Nitric Oxide, Prostanoids, Cyclooxygenase, and Angiogenesis in Colon and Breast Cancer

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ABSTRACT

Purpose: Several studies have shown an overexpression of cyclooxygenase-2 (COX-2) and elevated levels of prostacyclin (PGI2) and thromboxane (TXA2) in colon cancer. In this report, we determined the distribution of inducible form of nitric oxide synthase (iNOS), PGI2, and TXA2 in cancerous and adjoining areas of specimens from human colon and breast cancer obtained during surgery. Additionally, we investigated differences in expression and histological localization of COX-2 in colon and breast cancer.

Experimental Design: Specimens were obtained during surgery, one centrally located, the second from an adjacent, cancer-free area. Activity of iNOS was determined, using the conversion of L-[14C]arginine to L-[14C]citrulline. PGI2 and TXA2 were measured as their stable metabolites, using enzyme immunoassay. A standard immunoperoxidase method was used for immunohistochemical expression of COX-2.

Results: Significant differences in iNOS, PGI2, and TXA2 expressions between colon and breast cancer were noted, with an enhanced expression of COX-2 in colon cancer, including the cancerous, adjoining, and stromatous fields.

Conclusions: Increased expression of iNOS and production of prostanoids in colon cancer parallels the increase in COX-2, confirming the importance of this enzyme in colon cancer. The overexpression of COX-2, prostanoids, and nitric oxide in areas adjoining the tumor indicates increased metastatic potential for neoplastic cells in this area. Inflammatory changes in the tissue adjoining the cancer may play a role. COX-2 may result in the formation of new blood vessels and the spread of cancer.

INTRODUCTION

Cancer is the result of genetic mutations and dysregulations of cellular pathways, which lead to the formation of new blood vessels (angiogenesis), an essential process for tumor growth and spread (1). In solid tumors, angiogenesis is the result of a wide variety of signaling systems which include angiogenic factors (2). In some cancers, particularly those of the colon, two cellular pathways have emerged as being closely associated with angiogenesis and tumor growth: (a) the COX2 pathways resulting in the formation of prostanoids from arachidonic acid; and (b) the NO pathway (3–6), which through the activity of NO synthases, oxidizes the guanido group of L-arginine to citrulline and NO. NO originates from the activity of two isozymes: (a) the constitutive; and (b) iNOS, the inducible form of NO synthase. Whereas the constitutive form is mainly produced by endothelial cells, the inducible form originates from the activity of cytokines and inflammatory cells, primarily macrophages (7,8). NO production occurs in several steps leading to neovascularization in some cancers (9, 10). In head and neck cancer, e.g., iNOS activity increases together with three to five cyclic guanosine monophosphate levels, resulting in increased microvessel density and iNOS activity together with lymph node metastases. Colon cancer tissue also expresses NOS mRNA (11).

COX play an important role in promoting angiogenesis in cancer (12, 13). COX, also known as prostaglandin H synthase, is a rate-limiting enzyme in the biosynthesis of prostaglandin and related eicosanoids. Two isoforms of COX have been identified and cloned (14, 15). COX-1 is constitutively expressed in most cell types and is involved in the maintenance of physiological functions. In contrast, COX-2 is inducible by inflammatory cytokines, tumor promoters, and growth factors (12). Reports have related COX-2 to breast and colon cancer (16, 17). Like NO, COX regulates angiogenesis (18). Primarily, COX-2, but not COX-1, gene expression is elevated in many human colorectal cancers (13, 19–22), whereas it is undetectable in normal colorectal mucosa (23). COXs are important for tumor biology because prostanoids, such as PGE2 and PGI2, possess angiogenic activity (24). Therefore, NSAIDs, particularly

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COX-2 inhibitors, are preventive and therapeutic agents for colon cancer (18–22, 25, 26). The overexpression of COX-2 in colon cancer (13, 20, 23, 27) explains the beneficial effects of NSAIDs. COX is also expressed in breast cancer (17, 28). Attempts have been made to achieve chemoprevention of breast cancer by COX inhibitors; according to some investigations, administration of the specific COX-2 inhibitor, celecoxib, suppressed malignant breast tumors in rats (29, 30). Human breast cancer may also be susceptible to NSAIDs (17, 28, 31). Growth factors, such as VEGF and eicosanoids, regulate angiogenesis (5). COX-2 influences a variety of angiogenic factors through prostanoid production from arachidonic acid (5).

The connection between iNOS and COX-2 activation is documented by a synergistic effect of NOS and of COX (32, 33). In cell cultures, NO plays an important role in the release of prostanoids by activation of COX: NO inhibitors also attenuate PGE2 release (32). Similarly, NO production by endothelial cells increases the formation of eicosanoids from arachidonic acid through action on COX synthesis (34).

It is the purpose of our study to determine the distribution of iNOS, PGI₂, and TXA₂ in cancerous and adjoining areas of colon and breast cancer obtained during surgery. Differences in expression and in histological localization of COX-2 in colon and breast cancer were also investigated.

MATERIALS AND METHODS

Patient Demographics

Specimens of tumors were obtained during surgery after signed permission of the patient and with approval of the Institutional Review Board of the Huntington Memorial Hospital. Paraffin slides were furnished by the Department of Pathology of the Huntington Memorial Hospital. Two specimens were obtained, one centrally located, the second from an adjacent, cancer-free area, ~2–3-cm distance from the tumor margins. The specimens were flash frozen. Pertinent demographics and tumor characterization are shown in Table 1. Paraffin-embedded blocks from the tumor and adjacent areas were sectioned at 4-μm thickness. None of the patients had received NSAIDs for 18 days before surgery.

### Table 1 Demographics and tumor characterization

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Colon</th>
<th>Breast</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Patient age (years)</td>
<td>P-2: 67</td>
<td>P-18: 74</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>M M M M M F F F F F F F F F F</td>
<td>M M M M M F F F F F F F F F F</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>4.7 5.5 3.0 2.7 13.0 3.2 5.0 3.6 3.0 4.6 4.6 4.8 ± 0.9</td>
<td>3.0 1.9 3.5 6.0 1.7 4.5 3.2 5.5 2.5 9.6 1.4 2.5 3.8 ± 0.7</td>
</tr>
<tr>
<td>Node positive (%)</td>
<td>N Y N Y N Y N Y N N N N N N</td>
<td>N N N N N N N N N N N N N N</td>
</tr>
<tr>
<td>Proximal colon (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Distal colon (%)</td>
<td>127</td>
<td>127</td>
</tr>
<tr>
<td>T1</td>
<td>X X X X X X X X X X X X X</td>
<td>X X X X X X X X X X X X X</td>
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<tr>
<td>T2</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>T3</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>T4</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>70.2 ± 3.1</td>
<td>64.8 ± 4.3</td>
</tr>
</tbody>
</table>

The specimens were flash frozen. Pertinent demographics and tumor characterization are shown in Table 1. Paraffin-embedded blocks from the tumor and adjacent areas were sectioned at 4-μm thickness. None of the patients had received NSAIDs for 18 days before surgery.

Biochemical Methods

**Assay of iNOS Activity.** The inducible form of NO synthase activity was determined as described previously using the conversion of L-[^14]C]arginine to L-[^14]C]citrulline (35, 36). Tissue (50–100 mg) was prepared from the frozen specimens received from Huntington Memorial Hospital. After homogenization of the tissue in 900 μl of cold Tris-HCl buffer [0.05 M (pH 7.4)] containing D,L-DTT (1 mM), leupeptin (10 μg/ml), phosphoramidon (25 μg/ml), and aprotonin (100 μg/ml; all from Sigma Chemical Co., St. Louis, MO) and sonication for 15 s, the homogenate was centrifuged (1,200 g, 5 min), and EDTA (0.5 mM) and NADPH (1 mM; Sigma Chemical Co.) were added to 65–75 μl of supernatant. L-[^14]C]arginine (200,000 cpm; Amersham Life Science, Buckinghamshire, United Kingdom) was added; the samples were incubated for 30 min in a 37°C water bath. The reaction was stopped by adding ice-cold Tris-HCl buffer (pH 5.5). Radiolabeled citrulline was separated from arginine by cation exchange chromatography (Dowex 50-WX8, 200–400 mesh, Na-form; Bio-Rad Laboratories, Hercules, CA). The eluate (~3.5 ml) was mixed in 10 ml of scintillation fluid and counted in triplicate in a Beckman LS 100SC scintillation counter. All values were corrected for protein content of the samples (determined by the modified Lowry Assay) and calculated in pmol/mg protein/min.

**Assay of PGI₂ and TXA₂.** PGI₂ and TXA₂ were measured as their stable metabolites, PGF₁α and TXB₂, by using EIA kits (Cayman Chemicals, Ann Arbor, MI) as described previously (36). In brief, 80–200 μg of tissue were prepared from...
Table 2  iNOS, prostacyclin, and thromboxane determinations in colon and breast cancer

<table>
<thead>
<tr>
<th>A. Colon cancer</th>
<th>P-#</th>
<th>iNOS (fmol/µg/min)</th>
<th>PGF1α (pg/mg)</th>
<th>TXB2 (pg/mg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Tumor</td>
<td>Adjacent</td>
<td>Tumor</td>
<td>Adjacent</td>
</tr>
<tr>
<td>P-2</td>
<td>0.184</td>
<td>41.25</td>
<td>162.21</td>
<td></td>
</tr>
<tr>
<td>P-4</td>
<td>0.814</td>
<td>199.36</td>
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<tr>
<td>P-15</td>
<td>0.131</td>
<td>35.84</td>
<td>53.41</td>
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<tr>
<td>P-17</td>
<td>0.192</td>
<td>981.38</td>
<td>154.86</td>
<td></td>
</tr>
<tr>
<td>P-19</td>
<td>0.248</td>
<td>6.11</td>
<td>0.45</td>
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</tr>
<tr>
<td>P-34</td>
<td>0.134</td>
<td>387.23</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>P-36</td>
<td>0.116</td>
<td>22.47</td>
<td>5.48</td>
<td></td>
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<tr>
<td>P-39</td>
<td>0.037</td>
<td>20.92</td>
<td>14.29</td>
<td></td>
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<tr>
<td>P-40</td>
<td>0.100</td>
<td>54.27</td>
<td>29.22</td>
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<tr>
<td>P-41</td>
<td>0.145</td>
<td>118.93</td>
<td>113.73</td>
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<tr>
<td>P-47</td>
<td>0.133</td>
<td>8.90</td>
<td>2.02</td>
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<tr>
<td>P-52</td>
<td>0.119</td>
<td>21.430</td>
<td>136.37</td>
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<tr>
<td>N</td>
<td>12</td>
<td>9</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.196</td>
<td>41.25</td>
<td>113.73</td>
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<tr>
<td>SE</td>
<td>0.058</td>
<td>0.463</td>
<td>6.63</td>
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<table>
<thead>
<tr>
<th>B. Breast cancer</th>
<th>P-#</th>
<th>iNOS (fmol/µg/min)</th>
<th>PGF1α (pg/mg)</th>
<th>TXB2 (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor</td>
<td>Adjacent</td>
<td>Tumor</td>
<td>Adjacent</td>
</tr>
<tr>
<td>P-22</td>
<td>0.071</td>
<td>26.36</td>
<td>1.88</td>
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<tr>
<td>P-26</td>
<td>0.094</td>
<td>1.92</td>
<td>0.75</td>
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<td>P-27</td>
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<td>1.74</td>
<td>0.09</td>
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<tr>
<td>P-28</td>
<td>0.107</td>
<td>5.40</td>
<td>2.27</td>
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<tr>
<td>P-32</td>
<td>0.177</td>
<td>14.21</td>
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<tr>
<td>P-33</td>
<td>0.141</td>
<td>31.39</td>
<td>21.10</td>
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<tr>
<td>P-35</td>
<td>0.060</td>
<td>7.28</td>
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<tr>
<td>P-44</td>
<td>0.082</td>
<td>20.80</td>
<td>26.17</td>
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<tr>
<td>P-45</td>
<td>0.091</td>
<td>4.06</td>
<td>0.46</td>
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<tr>
<td>P-48</td>
<td>0.054</td>
<td>22.91</td>
<td>49.56</td>
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<tr>
<td>P-51</td>
<td>0.098</td>
<td>69.94</td>
<td>41.17</td>
<td></td>
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<tr>
<td>N</td>
<td>11</td>
<td>9</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.096</td>
<td>18.73</td>
<td>13.31</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>0.011</td>
<td>0.110</td>
<td>5.51</td>
<td></td>
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</table>

a iNOS, colon vs. breast; P = 0.009.

b PGF1α, colon vs. breast; P = 0.010.
c TXB2, colon vs. breast; P = 0.018.
d iNOS, colon adjacent vs. colon tumor, P = 0.002.
e PGF1α, breast adjacent vs. breast tumor; P = 0.015.
f TXB2, breast adjacent vs. breast tumor; P = 0.055.

each of the two specimens. The tissue from each sample was weighed and homogenized in 2 ml of ethanol. The homogenate was stored at 4°C for 5 min, then centrifuged (1,000 g, 15 min) to remove precipitate. The supernatant was added to 8 ml of double distilled water, and pH was adjusted to 4.0 with dilute HCl. The sample was passed through the C-18 reverse phase cartridge (Sep-Pak Cartridge; Waters Corp., Milford, MA), and the cartridge was rinsed with 5 ml of double distilled water, followed by 5 ml of high-performance liquid chromatography grade hexane (Sigma Chemical Co.). PGF1α and TXB2 were eluted with 5 ml of ethyl acetate containing 1% methanol and evaporated under a stream of dry nitrogen. The dried samples were reconstituted by EIA buffer and used for EIA analysis.

COX-2. A standard immunoperoxidase method was used for immunohistochemical expression of COX-2 with additional TSA using the Vectastain Elite ABC (Vector) and the TSA Biotin System (NEN Life Science Products, Inc., Boston, MA) kits. In brief, sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol before blocking with normal serum. Incubation with anti-PGHS-2 (anti-COX-2) primary polyclonal antibody (Oxford Biomedical Research, Oxford, MI) was performed at 1:200 dilution overnight at room temperature in a humidified chamber. After washing in PBS and incubation with biotinylated secondary antibody (30 min at room temperature), amplification was performed using the TSA Biotin System (NEN Life Science Products, Inc.). Bound antibodies were detected with 3,3’-diaminobenzidine (Vector). Slides were counterstained with filtered Mayer’s (Lillie’s Modification) hematoxylin (Dako) for 15 min, mounted with VectaMount (Vector), and photographed. Field size of observation was 0.8 mm². The area of highest expression (hotspot) was selected for examination. COX-2 expression was compared in cancer, adjacent, and stroma areas of colon and breast cancer. The adjacent areas...
were free of cancer. Counts were performed by at least two investigators who were blind to the experimental design.

Statistical Methods and Evaluation

For statistical comparisons, a permutation test was used (Refs. 37 and 38; Table 2). $P/H_{11021} < 0.05$ was considered significant.

RESULTS

Table 2 and Fig. 1 show the values for iNOS, PGF$_{1\alpha}$, and TXB$_2$ in colon and breast cancer in both cancerous and adjacent areas. Values were statistically higher in cancer and adjoining areas of colon as compared with breast cancer (Table 2; Fig. 1). Because the remote areas were free of cancer tissue, inflammatory reaction in these areas may have contributed to the elevation of iNOS and PGF$_{1\alpha}$. This view is supported by the occasional presence of macrophages in the adjacent areas of colon cancer (data not shown) and by the higher intensity for COX-2 immunoreactivity in stroma of the adjacent areas of colon cancer (Fig. 2, a and b; Table 3).

Fig. 3 compares the values in the cancer area for iNOS, PGF$_{1\alpha}$, and TXB$_2$ in individual patients. In colon cancer, the number of patients with higher values exceed those in breast cancer.

Immunohistochemical presentation for COX-2 in cancerous and adjacent tissue is shown in Fig. 2, a and b and Table 3. Table 3 illustrates enhanced expression of COX-2 in the cancer, adjacent, and stroma areas of colon cancer. It also illustrates enhanced expression of COX-2 in the stroma of the cancer areas of colon cancer as compared with breast cancer (Fig. 4a; Table 3). In the cancer, adjacent, and stroma areas, COX-2 expression was higher in colon than in breast cancer (Table 3). Equally, the paired differences between cancer and adjoining tissue were significantly higher in colon cancer ($P = 0.020$).

DISCUSSION

This study examines the activation of iNOS and the production of PGI$_2$, TXA$_2$, and COX-2 in cancer and noncancer adjacent tissue of patients with colon and breast cancers. Significant differences in iNOS, PGI$_2$, and TXA$_2$ expression between the two types of cancer are noted (Fig. 1; Table 2). In colon cancer, mean values for iNOS, PGF$_{1\alpha}$, and TXA$_2$ statistically exceeded those in breast cancer (Fig. 1; Table 2). A similar relationship was also found when comparing the adjacent areas in breast and colon cancer (Table 2). Fig. 3 shows the distribution of individual values in colon and breast cancer; not all patients with colon cancer showed an increase in iNOS, PGF$_{1\alpha}$, and TXA$_2$.

Our observations on expression of COX-2 in breast and colon cancer are shown in Figs. 2 and 4 and Table 3. The pertinent finding is the enhanced expression of COX-2 in colon cancer, including the cancerous, adjoining, and stromatous fields (Table 3). These findings strongly point toward a prominent role of COX-2 in colon cancer. A number of studies has shown an overexpression in COX-2 in both colon and breast cancer, e.g., COX-2 and not COX-1 expression is markedly elevated in human colorectal cancer (13). COX also regulates colon carcinoma-induced angiogenesis by production of angiogenic factors (18). Pertinent in this respect are the findings of Williams for malignant transformation (39) who implanted Lewis lung cancer cells into mice that had been genetically engineered to lack either COX-2 or COX-1. They observed a dramatic suppression of tumor growth and angiogenesis in the animal that lacked COX-2. They observed that COX-2 is expressed primarily in stromal cells of the tumor. It is to be noted, however, that COX-2 is also expressed in breast cancer (Table 3).

TXA$_2$ plays an important role as mediator for COX-2-dependent angiogenesis (40, 41). Elevated levels of prosta
glandin have also been shown in colon cancer and particularly in
benign adenomatous polyps with an additional increase in cancer (25). It is therefore not surprising that NSAIDs influence the growth of colon cancer. Studies have used combinatorial chemoprevention of intestinal neoplasia by a NSAID (42), and there have been several observational studies of the effect of exposure to NSAIDs on the subsequent development of colorectal cancer (43). Recently, specific inhibitors of COX-2 have been used against colon carcinogenesis. One of these, celecoxib, has suppressed the incidence and multiplicity of colon tumors and the total tumor burden (19–21, 24–26).

Marked induction of iNOS was found in colon cancer (Fig. 1; Table 2). It has been suggested that NO synthase activity is involved in tumor growth (9). Therefore, a role for NO in the development of collateral vessels has been postulated particularly because VEGF-R2 may be dependent on NO formation for angiogenesis and vasodilator effects of VEGF (44). Additional reports confirm the role of NO in tumor angiogenesis. NO inhibitors prevent tumor-induced angiogenesis in mammary tumors (45), and therapy of cancer metastases can be accomplished by the inactivation of iNOS (46). NO may also partially mediate tumor angiogenesis and increase tumor blood flow (47); the antitumor effects of a NO inhibitor, 1-NAME, are partially mediated by reduced tumor angiogenesis (45). NO also plays a role in the biology of human breast cancer (8), whereas in certain colon cancer cell lines, NOS gene may be expressed constitutively (48). Overproduction of NO in tumors leads to cytotoxicity, which by itself results in replication (3). Through a variety of mechanisms, cells exposed to NO undergo DNA damage, which may result in a combination of cell deaths and mutagenesis (3).

Increased activity of iNOS and COX-2 in colon cancer suggests an interrelationship between the two enzymes. This finding is supported by the fact that a NO donor increased PGE 2 formation in hypothalamic fragments and that released NO stimulated the synthesis of a series of prostanoids (33). NOS and COX pathways also interact in mesangial cells (33). Salvemini et al. (32) distinguished between a synergistic effect (NO production and activation of COX) and between interaction of the enzymes. COX is a potential target for NO because it contains an iron heme center at its active site (49). Inhibitors of NO attenuate PGE 2 release (33). Similarly, Davidge described that NO produced by endothelial cells increased the production of eicosanoids through the activation of COX synthesis (34).

Increased expression of iNOS and production of prostanoids in colon cancer parallel the increase in COX-2 as determined by immunohistochemistry and illustrates the importance of this enzyme in colon cancer. A sporadic increase in NO and prostanoids in colon cancer may reflect different stages of

![Fig. 2](image_url)
development in the population examined. One possibility for the predominance of expression of iNOS, PGi2, and TXA2 in the area adjacent to cancer may be the presence of inflammatory cells. In the absence of macrophages, inflammatory changes may affect fibroblast and endothelial cells and may also have contributed to the formation of prostanoids and iNOS. Whether this suggests vulnerability of noncancerous areas for malignant transformation is not certain.

The overexpression of COX-2, prostanoids, and NOs in areas adjoining the tumor may be important because it may increase the metastatic potential for neoplastic cells in this area (Figs. 2, a and b and 4, a and b; Table 3). Enhanced expression of COX-2 in cancer, stroma, and adjoining areas of colon cancer emphasizes the important role of this enzyme. COX-2 expression, particularly in the stroma cells, is an essential event in the earliest stage of polyp formation (21, 50). Furthermore, prostaglandins exert a positive effect on angiogenesis and carcinogenesis resulting from expression of COX-2 in cells adjacent the cancer (50). It has been shown that prostaglandins induce VEGF secretion in target cells (51, 52), and the stromal cells in tumors demonstrate active transcription of the VEGF gene (50). The question has been raised whether the enhanced COX expression in the stroma is an initial event in carcinogenesis or whether COX-2 is later found in inflammatory cells in or near the tumor, in the endothelial cells of the neovasculature, and in the carcinomatous epithelial cells (53). Inflammatory changes in the tissue adjoining the cancer may play a role, but cancer is the result of many factors, genetic mutations, and dysregulation of cellular pathways. Therefore, COX-2 may be just one of the many factors leading to formation of new blood vessels and spread of the cancer.

In summary, we have compared activation of iNOS and expression and formation of PGi2 and TXA2 in cancerous and adjacent areas of patients with colon and breast cancer. In colon cancer, there was a significantly greater induction of iNOS and of PGi2 and TXA2 production in cancer and adjoining cells. In breast cancer, there is less expression of COX-2 in the adjacent area as compared with colon cancer (2b).
addition, COX-2 was overexpressed in the cancerous, adjoining, and stromatous areas of colon cancer. Increases in iNOS, PGl₂, and TXA₂ was not found in all patients with colon cancer. This may be of importance in choosing therapy by NSAIDs because only patients with high expression of COX-2 activity would benefit from this treatment.

The results open interesting options for research and therapy of breast cancer. An up-regulation of COX-2 expression in breast cancer may increase the susceptibility of this tumor to the action of NSAIDs.

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REFERENCES

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