Expression of Cyclooxygenase-2 (COX-2) in Human Invasive Transitional Cell Carcinoma (TCC) of the Urinary Bladder

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Abstract

Cyclooxygenase (COX)-inhibiting drugs have antitumor activity in canine and rodent models of urinary bladder cancer. Two isoenzymes of COX have been identified, COX-1 and COX-2. The purpose of this study was to characterize COX-1 and COX-2 expression in human invasive transitional cell carcinoma of the urinary bladder by immunohistochemistry and Western blot analysis. COX-2 was not expressed in normal urinary bladder samples but was detected in 25 of 29 (86%) invasive transitional cell carcinomas of the urinary bladder and in 6 of 8 (75%) cases of carcinoma in situ. These results indicate that COX-2 may play a role in bladder cancer in humans and support further study of COX-2 inhibitors as potential antitumor agents in human bladder cancer.

Introduction

More than 50,000 people are diagnosed with TCC of the urinary bladder each year in the United States alone, and 10,000 of these patients are expected to die from the cancer (1). Most bladder cancer deaths result from invasive, metastatic TCC that is resistant to chemotherapy. More effective therapies are needed to treat or to prevent the development and progression of invasive TCC. COX-inhibiting drugs have induced remission of chemically induced bladder tumors in rodents and naturally occurring invasive TCC in dogs (a relevant model of human invasive urinary bladder cancer; Refs. 2–5). Two COX isoenzymes have been identified: COX-1, which is constitutively and relatively ubiquitously expressed; and COX-2, shown to be induced during inflammation (6). Prominent COX-2 expression has also been described recently in several forms of human cancer including carcinoma of the colon (7), breast (8), and lung (9). Specific COX-2 inhibitors have been developed and may be evaluated as antitumor agents in cancers that overexpress COX-2. The purpose of this study was to characterize the expression of COX-1 and COX-2 in human invasive TCC, noninvasive TCC, and CIS.

Materials and Methods

Tissue Samples. Formalin-fixed urinary bladder tissue samples from patients with invasive TCC, noninvasive TCC, CIS, and normal bladders were obtained from the Department of Urology, Indiana University School of Medicine, and from the Southern Division of the Cooperative Human Tissue Network. Snap-frozen bladder sections from patients undergoing cystectomy for TCC at the Indiana University School of Medicine were also evaluated for COX-1 and COX-2 protein by Western blot analysis. Complete pathology reports were available on all tissue samples. Tumors were graded 1, 2, or 3 according to criteria adopted by WHO. Information on distant spread of the cancers was not available on samples from the Cooperative Human Tissue Network, precluding analyses involving complete TNM staging.

Immunohistochemical Methods. Sections (4 μm) were cut from paraffin-embedded tissues and mounted on positively charged Superfrost slides (Fisher Scientific, Chicago, IL). Tissues were deparaffinized, rehydrated through graded alcohols, and then blocked for endogenous peroxidase and avidin/biotin in 3% hydrogen peroxide in methanol and avidin/biotin blocking kits (Vector Laboratories, Inc., Burlingame, CA), respectively. All tissues were preblocked in Tris-buffered saline containing 0.3% Triton, 0.2% saponin, and 0.5% blocking agent (NEN Life Sciences; Boston, MA) and incubated in primary antibody overnight at 4°C. Antisera specific for COX-1 (Cayman Chemical Co., Ann Arbor, MI) and COX-2 (Oxford Biomedical Research, Inc., Oxford, MI) were diluted 1:250 in Tris-buffered saline containing 0.3% Triton, 0.2% saponin, and 0.5% blocking agent. Immunoreactive complexes were detected using tyramide signal amplification (TSA-indirect; NEN Life Sciences) and visualized with the peroxidase substrate AEC (Zymed Laboratories, San Francisco, CA). Slides were counterstained briefly in hematoxylin–1 (Richard-Allan Scientific, Kalamazoo, MI), mounted in crystal mount, and covered with 50:50 xylene/permount (Fisher Scientific). Control slides were treated with either no primary antibody or with isotype-matched IgG serum. Specificity for COX-2 was confirmed by preincubating COX-2 antibody with 100× human recombinant COX-2 protein prior to addition to slides.

All slides were reviewed independently by two pathologists (D. Bostwick and N. Khan). The intensity of COX-1 and COX-2 immunostaining was graded on a scale of 0–3 where 0 = no staining, 1 = equivocal staining, 2 = moderate to intense staining, and 3 = highest intensity staining. The proportions of epithelial cells with COX-1 and COX-2 expression in TCC, CIS, and adjacent “normal” tissue were expressed as the percentage of epithelial cells with positive staining.

Western Blotting. Frozen tissue samples were immersed in ice-cold lysis buffer containing 150 mM NaCl, 100 mM Tris-buffered saline (pH 8), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Tissues were homogenized on ice for 2 min and centrifuged at 10,000 × g for 10 min at 4°C. The supernatants were removed, and the protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The protein (20 μg per lane) was loaded and separated by SDS-PAGE under reducing conditions and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The transfer of protein and equal loading in all lanes were verified using reversible staining with Ponceau S (Sigma Chemical Co., St. Louis, MO). Membranes were blocked using gelatin from cold-water fish (Sigma). Blots were incubated with the same COX-1- and COX-2-specific antisera used for immunohistochemistry at a dilution of 1:1000 overnight at 4°C. This was followed by application of sheep antimouse or goat antirabbit secondary antibody conjugated to horseradish peroxidase (Dako, Carpinteria, CA). COX-1 or COX-2 protein was detected by chemiluminescence using exposure to Kodak BioMax MR film (Rochester, NY). Positive controls included

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The abbreviations used are: TCC, transitional cell carcinoma; COX-1 and -2, cyclooxygenase-1 and -2; CIS, carcinoma in situ.

COX-1 and COX-2 electrophoresis standards (Cayman Chemical Co., Ann Arbor, MI) and protein from stimulated human peripheral blood monocytes.

**Results and Discussion**

Forty tissue samples from patients with urinary bladder cancer and 10 tissue samples from patients without urinary bladder disease were examined by immunohistochemistry. Patient and tumor characteristics are summarized in Table 1. In several slides, areas of invasive TCC, CIS, and adjacent morphologically normal epithelial tissue were present on the same slide. Positive immunoreactivity to COX-2 was noted in 25 of 29 (86%) invasive TCC samples, 7 of 9 (78%) noninvasive TCC samples, and in 6 of 8 (75%) CIS samples (Table 2 and Fig. 1). Epithelial cells that were positive for COX-2 immunostaining had granular eosinophilic staining of the cytoplasm (Fig. 1). Nuclear staining was not observed. In CIS, the COX-2 staining was generally localized to the apical cytoplasm (Fig. 1B). There was noticeable heterogeneity in the percentage of COX-2-positive cells within the tumor tissues, ranging from <10 to 90% (Table 2). In a few cases of invasive TCC, COX-2 immunoreactivity was detected in endothelial cells within the tumor. Low intensity staining was observed in smooth muscle tissue in some sections. COX-2 was also detected in 9 of 17 samples (53%) of morphologically normal epithelium adjacent to TCC (Fig. 1C). Immunoreactivity to COX-2 antibody was not detected in epithelial cells in any normal urinary bladder tissues studied.

All isotype control slides for COX-2 (rabbit IgG) were negative. Preincubating the COX-2 antisera with 100× human recombinant COX-2 dramatically reduced (90–95%) COX-2 immunoreactivity. The staining of COX-2 in these slides was either very weak (<1+) or was completely absent, confirming the specificity for COX-2.

Using the described immunohistochemical methods, we detected COX-1 expression less frequently in tumors (data not shown). Western blot analyses performed on six representative TCC samples and epithelium adjacent to the TCC showed COX-1 protein in both the tumors and normal adjacent tissues in all samples (Fig. 2).

COX-2 was expressed in the majority of invasive TCC and CIS samples but was absent in the epithelium of all normal urinary bladders. These findings support the further study of COX-2 as a potential target for therapy of invasive bladder cancer. The presence of COX-2 in CIS samples is important because CIS is recognized as a precursor of invasive TCC (10). This observation implies that COX-2 may play an early role in bladder cancer development. The heterogeneity, however, in regard to the percentage of positive cells in positive tumors should be noted.

COX-2 immunoreactivity was noted in approximately half of the sections of morphologically normal epithelium adjacent to the TCC. This may be a manifestation of the "field" effect (11). The field effect is a theory that exposure to carcinogens in urine results in biochemical changes and tumor initiation throughout the entire bladder epithelium. Epithelial cells away from the tumor may look morphologically normal but may have acquired mutations and biochemical changes similar to those in the cancer cells. Alternatively, increased COX-2 in adjacent normal epithelium may indicate neoplastic cells exerting a paracrine effect through the release of cytokines and/or growth factors.

This and previous studies (7–9, 12) have characterized COX-2 expression in multiple epithelial malignancies. The functional role of COX-2 in these cancers, however, remains to be completely defined. COX-1 and COX-2 convert arachidonic acid to prostaglandins and thromboxanes. Prostaglandin E2 is produced by many forms of cancer and has been associated with increased cancer cell proliferation (13, 14), resistance to apoptosis (13), host immunosuppression (15), and tumor angiogenesis (16). Although the functional role of COX-2 in cancer is not defined, drugs that inhibit COX (both COX-1 and COX-2) have had chemopreventative and antitumor effects in epidemiological studies in humans (17), experimental studies in laboratory animals (4), and clinical studies in pet dogs (2, 5) and humans (18). Inhibition of COX is likely involved in the antitumor effects of these

<table>
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<tr>
<th>Type of bladder cancer:</th>
<th>No. of patients</th>
<th>Positive epithelial cells in positive tumor</th>
<th>No. of patients with stain intensity</th>
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<tr>
<td>Invasive TCC</td>
<td>29</td>
<td>5–10, 11–40, 41–70, 71–100</td>
<td>1+</td>
</tr>
<tr>
<td>Noninvasive TCC</td>
<td>9</td>
<td>5–10, 11–40, 41–70, 71–100</td>
<td>1+</td>
</tr>
<tr>
<td>CIS</td>
<td>8</td>
<td>5–10, 11–40, 41–70, 71–100</td>
<td>1+</td>
</tr>
<tr>
<td>Normal cells adjacent to tumor</td>
<td>17</td>
<td>5–10, 11–40, 41–70, 71–100</td>
<td>1+</td>
</tr>
<tr>
<td>Normal bladder</td>
<td>4</td>
<td>5–10, 11–40, 41–70, 71–100</td>
<td>1+</td>
</tr>
</tbody>
</table>

Table 1 Patient and tumor characteristics

<table>
<thead>
<tr>
<th>Type sample evaluated</th>
<th>No.</th>
<th>No. positive samples</th>
<th>Positive epithelial cells in positive tumor</th>
<th>No. of patients with stain intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive TCC</td>
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<td>25</td>
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<tr>
<td>Noninvasive TCC</td>
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<td>7</td>
<td>5–10, 11–40, 41–70, 71–100</td>
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<tr>
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<tr>
<td>Normal bladder</td>
<td>4</td>
<td>0</td>
<td>5–10, 11–40, 41–70, 71–100</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2 Results of COX-2 immunohistochemistry
Specific COX-2 inhibitors may have antitumor activity with less toxicity than drugs that inhibit both COX-1 and COX-2 (6). Although reports of COX-2 inhibitors in bladder cancer are limited, the COX-2 inhibitor, nimesulide, had chemopreventive effects in a rodent model of superficial bladder cancer (19). Another animal model suited to the study of COX-2 inhibitors in bladder cancer is spontaneous canine invasive bladder cancer. Canine TCC has great similarity to human invasive TCC in COX-2 expression, histopathological characteristics, biological behavior including metastasis, and response to chemotherapy (3).4

Fig. 1. COX-2 immunohistochemistry of human invasive TCC (A), CIS (B), epithelium adjacent to the TCC (C), and normal human urinary bladder epithelium (D). COX-2 is expressed (note eosinophilic staining) in the invasive TCC (A), CIS (B), and epithelium adjacent to the tumor (C). COX-2 is not expressed in the normal urinary bladder (D). Bar = 40 μm.
Taken together, these results suggest COX-2 may play a role in bladder cancer development and/or progression and imply that COX-2 inhibitors may be investigated for the prevention or treatment of human invasive bladder cancer. Prior to the launch of human clinical trials, studies to evaluate the efficacy of COX-2 inhibitors in relevant animal models of invasive bladder cancer are needed. In addition, studies to define the functional role of COX-2 in bladder cancer and the mechanisms by which COX-2 inhibitors exert antitumor activity are essential.

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References
