucosa. By a paired t-test, the mean of the proportion in cancer tissues and adenoma is significantly higher ($p < 0.01$) than that in normal mucosa. On the other hand, that in hyperplastic polyps is not significantly different from that in normal mucosa ($p < 0.05$).

In several cases, colonic tissues from colorectal cancers and normal tissues were evaluated for immunoreactive COX-1 and COX-2 with specific antisera. The intensity of immunostaining was evaluated as shown in Table 1. In tissues of colorectal cancers, marked expression of immunoreactive COX-2 was shown whereas staining of immunoreactive COX-1 was very weak.

The proportion of COX-2 mRNA to total COX was classified according to degree of differentiation, location of the cancer and clinical stage (Fig. 5). No conditions had a significant effect on the proportion of COX-2 ($p < 0.05$) (by the Mann–Whitney test).

**DISCUSSION**

Recent clinical and epidemiological observations have suggested that NSAIDs decrease the incidence of colorectal cancer (2–4,25). In patients with familial adenomatous polyposis, administration of sulindac resulted in a striking reduction in adenoma size and number (26). Moreover, in animal models of colon carcinogenesis, COX inhibitors including indomethacin, sulindac and piroxicam exhibit chemopreventive effects as judged by reductions in the number of tumors per animal. COX catalyzes the committed step in the formation of prostaglandins and thromboxane from arachidonic acid and also catalyzes the oxidation of a wide range of biological materials including several classes of chemical carcinogens (27,28). The synthesis of COX, especially COX-2, is stimulated by growth factors or cytokines such as interleukin 1, tumor necrosis factor, epidermal growth factor and platelet-derived growth factor (29,30) and prostaglandins also seem to modulate cellular proliferation in a variety of cell types (31,32). Although the precise mechanism by which NSAIDs inhibit colon carcinogenesis is unknown, prostaglandins and COX enzymes may be involved in the process of carcinogenesis because a major action of NSAIDs is the inhibition of COX.
expression was observed in most colorectal cancers and adenomas but not in hyperplastic polyps. The results suggested that the up-regulation of COX-2 expression might be an early event in the disease process. We also showed that the increased proportion of COX-2 expression was consistently observed in any stage, tumor location or degree of differentiation.

In mammalian cells COX-1 and COX-2 proteins are encoded by two separate genes (5,6). The deduced amino acid sequences of COX-2 exhibited about 60% similarities to the sequences of COX-1 proteins and 81–88% similarities among the COX-2 enzymes of different species. The amino acid residues established to be important in catalysis by COX-1 are conserved in COX-2 (5,6). In particular, since human COX-2 exons 4–10 show over 70% identity to COX-1 exons at the amino acids level, we selected PCR primers in exons 5, 6 and 7. COX-2 cDNA, which encodes a polypeptide of 604 amino acids, is about 58% identical with the COX-1 cDNA in the nucleotide sequence level (21–23).

The present procedure is essentially based on the assumption that COX-1 expression is constant in tissues. Reportedly, COX-1 is present in most tissues and mostly constant in any physiological conditions (7–9). This means that our procedure detects only relative ratios of COX-2 expression against COX-1 expression. However, COX-1 might be an adequate control to detect the COX-2 expression level. Immunohistochemical staining could not detect COX-1 in some cases and was not consistent with the result of RT-PCR–SSCP. It is less efficient and has lower sensitivity. It is also subjective and difficult to make quantitative. The slight inconsistency between the proportion of COX-2 estimated by RT-PCR–SSCP and COX-2 intensity of immunostaining might be due to the above reasons.

The present procedure for determining relative ratios of COX-2 expression has the following advantages. Template concentrations and PCR cycles do not affect the proportions of COX-2 in the product if the amplified products have sufficient fluorescence signals, as was shown in our previous report (18). It has good reproducibility and linearity. No specific additional steps are required after PCR except for Klenow treatment and electrophoresis on a DNA sequencer. The method also lends itself to the simultaneous examination of multiple samples. The main advantages are its simplicity and relatively high sensitivity. Current PCR-based techniques can be applied to biopsied specimens of colorectal polyps and cancer tissues in order to investigate some properties of cancer and the preventive effects of NSAIDs on carcinogenesis. Our subsequent efforts will be directed towards a comprehensive analysis of COX-2 expression and clinical time course, including carcinogenesis, in human colorectal cancers, adenomas and polyps before and after administration of NSAIDs such as COX-2 specific inhibitor or non-specific COX inhibitor.

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References


