Enhanced Expression of Cyclooxygenase-2 in High Grade Human Transitional Cell Bladder Carcinomas

Martin Kömhoff,* Youfei Guan,* Heidi W. Shappell,† Linda Davis,* Greg Jack,§ Yu Shyr,‡ Michael O. Koch,§ Scott B. Shappell,¶ and Matthew D. Breyer*¶

From the Division of Nephrology,* Department of Medicine, and the Departments of Pathology,† Preventive Medicine,‡ and Urological Surgery,§ Vanderbilt University, Nashville; the Vanderbilt-Ingram Cancer Center,¶ Nashville; and the Nashville VA Medical Center,¶ Nashville, Tennessee

Studies in human and animal models have shown that cyclooxygenase (COX)-2 is up-regulated in several epithelial carcinomas including colon, breast, and lung. To elucidate the possible involvement of COX-2 in human bladder cancer we examined the expression of COX isoforms in benign tissue and in bladder carcinoma specimens. Paraffin embedded tissues from 75 patients with urothelial carcinomas were immunostained with specific antibodies raised against COX-1 and COX-2. COX-1 expression was detected in smooth muscle cells in both benign and malignant bladders. COX-2 immunoreactivity was absent in benign tissue and in specimens with low-grade urothelial carcinoma (0/23). In contrast, expression of COX-2 was detected in malignant epithelial cells in 38% (17/47) of specimens with high-grade urothelial carcinoma. Expression of COX-2 in high-grade bladder cancer was confirmed by radioactive in situ hybridization using a COX-2-selective riboprobe. Both immunohistochemistry and in situ hybridization showed COX-2 expression in a small subset of malignant cells. COX-2 mRNA was also expressed in three out of seven malignant urothelial cell lines. These data demonstrate elevated expression of COX-2 in a high percentage of high-grade bladder carcinomas, suggesting a possible role of COX-2 in the progression of bladder urothelial carcinoma and supporting its potential as a therapeutic target in human bladder carcinoma.

Materials and Methods

Case Selection and Histopathology

Cases were retrieved from the surgical pathology files of the Department of Pathology, Vanderbilt University Medical Center. Seventy-five separate tissue specimens from 69 patients (24 females and 45 males) were analyzed. Cases were selected to achieve a representative mixture of tumor grades and stages and included 29 transurethral resection biopsy specimens and 42 radical cystectomy specimens. All tissues were formalin-fixed and paraffin-embedded using standard conditions. In addition to bladder cancer sections with benign urothelium and urothelial carcinomas, one lymph node with metastatic high-grade urothelial carcinoma, one cystectomy with squamous cell carcinoma, one cystectomy with an intestinal type adenocarcinoma, one renal pelvis urothelial carcinoma, and two ureter urothelial carcinomas were also examined. In addition to review of pathology reports, slides from all cases were re-examined for uniform assignment of grade and stage and other histopathological features. Tumor histological grading was performed according to both the most widely used three-tiered (Grade 1 to 3) WHO scheme for TCC,16 and the recently recommended WHO/International Society of Urological Pathology revised two-tiered (low- and high-grade) scheme for urothelial carcinoma.17 Tumor staging was performed according to the American Joint Commission for Cancer-Union Internationale contre le Cancer (AJCC-UICC) classification.18 Approval by the local ethics committee was obtained.

Immunostaining

Sections were cut at 7 μm thickness, deparaffinized in xylene, and incubated for 30 minutes in methanol containing 0.3% H2O2 to block endogenous peroxidase activity. Primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); goat polyclonal anti-human COX-1: C-20, sc#1752 and COX-2: C-20, sc#1745 antibodies) and Cayman Chemical Company (Ann Arbor, MI; rabbit polyclonal anti-mouse COX-2 160106). All samples were studied with the Santa Cruz antibody (sc#1745) and immunoreactivity was confirmed in some sections with the Cayman antibody (160106). For detection of COX-2-immunoreactive protein, sections were microwaved for 3 minutes in phosphate buffered saline containing 0.1 mol/L sodium citrate, pH 6.0. Polyclonal anti COX-1 and anti COX-2 antibodies were diluted 1:200 in TBST (50 mmol/L Tris, pH 7.5, 300 mmol/L NaCl, and 0.05% Tween 20) containing 1% bovine serum albumin, 5% normal horse serum, and 1% nonfat dry milk. Immunolabeling was detected using a biotinylated rabbit anti-goat antibody followed by visualization with an avidin-biotin horseradish peroxidase labeling kit (Vectastain ABC kit) and diaminobenzidine staining. The specificity of the antibodies was demonstrated in the following control experiments: (i) omission of the primary antibodies resulted in unstained sections; (ii) in normal human kidney, the COX-1 antibody but not the COX-2 antibody strongly reacted with medullary collecting duct cells, which exclusively express COX-1 protein,19; (iii) double immunofluorescence using the goat and rabbit polyclonal anti-COX-2 antibodies revealed colocalization; (iv) pre-incubation of the goat polyclonal anti-COX2 antibody with the peptide to which it was generated, blocked immunostaining; and (v) the COX-2 ir-protein colocalized with mRNA expression by in situ hybridization.

In Situ Hybridization

In situ hybridization was performed as previously described.20 Briefly, before hybridization, tissue sections were deparaffinized, rehydrated in paraformaldehyde, treated with proteinase K (20 μg/ml), washed with phosphate-buffered saline, rehydrated in 4% paraformaldehyde, and treated with triethanolamine plus acetic anhydride (0.25% v/v). Finally, sections were dehydrated with 100% ethanol. 35S-labeled antisense and sense riboprobes synthesized from the COX-2-specific 3’ untranslated region of the human COX-2 cDNA (471 bp) were hybridized to the section at 55°C for 18 hours. After hybridization, the sections were washed at 65°C once in 5× SSC plus 10 mmol/L β-mercaptoethanol (BME), once in 50% formamide, 2× SSC, and 100 mmol/L BME for 30 minutes. After additional washes in 10 mmol/L Tris, 5 mmol/L EDTA, 500 mmol/L sodium chloride (TEN) at 37°C, the sections were treated with RNase A (10 μg/ml) at 37°C for 30 minutes, followed by another wash in TEN at 37°C. Sections were then washed twice in 2× SSC, and twice in 0.1× SSC at 65°C. Slides were dehydrated with graded ethanol containing 300 mmol/L ammonium acetate. Slides were then dipped in photoemulsion (Ilford K5, Knutsford, Cheshire, UK) diluted 1:1 with 2% glycerol and exposed for 4 to 5 days at 4°C. After developing in Kodak D-19, slides were counterstained with hematoxylin. Photomicrographs were viewed with a Zeiss Axioskop microscope using either bright- or dark-field optics (Micro Video Instruments, Avon, MA). Pictures were captured with a digital camera (Spot-Cam, Diagnostic Instruments, Sterling Heights, MI) and color composites were generated using Adobe Photoshop v4.0 on a Power Macintosh.

Cell Lines and RNase Protection Assay

Human bladder cancer cell lines T24, HT1376, RT4, HT1197, HUC-SV1/2, and 5637 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in the recommended media. Cells were harvested at confluence, RNA isolated by a modified chloroform/methanol extraction, and RNase protection assay for COX-2 performed as described previously.21

Statistical Analysis

Statistical analysis was performed using Fisher’s exact test. P values <0.05 were considered significant.
Table 1. Summary of Results

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<th>Case characteristics</th>
<th>COX-2-neg.</th>
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<td>T1–T4</td>
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<td>18</td>
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TCC, transitional cell carcinoma; squam, squamous cell; gland, glandular; Adeno, adenocarcinoma.

**Results**

**Immunohistochemistry**

The expression pattern of COX-2 immunoreactive protein in bladder carcinomas is summarized in Table 1. Patient age ranged from 36 to 96 years; median age was 63.49 ± 12.93 years. There was no correlation between COX-2 expression and either patient gender or age. Results were similar whether tissue was obtained by transurethral bladder biopsy or cystectomy. There was a significant correlation between COX-2 immunoreactivity and tumor grade. COX-2 was detected in no grade I bladder cancers (0/12) and only 1 out of 18 samples with grade 2 cancers exhibited COX-2-immunoreactive protein in malignant epithelial cells. In contrast, 38% of samples with grade 3 tumors exhibited COX-2 immunoreactivity with COX-2 observed predominantly in malignant cells. Results were also analyzed using the recently revised two-tiered scheme for grading urothelial carcinoma. By this analysis, COX-2 immunoreactive protein was not observed in any of the 23 cases with low-grade histology. In contrast, 38% (17/45) of high-grade samples were positive for expression of COX-2 immunoreactive protein in malignant epithelial cells. In addition to the association of COX-2 expression in high-grade bladder urothelial cancers including stomach, colon, lung, breast, and pancre-

Expression of COX-2 mRNA in human urothelial cell lines was examined by nuclease protection assays (Figure 3). Constitutive expression was observed in three out of five urothelial cancer cell lines, including T24, 5637, and HT11376 cell lines. COX-2 mRNA was not detected in RT4 or HT1197 cells. Nor was COX-2 mRNA detected in SV-40 transformed human urothelial cells (HUC-SV1 and HUC-SV2 cells).

**Discussion**

Recent studies have demonstrated COX-2 expression is aberrantly increased in several types of epithelial cancers including stomach, colon, lung, breast, and pancre-
Figure 1. A–D: Expression of COX-2 mRNA and immunoreactive-protein in malignant transitional cells in two patients (A and B, C and D) with grade III/high-grade histology. A and B: 1000 bright-field and dark-field illumination of involved human bladder. A: COX-2 immunostaining showing brown reaction product, indicating COX-2-immunoreactive protein seen predominantly over infiltrating malignant transitional cells. No significant immunoreactivity is detected in surrounding stromal tissue. B: in situ hybridization using a COX-2-selective riboprobe. Black grains depict hybridization over submucosal infiltrating malignant transitional cells. Note that distribution of the COX-2 mRNA hybridization signal in B is almost superimposable over the COX-2 immunoreactivity shown in A. C and D: 2000 bright-field illumination of involved human bladder cancer from a patient with grade III/high-grade TCC. C: COX-2-immunoreactive protein is seen within islands of malignant epithelial cells. Surrounding stromal cells are COX-2-negative. D: Similar to the tissue section A and B, in situ hybridization shows COX-2 mRNA in cells that are COX-2 ir-protein-positive.
The significance of COX-2 expression in these cancers has been most thoroughly studied in colon cancer, where increased COX-2 expression has been correlated with reduced patient survival. Furthermore, ingestion of COX-2-inhibiting NSAIDs is associated with reduced risk of de novo colon cancer, suggesting COX-2 plays a role in promoting colon cancer. The present study demonstrates that COX-2 expression is also increased in human bladder TCC and that this expression correlates with tumor grade and invasion.

Serial sections from several specimens demonstrate by in situ hybridization that COX-2 mRNA and COX-2 immunostaining colocalized. This independent confirmation of COX-2 distribution at both mRNA and protein levels is significant, because cross-reactivity of anti COX-2-antibodies with COX-1 has been reported. Furthermore, immunostaining with an anti-COX-1 antibody yielded a distinctly different expression pattern (ie, in smooth muscle) also supporting the specificity of the COX-2 immunostain. The expression of COX-2 immunoreactivity in human TCC was predominantly in subpopulations of infiltrating malignant cells. The heterogeneity of cellular COX-2 expression in TCC suggests significant differences in cells comprising these tumors. Interestingly, heterogeneity of COX-2 expression was also observed in cultured TCC cell lines, with only three of seven exhibiting detectable COX-2.

The significance of COX-2 expression in human bladder cancers is uncertain. Administration of both nonselective COX-1/2-inhibiting NSAIDs and a COX-2-selective inhibitor reduced the incidence of bladder cancer in chemical carcinogenesis models in rodents. These findings suggest that COX-2 activity may promote carcinogenesis. Studies in colon cancer also support a role for COX-2 as a tumor promoter. COX-2 knockout mice bearing both mutations in the familial adenomatous polyposis coli (APC) gene product and COX-2 knockout mice exhibit a profound reduction in spontaneous poly development compared to their COX-2 knockout littermates. In vitro studies suggest COX-2 overexpression may favor the growth of malignant epithelial cells by reducing apoptosis, increasing cell adhesion, and promoting angiogenesis. Finally, growth of COX-2 expressing human colon cancer xenografts in nude mice is markedly reduced by a COX-2 inhibitor. Whether COX-2 expression also promotes transitional cell cancer in humans remains to be clarified. The expression of COX-2 in bladder cancer cell lines documented herein suggests that these cells may provide useful tools for investigating the contribution of COX-2 to bladder cancer growth in experimental models.

During the preparation of this manuscript Mohammed et al reported the presence of COX-2 in human bladder TCCs. Those studies examined 40 cases and demonstrated COX-2 immunostaining in a high percentage of invasive carcinomas. They documented COX-2 expression exclusively by immunohistochemistry and did not report a correlation with tumor stage or grade. We have now confirmed the expression of COX-2 in TCCs docu-
menting its expression by using both in situ hybridization and immunohistochemistry.

Furthermore, the present findings suggest that COX-2 expression correlates with tumor stage and grade. We failed to detect COX-2 in any of the 23 low-grade carcinomas studied by immunostain. We also observed a significant difference in the COX-2 expression bladder TCCs without invasion of the lamina propria (TA) versus invasive cancers (T1–4). Although Mohammed et al did not observe COX-2 in normal bladders, they did detect COX-2 immunoreactivity in 7 of 9 low-grade tumors.29 The differences between these two reports could be due in part to differences in sensitivity of immunostaining technique, given the higher percentage of COX-2-expressing tumor cells noted by those investigators. Nonetheless, the percentage of COX-2-positive cells observed in our studies was similar by both immunostaining and in situ hybridization, and by the present technique allows discrimination between low- and high-grade cancers.

The observed immunostaining of high-grade tumors including carcinoma in situ reported by Mohammed et al corresponds with our observations of increased COX-2 expression in high-grade tumors and suggests that increased COX-2 could be an early event in at least some biologically and cytologically high-grade bladder tumor processes. Conversely, the restriction of COX-2 expression to a subset of tumor cells in high-grade tumors, as well as its decreased expression in low-grade carcinomas, suggests that COX-2 up-regulation could also occur at later stages of tumor progression from low- to high-grade tumors. These considerations may have relevance to the potential for treating bladder carcinoma with COX-2-selective inhibitors. A population-based study of patients ingesting aspirin failed to reveal a reduced incidence of bladder cancer in humans, despite its beneficial effect on colon cancer.12,30 Because aspirin is a relatively poor COX-2 inhibitor,31 the possibility that more potent COX-2 inhibitors might provide an effective antitumor therapy for high-grade bladder cancers remains open.

In summary, we have shown that COX-2 is expressed in human TCCs and, for the first time, report that its expression correlates with tumor grade. The availability of selective COX-2 inhibitors, which have been successfully used in animal models of cancer,3 and the poor prognosis of high-grade human urothelial carcinoma2 warrant further investigation to determine the role of COX-2 in human urothelial carcinoma.

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References


