

## Expression of Cyclooxygenase-1 and Cyclooxygenase-2 in Human Breast Cancer

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**Background:** Numerous studies have demonstrated that the levels of prostaglandins are greater in various cancers, including breast cancer and colon cancer, than in normal tissues. In particular, the inducible form of cyclooxygenase (COX), the rate-limiting enzyme in prostaglandin biosynthesis, is overexpressed in colon tumors. Epidemiologic studies have demonstrated that the use of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of colon cancer and, to a lesser extent, the risk of breast cancer. NSAIDs are known to inhibit COX, suggesting that the beneficial effect of NSAIDs in colon cancer may be related to COX overexpression in this disease. This possibility led us to ask whether COX is also overexpressed in breast cancers. **Methods:** Surgical specimens from 44 patients with breast cancer who had undergone lumpectomy or mastectomy were analyzed by immunoblot analysis and immunohistochemical analysis to determine the expression profile of the constitutively expressed form of cyclooxygenase (COX-1) and the inducible form (COX-2); the specimens from 14 patients included normal breast tissue. **Results:** Expression of COX-1 protein was substantially higher in 30 of 44 tumor samples than in any of the 14 normal tissue specimens. Immunoblot analysis revealed extremely high levels of COX-2 protein in two tumor samples. Immunohistochemical staining of specimens that expressed COX-1 and/or COX-2 revealed that COX-1 was localized in stromal cells adjacent to the tumor but not in tumor cells. In contrast, COX-2 was localized primarily in tumor cells but also appeared in stromal cells. **Conclusion:** Our results suggest that overexpression of COX may not be unique to colon cancer and may be a feature com-

mon to other epithelial tumors. [J Natl Cancer Inst 1998;90:455-60]

Various epidemiologic studies have revealed that the use of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of colon cancer (1-4). Epidemiologic studies investigating the effects of NSAIDs on cancers other than colon cancer have been limited and have yielded inconclusive results. Three prospective studies (1,5,6) and one case-control study (7) revealed no statistically significant relationship between the use of NSAIDs and the risk of breast cancer. However, a case-control study (3) and a prospective study (8) showed a statistically significant reduction of breast cancer risk with the use of NSAIDs.

Cyclooxygenase (COX) (otherwise known as prostaglandin endoperoxide synthase) catalyzes the conversion of arachidonic acid to prostaglandin endoperoxide (prostaglandin  $H_2$ ). This is the rate-limiting step in prostaglandin and thromboxane biosynthesis. Two isoforms of COX have been cloned from various animal cells: 1) constitutively expressed COX-1 (9-13) and 2) mitogen-inducible COX-2 (14-19). Numerous studies (20-25) have demonstrated that the levels of prostaglandins in various tumors or the tumor's biosynthetic capacity for prostaglandins is greater than that of normal tissues. Recently, it has been shown that the inducible form of COX is overexpressed in colon tumor tissues (26-29). Malignant human breast tumors produced more prostaglandin-like material than did benign tumors or normal breast tissues (20). Thus, it needs to be determined whether the increased prostaglandin production in tumors other than colon cancer is also due to enhanced expression of COX.

In this study, we evaluated the profile of COX expression in mastectomy and lumpectomy specimens from patients with breast cancer to determine whether overexpression of COX is unique to colon cancer or whether it also occurs in breast cancer.

## Materials and Methods

### Tissue Samples

Lumpectomy and mastectomy specimens from patients with breast cancer were separated into tu-

mor tissue and predominantly normal tissue by a pathologist. They were then quickly frozen in liquid nitrogen, and portions of the specimens were embedded in paraffin. Samples were analyzed from 44 patients, including 14 patients whose specimens included both tumor and normal breast tissues with no histologic evidence of presence of cancer cells.

### Immunoblot Analysis

Frozen tissues were homogenized in lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 1 mM diethyldithiocarbamic acid, 1 mM ethylenediaminetetraacetic acid [EDTA], 1% Tween 20, 10  $\mu$ M leupeptin, and 1 mM phenylmethylsulfonyl fluoride) on ice. The lysates were sonicated at 35 strokes, then centrifuged at 10000g at 4°C for 20 minutes. After centrifugation, the supernatant was used for immunoblot analysis. Immunoblot analysis for COX-1 and COX-2 was carried out essentially as described in our previous study (30). Briefly, solubilized proteins were separated in a sodium dodecyl sulfate-polyacrylamide gel (8% gel) and transblotted onto a polyvinylidene difluoride transfer membrane. The membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20. The membrane was analyzed by the ECL detection system (Amersham Life Science Inc., Arlington Heights, IL). Polyclonal anti-COX-2 antibodies were prepared as described in our previous study (31). To prepare polyclonal antibodies for COX-1, we immunized three rabbits with COX-1 protein purified from ram seminal vesicle (Oxford Biomedical, Oxford, MI). Antiserum from one of the rabbits showed no cross-reactivity with COX-2 protein as assessed by western blot analysis (Fig. 1, C). The anti-COX-1 antibodies recognized COX-1 from human platelets, rat alveolar macrophages, and murine peritoneal macrophages. To prepare polyclonal anti-COX-2 antibodies, we used a 19-amino acid polypeptide conjugated to thyroglobulin, as described in our previous study (30). This sequence is present in the carboxyl-terminal region of murine COX-2 but not in COX-1. These anti-COX-2 antibodies recognize COX-2 from humans, rats, and mice. Polyclonal anti-actin antibody was purchased from Sigma Chemical Co., St. Louis, MO.

### Immunohistochemical Analyses

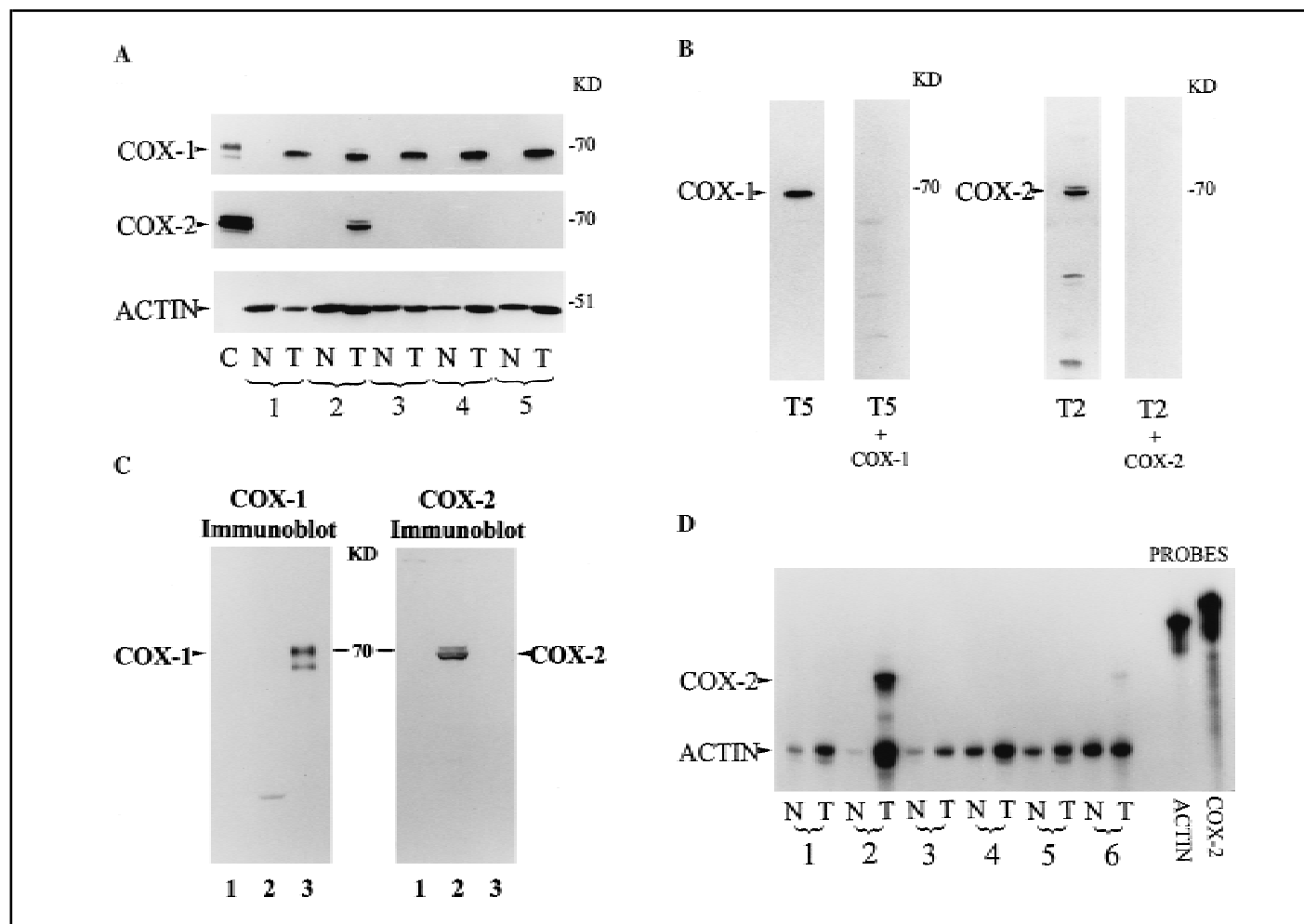
Formalin-fixed, paraffin-embedded tissues were cut to 4  $\mu$ m, and sections were deparaffinized, hydrated through graded xylene and alcohol, and treated with 0.05% saponin in PBS. Sections were incubated in a humidified chamber at 37°C with the

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See "Notes" following "References."

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**Fig. 1.** Expression of cyclooxygenase (COX)-1 and COX-2 in breast tumor tissues compared with that in matched normal tissues. A portion of the frozen tissue was homogenized in lysis buffer, and the solubilized lysate obtained after centrifugation (10 000g for 20 minutes at 4 °C) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8% gel) and immunoblot analysis for COX-1 and COX-2, as described in the “Materials and Methods” section. **A)** Results obtained for five representative tumor (T) and normal (N) tissues are shown. Solubilized lysates of human platelets and of a monocytic cell line (RAW 264.7) that had been stimulated with lipopolysaccharide (LPS) were used as positive controls (C) for COX-1 and COX-2, respectively. For actin, no positive control was used. Samples 1, 2, 3, 4, and 5 correspond to patients 4, 6, 9, 10, and 11, respectively, in Table 1. **B)** Tumor samples that had been incubated with COX-1 or COX-2 antiserum, with or without pretreatment with the respective antigen (purified COX-1 protein from ram seminal vesicle for COX-1 and a

19-amino acid peptide used for immunization for COX-2, respectively). T5 and T2 are the same tumor samples used in **A**. **C)** COX-1 and COX-2 immunoblots to test whether COX-1 antibodies cross-react with COX-2 and whether COX-2 antibodies cross-react with COX-1. Lane 1, cell lysate of RAW 264.7 cells without LPS treatment; lane 2, cell lysate of RAW 264.7 cells treated with LPS (this lysate contains only COX-2); lane 3, cell lysate of human platelets (this lysate contains only COX-1). **D)** Expression of COX-2 messenger RNA (mRNA) in breast tumor samples that express COX-2 protein. Total RNA was extracted from frozen tissues, and the abundance of COX-2 mRNA was determined by the ribonuclease (RNase) protection assay as described in the “Materials and Methods” section. The same samples that were analyzed in Fig. 1, A, and another set (#6) in which tumor tissue (patient 29 in Table 1) expresses COX-2 protein were analyzed by use of the RNase protection assay. KD = kilodaltons.

following reagents at dilutions and times indicated: normal goat serum (1 : 200), 30 minutes; rabbit anti-COX-1 (1 : 200) or anti-COX-2 (1 : 50), 60 minutes; biotinylated goat anti-rabbit serum (1 : 200; Vector Laboratories, Inc., Burlingame, CA), 30 minutes; 0.3% H<sub>2</sub>O<sub>2</sub> in PBS (pH 7.3), 10 minutes; and horseradish peroxidase-labeled avidin–biotin complex (1 : 50; Vector Laboratories, Inc.), 30 minutes; there were two washes in PBS, 5 minutes each, between each step. Peroxidase staining was generated by the addition of H<sub>2</sub>O<sub>2</sub> in PBS for 10 minutes, with amino-ethyl carbazole as the chromogen (Peroxidase Substrate Kit; Vector Laboratories, Inc.); the sections were counterstained with Gill’s hematoxylin, overlaid with an aqueous mounting medium (Aquamount; Lerner Laboratories, Pittsburgh, PA), and placed under a coverslip.

### Ribonuclease Protection Assay

Total cellular RNA was isolated by TRIZOL reagent [Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD] and quantitated by its absorbance at 260 nm. The probes for human COX-2 (nucleotides 574–732, 158 base pairs [bp]) and COX-1 (nucleotides 1076–1385, 309 bp) were obtained from Continental Scientific, Inc., San Diego, CA, and the probe for actin (nucleotides 820–946, 127 bp) was obtained from Ambion, Austin, TX. Probes were labeled with 800 Ci/mmol [<sup>32</sup>P]uridine triphosphate (Du Pont NEN, Wilmington, DE) by use of Ambion’s MAXIScript kit. Ten micrograms of total RNA was hybridized to <sup>32</sup>P-labeled antisense riboprobes by use of Ambion’s RPA II kit. The ribonuclease

(RNase) protection assay was performed as described previously (31).

### Results

#### Immunoblot Analysis of COX in Breast Tumor Tissues and Matched Normal Tissues

Surgical specimens from 44 patients were analyzed; specimens from 14 patients, mostly those who underwent mastectomy, included normal breast tissue. Substantially higher levels of constitutively expressed cyclooxygenase (COX-

1) protein were detected in 30 of 44 tumor tissues as compared with the levels in 14 normal tissues, whereas exceedingly high levels of COX-2 protein were detected in two tumor tissues. The levels of COX-1 or COX-2 protein in normal tissues were below the detection limit of the immunoblot analysis used in the present study. However, if the film was exposed to the membrane for a longer period of time, COX-1 protein was clearly detected (data not shown), indicating that COX-1 is expressed in the normal breast tissue as well. Results of the immunoblot analyses of five representative tumor tissues and matched normal tissues are shown in Fig. 1, A. COX-1 and COX-2 antibodies, pre-treated with the respective antigen, were unable to detect COX-1 or COX-2 protein from the tumor samples (Fig. 1, B), demonstrating the specificity of the antibodies.

In addition, anti-COX-1 antibody showed negligible cross-reactivity with COX-2, as assessed by western blot analysis (Fig. 1, C). Platelets are known to contain only COX-1. However, the monocytic cell line RAW 264.7 does not express COX-1 but selectively expresses COX-2 in response to lipopolysaccharide (LPS), as shown in our previous study (30). Thus, a cell lysate of human platelets or of RAW 264.7 cells that had been stimulated with LPS was used as a standard for COX-1 or COX-2, respectively. Anti-COX-2 antibodies did not cross-react with COX-1 in human platelets (Fig. 1, C).

#### RNase Protection Assay of COX-2 Messenger RNA in Breast Tumor Samples That Overexpress COX-2 Protein

The same sets of samples analyzed in Fig. 1, A, and an additional set (from patient 29; lane 6, Fig. 1, D) in which the tumor tissue expresses COX-2 protein were assayed by use of the RNase protection assay. COX-2 messenger RNA (mRNA) was detected in two tumor tissues (2T and 6T in Fig. 1, D) but not in normal tissues. The levels of COX-1 mRNA were below the detection limit of the RNase protection assay we used. However, COX-1 mRNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) by use of the primer sequences of TGCATGTGGCTGTG-GATGTCATCAG (forward) and CA-CAGCAGGCCCATCATCCTGAC (reverse) (data not shown).

#### Immunohistochemical Analysis of Breast Tumor Tissues for COX-1 and COX-2

Immunohistochemical staining of the formalin-fixed, paraffin-embedded tumor tissue (from patient 27 in Table 1) with

the use of anti-COX-1 antiserum revealed that COX-1 was localized in stromal cells (probably including macrophages) adjacent to the tumor cells but not in the tumor cells themselves (Fig. 2, b). In contrast, COX-2 was localized primarily in tumor cells, as well as in some stromal

**Table 1.** Characterization of surgical specimens from breast cancer patients

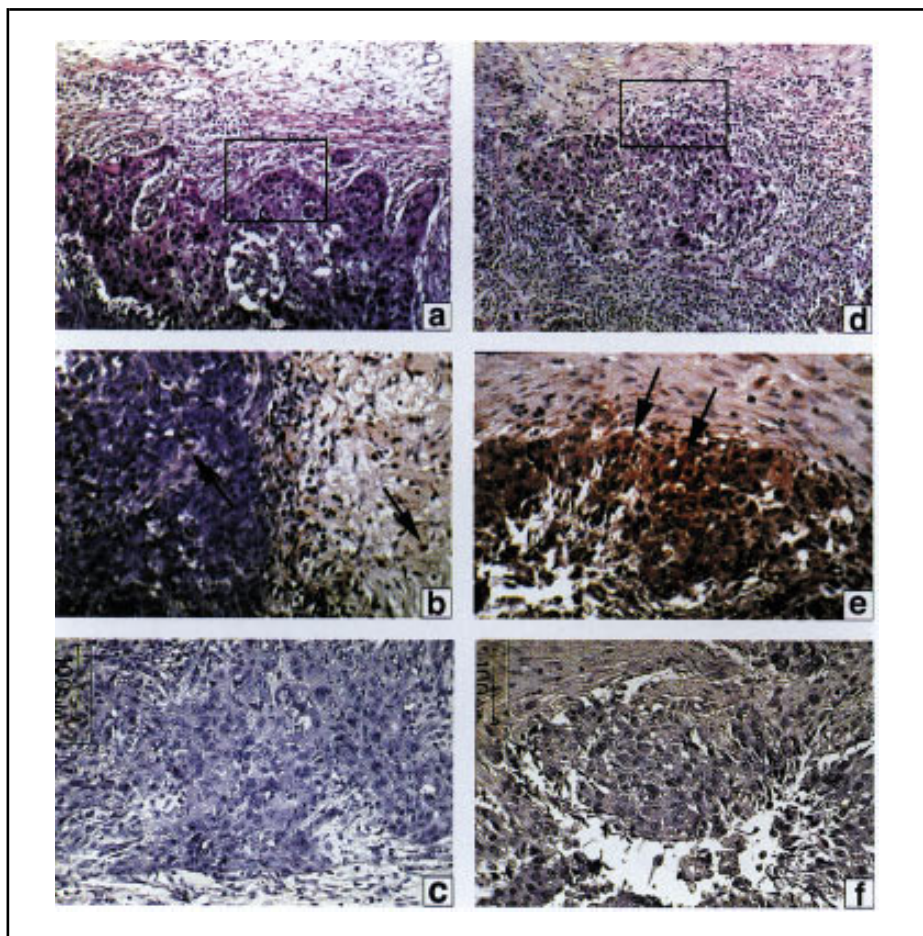
Patient No. and tissue*	Tumor type	Cyclooxygenase-1†	Cyclooxygenase-2‡
1N			
1T	Infiltrating ductal carcinoma	+	
2N			
2T	Infiltrating ductal carcinoma	+	
3N			
3T	Infiltrating ductal carcinoma	+	
4N			
4T	Infiltrating ductal carcinoma	+	
5N			
5T	Infiltrating ductal carcinoma	+	
6N			
6T	Invasive medullary	+	+
7N			
7T	Infiltrating lobular	+	
8N			
8T	Infiltrating ductal carcinoma	+	
9N			
9T	Intraductal carcinoma <i>in situ</i>	+	
10N			
10T	Infiltrating ductal carcinoma	+	
11N			
11T	Infiltrating ductal carcinoma	+	
12N			
12T	Infiltrating ductal carcinoma	+	
13N			
13T	Infiltrating ductal carcinoma	+	
14N			
14T	No report‡	+	
15T	Infiltrating ductal carcinoma	+	
16T	Infiltrating ductal carcinoma	+	
17T	Infiltrating ductal carcinoma		
18T	Infiltrating ductal carcinoma		
19T	Infiltrating ductal carcinoma		
20T	Infiltrating ductal carcinoma	+	
21T	Infiltrating ductal carcinoma	+	
22T	Infiltrating ductal carcinoma		
23T	Infiltrating ductal carcinoma		
24T	Infiltrating ductal carcinoma		
25T	No report‡		
26T	Infiltrating ductal carcinoma		
27T	Infiltrating ductal carcinoma	+	
28T	Infiltrating ductal carcinoma	+	
29T	Infiltrating ductal carcinoma	+	+
30T	Infiltrating ductal carcinoma		
31T	Infiltrating ductal carcinoma		
32T	Infiltrating ductal carcinoma		
33T	Infiltrating ductal carcinoma	+	
34T	Infiltrating ductal carcinoma	+	
35T	Infiltrating ductal carcinoma	+	
36T	Infiltrating ductal carcinoma		
37T	Lobular carcinoma		
38T	Adenocarcinoma		
39T	Ductal papillary carcinoma	+	
40T	Infiltrating ductal carcinoma	+	
41T	Infiltrating ductal carcinoma	+	
42T	Infiltrating ductal carcinoma	+	
43T	Infiltrating ductal carcinoma	+	
44T	Infiltrating ductal carcinoma	+	

\*N = normal tissue; T = tumor tissue.

†+ = high level of expression.

‡Pathology report of specimen unavailable in database.





**Fig. 2.** Immunohistochemical analysis of breast tumor tissues for cyclooxygenase (COX)-1 and COX-2. **a)** Hematoxylin–eosin-stained sections of the formalin-fixed, paraffin-embedded tumor tissue (specimen from patient 11 in Table 1) show a portion of the tumor and adjacent stroma. **b)** Immunohistochemical staining with the use of anti-COX-1 antiserum of deeper sections of the area are indicated as a rectangular box in **a**. Arrows indicate stromal cells. **c)** Immunohistochemical staining of the tumor specimen used in **b** with the preimmune rabbit serum for COX-1. **d)** Hematoxylin–eosin-stained sections of a specimen (from patient 6 in Table 1) demonstrate the tumor cells and stroma. **e)** Immunohistochemical staining of deeper sections of the area indicated as a rectangular box in **d** with the use of anti-COX-2 antiserum. Arrows indicate tumor cells. **f)** Immunohistochemical staining of the same tumor specimen as **d** with the COX-2 preimmune serum.

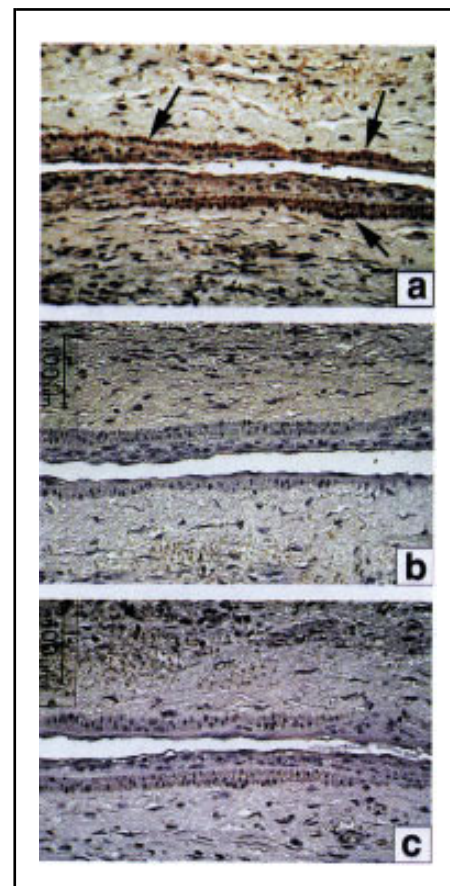
cells, in a specimen from patient 6 in Table 1 (Fig. 2, e). Immunohistochemical staining of a longitudinal section from patient 6 of an artery in tumor tissue that expresses COX-2 (T2 in Fig. 1, A) also demonstrated intense staining of the smooth muscle cells with anti-COX-2 antibodies (Fig. 3, a), whereas the smooth muscle cells did not stain with COX-2 preimmune serum or anti-COX-1 antiserum (Fig. 3, b and c).

## Discussion

In our previous studies, COX-1 mRNA was not detected by the RNase protection assay despite the presence of COX-1 protein in rat alveolar macrophages (30); however, COX-1 mRNA was detected by RT-PCR (31). Thus, it is not uncommon to see high levels of expression of COX-1

protein in the presence of trace amounts of its mRNA.

Parrett et al. (32) recently showed that COX-2 mRNA was detected by RT-PCR in all 13 breast tumor samples that they studied. However, they did not determine the frequency of COX-2 and COX-1 protein expression in their samples. The discrepancy in the frequency of COX-2 expression between the study by Parrett et al. (32) and our study is dramatic (100% [13 of 13] versus 5% [two of 44], respectively). This discrepancy needs to be evaluated on the basis of analytic methods used and the criteria by which expression frequency is determined. We based the frequency of COX-2 expression on both increased protein levels, as determined by immunoblot analysis, and a parallel increase in endogenous levels of mRNA, as



**Fig. 3.** Immunohistochemical staining of the tissue specimen from patient 6. **a)** Staining with anti-cyclooxygenase (COX)-2 antiserum; perivascular smooth muscle cells (arrows). **b)** Staining with the preimmune serum for COX-2. **c)** Staining with anti-COX-1 antiserum.

determined by the RNase protection assay instead of RT-PCR.

The pattern of COX expression in breast tumor tissues was quantitatively different from that in colon tumor tissues. Kargman et al. (28) showed that COX-2 protein was overexpressed in 19 of 25 colon tumor tissues, whereas COX-1 protein was detected in all normal and tumor tissues. In our study, much greater levels of COX-1 protein were detected in 30 of 44 tumor samples than in 14 normal tissues. The frequency of COX-2 expression in our study (two of 44 breast tumors), determined by immunoblot analysis, was much lower than that for colon cancer. The finding of marked expression of COX-2 by smooth muscle cells of a small artery near tumor tissue that overexpressed COX-2 is itself intriguing. One possible explanation for this result is that cancer cells may release paracrine factor(s) that can induce the expression of COX-2 in arterial smooth muscle cells

and perhaps in other cell types in tumor-bearing tissues.

Our results indicate that the overexpression of COX-1 protein occurs more often than that of COX-2 protein in most breast tumors analyzed. Since COX-1 protein is localized mainly in stromal cells instead of tumor cells, these results suggest the possibility that tumor cells may release factors that stimulate the recruitment of cells expressing COX-1 or that stimulate neighboring stromal cells to express COX-1 in addition to COX-2. Prostaglandins derived from stromal cells can act on tumor cells in a paracrine fashion, or vice versa. It has been demonstrated that the expression of COX-2 can be induced by cyclic adenosine monophosphate (cAMP) in mouse osteoblastic cells (33), suggesting that COX-2 expression can be increased in response to its catalytic product—prostaglandin E<sub>2</sub>—via the cAMP-signaling pathway. The COX-1 gene is considered to be a house-keeping gene. However, details of the regulation of COX-1 expression are virtually unknown.

Since the COX expressed in the breast tumor cells in our studies is primarily COX-2, COX-2 may be responsible for the production of prostaglandins during the tumor development. As the tumor progresses, COX-1 may become expressed in stromal cells, even when nearby tumor cells do not overexpress COX-2. This expression of COX-1 in stromal cells may play an important role in producing prostaglandins in later stages of tumor development. This implies that there may be a difference in the potential efficacy of chemoprevention between specific inhibitors of COX-2 and general inhibitors of COX. Constitutively expressed COX-1 and mitogen-inducible COX-2 are separate gene products, but both isozymes are structurally homologous and possess similar kinetic properties (34). Thus, although the regulatory mechanism for the expression of each isozyme is quite different, the net effect of the expression of either isozyme would be similar, i.e., an enhanced capacity of tissues to produce prostaglandins. For those tumors for which aspirin or NSAIDs are known to be beneficial in reducing the risk, the differential expression of COX-1 and COX-2 at various stages of tumor development could have profound implications for selecting the

proper type of COX inhibitor to test as a potential chemopreventive agent.

The mechanism by which aspirin and other NSAIDs act to reduce the risk of colon cancer is not well understood. Since the well-documented pharmacologic action of aspirin and other NSAIDs is to inhibit cyclooxygenase, it can be inferred that the beneficial effect of NSAIDs may be mediated through the inhibition of prostaglandin biosynthesis by NSAIDs. A corollary to this is that any dietary or pharmacologic agent that suppresses the production of prostaglandins may also help to reduce the risk of colon cancer. However, experimental evidence supporting this hypothesis has not been demonstrated in humans.

The factors that cause the expression of COX-2 in tumor cells and stromal cells and the role that COX-2 plays in tumorigenesis are not well understood. Results from a recent study (35) with transgenic mice provide genetic evidence that the induction of COX-2 is an early, rate-limiting step for adenoma formation. Furthermore, a specific inhibitor of COX-2 markedly reduces the number of polyps in APC<sup>Δ716</sup> (+/−) heterozygous mice. Tsujii and DuBois (36) showed that rat intestinal epithelial cells transfected with COX-2 complementary DNA (cDNA) are resistant to apoptosis. They have also shown that COX-2 expression in human colon cancer cells increases the metastatic potential (36). These results suggest that COX-2 overexpression leads to phenotypic changes in cells that can enhance the cells' tumorigenic and metastatic potential. Recently, Narko et al. (37) demonstrated that immortalized endothelial cells transfected with COX-1 cDNA become tumorigenic when they are injected into nude mice. Our results, which demonstrate an increased expression of COX-1 and COX-2 in breast tumors, underline the need for further study of the role of COX overexpression in breast tumors and the effects of NSAIDs on the risk of breast cancer.

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## Notes

Supported by Public Health Service grant DK41868 from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services; and by grant 93–37200–8961 from the Department of Agriculture.

We thank Ellen Arbour, Jin Kyung Kim, and Angelina Deming for their technical assistance and Dr. Donna Ryan for reading the manuscript.

Manuscript received September 4, 1997; revised December 10, 1997; accepted January 15, 1998.