[CANCER RESEARCH 62, 1676–1681, March 15, 2002]

Cyclooxygenase-2 Expression in Human Breast Cancers and Adjacent Ductal Carcinoma *in Situ*[1]

Elizabeth Half, Xi Ming Tang, Karin Gwyn, Aysegul Sahin, Kyle Wathen, and Frank A. Sinicrope[2]

Departments of Gastrointestinal Medicine and Nutrition [E. H., X. M. T., F. A. S.], Breast Medical Oncology [K. G.], and Biomathematics [K. W.] and Division of Pathology [A. S.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Cyclooxygenase-2 (COX-2) is an inducible enzyme that converts arachidonic acid to prostaglandins. Overexpression of the *COX-2* gene in mammary glands of transgenic mice was sufficient to induce tumorigenesis. We analyzed COX-2 expression in human breast cancers (and breast cancer cell lines) and adjacent ductal carcinoma *in situ* (DCIS) as well as its association with HER2/neu and clinicopathological variables. Archival primary breast carcinomas ($n = 57$), adjacent DCIS ($n = 14$) and DCIS alone ($n = 2$) were analyzed for COX-2 and HER2 expression by immunohistochemistry using specific monoclonal antibodies. An immunohistochemical scoring system was used. *HER2* gene amplification had been analyzed previously by fluorescence *in situ* hybridization ($n = 20$). Histology of carcinomas included infiltrating ductal ($n = 44$), lobular ($n = 2$), and other ($n = 7$). Frozen breast cancers and adjacent normal tissue pairs ($n = 9$) were analyzed for *COX-2* mRNA by reverse transcription-PCR. COX-2 and HER2 expression were also analyzed in human breast cancer cell lines (MCF-7, MCF-7/*HER2*, SK-BR-3, and MDA-MB-231) by immunoblotting. Cytoplasmic COX-2 expression was detected at an intermediate or high level in epithelial cells in 18 of 42 (43%) invasive breast cancers and in 10 of 16 (63%) cases of DCIS. Normal-appearing breast epithelia adjacent to cancer expressed COX-2 in 81% of cases and was generally focal and of similar or decreased intensity relative to adjacent neoplastic epithelia. *COX-2* mRNA was detected in all samples analyzed by reverse transcription-PCR and was increased in eight of nine breast cancers relative to paired normal tissue. In archival tumors, no significant correlation was found between COX-2 and HER2 expression/amplification and clinicopathological variables. COX-2 expression was induced in MCF-7 cells stably transfected with *HER2*, in contrast to parental MCF-7 cells, and was detected in MDA-MB-231, but not SK-BR-3 cells. COX-2 is frequently overexpressed in invasive breast cancers and in adjacent DCIS and, thus, may be an early event in mammary tumorigenesis. Forced HER2 expression in MCF-7 cells was shown to up-regulate COX-2, although no association was found in human tumors. Our results suggest that nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors may be useful in the chemoprevention and therapy of human breast cancer.

INTRODUCTION

Elevated levels of PGE_2[3] have been detected in cultured human breast cancer cells as well as in invasive human breast cancers (1–4) and are associated with negative hormone receptor status and increased metastatic potential (1). COX enzymes catalyze the conversion of arachidonic acid to PGs and related eicosanoids. Two isoforms of the *COX* gene have been identified, which encode for constitutively expressed COX-1 and inducible COX-2 (5).

COX-2 is an intermediate response gene that encodes a 71-kDa protein which is up-regulated at sites of inflammation (6) and in some epithelial cancers, including human colon (7, 8), gastric (9), esophageal (10, 11), and lung (12) adenocarcinomas. Increases in COX-2 and PG production were induced by viral and *Ras*-mediated transformation of mammary epithelial cells (13). Elevated levels of *COX-2* mRNA and protein have been reported in breast cancer cell lines (1), and limited and conflicting data exist regarding the frequency of COX-2 expression in human breast cancers. Using RT-PCR, Parrett *et al.* (14) detected *COX-2* expression in all 13 breast cancers examined in contrast to normal human breast tissue. Soslow *et al.* (15) reported that 7 of 17 invasive breast cancers expressed COX-2 proteins. In contrast, Hwang *et al.* (16) detected COX-2 protein by immunoblot analysis in only 2 of 44 human breast cancer specimens.

In a transgenic mouse model, overexpression of COX-2 in mammary epithelial cells resulted in the development of mammary tumors, indicating that COX-2 was sufficient to induce mammary tumorigenesis (17). The importance of COX-2 in intestinal tumorigenesis was shown by mating *COX-2* knockout mice to ^{Apc}Min mice, which resulted in a dramatic reduction in the number of intestinal polyps in double knockouts (18). The effect of selective COX-2 inhibitors on mammary carcinogenesis has been reported. Treatment with celecoxib significantly delayed mammary tumor development in DMBA-treated rats and was found to be more effective than the COX inhibitor ibuprofen (19). Furthermore, nimesulide was shown to significantly reduce tumor size, multiplicity, and to a lesser extent, incidence in experimental mammary tumors (20). The effect of celecoxib on established breast cancer was also studied in a study in which rats were maintained for 4 months after DMBA treatment and subsequently fed celecoxib or control for 6 weeks (21). Mean tumor volume was decreased by 32% in the treatment group, but increased dramatically (518%) in controls.

Adenocarcinoma of the breast is the most common cancer in women in the United States and is second only to lung cancer as a cause of cancer-related mortality (22). Epidemiological studies suggest that regular intake of NSAIDs may protect against breast cancer (23–25). In a case-control study, a 40% reduction in breast cancer incidence was observed in women who reported daily intake of aspirin, ibuprofen, or other NSAIDs for at least 5 years (24). In a Canadian study of 5882 women, regular intake of NSAIDs for 2–5 years was associated with a 24% reduction in invasive breast cancer (25). The Nurses Health Study, however, found no difference in aspirin intake among women who developed breast cancer and those who did not (26). Regular and prolonged intake of NSAIDs is associated with a 40–50% reduction in colorectal cancer incidence (23, 27, 28) and also reduces mortality related to this disease (29). NSAIDs inhibit COX enzymes and are potent chemopreventive agents against colon (30–32) and some mammary carcinoma models. In this regard, indomethacin (33–35) and ibuprofen (36, 37) were both found to reduce the incidence of DMBA-induced mammary tumors in rats. In 2-amino-1-methyl-6-phenylimidazopyridine- and *N*-methyl-*N*-nitrosourea-induced breast cancer models, aspirin (38) and flurbiprofen (39) were shown to reduce mammary tumorigenesis. In other studies, however, indomethacin (40) and piroxicam (41) failed to exert a

Received 8/31/01; accepted 1/8/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by a Kadoorie Human Cancer Genetics Award (to F. A. S.)

[2] To whom requests for reprints should be addressed, at Department of Gastrointestinal Medicine and Nutrition, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. E-mail: fsinicro@mdanderson.org.

[3] The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; RT-PCR, reverse transcription-PCR; DMBA, dimethylbenz[*a*]anthracene; NSAID, nonsteroidal anti-inflammatory drug; DCIS, ductal carcinoma *in situ*; IHC, immunohistochemistry; MAB, monoclonal antibody.

chemopreventive effect against DMBA-induced breast tumors in female rats.

We analyzed COX-2 expression in human breast cancer cell lines and in normal and neoplastic breast epithelia. Specifically, we determined the frequency and immunolocalization of COX-2 expression in a series of human breast cancers and its association with clinicopathological variables, including tumor stage, hormone receptor status, and HER2 expression/amplification. HER2 is a 185-kDa receptor tyrosine kinase encoded by the *neu* proto-oncogene that is overexpressed in 25–30% of human breast cancers and is associated with an increased risk of relapse and death (42). HER2 lacks a ligand, but amplifies the signal provided by other HER family receptors by heterodimerizing with them (43). Interestingly, Vadlamudi *et al.* (44) showed that activation of the HER2/HER3 pathway by heregulin in colon carcinoma cells induced the activity of the *COX-2* promoter, the expression of *COX-2* mRNA and COX-2 protein, and accumulation of PGE_2. Moreover, inhibition of HER2/HER3 signaling by an anti-HER3 antibody decreased HER2/HER3 heterodimers and COX-2 expression (44). These *in vitro* results suggest that a coordinate relationship may exist between the regulation of the COX-2 and HER2 pathways.

MATERIALS AND METHODS

Tissue Samples. We analyzed a nonconsecutive series of primary human breast carcinomas ($n = 57$), adjacent DCIS ($n = 14$) or DCIS alone ($n = 2$), and normal-appearing breast epithelia ($n = 41$) that were obtained from the surgical pathology laboratory at The University of Texas M. D. Anderson Cancer Center. Tissue specimens were formalin fixed, paraffin embedded, and represented different tumor stages [stage 1 ($n = 15$), stage 2 ($n = 24$), stage 3 ($n = 12$), stage 4 ($n = 1$), or unknown ($n = 3$)], according to the TNM classification (45). Consecutive 4–6-μm tissue sections were cut from paraffin blocks and placed on polylysine-coated slides for IHC analysis. An additional nine pairs of frozen, invasive breast cancer and normal tissue samples were obtained from the Institutional Tissue Bank.

Tumor Cell Lines. We analyzed the human breast adenocarcinoma cell lines MDA-MB-231, SK-BR-3, MCF-7, and MCF-7/*HER2*. MCF-7 cells had been stably transfected with the *HER*2 cDNA (gift from Dr. Mien Chi Hung, University of Texas M. D. Anderson Cancer Center). The HCA-7 colorectal carcinoma cell line was obtained from Dr. S. Kirkland (Imperial Cancer Research Fund, London, United Kingdom), and the HCT116 colon cancer cell line was from the American Type Culture Collection (Rockville, MD). All cells were grown in DMEM:F-12 (Life Technologies, Inc., Frederick, MD) supplemented with heat-inactivated 10% FCS and 10 units/ml penicillin and streptomycin. Cultured cells were maintained in a humidified environment at 37°C with 5% CO_2.

IHC. Slides were deparaffinized, and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 30 min at room temperature. Sections were then microwaved in PBS (pH 7.4) for 4 min for antigen retrieval. Immunoreactivity was detected by an immunoperoxidase method (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA). Nonspecific binding was blocked with avidin then biotin (Vector) for 15 min each. The primary MAb against human COX-2 (Cayman Chemical, Ann Arbor, MI) recognizes a 19-amino acid sequence at the COOH terminus that is absent in COX-1. This antibody was applied at a dilution of 1:500 for 2 h at room temperature. After slides were rinsed in PBS, the biotinylated secondary IgG antibody was applied for 30 min at room temperature. Slides were rinsed in PBS, and avidin conjugated to horseradish peroxidase (ABC reagent) was applied for 45 min at room temperature. The chromogen 3,3′-diaminobenzidine (Research Genetics, Huntsville, AL) was subsequently added, and the color reaction was observed with light microscopy. The reaction was stopped by immersing slides in deionized water; slides were then counterstained with hematoxylin and mounted.

As a control for nonspecific staining, a COX-2-specific blocking peptide (Cayman Chemical) derived from the human *COX-2* cDNA sequence was mixed with the COX-2 MAb and applied to sections. In all cases, immunoreactivity was completely suppressed. As a negative control, the primary MAb was omitted and PBS was applied, with all other steps of the procedure included. The HCA-7 colon cancer cell line was used as a positive control for COX-2.

For HER2 immunostaining, a modification of the procedure used for COX-2 was used. Antigen retrieval was performed by microwave treatment of slides in citrate buffer (pH 6.0) for 4 min on the high setting and an additional 6 min at lower power. Slides were incubated with a monoclonal anti-c-erbB-2/HER2 antibody (clone e2-4001+B5; Neomarkers, Fremont, CA) at a 1:200 dilution. A series of breast cancers were stained with this MAb, and the results were compared with these same cases stained with another anti-c-erbB-2/HER2 MAb (Oncogene Science, Manhasset, NY). Identical results were found (data not shown). A human breast cancer with HER2 overexpression and amplification was included with all slide runs. Additional controls included SK-BR-3 and MDA-MB-231 cells. Negative control slides omitted the primary antibody but included all other steps of the procedure.

IHC Scoring. For COX-2, weighted score was computed that represented the product of percentage of tumor cell positivity and intensity, as described previously (46). Percent tumor cell positivity was categorized as follows: 0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%, and 4, >75%. Staining intensity was graded as absent (0), weak (1+), medium (2+), or strong (3+). The intensity of the invasive tumor and of DCIS (when it existed on the same slide) were determined separately. On the basis of weighted scores, expression was categorized as negative or low (0–4), intermediate (5–8), or high (9–12). Localization of HER2 staining was described as cytoplasmic or membranous. Cases considered positive for HER2 had membranous staining in at least 10% of tumor cells and intensity scored as 0–3+. Before the study specimens were scored, representative sections displaying each of the four staining intensities were reviewed by two examiners.

Immunoblotting. Frozen tissue samples were homogenized in Tris-HCl buffer (pH 7.4) containing 0.5% NP40 and protease inhibitors (Boehringer Mannheim, Indianapolis, IN). Cultured cell lines were processed according to standard procedures, and a cell lysate was prepared. Protein extraction was performed on tissue and cell lysates, and the protein concentration was determined in soluble supernatants in each sample. We then loaded 40 μg of protein per lane onto a SDS-polyacrylamide gel, and proteins were then transferred onto an Immobilon membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat dry milk. Blots were incubated overnight at 4°C with MAb against COX-2 (Cayman Chemical) at a dilution of 1:1000 and actin (Oncogene Science, Uniondale, NY). The level of protein expression was measured with the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Actin levels were analyzed as controls for protein loading.

RT-PCR. Total RNA was extracted and purified from human breast tumors and paired normal tissues using the RNeasy mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (1 μg) was converted to cDNA by Superscript^{II} reverse transcriptase (Life Technologies, Inc., Rockville, MD) in a total reaction volume of 20 μl. The cDNA was PCR-amplified with AmpliTaq DNA polymerase (Perkin-Elmer/Applied Biosystems, Foster City, CA). Relative expression of *COX-2* mRNA was analyzed using quantitative multiplex RT-PCR with *β-actin* as an internal control. The reaction mixture contained 1 unit of Hotstart polymerase (Qiagen, Hilden, Germany), 10 mM deoxynucleotide triphosphates, 1 μl of DMSO, 50 ng of forward (F) and reverse (R) primers, and 1 μl of cDNA in a final volume of 10 μl. The primer sequences and PCR product sizes were as follows: for *COX-2*, (F) 5′-GCTGAGCCATACAGCAAATCC-3′, (R) 5′-GGGAGTCGGGCAATCATCAG-3′ (386-bp product); for *β-actin*, (F) 5′-GTTGCTATCCAGGCTGTGC-3′ and (R) 5′-GCATCCTGTCGGCAATGC-3′ (540-bp product). The reaction was performed according to the following program: 15 min at 94°C for activating the polymerase, 38 cycles (28 cycles for *β-actin*) at 94°C for 30 s for denaturation, 1 min at 61°C for annealing, and extension for 1 min at 72°C, followed by an additional extension step at 72°C for 7 min. The PCR products were run on a 2.0% agarose gel and then stained with 0.5 μg/ml ethidium bromide. Stained bands on the gel were visualized under UV light and then photographed.

RESULTS

Patient Characteristics. We evaluated archival tissue specimens from 57 women with invasive breast cancer, as shown in Table 1. Age

Table 1 *Clinicopathological variables in human breast neoplasms*

	n
Clinical stage	
0 (DCIS)	2
I	15
II A, B	24
III	12
IV	1
Neoadjuvant treatment	
Chemotherapy ± hormonal	16
None	41
Nuclear grade	
1	7
2	22
3	25
Lymphovascular invasion	
No	28
Yes	19
Histology	
Ductal	44
Lobular	2
DCIS[a]	16
Other	7
Estrogen receptor	
Positive	36
Negative	13
ND[b]	8
Progesterone receptor	
Positive	26
Negative	22
ND	9

[a] Includes stage 0 and DCIS adjacent to invasive cancer.
[b] ND, not done.

at diagnosis ranged from 25 to 78 years (median age, 47 year), and 36 (63%) women were less than age 50. Eighty percent of the tumors were infiltrating ductal adenocarcinomas. Adjacent DCIS was found in 13 of the 57 cases. Two patients had exclusively DCIS and no invasive cancer. Patient characteristics, including tumor stage, nuclear grade, hormonal receptor status, and preoperative treatment history, are outlined in Table 1. At a median follow-up of 19 months (range, 1–86 months), all but two patients were alive.

COX-2 Immunoreactivity. Cytoplasmic COX-2 expression was detected at an intermediate or high level by a semiquantitative IHC method in neoplastic epithelial cells in 18 of 42 (43%) primary breast cancers (Table 2). Negative or low-level staining for COX-2 was observed in 57% of these invasive tumors. COX-2 was detected at an intermediate or high level in 10 of 16 (62.5%) cases of DCIS adjacent to cancer. The two cases of DCIS alone showed an intermediate level of COX-2. COX-2 staining was granular and localized to the tumor cytoplasm (Fig. 1). COX-2 expression was less heterogeneous in DCIS cases than in invasive cancers. Within the same tissue sections, COX-2 expression in invasive breast tumors and adjacent DCIS were highly correlated ($P = 0.019$). COX-2 was expressed in benign ductular epithelial cells adjacent to tumor in 81% of cases (Table 2). Staining in normal-appearing epithelia was often focal and of similar or reduced intensity relative to neoplastic epithelia. Stromal staining for COX-2 in fibroblasts and other interstitial cells was infrequently detected. Specificity of staining was confirmed by use of a COX-2 sequence-specific blocking peptide that completely suppressed staining in all cases.

***COX-2* mRNA by RT-PCR.** *COX-2* mRNA expression was analyzed in paired tumor and normal tissue specimens from breast cancer patients ($n = 9$), using COX-2 specific primers and RT-PCR. As shown in Fig. 2, COX-2 was detected in tumor and normal breast tissue from all nine patients. The level of *COX-2* mRNA in tumors was shown to exceed that in normal tissues in eight of these paired specimens from the same patients.

HER2/neu Expression and Gene Amplification. HER2 was analyzed by IHC in all breast cancer cases. Membranous staining of 3+ intensity was detected in 17 of 55 (31%) invasive breast cancers (Fig. 1*E*), and was 2+ in 4 and 1+ in 5 cases. Results for *HER2* gene amplification by FISH were available on 20 cases. Five of 7 (72%) tumors with 3+ membranous staining and all 2+ tumors ($n = 4$) were amplified. *HER2* gene amplification was not found in the cases that stained 1+ or were negative for HER2. A significant correlation was observed between membranous HER2 staining and gene amplification ($P = 0.003$). Cytoplasmic HER2 staining was detected in 15 of 55 (27%) cancers; 23 (42%) cases were negative for HER2. Cytoplasmic staining was granular, and the intensity was 2+ or 3+. HER2 was not amplified in these cytoplasmic or the negative cases.

Association of COX-2 with Clinicopathological Variables. COX-2 protein expression in invasive breast cancers or adjacent DCIS did not significantly correlate with any of the clinicopathological variables examined, including hormonal status. No difference in the frequency of COX-2 expression was found in tumors from patients receiving neoadjuvant therapy ($n = 16$) *versus* untreated patients ($P = 0.16$). No association was found between COX-2 and HER2 immunostaining or gene amplification.

COX-2 in Breast Carcinoma Cell Lines. Human breast cancer cell lines (SK-BR-3, MDA-MB-231, and MCF-7) were analyzed for COX-2 and HER2 expression by immunoblotting. We studied the estrogen-dependent, poorly invasive, and nonmetastatic MCF-7 cell line (47) and the estrogen-independent, highly invasive, and metastatic MDA-MB-231 cell line (48). SK-BR-3 cells are less invasive and estrogen dependent (49). SK-BR-3 and MCF-7 cells were found to lack COX-2 proteins, and MDA-MB-231 cells displayed a low level of COX-2 (Fig. 3, *A* and *B*). To determine whether HER2 can regulate the expression of COX-2, we analyzed MCF-7 cells stably transfected with the *HER2.* cDNA MCF-7/*HER2* transfectants were found to express COX-2 in contrast to parental cells, suggesting induction of COX-2 by *HER2* (Fig. 3*B*). As shown previously, SK-BR-3 cells overexpressed HER2 in the cellular membrane (Fig. 1*F*) and displayed *HER2* amplification (Fig. 3*A*; Ref. 49); MDA-MB-231 cells expressed a low level of HER2 proteins (Ref. 48; Fig. 3*A*). The HCA-7 colorectal cancer cell line is known to constitutively express COX-2 and was used as a positive control (50). The COX-2-negative HCT116 colon cancer cell line (8) was used as a negative control for COX-2 protein expression. HCA-7 cells were found to overexpress COX-2 and HER2 proteins (Fig. 3*A*). HCT116 cells lacked COX-2 but expressed HER2 proteins (Fig. 3*A*). Immunostaining of these cell lines was concordant with immunoblotting results (data not shown). Neither HCA-7 nor HCT116 cells had *HER2* amplification (Fig. 3*A*).

DISCUSSION

We detected frequent expression of COX-2 in epithelial cells of human breast adenocarcinomas and adjacent DCIS, which may ac-

Table 2 *Analysis of COX-2* [a] *expression by IHC in human breast tissues*

	n (%)
Invasive cancer ($n = 42$)	
Negative or low	24 (57)
Intermediate	8 (19)
High	10 (24)
DCIS ($n = 16$)	
Negative or low	6 (37)
Intermediate[b]	8 (50)
High	2 (13)
Normal epithelium ($n = 48$)	
Negative	9
Positive	39 (81)

[a] COX-2 expression was categorized based on weighted scores (product of staining intensity and percentage of tumor cell positivity).
[b] Includes DCIS alone (n=2).

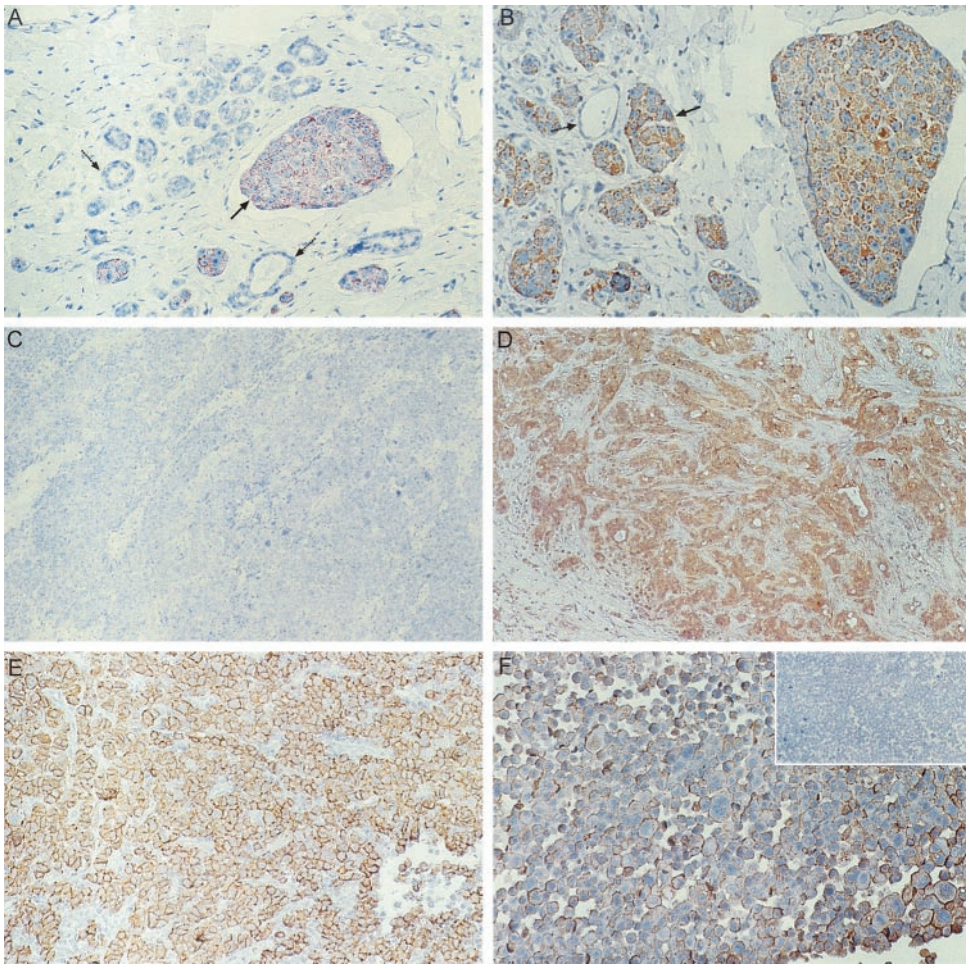

Fig. 1. *A* and *B*, COX-2 staining is detected in the cytoplasm of tumor cells within DCIS. Normal breast ductular epithelium (*dotted arrow*) shows reduced intensity of COX-2 staining relative to DCIS (*solid arrow*). *C*, invasive breast carcinoma lacking COX-2 staining. *D*, infiltrating ductal adenocarcinoma of the breast with a high level of COX-2 expression. *E*, membranous HER2 staining (3+) in an infiltrating ductal breast carcinoma. *F*, SK-BR-3 breast cancer cells overexpress HER2 in the cell membrane in contrast to MDA-MB-231 cells (*inset*).

count for the elevated levels of PGs, including PGE_2, found in these tumors. We report the largest series to date using a semiquantitative IHC approach. We found that COX-2 is expressed at intermediate or high levels in 43% of human breast carcinomas and in 62.5% of DCIS. The higher frequency of COX-2 in DCIS *versus* invasive cancer suggests that up-regulation of COX-2 is a relatively early event in mammary carcinogenesis. Given that DCIS was adjacent to invasive tumor in all but two cases (which expressed COX-2), further study of COX-2 in patients with DCIS alone is warranted. Our findings in breast neoplasms differ from those reported in human colorectal adenomas, where COX-2 was detected at a reduced frequency relative to invasive cancers (7, 8). COX-2 staining was detected in normal-appearing breast epithelium in 81% of cases and was often focal and of similar or reduced intensity relative to tumor. Despite frequent expression of COX-2 in normal epithelia from breast cancer cases, *COX-2* mRNA in eight of nine tumors, was increased in eight of nine tumors relative to paired normal tissues from the same patients.

COX-2 expression was unrelated to the clinicopathological variables examined, including clinical stage and hormonal status. Given data indicating that activation of the HER2/HER3 pathways in colon cancer cell lines can induce *COX-2* mRNA and protein as well as PGE_2 biosynthesis (44), we compared the expression of COX-2 and HER2 in breast cancers by IHC. No association between COX-2 and HER2 expression or amplification was found; however, COX-2 is regulated by multiple factors, including cytokines, growth factors, and tumor promoters; consequently, a direct relationship between COX-2 and HER2 may not be readily observed. In addition, HER2 can undergo posttranslational modification, *i.e.*, tyrosine phosphorylation, resulting in its activation. Therefore, the level of HER2 expression may not correlate with activity and its ability to induce COX-2. Use of an anti-HER-2 antibody may allow further examination of the relationship between HER-2 and COX-2.

Forced expression of HER2 in MCF-7 cells, which lack endogenous COX-2 proteins, resulted in low-level COX-2 expression, indicating that COX-2 can be induced by HER2. HCA-7 colon cancer cells, which constitutively express COX-2 (50), were found to overexpress HER2. Growth of these cells in culture has been shown to be inhibited by anti-HER2 antibodies and celecoxib, as were HCA-7 xenografts grown in nude mice (50). Furthermore, inhibition of HER2/HER3 signaling by an anti-HER3 antibody decreased both HER2/HER3 heterodimerization and COX-2 expression (44). Taken

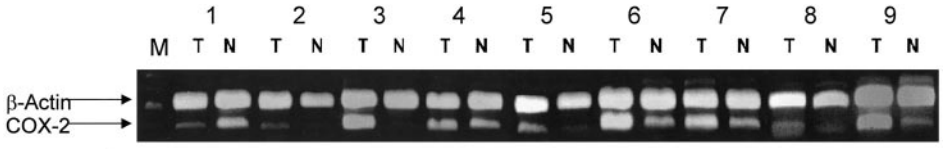

Fig. 2. RT-PCR analysis of *COX-2* mRNA expression in infiltrating ductal adenocarcinomas of the breast. Total RNA was extracted from tumors (*T*) and their paired normal (*N*) tissues. PCR product sizes (in bp) are 386 for *COX-2* and 540 for *β-actin*; *Lane M*, DNA markers.

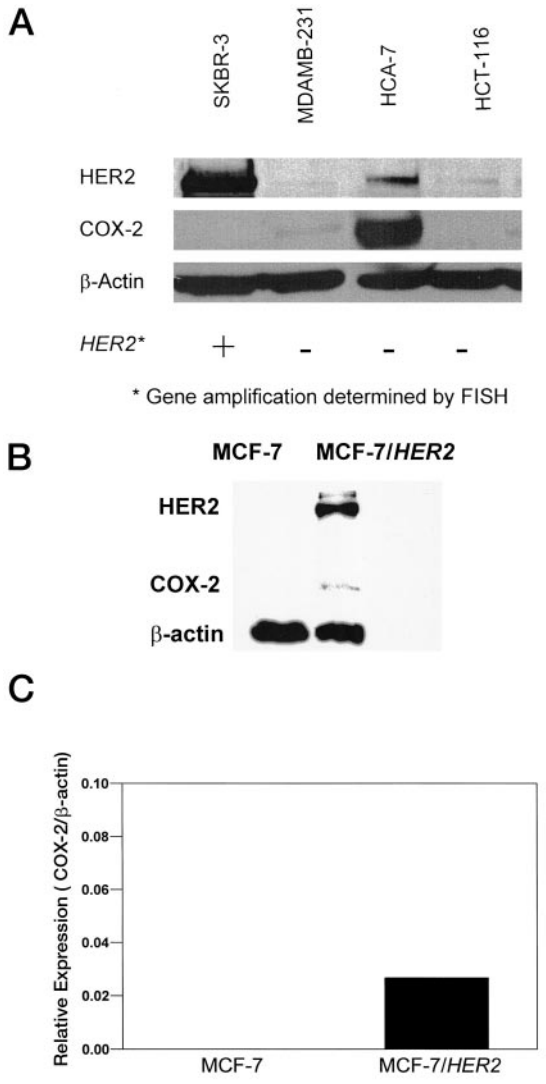

Fig. 3. *A*, immunoblot of COX-2 (70 kDa) and HER2 (185 kDa) protein levels in human breast (*SK-BR-3* and *MDAMB-231*) and colorectal (*HCA-7* and *HCT-116*) carcinoma cell lines. HCA-7 cells constitutively express COX-2 (positive control) and HCT116 cells lack COX-2 proteins (negative control). *HER2* gene amplification results, determined by fluorescence *in situ* hybridization (*FISH*), are shown for these same cell lines. *B*, MCF-7 breast cancer cells stably transfected with the *HER-2* cDNA show induction of COX-2 proteins in contrast to parental MCF-7 cells. *C*, Densitometry analysis of COX-2 protein expression relative to *β-actin* in MCF-7/HER2-transfected cells compared with parental MCF-7 cells.

together, these *in vitro* results suggest that a coordinate relationship may exist between the regulation of the COX-2 and HER2 pathways that can potentially be exploited for therapeutic advantage. In this regard, the combination of an anti-HER2 antibody and celecoxib showed greater efficacy than either agent alone in inhibiting growth of cultured HCA-7 cells and HCA-7 tumor xenografts (50).

Data in human breast cancers, including our results, as well as animal model studies indicate that COX-2 up-regulation and elevated PGE_2 levels are involved in breast carcinogenesis and that their inhibition by NSAIDs may reduce breast cancer incidence. (1, 4). Moreover, in transgenic mice, forced expression of the *COX-2* gene in mammary glands was sufficient to induce mammary tumorigenesis (17). In that study, COX-2-induced tumors had reduced levels of proapoptotic proteins (Bax and Bcl-xL) and an increase in the anti-apoptotic Bcl-2 protein, suggesting that reduced apoptosis is associated with tumorigenesis (17). Experimental data indicate that increased COX-2 plays a role in conferring resistance to apoptosis (51) and that NSAIDs can induce apoptosis in colon carcinoma cell lines (52). NSAIDs have been shown to inhibit breast and intestinal tumors in animal models, and the selective COX-2 inhibitor celecoxib has been shown to significantly reduce the number of polyps in patients with colorectal familial adenomatous polyposis (53). Taken together, inhibition of COX-2 by NSAIDs may be an important mechanism-based strategy for the prevention and treatment of human breast cancer.

REFERENCES

1. Liu, X., and Rose, D. Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines. Cancer Res., *56:* 5125–5127, 1996.
2. Schrey, M. P., and Patel, K. V. Prostaglandin E_2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. Br. J. Cancer, *72:* 1412–1419, 1995.
3. Rolland, P. H., Martin, P. M., Jacquemier, J., Rolland, A., and Toga, M. Prostaglandins in human breast cancer: evidence suggesting that elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. J. Natl. Cancer Inst. (Bethesda), *64:* 1061–1070, 1980.
4. Karmali, R. A., Welts, S., Thaler, H. T., and Lefevre, F. Prostaglandins in breast cancer. Relationship to disease stage and hormone status. Br. J. Cancer, *48:* 689–696, 1983.
5. Smith, W., Garavito, M., and DeWitt, D. Prostaglandin endoperoxide H synthase (cyclooxygenase)-1 and -2. J. Biol. Chem., *271:* 33157–33160, 1996.
6. Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. Molecular cloning of human prostaglandin endoperoxidase synthase type 2 and demonstration of expression in response to cytokines. J. Biol. Chem., *268:* 9049–9054, 1993.
7. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrebach, S., and Dubois, R. N. Up-regulation of cyclooxygenase gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology, *107:* 1183–1188, 1994.
8. Sinicrope, F. A., Lemoine, M., Xi, L., Lynch, P. M., Cleary, K. R., Shen, Y., and Frazier, M. L. Reduced expression of cyclooxygenase 2 proteins in hereditary nonpolyposis colorectal cancers relative to sporadic cancers. Gastroenterology, *117:* 350–358, 1999.
9. Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res., *57:* 1276–1280, 1997.
10. Zimmermann, K. C., Sarbia, M., Weber, A., Bochard, F., Gabbert, H. E., and Schror, K. Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res., *59:* 198–204, 1999.
11. Wilson, K. T., Fu, S., Ramanujam, K. S., and Meltzer, S. J. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. Cancer Res., *58:* 2929–2934, 1998.
12. Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozaki, K., Nakamura, S., Ogawa, M., Mitsudomi, T., Sogiura, T., and Takahashi, T. Increased expression of cyclooxygenase-2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res., *58:* 3761–3764, 1998.
13. Subbaramaiah, K., Telang, N., Bansal, M. B., Weksler, B. B., and Dannenberg, A. J. Cyclooxygenase-2 gene expression is upregulated in transformed mammary epithelial cells. Ann. NY Acad. Sci., *833:* 179–185, 1997.
14. Parrett, M., Harris, R., Joarder, F., Ross, M., Clausen, K., and Robertson, F. Cyclooxygenase-2 gene expression in human breast cancer. Int. J. Oncol., *10:* 503–507, 1997.
15. Soslow, R., Dannenberg, A., Rush, D., Woerner, B. M., Khan, K. N., Masferrer, J., and Koki, A. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. Cancer (Phila.), *89:* 2637–2645, 2000.
16. Hwang, D., Scollard, D., Byrne, J., and Levine, E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. J. Natl. Cancer Inst. (Bethesda), *90:* 455–460, 1998.
17. Liu, C. H., Chang, S., Narko, K., Trifan, O. C., Wu, M., Smith, E., Haudenschild, C., Lane, T. F., and Hla, T. Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. J. Biol. Chem., *276:* 18563–18569, 2001.
18. Oshima, M., Dinchuk, J. E., Kargman, S. L., and Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in Apc716 knockout mice by inhibition of cyclooxygenase-2 (COX-2). Cell, *87:* 803–809, 1996.
19. Harris, R. E., Alshafie, G. A., Abou-Issa, H., and Seibert, K. Chemoprevention of breast cancer in rats by celecoxib, a cyclooxygenase 2 inhibitor. Cancer Res., *60:* 2101–2103, 2000.
20. Nakatsugi, S., Ohta, T., Kawamori, T., Mutoh, M., Tanigawa, T., Watanabe, K., Sugie, S., Sugimura, T., and Wakabayashi, K. Chemoprevention by nimesulide, a selective cyclooxygenase-2 inhibitor, of 2-amino-1-methyl-6-phenylimidazo[4,5,-*b*]-pyridine (PhIP)-induced mammary gland carcinogenesis in rats. Jpn. J. Cancer Res., *91:* 886–892, 2000.
21. Alshafie, G. A., Abou-Issa, H., Seibert, K., and Harris, R. Chemotherapeutic evaluation of celecoxib, a cyclooxygenase-2 inhibitor, in rats mammary tumor model. Oncol. Rep., *7:* 1377–1381, 2000.
22. Greenlee, R., Hill-Harmon, M. B., Murray, T., and Thun, M. Cancer statistics, 2001. CA Cancer J. Clin., *51:* 15–36, 2001.

23. Schreinemachers, D. M., and Everson, R. B. Aspirin use and lung, colon, and breast cancer. Incidence in a prospective study. Epidemiology, *5:* 138–146, 1994.
24. Harris, R. E., Namboodiri, K. K., and Farrar, W. B. Non-steroidal anti-inflammatory drugs and breast cancer. Epidemiology, *7:* 203–205, 1996.
25. Sharp, C. R., Collet, J. P., McNutt, M., Belzile, E., Boivin, J. F., and Hanley, J. A. Nested case control study of the effect of non-steroidal anti-inflammatory drugs on breast cancer risk and stage. Br. J. Cancer, *83:* 112–120, 2000.
26. Egan, K., Stampfer, M., Giovannucci, E., Rosner, B., and Colditz, G. Prospective study of regular aspirin use and the risk of breast cancer. J. Natl. Cancer Inst. (Bethesda), *88:* 988–993, 1996.
27. Rosenberg, L., Palmer, J. R., Zauber, A. G., Warshaver, M. E., Stolley, P.D., and Shapiro, S. A hypothesis: nonsteroidal anti-inflammatory drugs reduce the incidence of large-bowel cancer. J. Natl. Cancer Inst., *83:* 355–358, 1991.
28. Thun, M. J., Namboodiri, M. M., and Heath, C. W., Jr. Aspirin use and reduced risk of fatal colon cancer. N. Engl. J. Med., *325:* 1593–1596, 1991.
29. Giovannucci, E., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., and Willett, W. C. Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. Ann. Intern. Med., *121:* 241–246, 1994.
30. Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res., *58:* 409–412, 1998.
31. Jacoby, R. F., Marshall, D. J., Newton, M. A., Novakovick, K., Tutsch, K., Cole, C. E., Lubet, R. A., Kelloff, G. J., Verma, A., Moser, A. R., and Dove, W. F. Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal anti-inflammatory drug piroxicam. Cancer Res., *56:* 710–714, 1996.
32. Boolbol, S. K., Dannenberg, A. J., Chadburn, A., Marticci, C., Guo, X. J., Ramonetti, J. T., Abreu-Goris, M., Newmark, H. L., Lipkin, M. L., DeCosse, J. J., and Bertagnolli, M. M. Sulindac prevents tumors in a murine model of familial adenomatous polyposis. Cancer Res., *56:* 2556–2560, 1996.
33. McCormick, L. D., Madigan, M. J., and Moon, R. C. Modulation of rat mammary carcinogenesis by indomethacin. Cancer Res., *45:* 1803–1808, 1985.
34. Noguchi, M., Taniya, T., Koyasaki, N., Kumaki, T., Miyazaki, I., and Mizukami, Y. Effects of prostaglandin synthetase inhibitor indomethacin on tumorigenesis, tumor proliferation, cell kinetics, and receptor contents of 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinoma in Sprague-Dawley rats fed a high or low fat diet. Cancer Res., *51:* 2683–2689, 1991.
35. Carter, C. A., Ip, M. M., and Ip, C. A comparison of the effects of prostaglandin synthesis inhibitors indomethacin and carprofen on 7,12-dimethylbenz(*a*)anthracene-induced mammary tumorigenesis in rats fed different amounts of essential fatty acids. Carcinogenesis (Lond.), *10:* 1369–1374, 1989.
36. Alshafie, G. A., Harris, R. E., Robertson, F. M., Parrett, M. L., Ross, M., and Abou-Issa, H. Comparative chemopreventive activity of ibuprofen and *N*-(4-hydroxyphenyl)retinamide against the development and growth of rat mammary adenocarcinomas. Anticancer Res., *19:* 3031–3036, 1999.
37. Robertson, F. M., Parrett, M. L., Joarder, F. S., Ross, M., Abou-Issa, H. M., Alshafie, G., and Harris, R. E. Ibuprofen-induced inhibition of cyclooxygenase isoform gene expression and regression of rat mammary carcinomas. Cancer Lett., *122:* 165–175, 1998.
38. Mori, H., Sugie, S., Rahman, W., and Suzui, N. Chemoprevention of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-induced mammary carcinogenesis in rats. Cancer Lett., *143:* 195–198, 1999.
39. McCormick, D. L., and Moon, R. C. Inhibition of mammary carcinogenesis by flurbiprofen, a non-steroidal anti-inflammatory agent. Br. J. Cancer, *48:* 859–861, 1983.
40. Abu-El-Ela, S., Prasse, K. W., Farrel, R. L., Carroll, R. W., Wade, A. E., and Brunce, O. R. Effects of D,L-2-difluoromethylornithine and indomethacin on mammary tumors promotion in rats fed high n-3 and/or n-6 fat diets. Cancer Res., *49:* 1434–1440, 1989.
41. Kitagawa, H., and Noguchi, M. Comparative effects of piroxicam and esculetin on incidence, proliferation, and cell kinetics of mammary carcinomas induced by 7,12-dimethylbenz[*a*]anthracene in rats on high and low fat diets. Oncology, *51:* 410–410, 1994.
42. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science (Wash. DC), *235:* 177–182, 1987.
43. Klapper, L. N., Kirschbaum, M. H., Sela, M., and Yarden, Y. Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. Adv. Cancer Res., *77:* 25–79, 2000.
44. Vadlamudi, R., Mandal, M., Adam, L., Steinbach, G., Mendelson, J., and Kumar, R. Regulation of cyclooxygenase-2 pathway by HER2 receptor. Oncogene, *18:* 305–314, 1999.
45. Sicher, K., and Waterhouse, J. A. Evaluation of TNM classification of carcinoma of the breast. Br. J. Cancer, *28:* 580–588, 1973.
46. Krajewska, M., Krajewski, S., Epstein, J. I., Shabaik, A., Sauvageot, J., Song, K., Kitada, S., and Reed, J. C. Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. Am. J Pathol., *148:* 1567–1576, 1996.
47. Price, J. M., and Zhang, R. D. Studies of human breast cancer metastasis using nude mice. Cancer Metastasis Rev., *8:* 285–297, 1990.
48. Kunisue, H., Kurebayashi, J., Otsuki, T., Tang, C. K., Kurosumi, M., Yamamoto, S., Tanaka, K., Doihara, H., Shimizu, N., and Sonoo, H. Anti-HER2 antibody enhances the growth inhibitory effect of anti-oestrogen on breast cancer cells expressing both oestrogen receptors and HER2. Br. J. Cancer, *82:* 46–51, 2000.
49. Van de Vijver, M. J., Mooi, W. J., Wisman, P., Peterse, J. L., and Nusse, R. Immunohistochemical detection of the neu protein in tissue sections of human breast tumors with amplified neu DNA. Oncogene, *2:* 175–178, 1988.
50. Mann, M., Sheng, H., Shao, J., Williams, C. S., Pisacane, P. I., Sliwkowski, M. X., and DuBois, R. N. Targeting cyclooxygenase 2 and HER-2/neu pathways inhibits colorectal carcinoma growth. Gastroenterology, *120:* 1713–1719, 2001.
51. Tsujii, M., and DuBois, R. N. Alteration in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell, *83:* 493–501, 1995.
52. Piazza, G. A., Rahm, A. L., Krutzsch, M., Sperl, G., Paranka, N. S., Gross, P. H., Brendel, K., Burt, R. W., Alberts, D. S., Pamukcu, R., *et al*. Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. Cancer Res., *55:* 3110–3116, 1995.
53. Steinbach, G., Lynch, P., Phillips, R., Wallace, M., Hawk, E., Gordon, G., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L., and Levin, B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polylposis. N. Engl. J. Med., *342:* 1946–1952, 2000.

Cyclooxygenase-2 Expression in Human Breast Cancers and Adjacent Ductal Carcinoma *in Situ*

Elizabeth Half, Xi Ming Tang, Karin Gwyn, et al.

Cancer Res 2002;62:1676-1681.