Cyclooxygenase-2 Expression Correlates with Local Chronic Inflammation and Tumor Neovascularization in Human Prostate Cancer

Wanzhong Wang,1 Anders Bergh,2 and Jan-Erik Damber1

Abstract

Purpose: Chronic inflammation is linked to the development of cancer in several organs, including the prostate. Up-regulated cyclooxygenase-2 (COX-2) may play a role in influencing cell proliferation, differentiation, apoptosis, or angiogenesis. This study aimed to derive data from human prostate cancer to investigate whether chronic inflammation and angiogenesis were correlated with the expression of COX-2.

Experimental Design: In this study, we did double-immunohistochemical analysis of a set of 43 human prostate cancer for COX-2 expression and the correlation with T-lymphocyte and macrophage densities and CD31-marked microvessel density (MVD) in situ.

Results: COX-2 positive staining was detected in 40/43 cancer samples with the very heterogeneous expression. Elevated COX-2 expression was associated with high Gleason score (P = 0.002). Foci of chronic inflammation were found in all 43 samples. COX-2 positive areas were noted with high T-lymphocyte and macrophage densities than COX-2 negative tumor areas (P < 0.0001 and P = 0.001, respectively). MVD were also found higher in COX-2 positive areas than in COX-2 negative tumor areas (P = 0.001).

Conclusions: This study shows a novel relationship between COX-2 expression and the local chronic inflammation within prostate cancer and the increased angiogenesis. It is likely that the proinflammatory cytokines, released by T-lymphocytes and macrophages, up-regulate COX-2 in adjacent tumor cells and stimulate the angiogenesis in stromal tissues. These findings suggest that COX-2 may be an effective therapeutic target in prostate cancer treatment.

Chronic inflammation of long-standing duration has been linked to the development of tumors in several organs, including the prostate (1). In fact, several studies have suggested that ~15% of malignancies worldwide are attributed to inflammation (2, 3). The inflammatory microenvironment, which is characterized by accumulation of different types of inflammatory cells in the tissue stroma and epithelium, may support the development of malignancy by secreting various proteases, mitogenic, antiapoptotic, and angiogenic factors (2). Elevated levels of proinflammatory cytokines may induce a network of regulatory factors that influence cell survival, growth, differentiation, and movement of both tumor and stromal cells (3) as well as induction of cyclooxygenase-2 (COX-2) expression.

COX is the enzyme responsible for the production of prostaglandins from arachidonic acid. Accumulating evidence shows that COX-2, through production of prostaglandins, may play a key role in tumorigenesis of a variety of human malignancies by stimulating cell proliferation (4, 5), inhibiting epithelial differentiation (6), enhancing cell invasiveness and tumor metastasis (7–9), inhibiting apoptosis (10), mediating immune suppression, and increasing the production of mutagens (11). COX-2 also contributes to angiogenesis (12–14), a crucial process for tumor growth and metastasis. Furthermore, epidemiologic studies have documented that the use of nonsteroidal anti-inflammatory drugs reduces colon cancer risk by 40–50% (15). The mechanism underlying the chemopreventive effects of nonsteroidal anti-inflammatory drugs may be related to their ability to inhibit COX-2.

Several lines of evidence suggest a connection between inflammation and prostate cancer. Polymorphisms and mutations in genes regulating the inflammatory process have been linked to prostate cancer risk (16). Prostatitis is apparently a risk factor for prostate cancer (16, 17) and the risk of biochemical relapse following radical prostatectomy is increased in patients with high-grade inflammation surrounding malignant glands (18); however, the mechanism by which inflammation influences the development and behavior of prostate cancer is unclear.

In a previous study, we have shown that COX-2 is locally up-regulated in prostate luminal epithelial cells in benign prostate hyperplasia if T-lymphocytes and macrophages are present.
Such COX-2 positive epithelial cells had higher levels of Bcl-2 immunostaining and higher proliferation when compared with COX-2 negative cells (19). In this study, we analyzed if COX-2 expression in human prostate cancer is associated with local inflammation using double staining immunohistochemistry technique. In addition, we assessed the relationship between COX-2 and Gleason score of prostate cancer and reported a new finding of a relationship between COX-2 expression and microvessel density (MVD) in prostate cancer.

**Materials and Methods**

**Tissue samples.** The material comprised 43 specimens of prostate cancer. All of the tissues had been fixed in formalin and processed routinely through graded alcohols to paraffin blocks. Tissues were obtained from patients with prostate cancer undergoing prostatectomy at the Department of Urology, Sahlgrenska University Hospital, Göteborg University, Sweden (n = 28) and at the Department of Urology, Shandong Provincial Hospital, China (n = 15). Histologic diagnosis of prostate cancer was based on H&E-stained sections. The Gleason system was used for histologic grading. A primary and secondary Gleason grade (1-5) was determined for each tumor, and the combined score (Gleason sum) was then calculated. To obtain sufficient quantities for statistical analysis, the tumors were grouped in secondary Gleason grade (1-5) was determined for each tumor, and the combined score (Gleason sum) was then calculated. To obtain sufficient quantities for statistical analysis, the tumors were grouped in two categories: low grade if the Gleason score was 7 or less (n = 28), and high grade if the Gleason score was >7 (n = 15).

**Antibodies.** COX-2 affinity–purified polyclonal antibody was obtained from Cayman Chemical Co. (Ann Arbor, MI). CD3 (Ready to Use), CD31 (working dilution 1:25), and CD68 (Ready to Use) were purchased commercially from NeoMarkers Co. (Fremont, CA).

**Immunohistochemical staining.** COX-2 immunohistochemical staining was done using the ABC kit (Vector Laboratories, Inc., Burlingame, CA). Briefly (19), deparaffinized sections (4 µm) were rinsed with methyl alcohol-hydrogen peroxide and then microwaved in citrate buffer (pH 6.0) to induce epitope retrieval. Diluted COX-2 primary antibody (1:100) was incubated on slides at +4°C overnight and then incubated with biotinylated secondary antibody at room temperature. For localization, avidin–biotin complex was applied at room temperature followed by an application of alkaline phosphatase–labeled polymer for 30 minutes at room temperature. COX-2 immunostaining was labeled with 3,3′-diaminobenzidine tetrahydrochloride as the chromagen. Slides were counterstained with Mayer hematoxylin.

The specificity of the antibody was evaluated by preadsorption of the COX-2 antibody with a COX-2–specific blocking peptide (Cayman) before the staining procedure. The rabbit polyclonal affinity-purified immunoglobulin G was raised against a peptide corresponding to amino acids 584 to 598 of murine COX-2. According to information given by the manufacturers, it cross-reacts with the human COX-2 and has negative reaction with COX-1. A COX-2–specific blocking peptide, which derived from the human COX-2 cDNA sequence, was used in conjunction with COX-2 polyclonal antibody (Cayman) to block antibody/protein complex formation during immunohistochemical analysis for COX-2. COX-2 blocking peptide was mixed with the COX-2 antibody in a 1:1 (w/w) ratio in a microfuge tube with the dilution of 1:100 and incubated for 1 hour at room temperature before application of the antibody to the slide. Then the manual immunohistochemistry was done as described above. In all cases, the immunoreactivity of COX-2 was completely blocked by COX-2 blocking peptide (Fig. 1) in line with other studies (19, 20).

**Double immunohistochemical staining.** To evaluate accurately the expression of COX-2 and the presence of related reagents on the same tissue section in situ, double immunohistochemical staining was done with the following combination of antibodies: COX-2/CD3 and COX-2/CD68 were used to mark the types of chronic inflammatory cells within prostate; COX-2/CD31 was designed to label the microvessels in condition of COX-2 staining (21). DAKO EnVision Doublestain System (Carpinteria, CA) was used in this procedure. Briefly (19), serially consecutive sections were treated for epitope retrieval as described above. After being incubated with peroxidase block for 5 minutes at room temperature, the slides were exposed to COX-2 primary antibody (1:100) and incubated at +4°C overnight. The slides were then incubated with horseradish peroxidase–labeled polymer for 30 minutes at room temperature. COX-2 immunostaining was labeled by applying 3,3′-diaminobenzidine tetrahydrochloride for 1 to 5 minutes. Next, doublestain block was added to the slides, and the slides were incubated for 3 minutes. Then, the slides were incubated with the second primary antibodies for 30 minutes at room temperature followed by an application of alkaline phosphatase–labeled polymer for another 30 minutes. The second substrate- chromagen solution, Fast Red, was incubated on the slides for 1 to 5 minutes. Slides were counterstained with Mayer hematoxylin and coverslipted with DAKO Glycergel.

**Cyclooxygenase-2 immunohistochemical expression scores.** COX-2 positive staining was identified by the presence of marked diffuse brown (3,3′-diaminobenzidine tetrahydrochloride) cytoplasm or perinuclear staining in prostate cancer cells. Quantitation of COX-2 immunohistochemical expression score was done using 10 to 20 ocular measuring fields chosen randomly under a microscope set at ×200 magnification. Each slide was scored independently and the results were summed. The immunostaining results were scored separately according to the criteria of Krajewska et al. (22). The extension of positive tumor cells was graded as follows: 0, none; 1, positive staining cells <1/3; 2, 1-3/3; 3, >2/3. The immunostaining intensity was rated as follows: 0, none; 1, weak staining; 2, moderate; 3, strong. A score was calculated in which the extension was multiplied by the intensity rating (score range from 0 to 9) for each case. The COX-2 immunohistochemical expression scores were categorized as follows: grade 1, negative (0); grade 2, low (1-3); grade 3, intermediate (4-6); and grade 4, high (>7).

![Fig. 1. COX-2 immunohistochemical staining in prostate cancer. Note the heterogeneous immunoreactivity in the cancer tissues (magnification: A, ×100; B, ×200; bottom-left insets, original × 400).](image-url)
Evaluation of double immunohistochemical staining. The two antigens staining in double immunohistochemical staining slides were identified by colors: the first antigen, COX-2, was stained brown (3,3′-diaminobenzidine tetrahydrochloride) and the second one was stained red (Fast Red). For example, apart from the brown immunostaining of COX-2 on prostate cancer cells in COX-2/CD31 double immunohistochemical staining slides, the endothelial cells were labeled with cytoplasm red staining. Any single endothelial cells or cluster of endothelial cells that was labeled with CD31 was regarded as a single microvessel (23). The quantification of inflammatory cells or microvessel densities was done by counting 20 to 40 randomly selected microscopic fields of prostate cancer at ×400 magnification [high power fields (HPF)] for each slide on COX-2/CD3, COX-2/CD68, or COX-2/CD31 double immunohistochemical staining sections and the values of the second antigen staining were regarded as inflammatory cell density or MVD.

Statistics. Statistical analysis was carried using SPSS 12.0 for Windows software. The COX-2 expression immunohistochemical score and the relationship with Gleason score, inflammatory cell density, and MVD were analyzed with Mann-Whitney U test. The strength of association between the COX-2 immunohistochemical score and inflammatory cell density or MVD was assessed using the Spearman rank correlation coefficient test.

Results

The heterogeneous cyclooxygenase-2 immunoreactivity. COX-2 immunohistochemical expression was observed showing a predominant cytoplasmic or perinuclear staining in prostate cancer cells. Adjacent stromal cells stained weakly positive. No COX-2 immunostaining was done in the vascular endothelium. COX-2 heterogeneous staining, both in the percentage of tumor cells stained and in staining intensity, was observed in most cases. Intense positive-staining cells, even the scattered positive tumor cells, were not always present (Fig. 1; refs. 24, 25). Accumulation of COX-2–positive tumor cells was particularly conspicuous in areas with chronic inflammation. In the surrounding nonmalignant prostatic glands, scattered COX-2–staining epithelium was detected in atrophic glands that were infiltrated by inflammatory cells (19).

Relationship between cyclooxygenase-2 expression and Gleason score. Overall, COX-2–positive tumor cells were detected in 40 of 43 cancer samples. The quantitative immunostaining data showed that majority of them (28 of 43, 65.1%) had the weak COX-2 immunostaining (COX-2 immunohistochemical score ≤ 3); 12 of 43 samples (27.9%) had the intermediate or strong COX-2 expression (COX-2 immunohistochemical score > 3). There was a significant association between elevated COX-2 expression and Gleason score. Quantification showed that the mean COX-2 immunohistochemical score was 1.13 (± 0.85) in low Gleason score specimens (n = 28). In contrast, the high Gleason score cases (n = 15) had increased COX-2 staining score (2.41 ± 1.95; P = 0.002).

Focal chronic inflammation and the up-regulation of cyclooxygenase-2 in adjacent tumor cells. Foci of chronic inflammation, with accumulation of T-lymphocytes and macrophages, were detected in all 43 prostate cancer samples (Fig. 2). Quantification of double labeling of COX-2/CD3 showed that the T-lymphocyte density was heterogeneous from 0 to 38 / HPF with a mean density of 7.95 (± 5.78) / HPF. No significant difference in T-lymphocyte density was found between the low and high Gleason score groups (P = 0.175). A higher T-lymphocyte density was found in COX-2–positive (9.51 ± 6.56 / HPF) than in COX-2–negative tumor fields (5.57 ± 3.15 / HPF; P < 0.0001). Further analysis showed that T-lymphocyte density was related to COX-2 expression in both the low and high Gleason score groups (P = 0.001 and 0.028, respectively). There was a significant positive correlation between T-lymphocyte density and COX-2 expression score.

Fig. 2. COX-2 expression in relation to chronic inflammation (×200; insets, original × 400). A, tumor cells in a Gleason score 5 patient only showing strong COX-2 immunostaining (brown, arrowhead) in the area with intense T-lymphocyte (red, arrow) infiltration. B, cancer cells showing intense expression of COX-2 (brown, arrowheads) in a Gleason score 9 patient. T-lymphocytes (red, arrow) are also present. C, tumor cells in a Gleason score 5 tumor showing focal COX-2 positive immunostaining (brown, arrowhead) in the area with macrophage (red, arrow) infiltration. D, tumor cells in a Gleason score 8 tumor with intense macrophage infiltration (red, arrow) showing strong COX-2 expression (brown, arrowheads).
CD68 immunostaining showed that the macrophage density varied widely within prostate cancer tissues. The mean macrophage density was 2.73 (± 2.15) / HPF with a median of 1.35 / HPF and ranging from 0 to 17 / HPF. Macrophage density was not significantly different between the low and high Gleason score specimens ($P = 0.118$). However, the macrophage density was higher in COX-2–positive tumor fields (2.22 ± 1.35 / HPF) than in COX-2–negative fields (3.89 ± 2.73 / HPF; $P = 0.001$). A significant positive correlation was shown between COX-2 immunohistochemical expression score and macrophage density (Spearman rank correlation coefficient test, $\rho = 0.358$, $P < 0.0001$). Interestingly, COX-2 expression was significantly related to macrophage density in low Gleason score specimens ($\rho = 0.492$, $P < 0.0001$) but not in high Gleason score specimens ($\rho = 0.175$, $P = 0.315$; Fig. 3B; Tables 1 and 2).

Cyclooxygenase-2 expression and microvessel density. The spatial relationship between COX-2–expressing cancer cells and CD31-marked microvessels was investigated using COX-2/CD31 double immunohistochemical staining. COX-2 protein expression was not detected in endothelial cells (Fig. 4). In all cases, the mean CD31-labeled MVD was 6.37 / HPF with a median MVD value of 5.73 (0-18) / HPF. MVD was significantly increased in high Gleason score cases (8.02 ± 2.95 / HPF) compared with low Gleason score cancer specimens (5.30 ± 1.52 / HPF, $P < 0.0001$). A significant difference in MVD was also noted between COX-2–negative (5.50 ± 2.10 / HPF) and COX-2–positive staining areas (7.06 ± 2.71 / HPF; $P = 0.001$). Spearman rank correlation coefficient test showed a significant correlation between MVD and COX-2 expression score ($\rho = 0.434$, $P < 0.0001$; Fig. 5; Table 3).

Discussion

In this study, we show, using double immunohistochemical techniques, that prostate tumor cells adjacent to areas with chronic inflammation up-regulate COX-2. We also show that vascular density is higher in inflamed than in noninflamed tumor areas, and COX-2 expression is increased in high Gleason score cancer specimens. Our finding of an increased number of COX-2 positive cells in high-grade cancer is generally in line with previous observations (25–27). These studies conclude that there is an up-regulation of COX-2 in prostate cancer and in some COX-2 staining is related to tumor differentiation (28, 29). A more recent study, however, showed that COX-2 protein expression is not consistently elevated in prostate cancer and does not correlate with established clinical-pathologic risk factors such as Gleason score and pathologic stage (24). In addition, the study noted that COX-2 protein was consistently observed in areas of postinflammatory atrophy, one kind of lesion that has been implicated in prostatic carcinogenesis. To date, however, there are few quantitative analyses of COX-2 expression in relation to Gleason score. In the present study, the COX-2 positive rate (93%) is higher than in any other report. This discrepancy can probably be explained by the fact that there is a very heterogeneous expression of COX-2 in prostate cancer tissues. A detailed quantitative analysis, based on the information of each microscopic field instead of the whole slide of each sample, may be the best way to interpret such heterogeneous findings.

**Table 1. Inflammatory cell densities in relation to Gleason score and COX-2 expression in prostate cancer tissue**

<table>
<thead>
<tr>
<th></th>
<th>T-lymphocyte density*</th>
<th>Macrophage density*</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 7</td>
<td>8.82 ± 6.75</td>
<td>3.19 ± 2.03</td>
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<tr>
<td>&gt; 7</td>
<td>6.56 ± 3.42</td>
<td>4.47 ± 3.21</td>
</tr>
<tr>
<td>COX-2 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>5.57 ± 3.15</td>
<td>2.22 ± 1.35</td>
</tr>
<tr>
<td>Positive</td>
<td>9.51 ± 6.56</td>
<td>3.89 ± 2.73</td>
</tr>
</tbody>
</table>

* T-lymphocyte and macrophage density mean the counting of CD3- or CD68-marked inflammatory cells under the HPF × 400.

1 COX-2 expression: negative means COX-2 immunohistochemical score (HPF × 400) is less than 1. Positive means COX-2 immunohistochemical score is 1 or >1.
Our data suggest that COX-2 expression is up-regulated focally in tumor areas with chronic inflammation, an observation that has heretofore not been noted. This observation is in line with our previous finding in benign prostate hyperplasia that COX-2 expression in benign prostate epithelium is correlated with local chronic inflammation, especially with accumulation of T-lymphocytes and macrophages (19). A significant correlation was found between COX-2 expression in tumor cells, especially in the low Gleason score specimens, and inflammatory cell density, both of T-lymphocytes and macrophages. The mechanism behind the association between inflammation and COX-2 expression in epithelial cells is not fully established. Several studies, however, have shown that COX-2 is up-regulated by factors such as interleukins 1, 4, 6, and 13, vascular endothelial growth factor, and tumor necrosis factor α (30–33), which are released by macrophages or activated T-lymphocytes (34). Moreover, in cell coculture experiments, COX-2 is induced by the presence of inflammatory cells (35). In normal prostate cells and prostate cancer cells, COX-2 protein levels are increased after tumor necrosis factor-α stimulation in vitro (36, 37). These observations suggest that the proinflammatory cytokines released by T-lymphocytes and macrophages may contribute to the up-regulation of COX-2 in adjacent tumor cells.

This study provides the first evidence of a direct link between COX-2 and angiogenesis in prostate cancer tissue. This correlation is consistent with observations in colon, breast, liver, and endometrial tumors (38–41). Angiogenesis

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>COX-2 expression</th>
<th>T-lymphocyte density</th>
<th>Macrophage density</th>
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</thead>
<tbody>
<tr>
<td>&lt;7 Negative</td>
<td>5.88 ± 3.45</td>
<td>2.22 ± 1.35</td>
<td></td>
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<tr>
<td>&lt;7 Positive</td>
<td>10.94 ± 7.73</td>
<td>3.89 ± 2.25</td>
<td>0.0001</td>
</tr>
<tr>
<td>&gt;7 Negative</td>
<td>4.98 ± 2.49</td>
<td>3.86 ± 2.93</td>
<td></td>
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<tr>
<td>&gt;7 Positive</td>
<td>7.45 ± 3.58</td>
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<td>0.533</td>
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</table>

*T-lymphocyte and macrophage densities mean the counting of CD3- or CD68-marked inflammatory cells under the HPF × 400.

1COX-2 expression: negative means COX-2 immunohistochemical score (HPF × 400) is less than 1. Positive means COX-2 immunohistochemical score is 1 or >1.

1P = 0.585.

1P = 0.042.

1P = 0.102.

1P = 0.698.
in prostate cancer is regulated by a variety of stimulatory factors like vascular endothelial growth factor, interleukin 8, tumor necrosis factor α, and inhibitors such as pigment epithelium–derived factor and thrombospondin 1 (42). These regulators are produced by tumor epithelial cells, stroma cells, and inflammatory cells (42). Interestingly, some of these factors could also induce COX-2 expression in vitro (30, 32, 37). The contribution of inflammatory mediators to the angiogenesis of tumors and their growth is becoming evident (25). COX-2 may stimulate angiogenesis through the production of angiogenic factors such as prostaglandins and vascular endothelial growth factor (5, 14). Angiogenesis of the tumor tissues can be suppressed by the COX-2 inhibitor celecoxib (25). Collectively, these observations suggest that the correlation between COX-2 expression and angiogenesis could be related either to the secretion of angiogenic prostaglandins from COX-2–expressing tumor cells or to the possibility that inflammatory cells secrete factors that directly stimulate angiogenesis.

The observation that up-regulation of COX-2 in nonmalignant prostate epithelial cells (19) and prostate cancer cells is related to chronic inflammation and increase of tumor angiogenesis may have chemopreventive and therapeutic implications in prostate cancer. It might be suggested that COX-2 inhibitors, through inhibition of the proinflammatory cytokines, inhibit the procession of precancerous lesions and the angiogenesis of tumors. In fact, studies in experimental models have shown that COX-2 inhibitors suppress the growth and angiogenesis of prostate cancer (7, 14, 37, 43, 44). In addition, several studies have already recognized that nonsteroidal anti-inflammatory drugs have a dramatic antitumor effect in prostate cancer both in vivo (45) and in vitro (10). Additionally, a cohort study (46) and a case-control study (47) report strong inverse associations between nonsteroidal anti-inflammatory drug intake and risk of prostate cancer.

### References


### Table 3. MVD in relation to Gleason score and COX-2 expression in prostate cancer tissues

<table>
<thead>
<tr>
<th>MVD</th>
<th>Mean ± SD</th>
<th>P</th>
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<tbody>
<tr>
<td>Positive</td>
<td>7.06 ± 2.71</td>
<td>0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>5.50 ± 2.10</td>
<td></td>
</tr>
<tr>
<td>≤7</td>
<td>5.30 ± 1.52</td>
<td></td>
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<tr>
<td>&gt;7</td>
<td>8.02 ± 2.95</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*MVD means the counting of CD31-marked microvessels under the HPF (HPF = 400).

1COX-2 expression: negative means COX-2 immunohistochemical score (HPF × 400) is less than 1. Positive means COX-2 immunohistochemical score is 1 or >1.


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