Correlation between Cyclooxygenase-2 Expression and Angiogenesis in Human Breast Cancer

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ABSTRACT

Purpose: Cyclooxygenase (COX)-2 is overexpressed in breast cancer and may have a role in regulating tumor growth via effects on angiogenesis, cell proliferation, or apoptosis. This study aimed to derive data from human breast carcinomas to help substantiate or refute these relationships.

Experimental Design: We performed immunohistochemical analysis of a set of 86 breast tumors for COX-2, estrogen receptor (ER), progesterone receptor (PGR), HER-2, Ki67 (a marker of proliferation), and CD31 (an endothelial cell marker of angiogenesis).

Results: COX-2 protein expression was detected in 79% of all tumors studied. ER was detected in 79% of all tumors studied, PGR was detected in 73% of all tumors studied, and HER-2 was detected in 16% of all tumors studied. COX-2 protein expression did not significantly correlate with tumor size, grade, axillary lymph node status, or the presence of vascular invasion. A significant negative correlation (P < 0.001) was observed between ER and Ki67. COX-2 expression showed a significant linear correlation with CD31 staining (P < 0.001). No significant correlations were observed between COX-2 and ER, PGR, or HER-2.

Conclusions: This study demonstrates a novel relationship between COX-2 expression and the neovasculature of human breast adenocarcinomas. If this is a functional relationship, it provides support for a potential therapeutic role of COX-2 inhibitors in human breast cancer tissue via their antiangiogenic properties.

INTRODUCTION

A number of studies over the past 25 years have assessed a possible link between use of NSAIDs and altered cancer incidence (1), and several have reported an inverse relationship for both colon cancer (2) and breast cancer (3). The main target of NSAID drug action is the COX enzyme, which catalyzes the conversion of arachidonic acid to prostaglandin precursors important in inflammatory processes. Two isoenzymes, which are the product of two separate genes, exist in humans: (a) COX-1, the constitutive form; and (b) COX-2, first identified in 1992 (4, 5), the inducible form. It is the COX-2 isoenzyme that is induced in response to bacterial endotoxin, cytokines, and growth factors (6, 7).

A substantial body of evidence supports a role for COX-2 in carcinogenesis. A number of studies have shown overexpression of COX-2 in solid malignancies including breast (8). The expression of COX-2 in human solid cancers is not confined to the epithelial component of the tumor; the neovasculature also demonstrates significant COX-2 expression (8). Angiogenesis is an important prognostic variable in breast cancer (9). The effects of specific COX-2 inhibitors have been tested in animal models of angiogenesis, and celecoxib, a specific COX-2 inhibitor, has been shown to cause inhibition of the angiogenic response in fibroblast growth factor-induced rat corneal angiogenesis (8). In addition to antiangiogenic effects, COX-2 may regulate tumor growth and progression via effects on proliferation, apoptosis, and immunological surveillance. The main COX-2 product, PGE2, has also been implicated in the regulation of intratumoral aromatase, providing a mechanism by which COX-2 overexpression may enhance tumor growth and progression in ER-positive tumors. The inhibition of COX-2 via specific COX-2 inhibitors has been shown to prevent mammary tumor development in rat models (10). In addition, forced overexpression of COX-2 is sufficient to induce tumorigenesis in transgenic mice (11). These data have provided a rationale for the use of selective COX-2 inhibitors for the chemoprevention of polyps in familial adenomatous polyposis patients, and studies of their effects in a number of solid tumors are ongoing.

In the present study, we have assessed the relationship between COX-2 expression and the most important biochemical features of breast cancer: proliferation; ER; PGR; HER-2; and angiogenesis. We report a novel finding of a strong relationship between COX-2 expression and neovasculature in breast carcinomas.

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MATERIALS AND METHODS

Tissue Samples. A panel of 100 breast tumors presenting chronologically between 1994 and 1995 at the Royal Marsden Hospital was identified using the hospital breast tumor database. Sequential patients with primary breast tumors containing invasive cancer who had not received treatment prior to surgery were selected. Of these, 86 were available for immunohistochemical assessment. Consecutive 4-μm tissue sections were cut and placed on polylysine-coated slides for IHC analysis.

IHC. The immunohistochemical methods other than those for COX-2 and CD31 have been described in detail elsewhere. In brief, measurement of cell proliferation used the MIB1 mouse monoclonal antibody to Ki67 (12). ER staining was by the Novocastra 6F11 mouse monoclonal antibody, and PGR staining was by the Novocastra 1A6 mouse monoclonal antibody (13). Assessment of HER-2 staining was performed using the DAKO Herceptest kit with strict adherence to the manufacturer’s instructions. Positive and negative controls (in which a tissue known to express the antigen was included, and the primary antibody was omitted, respectively) were used in all cases.

HER-2 FISH Analysis. Tumors that were scored 2+ for membranous staining using the DAKO Herceptest kit were subjected to FISH analysis using the Vysis PathVysion kit, which incorporates a control probe for chromosome 17 as well as the test probe for the HER-2 gene, according to the manufacturer’s instructions. In brief, 4-μm paraffin-embedded sections were dewaxed, taken to absolute ethanol, and air dried. They were then placed in 0.2 M HCl at room temperature for 20 min and in pretreatment solution at 80°C for 30 min and then underwent a proteolytic digestion at 37°C for 25 min. The sections were then denatured in formamide at 72°C for 5 min before incubation in the PathVysion HER-2/17 probe overnight in the dark at 37°C. The following day, the sections were washed in posthybridization buffer for 2 min at 72°C, air dried in the dark, and then mounted in 4,6-diamidino-2-phenylindole.

CD31 Staining. CD31 staining was performed using the antibody JC70 (anti-CD31; Dako). Briefly, paraffin-embedded sections were cut into 4-μm sections, mounted onto slides, dewaxed in xylene, and rehydrated in alcohol, and then endogenous peroxidase activity was blocked with 10% hydrogen peroxide in water for 5 min. Antigen retrieval was performed by microwave treatment of the sections in citrate buffer for 10 min, followed by treatment with avidin/biotin (Vector Blocking Kit). Sections were treated with TNB-BB (0.5% blocking agent in PBS) and incubated with a 1:500 dilution of COX-2-specific antibody (PG-27B; Oxford Biomedical Research Inc.). Specificity of the antibody was determined by the observation that excess antigen (Santa Cruz Biotechnology) was able to negate staining. Immunoreactive complexes were detected using tyramide signal amplification (TSA-indirect) and visualized with the peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride. Slides were counterstained with hematoxylin.

IHC Scoring. In all areas, only malignant cells were scored. ER and PGR expression was assessed semiquantitatively by assessing the intensity (scored 0–3) and percentage of cells positive at each intensity in 10 high-powered fields. The two measurements were multiplied to give an H-score varying from 0 to 300. Samples were considered positive for either receptor if the score exceeded 20. The results for Ki67 were expressed as the percentage of positive cells. CD31 expression was determined using a Chalkley count as described previously (14). COX-2 expression was evaluated semiquantitatively by assessing the intensity (scored 0–3) and by estimating the percentage of cells positive at each intensity in 10 high-powered fields. The two measurements were multiplied and summed to give a score varying from 0 to 300. A score of 100 or greater was considered strong positivity. For HER-2, tumors that exhibited membranous staining of 3+ intensity or were 2+ but showed gene amplification by FISH analysis were considered positive. Sections were scored by one investigator (G. D.) and subjected to review by a second (J. S.).

Statistical Analysis. Analysis was performed using the Mann-Whitney test for categorical analyses. For continuous variables, a Spearman rank-correlation was used.

RESULTS

Patient Characteristics. The age at diagnosis ranged from 29 to 79 years (mean age, 59 years), and 27 (31%) of the
women were less than age 50 years. Eighty-five percent of the tumors were infiltrating ductal carcinomas. Patient characteristics, including tumor stage, nuclear grade, and preoperative treatment history, are shown in Table 1.

**COX-2 Immunoreactivity.** Cytoplasmic COX-2 expression was detected by semiquantitative scoring in 63 of 80 (79%) tumors studied, with 54% staining with a score of 100 or greater indicating strong positivity. Six tumors could not be reproducibly scored due to excessive background staining and were excluded from the analysis. Staining was cytoplasmic, granular, and perinuclear, with staining localized to the tumor cells and not the surrounding stroma (Fig. 1). The four categories of intensity of COX-2 staining (none, weak, moderate, and strong) are shown in Fig. 2. In addition, the neovascularity surrounding tumor cells also demonstrated significant COX-2 expression, although this was not scored independently. COX-2 was not expressed in normal breast tissue (Fig. 1).

**HER-2/neu Expression and Gene Amplification.** HER-2 was analyzed by IHC in all breast cancer cases. Membranous staining of 3+ intensity was detected in 11 of 86 (13%) invasive breast cancers, membranous staining of 2+ intensity was detected in 7 cases, and membranous staining of 1+ intensity was detected in 24 cases. The remaining 44 cases showed no membranous staining. Cases that were scored as 2+ were subjected to FISH analysis for HER-2 gene amplification. Three of seven tumors with 2+ membranous staining were amplified. Thus, a total of 14 (16%) tumors were considered HER-2 positive.

**ER, PGR, and Ki67 Expression.** ER and PGR positivity as defined by an H-score of 20 or greater was detected in 68 and 63 of 86 breast tumors (79% and 73%), respectively. The median value for Ki67 was 8.9% positive cells.

**Association of COX-2 with Clinicopathological Variables.** COX-2 protein expression did not significantly correlate with tumor size, grade, axillary lymph node status, or the presence of vascular invasion. As expected, a significant negative correlation \((r = -0.37; P < 0.001)\) was observed between ER expression and Ki67. COX-2 expression showed a significant correlation with CD31 staining assessed by Chalkley scoring \((r = 0.57; P < 0.001); \) Fig. 3). Vessels from closely adjacent tumour sections stained positively for both COX-2 and CD31 (Fig. 4). No significant correlations were observed between COX-2 and ER, PGR, or HER-2. These results are shown in Table 2.

**DISCUSSION**

This was a descriptive analysis of COX-2 staining to evaluate its relationship with key pathological indices in breast cancer and to allow consideration of the consistency of these
data with preclinical studies. We detected significant expression of COX-2 in the epithelial cells and neovasculature of human breast adenocarcinomas. COX-2 expression was detected in 79% of human breast carcinomas, with scores of 0–100 in 54% of human breast carcinomas. These data are consistent with previous findings (15, 16). Expression was granular, cytoplasmic, and perinuclear, also as reported previously. Staining was lost on preincubation of antibody with excess antigen, supporting its validity. No expression of COX-2 was detected in normal breast tissue. COX-2 expression was unrelated to the clinicopathological variables examined, including histopathological grade, clinical stage, and axillary nodal status. We also found no relationship between COX-2 and hormonal status (ER, PGR) or HER-2 status.

Fig. 2 COX-2 staining in breast cancer tissue sections. a, no staining. b, weak staining. c, moderate staining. d, strong staining.

Fig. 3 The relationship between COX-2 (score of 0–300) and CD31 expression (Chalkley count) in human breast cancer (p = 0.33; P < 0.001).
tion as measured by Ki67 was detected in our study group. This and the rates of ER (79%) and HER-2 (16%) positivity are consistent with expectations and indicate that the sample set is an unselected breast cancer population.

There was a statistically significant relationship between COX-2 and angiogenesis as measured by CD31. A number of studies have suggested that the intensity of angiogenesis may be inversely correlated with survival (17, 18), although not all studies have found this association (19). CD31 (also known as platelet-endothelial cell adhesion molecule-1) is a member of the immunoglobulin superfamily that plays a role in a number of endothelial cell functions including migration, angiogenesis, and transmigration of leukocytes across endothelium. This is the first evidence of a direct link between COX-2 and angiogenesis as assessed by CD31 staining in breast cancer tissue. Given the relatively small size of our data set, any significant relationship between COX-2 and angiogenesis is likely to be an important one in vivo. A similar association has recently been reported in a rat corneal model of angiogenesis (20). Leahy et al. (20) clearly demonstrated the colocalization of COX-2 and CD31 within the vascular endothelial cells using a double staining immunohistochemical technique. In addition, a correlation has been found between COX-2 and neovasculature in human colorectal cancer, a disease for which there is clear evidence of a chemopreventive effect of COX-2 inhibitors (17).

Table 2  Relationship between COX-2 and clinicopathological variables

<table>
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<tr>
<th>A. With two categories by Mann-Whitney analysis</th>
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<tr>
<td>Variable</td>
</tr>
<tr>
<td>Lymph node +</td>
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<td>Vascular invasion +</td>
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<th>B. By Spearman rank correlation for other variables</th>
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<tbody>
<tr>
<td>Variable</td>
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<tr>
<td>Tumor size</td>
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<tr>
<td>Tumor grade</td>
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<tr>
<td>ER</td>
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<td>PGR</td>
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COX-2 inhibitors retard tumor progression in cell model systems by affecting tumor cell migration and invasion as well as angiogenesis (18). However, the mechanism by which COX-2 induction affects angiogenesis is still unclear. In breast cancer, tumor invasion into the local tissue and tumor growth at metastatic sites are preceded by tumor-induced proliferation of a predominantly vascular stroma (19). VEGF regulates vascular permeability, is an important mediator of vasculogenesis and angiogenesis (21), and is expressed in breast cancer (22, 23). Inhibition of the receptor kinase activity of VEGF, together with fibroblast and platelet-derived growth factors, both suppresses tumor growth and enhances tumor radiation response in mammary tumor xenografts (24). The main COX-2 product, PGE2, induces VEGF and basic fibroblast growth factor (25). In addition, in a VEGF-induced mouse corneal model of angiogenesis, the selective COX-2 inhibitor NS-398 inhibited angiogenesis (26), an effect that was reversed by PGE2. If the data derived in our study denote a similar functional relationship between COX-2 and angiogenesis, inhibitors of the enzyme may be applicable for blocking angiogenesis in breast cancer. These agents are well tolerated and would be attractive as potential preventive approaches or as adjuncts to conventional antitumor agents because they would be expected to prevent the expansion of tumors that is dependent on the development of neovasculature.

Fig. 4  Serial sections demonstrating (a) COX-2-positive and (b) CD31-positive endothelial cells.
REFERENCES

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