Transitional Cell Hyperplasia and Carcinomas in Urinary Bladders of Transgenic Mice with Keratin 5 Promoter-Driven Cyclooxygenase-2 Overexpression

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Abstract
The inducible form of cyclooxygenase (COX), COX-2, is up-regulated in many epithelial cancers and its prostaglandin products increase proliferation, enhance angiogenesis, and inhibit apoptosis in several tissues. Pharmacologic inhibition and genetic deletion studies showed a marked reduction of tumor development in colon and skin. COX-2 has also been strongly implicated in urinary bladder cancer primarily by studies with nonselective COX- and COX-2-selective inhibitors. We now show that forced expression of COX-2, under the control of a keratin 5 promoter, is sufficient to cause transitional cell hyperplasia (TCH) in 17% and 75% of the heterozygous and homozygous transgenic lines, respectively, in an age-dependent manner. TCH was strongly associated with inflammation, primarily nodules of B lymphocytes; some T cells and macrophage infiltration were also observed. Additionally, transitional cell carcinoma was observed in ~10% of the K5.COX-2 transgenic mice; no TCH or transitional cell carcinoma was observed in wild-type bladders. Immunohistochemistry for vascular proliferation and vascular endothelial growth factor showed significant increases above that in wild-type urinary bladders. Our results suggest that overexpression of COX-2 is sufficient to cause hyperplasia and carcinomas in the urinary bladder. Therefore, inhibition of COX-2 should continue to be pursued as a potential chemopreventive and therapeutic strategy. (Cancer Res 2005; 65(5): 1808-13)

Introduction
The prostaglandin G/H synthases, cyclooxygenase-1 (COX-1) and COX-2, are key enzymes in the synthesis of prostaglandins from arachidonic acid. COX-1 is expressed in nearly all tissues and is thought to be responsible for the low levels of prostaglandins needed for tissue maintenance and homeostasis. COX-2 is not expressed in most tissues under normal conditions, but expression is rapidly induced by growth factors or agents that cause tissue irritation or inflammation (1). COX-2 is expressed in many cancers (2, 3), and prostaglandins have been reported to increase proliferation, enhance angiogenesis, promote invasion, and inhibit apoptosis and differentiation. Genetic deletion or chemical inhibition of COX-2 in mice results in a decrease in several types of tumors (4–7). Transgenic overexpression of COX-2 in mice results in the spontaneous development of mammary tumors in multiparous mice (8), induces proliferative epithelial lesions in virgin mammary glands (9), and enhances dimethylbenz(a)anthracene-induced skin carcinogenesis (10).

There is strong evidence that COX-2 expression and activity is important in the development of urinary bladder cancers. COX-2 is up-regulated in invasive and noninvasive transitional cell carcinoma (TCC) in the human urinary bladder (11–13), and the level of COX-2 expression is positively associated with increasing stage and grade of TCC (14–16). Elevated COX-2 expression has also been reported in rat and canine models of bladder cancer (17, 18), and COX inhibitors reduce tumor growth and incidence in these models (19, 20). Collectively, the results from expression studies in humans and preclinical studies in animals support the hypothesis that COX-2 plays an important role in TCC development and progression (21).

Although compelling, the data on the involvement of COX-2 and prostaglandin synthesis in bladder cancer development has been primarily correlative in nature. To determine if COX-2 overexpression could be causally related to cancer development in these tissues, we utilized a transgenic mouse line (K5.COX-2 mice) that expresses a COX-2 transgene under control of a keratin 5 promoter (22). The COX-2 transgene of K5.COX-2 mice is expressed in the basal epithelial cells of skin and in other tissues with simple and stratified epithelium, including the urinary bladder. We analyzed urinary bladder tissues from two high expressing K5.COX-2 lines for evidence of spontaneous pathologic changes. We report that COX-2 overexpression in basal epithelial cells of K5.COX-2 mice is sufficient to cause transitional cell hyperplasia (TCH) and TCC of the urinary bladder. Furthermore, we provide evidence that COX-2 expression in the urinary bladder is proinflammatory and enhances expression of vascular endothelial growth factor (VEGF) and vascular proliferation.

Materials and Methods
Materials. Purified rat-monoconal anti-CD3, anti-CD45R, and anti-mouse F4/80 were from Serotec (Raleigh, NC); biotinylated rabbit-anti-rat IgG purified rat-monoconal anti-mouse Ki-67 were from DakoCytomation (Carpinteria, CA); rabbit polyclonal anti-cytokeratin-5 was from Covance/BabCo (Princeton, NJ); goat polyclonal anti-VEGF was from Santa Cruz Biotechnology; mouse anti- Factor VIII was from DakoCytomation; mouse anti-COX-2 and the prostaglandin E2 (PGE2) immunoassay kit were from

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Caygenex (San Ramon, CA); alkaline phosphatase–conjugated anti-rabbit IgG was from Dianova (Hamburg, Germany); and biotinylated goat-anti-rabbit IgG, biotinylated rabbit-anti-rat IgG, and avidin-biotin complex–horseradish peroxidase were from Vector (Burlingame, CA).

Transgenic Mouse Lines. Wild-type NMRI mice (outbred strain from RCC, Füllinsdorf, Switzerland) and K5.COX-2 transgenic lines 667/+ and 675/+-, generated as described (22), were kept in Deutsches Krebsforschungszentrum (Heidelberg, Germany) under an artificial day/night rhythm and fed Altromin standard food pellets and water ad libitum. All animal experiments were approved by the Governmental Committee for Animal Experimentation (License 053/00).

Tissue Preparation and Histologic Analysis. The urogenital tracts, including the bladder and all prostatic lobes, were excised and fixed in PBS-buffered 10% paraformaldehyde. The fixed urogenital tracts were then bisected longitudinally through the bladder and urethra and positioned with the cut faces oriented in the same direction for paraffin embedding. Five-micrometer sections were stained with H&E by standard protocols. These sections included a cross section of the entire bladder and at least one section per mouse was analyzed for histopathology by a veterinary pathologist. Immunohistochemical staining was done using modified avidin-biotin complex techniques. Deparaffinized and hydrated sections were blocked with 3% hydrogen peroxide and, with the exception of the sections to be stained for VEGF and Factor VIII, were subjected to microwave antigen retrieval in 10 mmol/L citrate buffer (pH 6.0) for 10 to 15 minutes. Sections were blocked using 10% species-appropriate serum and incubated with primary antibody, Primary antibody dilutions and treatments were as follows: Ki-67, 1:200, overnight at 4°C; keratin 5, 1:500, 1 hour at room temperature (RT); CD3, 1:100, 2 hours at RT; CD45R, 1:2,000, 1 hour at RT; F4/80, 1:25, 2 hours at RT; COX-2, 1:2,000 overnight at 4°C; VEGF, 1:75, incubated 30 minutes at RT and then overnight at 4°C; Factor VIII, 1:500, incubated 30 minutes at RT. Species-appropriate biotinylated secondary antibodies were used at 1:200 for 2 hours at RT followed by avidin-biotin complex–horseradish peroxidase or streptavidin–horseradish peroxidase treatment for 30 minutes at RT. Slides were developed with 3,3′-diaminobenzidine for ~5 minutes. Ki-67-positive cell counts were divided by bladder epithelium circumference measured using calibrated Spot V4.0.5 imaging software (Diagnostic Instruments, Sterling Heights, MI). Immunoblot Analysis. Immunoprecipitation of COX-2 and COX-1 and subsequent immunoblot analysis were carried out by using COX isozyme–specific antisera as described (10).

5-Fluorouracil Measurements. Endogenous PGE2 levels in bladder tissue were determined by enzyme immunoassay as previously described (10). Briefly, bladder samples were pulverized and suspended in 2 mL of ice-cold ethanol. After centrifugation, the supernatant was collected and the dried lipids were then analyzed according to Cayman Chemical (Ann Arbor, MI). Streptavidin–horseradish peroxidase was from Biogenex (San Ramon, CA); alkaline phosphatase–conjugated anti-rabbit IgG was from Dianova (Hamburg, Germany); and biotinylated goat-anti-rabbit IgG, biotinylated rabbit-anti-rat IgG, and avidin–biotin complex–horseradish peroxidase were from Vector (Burlingame, CA).

Results

Transgenic Expression of COX-2 in the Urinary Bladders of K5.COX-2 Mice. The expression of COX-2 in K5.COX-2 mice is driven by the bovine cytokeratin 5 promoter in the basal cells of simple and stratified epithelia in a variety of tissues including the urinary bladder (9, 10). COX-2 expression in urogenital tissues of K5.COX-2 transgenic mice was confirmed by immunohistochemistry on tissues from homozygous line 675 and hemizygous line 667. As can be seen in Fig. 1A, little or no COX-2 protein is expressed in bladder tissue of wild-type NMRI mice. However, intense staining of COX-2 protein was observed in bladder epithelium from K5.COX-2 mice of both transgenic lines (Fig. 1B and data not shown). In hyperplastic regions, the expression of COX-2 extends into the suprabasal cell layers, similar to the expression of keratin 5 (data not shown). Analysis of bladder tissue for COX-2 expression by immunoblot analysis confirmed that little or no COX-2 protein is present in wild-type bladder tissue, but K5.COX-2 bladders express high levels of COX-2 protein (Fig. 1C). COX-1 steady state levels were similar in wild-type and transgenic bladders (Fig. 1C). The elevated expression of COX-2 resulted in an ~5-fold increase in PGE2 contents in the bladders of K5.COX-2 transgenics compared with wild-type mice (Fig. 1D).

Inflammation, Hyperplasia, and Carcinoma in K5.COX-2 Urinary Bladders. A histopathologic analysis was conducted on urinary bladders from the two K5.COX-2 transgenic lines and age-matched wild-type controls. H&E-stained sections from a total of 67 urinary bladders from 3- to 16-month-old male mice were examined. From the results of this analysis, it is clear that overexpression of COX-2 in the epithelial cells of the urinary bladder is associated with the development of inflammation, TCH, and TCC (Fig. 2). Interestingly, the initial lesion in K5.COX-2 urinary bladders seemed to be inflammation consisting of perivascular infiltrates of lymphocytes and plasma cells. By 6 months of age, there were nodules of lymphoid proliferation that were more pronounced between 10 and 16 months of age (Fig. 2B and C). The incidence and severity of the perivascular infiltrates and lymphoid nodular proliferation were increased in the homozygous founder line 675 compared with hemizygous line 667. Characterization of these lymphoid nodules by immunohistochemistry revealed that they are primarily composed of B lymphocytes. Most of the cells within the nodules are positive for the B-cell marker, CD45R (Fig. 3A). Few cells within these nodules stained for the T-cell marker CD-3 (Fig. 3B), whereas F4 staining...
indicated a few areas of macrophage infiltration outside of the lymphoid nodules (Fig. 3C).

The earliest TCH observed was at 6 months, but incidence and severity of these lesions increased with age. TCH occurred in 17% and 75% of the heterozygous and homozygous K5.COX-2 mice, respectively, in animals 10 months of age or older (Table 1).

Both papillary and nodular TCH was observed often within the same section. TCC occurred with an incidence of 8% and 10% in heterozygous and homozygous K5.COXR-2 transgenic founder lines, respectively. The time to tumor formation seemed to be ~12 to 16 months as no animals younger than 12 months presented with TCC. The TCC tumors in K5.COXR-2 mice were anaplastic, had numerous mitotic cells, and had invaded the muscle wall of the urinary bladder (Fig. 2D). No TCH or TCC lesions were observed in any of the urinary bladders from wild-type NMRI mice.

**Increased Proliferation Associated with TCH of the Urinary Bladder.** Tissue hyperplasia can arise from an increase in cellular proliferation and a decrease in apoptosis. In normal bladders from wild-type mice, we observed very few apoptotic cells as determined by terminal deoxynucleotidyl transferase–mediated nick end labeling. There was no apparent reduction in apoptosis in hyperplastic K5.COX-2 urinary bladder tissue (data not shown).

To determine if there was an increase in proliferating epithelial

Figure 2. Expression of the COX-2 transgene leads to TCH and TCC in the bladders of K5.COXR-2 transgenic mice. Bladders from wild-type (A) and K5.COXR-2 (B-D) mice were stained with H&E. Note the thin epithelium (black arrow) in wild-type bladders in A (×40). Papillary TCH denoted by extensive papillary extensions into the bladder lumen (B, ×40) and thickened hyperplastic epithelium (black arrows, B and C, ×200) in a K5.COXR-2 bladder. Note also the nests of darkly stained inflammatory cells (white arrowheads, B). D, invasive TCC in a K5.COXR-2 bladder (×200); darkly stained tumor cells can be seen invading through the muscle (black arrowhead).

Figure 3. Inflammatory cells in K5.COXR-2 bladders are predominately B-cell lymphocytes. A, bladders from K5.COXR-2 mice were stained with anti-CD45R to test for the presence of B lymphocytes. Most of the cells in the large nests of inflammatory cells stained positive for CD45R antigen (arrows), indicating the presence of large numbers of B lymphocytes. B, staining with anti-CD3 to detect the presence of T-lymphocytes revealed the presence of small numbers of these cells within the inflammatory cell nests (arrow). C, bladders were also stained with anti-F4 to detect the presence of macrophages. Macrophages seemed sporadically in small numbers throughout the stroma and epithelium, and less frequently as patches of multiple cells (arrow).
cells in hyperplastic urinary bladders from K5.COX-2 transgenic mice compared with wild-type controls, a subset of tissue sections were analyzed for expression of the proliferation marker Ki-67 (Fig. 4). Sections of bladders from six wild-type mice, six K5.COX-2 mice with nonhyperplastic urinary bladder tissue, and six K5.COX-2 mice with bladders exhibiting TCH were analyzed. In nonhyperplastic urinary bladder tissues (Fig. 4A), very few Ki-67 positively stained epithelial cells were observed (mean, 0.24 cells/mm; 95% confidence interval, 0.08-0.40). There was no increase in Ki-67-positive epithelial cells in nonhyperplastic K5.COX-2 urinary bladders compared with wild-type bladders. In urinary bladder tissues with TCH (Fig. 4B), there was an ~40-fold increase in the number of Ki-67-positive staining epithelial cells (mean, 8.0 cells/mm; 95% confidence interval, 3.8-12.2) compared with nonhyperplastic tissues. These results indicate that increased proliferation is the primary mechanism by which COX-2 expression is inducing hyperplasia in the mouse urinary bladder.

Vascular Proliferation and VEGF Expression in K5.COX-2 Urinary Bladders. In mice 10 to 16 months of age, vascular proliferation within the lamina propria (stroma) was observed in ~50% of urinary bladders from K5.COX-2 line 667 and 75% of urinary bladders from line 675. In contrast, only one urinary bladder section from a wild-type mouse (9% of total) had any evidence of vascular proliferation. The presence of vascular proliferation was strongly associated with TCH as 16 of 17 urinary bladders with TCH also had vascular proliferation. Only 6 of 25 bladders without TCH had evidence of vascular proliferation. Immunohistochemical staining of urinary bladder tissue for Factor VIII showed the extent of vascular proliferation in hyperplastic bladders (Fig. 5B) compared with nonhyperplastic bladder tissue (Fig. 5A). The number of blood vessels present in the lamina propria underlying areas of hyperplastic epithelium was greatly increased, and numerous abnormally large blood vessels were found to be present.

Because PGE₂ has been reported to induce VEGF expression (23), we stained adjacent sections for VEGF. Very little expression of VEGF was seen in nonhyperplastic bladder epithelium of wild-type mice (Fig. 5C), whereas strong staining for VEGF was found throughout the hyperplastic epithelium of K5.COX-2 urinary bladders (Fig. 5D).

Discussion

There is substantial correlative evidence implicating COX-2 activity in the development of bladder cancer. Whereas COX-1 expression is not elevated in TCC and is confined to the stroma in benign and malignant bladders (11), COX-2 is elevated in dysplasia, carcinoma in situ, and invasive urinary bladder cancers (21). Expression of COX-2 correlates with tumor stage and grade in human bladder TCC (12, 15). COX-2 activity in urothelial mucosal tissues was reported to be elevated in smokers compared with nonsmokers and to correlate with the number of cigarettes smoked by bladder cancer patients (24). In one case-control study, nonsteroidal anti-inflammatory drug users were found to have a decreased risk for urinary bladder cancer (25).

Evidence for a functional role of COX-2 in bladder cancer is primarily from preclinical models. In a rat bladder cancer model, COX-2-selective inhibitors effectively inhibited tumor growth and incidence (19). The COX inhibitor piroxicam reduced tumor volumes and enhanced remission of spontaneous urinary bladders tumors in dogs (20). Although supportive of a functional role of COX-2 in the etiology of bladder cancer, these inhibitors also have COX-independent effects.

Transgenic mice offer a unique opportunity to study tissue-specific effects by expressing putative cancer causing genes in an in vivo system. Transgenic expression of COX-2 using a mouse mammary tumor virus promoter is sufficient to induce focal mammary gland hyperplasia, dysplasia, and metastatic tumors in multiparous female mice (8). The primary mechanisms by which COX-2 causes tumorigenesis in this model seem to be through regulation of genes involved in apoptosis and angiogenesis (23). We have reported on the ability of keratin 5 promoter–driven expression of COX-2 in K5.COX-2 transgenic mice to enhance skin tumorigenesis following treatment with dimethylnbenz(a)anthracene. Interestingly, COX-2 was not sufficient to initiate skin carcinogenesis in this model but was sufficient to “autopromote” tumorigenesis in skin initiated by a subcarcinogenic dose of a genotoxic carcinogen (10). COX-2 expression is elevated in basal keratinocytes in K5.COX-2 mice and results in abnormal epidermal differentiation leading to hyperplasia, dysplasia, and increased vascularization (22). Because basal epithelial cells in the urinary bladder also express keratin 5 (26), we used K5.COX-2 mice to determine the effect of COX-2 expression on histopathology in these tissues.

Strong expression of COX-2 and an increase in PGE₂ levels were observed in the urinary bladders of both of the K5.COX-2 lines.
The expression of VEGF is enhanced in epithelial cells of K5.COX-2 bladders, and the number of blood vessels in the bladder stroma is greatly increased. Wild-type (A and C) and K5.COX-2 (B and D) bladders were stained with anti-Factor VIII (A and B) or anti-VEGF (C and D). Factor VIII staining of the wild-type bladders (A) showed relatively few blood vessels in the bladder stroma (arrowhead). In striking contrast, many blood vessels are revealed in K5.COX-2 bladder stroma (B, brown stain), including some relatively large vessels (arrowheads). Whereas staining of the epithelium of the wild-type bladders (C) for VEGF protein was either absent or very weak (arrow), the K5.COX-2 bladders (D) were stained very strongly throughout the hyperplastic epithelium (arrow).

examined whereas COX-1 levels were unchanged. Inflammation was strongly associated with the presence of TCH in bladders from K5.COX-2 mice. The highest incidence of inflammation and TCH occurred in line 675, which is maintained as a homozygous line. Mice from the hemizygous line 667 exhibited similar lesions, although with a lower incidence, indicating that the bladder phenotype was not due to an insertional effect of the transgene. Because prostaglandins have been reported to be proinflammatory, it is possible that COX-2-induced inflammation is a causative factor in the development of the TCH in these mice. The significance of the finding that B lymphocytes are the predominant cells in the lymphoid nodules is not clear. PGE$_2$ has been reported to synergize with interleukin (IL)-4 to cause differentiation, including antibody isotype switching (from immunoglobulin M to immunoglobulin E), and to mildly inhibit proliferation of B lymphocytes in vitro (27). PGE$_2$ can also induce a switch from T-helper 1-type cytokines (e.g., IL-2, IFN-γ, and IL-12) to the production of T-helper 2-type cytokines (e.g., IL-4, IL-5, and IL-10; ref.28). However, these activities of PGE$_2$ seem to be dependent on the site and mode of activation of the lymphocytes (29). Further analysis of the levels of individual prostaglandins and cytokines present in K5.COX-2 bladder tissue will be necessary to better understand the mechanisms behind the observed inflammatory response.

The development of TCH in the bladder of K5.COX-2 mice was associated with a dramatic increase in proliferating cells in the basal cell layers of the urothelium. Prostaglandin synthesis via COX-2 is necessary for proliferation induced by growth factors and tumor promoters in a number of tissues and cell types (30–33); however, it is unclear if the hyperplasia observed in K5.COX-2 bladders was due solely to prostaglandins or if inflammatory cytokines also were contributory. No decrease in apoptosis was observed by terminal deoxynucleotidyl transferase–mediated nick end labeling analysis in the urothelium of K5.COX-2 mice. In contrast, mouse mammary tumor virus–human COX-2 transgenic mice exhibited decreased apoptosis in involuting mammary epithelium compared with wild-type littermates (8). The low number of apoptotic cells in the wild-type bladders may preclude finding any reduction in transgenic mice by the methods utilized in this study. In concordance with the findings in the mouse mammary tumor virus–human COX-2 mammary model and the skin phenotype of K5.COX-2 mice, a dramatic increase in vascularization was observed in the lamina propria of the urinary bladder. Neovascularization depends on an increase in proangiogenic factors relative to antiangiogenic factors. The proangiogenic factor VEGF was clearly elevated in the urothelium of K5.COX-2 mice and likely contributed to the observed increase in vascularization.

Although bladder TCH developed in mice as young as 6 months and in 75% of line 675 mice, TCC developed only in mice aged 10 to 16 months and in just 10% of line 675 and 8% of line 667 mice. The low incidence of TCC and the relatively late occurrence implies that additional mutations are required for progression from TCH to TCC. One possible mechanism by which elevated COX enzyme activity could lead to genetic mutations is through increased formation of DNA adducts resulting from increased lipid peroxidation (34). Malondialdehyde is generated from the peroxidation of membrane phospholipids (35) and is also produced as a by-product of prostaglandin synthesis (36). Malondialdehyde has been shown to react with DNA to form adducts (37) and to be mutagenic (38). Another DNA lesion that occurs as a result of oxidative stress is 8-hydroxydeoxyguanosine. The level of 8-hydroxydeoxyguanosine in DNA is increased in inflammation (39). COX-2-selective inhibitors have been shown to decrease the formation of 8-hydroxydeoxyguanosine in a rat model of liver carcinogenesis (39) and a rat model of colonic inflammation (40), suggesting that the reactive oxygen species may come from either COX-mediated oxidative metabolism of arachidonic acid or from inflammatory cells activated by prostaglandin products of COX. Further studies will be needed to determine if the elevated COX-2 expression in K5.COX-2 mice is sufficient to cause an increase in DNA adduct formation.

It is clear from our data that transgenic expression of COX-2 is sufficient to cause TCH and ultimately TCC in the mouse bladder. Although further studies are necessary to fully elucidate the mechanisms involved, COX-2 expression resulted in inflammation, increased proliferation of the basal urothelium, and enhanced vascularization of the lamina propria. These findings support the hypothesis that COX-2 can play a functional role in urinary bladder carcinogenesis. Indeed, they further validate the rationale for conducting the current phase III trial testing the ability of the COX-2-selective inhibitor celecoxib to prevent bladder cancer recurrence in high-risk patients having undergone transurethral resection (21).

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