

Effects of Short-term Celecoxib Treatment in Patients with Invasive Transitional Cell Carcinoma of the Urinary Bladder

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Abstract

High-grade invasive transitional cell carcinoma (InvTCC) kills >14,000 people yearly in the United States, and better therapy is needed. Cyclooxygenase-2 (Cox-2) is overexpressed in bladder cancer. Cox inhibitors have caused remission of InvTCC in animal studies, and cancer regression was associated with doubling of the apoptotic index in the tumor. The purpose of this study was to determine the apoptosis-inducing effects of celecoxib (a Cox-2 inhibitor) in InvTCC in humans. Patients (minimum of 10 with paired tumor samples) with InvTCC who had elected to undergo cystectomy were enrolled. The main study end point was induction of apoptosis in tumor tissues. Patients received celecoxib (400 mg twice daily p.o. for a minimum of 14 days) between the time of diagnosis [transurethral resection of bladder tumor (TURBT)] and the time of cystectomy (standard frontline treatment for InvTCC). Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling assay and immunohistochemistry were done on TURBT and cystectomy samples. Of 13 cases treated with celecoxib, no residual invasive cancer was identified in 3 patients at the time of cystectomy (post celecoxib). Of the 10 patients with residual cancer, 7 had induction of apoptosis in their tumor. Induction of apoptosis was less frequent (3 of 13 cases; $P < 0.04$) in control patients not receiving a Cox inhibitor. Expression of vascular endothelial growth factor in the tumor cells decreased more frequently ($P < 0.026$) in the treated patients as compared with nontreated control cases. The biological effects of celecoxib treatment (increased apoptosis) justify further study of the antitumor effects of Cox-2 inhibitors in InvTCC. *Mol Cancer Ther*; 9(5); 1371–7. ©2010 AACR.

Introduction

Invasive urinary bladder cancer kills more than 14,000 people each year in the United States (1). Most of those deaths are due to high-grade invasive transitional cell carcinoma (InvTCC) that has metastasized and is resistant to chemotherapy. Cyclooxygenase-2 (Cox-2) is differentially overexpressed in many cancers (including InvTCC) as compared with the corresponding normal tissue, thereby making it an attractive target for cancer therapy (2–10). Cox inhibitors have induced apoptosis and have caused tumor regression in athymic mice bearing human bladder cancer xenografts and in pet dogs with

naturally occurring InvTCC (11–13). The antitumor activity of Cox inhibitors is thought to be due, at least in part, to inhibition of Cox and the resulting decrease in Cox products (prostaglandins and thromboxanes), although Cox-independent effects have also been reported (10, 14, 15). One of the key events identified to date in the antitumor activity of Cox inhibitors *in vivo* in animals is induction of apoptosis (10, 11). A significant association between increase in apoptotic index and tumor regression induced by the Cox inhibitor piroxicam was noted in dogs with naturally occurring InvTCC (12). Naturally occurring InvTCC in dogs is very similar to human InvTCC in histopathology, molecular features, biological behavior including frequency and sites of metastasis, and response to chemotherapy (16). The compressed life span of the dog along with intact immune system and body processes makes it a very attractive tool to study response to drugs and to study their mechanisms of action associated with tumor regression.

Before considering large, longer-term trials in humans, a prospective pilot study was done to determine the effects of short-term Cox-2 inhibitor (celecoxib) treatment in inducing apoptosis in people with InvTCC. Tissue analyses were also conducted to explore some of the possible mechanisms involved in the induction of apoptosis.

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Materials and Methods

Pilot study. The pilot study was done at the Indiana University School of Medicine with the approval of and following the guidelines of the Indiana University Institutional Review Board. A prospective pilot study was done in patients with bladder-confined InvTCC who had elected to undergo cystectomy, as part of standard care for the treatment of their cancer. A Cox-2 inhibitor, celecoxib, was scheduled to be given for a minimum of 2 weeks and a maximum of 6 weeks between the time of diagnosis [transurethral resection of bladder tumor (TURBT)] and cystectomy. Tissues collected at the time of TURBT (pre celecoxib) and at the time of cystectomy (post celecoxib) were studied. The main end point was induction of apoptosis in the tumor with celecoxib treatment. To determine that changes in apoptotic index were most likely due to celecoxib and not other causes, samples from similar patients undergoing cystectomy at the same institution and who were not receiving any Cox inhibitor were studied for comparison. Immunohistochemistry was used to investigate proteins of potential importance in Cox inhibitor-induced apoptosis (17–25). Urine samples collected before and during celecoxib treatment were analyzed for Cox metabolites [prostaglandin E₂ (PGE₂) and thromboxane B₂ (TBXB₂)] as an indication of Cox activity.

Entry requirements included (a) patients more than 35 years of age with confirmed InvTCC localized to the bladder who were planning to undergo cystectomy; (b) serum creatinine concentrations ≤ 1.5 mg/dL, aspartate aminotransferase (SGOT) ≤ 45 IU/L, and alanine aminotransferase (SGPT) ≤ 35 IU/L; (c) no current use of Cox inhibitors and no recent Cox inhibitor use that had lasted for a month or longer; (d) no known hypersensitivity to celecoxib, other Cox inhibitors, or sulfonamides; (e) tissue available from the TURBT and cystectomy; and (f) informed patient consent in writing.

Patient evaluation and monitoring included (a) CBC and serum biochemical profile before treatment and serum creatinine, SGOT, and SGPT after 3 weeks of treatment; (b) interview with the study nurse before and after 3 and 6 weeks of treatment; (c) and a diary in which the patient was asked to record when celecoxib was taken and any unusual symptoms noted by the patient. For patients who received less than 3 weeks of treatment, the follow-up laboratory work and interview with the study nurse were conducted at the end of the treatment period.

Treatment consisted of celecoxib (Celebrex, Pfizer) given p.o. at a dose of 400 mg twice daily.

Samples. Tissues examined included formalin-fixed tissues collected at the time of TURBT and cystectomy. The analyses done on tissues included terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and immunohistochemistry for Cox-2 (Oxford Biochemical Research, Inc.), Ki67 (Zymed), p53 (Signet), vascular endothelial growth factor (VEGF; Zymed), basic fibroblast growth factor (bFGF; Upstate), and survivin

(Lab Vision). Urine samples from healthy control patients and urine samples from InvTCC patients before and after celecoxib were analyzed for PGE₂ and TBXB₂.

Sample size and analyses. The plan was to enroll a minimum of 10 patients receiving celecoxib. This number was based on the expectation that positive results (induction of apoptosis) would be noted in one or more of the patients should celecoxib have a true apoptosis-inducing activity rate of at least 20% of patients (26). Induction of apoptosis was defined as doubling or more of the apoptotic index, as this level of change in apoptotic index has been considered biologically or clinically meaningful (12, 27). If no cases had induction of apoptosis, then further study would not be indicated. SAS 9.1.3 was used for all statistical analyses. Due to the small sample size, Fisher's exact test was used to compare differences in the frequency of the categorical responses. A *P* value of <0.05 was considered significant.

Determination of apoptosis by TUNEL. Apoptosis was measured quantitatively with the use of the TUNEL assay (28). The ApopTag *In situ* Apoptosis Detection kit (Chemicon) was used following the manufacturer's instructions. The percent of positive tumor cells was determined in five different high-power fields, and the average recorded.

Immunohistochemistry. Immunohistochemistry was done as described previously (12). Briefly, 5- μ m sections were cut from paraffin-embedded human InvTCC tissues and placed on Superfrost slides. Sections were dewaxed in xylene and rehydrated in descending percentages of alcohol. Target Retrieval solution (Dako Corp.) was used according to the manufacturer's instructions. The sections were then immersed in 3% hydrogen peroxide in methanol to block the endogenous peroxidase and then blocked for avidin and biotin (Vector Laboratories, Inc.). The sections were permeabilized in TNB-BB [0.1 mol/L Tris (pH 7.5)/0.15 mol/L NaCl/0.5% blocking agent/0.3% Triton-X, 0.2% saponin] and incubated in primary antibody overnight at 4°C. Cox-2 immunoreactive complexes were detected using tyramide signal amplification (TSA-indirect, NEN Life Sciences) and visualized with the peroxidase substrate AEC (Zymed Laboratories), whereas the 3,3'-diaminobenzidine substrate (Vector Laboratories) was used to visualize all other immunocomplexes (Ki67, VEGF, survivin, p53, and bFGF). Slides were counterstained with hematoxylin-1 (Richard-Allan Scientific), mounted in crystal mount, and coverslipped in 50:50 xylene/Permount (Fisher Scientific).

Two pathologists who were blinded to treatment status reviewed slides independently. The percentages of cancer cells with immunoreactivity to Cox-2, Ki67, VEGF, survivin, and p53 were determined. The intensity of immunostaining was graded on a scale of 0 to 3 (0, no staining; 1, equivocal staining; 2, moderate to intense staining; 3, highest intensity staining). A change (between TURBT and cystectomy samples) of $\geq 20\%$ in the percent of cells expressing the protein of interest was considered meaningful.

Table 1. Characteristics of control and celecoxib treatment subjects

	Control (n = 13)	Celecoxib treatment (n = 13)
Gender		
Male	9	8
Female	4	5
Age		
Mean	68	61
Median	67	63
Range	52–79	37–83
Grade		
2/3	1	2
3/4	12	11
LVI	4	4*
TNM stage classification at cystectomy (6th edition 2003–2009)		
T ₀ N ₀ M ₀	0	1 [†]
T ₀ N ₁ M ₀	0	1 [†]
T _{is} N ₀ M ₀	1 [‡]	1 [†]
T ₁ N ₀ M ₀	0	1
T _{2a} N ₁ M ₀	1	0
T _{2b} N ₀ M ₀	0	1
T _{3a} N ₀ M ₀	3	3
T _{3a} N ₁ M ₀	2	0
T _{3a} N ₂ M ₀	1	2
T _{3a} N ₃ M ₀	1	0
T _{3b} N ₃ M ₀	1	0
T _{4a} N ₀ M ₀	2	1
T _{4a} N ₁ M ₀	0	1
T _{4a} N ₃ M ₀	0	1

NOTE: TNM stage was classified according to WHO TNM classification (6th edition, 2003–2009) and was based on cystectomy samples. No significant differences (*t* test/Wilcoxon rank sum for continuous variables, Fisher's exact test for categorical variables) were found between the patients in the control versus the treated group with regard to gender, age, grade, lymphovascular invasion, or stage. Abbreviation: LVI, lymphovascular invasion.

*Invasive cancer was not detected at cystectomy for 3 patients; *n* = 10 samples assessed for LVI (*n* = 10).

[†]Invasive cancer was not detected at cystectomy. All 3 cases had T₂ cancer at the time of TURBT.

[‡]Invasive cancer was not detected at cystectomy. This patient had T₁ cancer at the time of TURBT.

PGE₂ and TBXB₂ concentrations. PGE₂ concentrations (PGE₂ ELISA Kit, Cayman Chemical Co.) and TBXB₂ concentrations (Thromboxane B₂ Enzyme Immunoassay Kit, Assay Designs) in urine were measured before and after treatment with celecoxib according to the manufacturers' protocols. The data were normalized to urine creatinine concentration and reported as picograms per milligram of creatinine.

Results

Pilot study. Subject characteristics are summarized in Table 1. Thirteen patients with high-grade InvTCC received celecoxib treatment in the study. The patients receiving celecoxib (*n* = 13) included 5 women and 8 men. The mean age was 61.4 years (range, 37–83 years). The nontreated control patients (*n* = 13) included 9 men and 4 women with a mean age of 68 years (range, 52–79 years). Of the 13 patients receiving celecoxib, 3 did not have residual InvTCC at the time of cystectomy. In 2 of these 3 patients, no evidence of malignancy was found at the time of the cystectomy. The third patient had carcinoma *in situ*, but no InvTCC detected at the time of cystectomy. Analyses were done on tumor tissues from the 10 treated patients who had InvTCC at the time of cystectomy. Samples from 13 nontreated control patients were analyzed. In treated patients, celecoxib was given for 20.2 ± 9.6 days. One of the patients received less than the planned minimum 14-day treatment (treated 9 days). Treatment was generally well tolerated. One patient reported fatigue, and one patient reported back and stomach aches while receiving celecoxib. No increases in serum creatinine, SGOT, or SGPT were observed.

Increase in apoptosis. Of the 10 treated patients with residual cancer at the time of cystectomy, the apoptotic index in the tumor doubled in 7 patients, increased (but did not double) in 1 patient, remained unchanged in 1 patient, and decreased in 1 patient (Table 2; Fig. 1). In 13 untreated control patients, the apoptotic index increased in 3 patients and remained unchanged or decreased in 10 patients. Induction of apoptosis occurred significantly more frequently (Fisher's exact test, *P* < 0.04) in celecoxib-treated patients than in control patients. In control untreated patients, the percentage of tumor cells undergoing apoptosis was 2.0 ± 1.5 (mean ± SD) at the time of TURBT and was 2.7 ± 3.3 at the time of cystectomy. In patients in the celecoxib treatment group, the percentage of tumor cells undergoing apoptosis increased from 6.2 ± 15.4 at the time of TURBT to 9.9 ± 21.2 at the time of cystectomy.

Expression of Cox-2, VEGF, survivin, p53, Ki67, and bFGF. The percentage of cells with immunoreactivity to Cox-2 ranged from <10% to 55% in treated and from <10% to 75% in nontreated patients. There were no consistent changes in Cox-2 expression with treatment. It was interesting to note that doubling of the apoptotic index following treatment with celecoxib even occurred in patients with relatively low expression of Cox-2 (those having <10% of tumor cells expressing Cox-2).

There was variability in the change in VEGF expression in the treated and nontreated patients (Table 3). Reduction in VEGF (≥20% reduction in percentage of tumor cells expressing VEGF) was significantly more frequent in patients receiving celecoxib (4 of 9) than in untreated patients (0 of 11; *P* < 0.027). VEGF, however, increased in 3 patients and remained unchanged in 2

Table 2. Change in apoptotic index

	Control (n = 13), n (%)	Celecoxib treatment (n = 10), n (%)
Apoptotic index doubled ($\geq 100\%$ increase)	3 (23)	7 (70)
Apoptotic index increased by 20–99%	0 (0)	1 (10)
Less than 20% change in apoptotic index	4 (31)	1 (10)
Apoptotic index decreased by $\geq 20\%$	6 (46)	1 (10)

NOTE: Change in apoptotic index between TURBT and cystectomy in patients receiving celecoxib or in patients not receiving a Cox inhibitor (controls). Induction of apoptosis occurred significantly more frequently (Fisher's exact test, $P < 0.04$) in celecoxib-treated patients than in control patients.

patients receiving celecoxib. In nontreated patients, VEGF expression increased (by $\geq 20\%$) in 7 patients and remained the same in 4 patients. There was no association between change in VEGF expression and change in apoptotic index. Immunoreactivity to bFGF was noted in endothelial cells in blood vessels within the tumor and in a small percentage of stromal cells in some cases. No differences in bFGF were noted between treated and nontreated patients, and no changes with celecoxib treatment were detected.

Of the 23 patients in the study with InvTCC at cystectomy, p53 was detected in 14 (60.8%) tumors. No significant differences were noted between treated and control patients at baseline or at the time of cystectomy. There were no significant differences between treated and control patients with regard to the expression of Ki67 or survivin or any changes with treatment. It was of interest to note, however, that the survivin located in the

nucleus increased in 6 of 10 patients receiving celecoxib, compared with 3 of 11 nontreated patients.

Urine PGE₂ and TBXB₂. Paired urine samples (pre- and post-treatment) were available from 9 patients treated with celecoxib. Stable metabolites of PGE₂ and TBXB₂ were measured in urine from the patients treated with celecoxib and from 30 and 8 patients, respectively, without urinary tract cancer or infection (Fig. 2). Interestingly, the PGE₂ and TBXB₂ concentrations did not decrease in all patients receiving celecoxib as expected. In fact, the PGE₂ concentrations increased in 5 of 9 patients, and TBXB₂ concentrations increased in 8 of 9 patients.

Discussion

There is continued and renewed interest in the use of Cox inhibitors in cancer treatment. Although enthusiasm for the use of Cox-2 inhibitors in primary cancer prevention has waned with the recognition of the thromboembolic risks (29), there is still substantial interest in using these drugs in cancer treatment and as agents to prevent cancer progression, especially where the outlook for the patient is guarded or worse. In InvTCC, for example, the median survival of patients with metastasis treated with chemotherapy is typically ≤ 14 months (30), and new therapy approaches are needed. In studies in dogs with InvTCC, Cox inhibitors have had considerable activity as single agents (remission in 18% of animals, inhibition of growth in an additional 55% of dogs) and have greatly enhanced the remission rate with chemotherapy (12, 31). Naturally occurring InvTCC in dogs closely mimics human InvTCC in its histopathology, molecular features (including Cox-2 expression), frequency and sites of metastases, and response to treatment (16, 32).

The purpose of the study was to determine the apoptosis-inducing effects of a Cox-2 inhibitor in humans with InvTCC. Although it would be intriguing to measure changes in tumor size in response to celecoxib, this would require longer-term treatment, which would not be possible within the accepted time frame for scheduling standard care (cystectomy). In addition, it is difficult to measure the size of InvTCC in the bladder in people, especially when flatter lesions in the bladder are

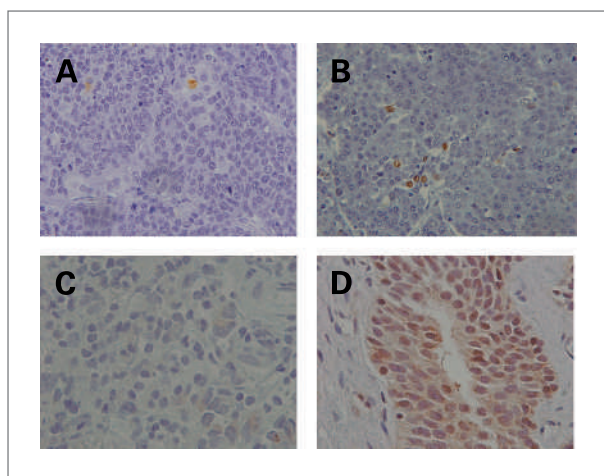


Figure 1. Photomicrographs of InvTCC tissues collected before and after celecoxib treatment. A and B, results of TUNEL assay (Chemicon) used to detect apoptotic tumor cells. A, photomicrograph from the pretreatment (TURBT) sample with minimal apoptosis detected. B, photomicrograph from the posttreatment sample from the same patient with multiple apoptotic cells noted (note brown nuclear staining). C and D, immunohistochemistry was used to detect survivin expression before and after celecoxib treatment. Note the marked increase in survivin expression between the pretreatment sample (C) and the posttreatment sample (D) from the same patient treated with celecoxib.

Table 3. VEGF expression (percentage of tumor cells expressing VEGF) at TURBT and cystectomy

VEGF expression	
TURBT	Cystectomy
Control (n = 11)	
0	10
10	30
10	55
20	50
25	50
30	60
57	70
70	70
70	82
75	62
79	80
Celecoxib treatment (n = 9)	
20	20
40	40
50	25
50	70
50	85
50	80
50	20
70	45
80	33

NOTE: In untreated control patients, the mean \pm SD of the percentage of cells expressing VEGF was 40.5 ± 29.9 at the time of TURBT and 56.3 ± 21.4 at the time of cystectomy. In patients treated with celecoxib, the mean \pm SD of the percentage of cells expressing VEGF was 51.1 ± 16.9 at the time of TURBT and 46.4 ± 25.6 at the time of cystectomy. Expression of VEGF in the tumor cells decreased (by $\geq 20\%$) more frequently ($P < 0.026$) in the treated patients as compared with nontreated control cases.

present. Therefore, another marker of celecoxib activity was selected. Induction of apoptosis was selected because it was significantly associated with antitumor activity of Cox inhibitor in dogs with InvTCC (12). The study results were very encouraging in that celecoxib use was significantly ($P < 0.04$) associated with induction of apoptosis. The induction of apoptosis was attributed to celecoxib because this same level of increase was not observed in control patients who had not received celecoxib or other Cox inhibitors.

Multiple mechanisms could be involved in the induction of apoptosis by celecoxib. Broad mechanisms reported in the literature include Cox-2-dependent and Cox-2-independent actions leading to effects on apoptosis via the mitochondrial pathway (17, 18) or activation of

caspases (17, 18); through inhibition of 3-phosphoinositide-dependent protein kinase 1 (18); and through secondary effects such as those related to a decrease in VEGF (12) or *VE-cadherin* gene expression (19) or changes in other genes (20, 33).

In prior studies in dogs with InvTCC, the antitumor effects of Cox inhibitor were associated with a reduction in angiogenic factors, especially urine bFGF. VEGF-C has been shown to be an important prognostic marker in human bladder cancer (34, 35) and its expression has been correlated with Cox-2 expression (21). In the current study, celecoxib treatment was associated with a decrease in the expression of VEGF-C (but not bFGF) in tumor samples of some patients. Reduction in VEGF has been reported with celecoxib treatment in gastric cancer (22), although not in pancreatic cancer (23). Multiple mechanisms are most likely involved in the effects induced by celecoxib (24).

It was interesting that nuclear survivin expression in the tumor increased in 6 of 10 patients receiving celecoxib. Survivin, a member of the inhibitor of apoptosis proteins, has been reported to block induction of apoptosis, interfere with the antitumor effects of chemotherapy and radiation therapy, to promote angiogenesis, and to be associated with a poor prognosis (25, 36). Survivin has cytoplasmic-nuclear shuttling activity, and its subcellular localization affects its regulatory function (25, 37–43). Although cytoplasmic survivin has consistently been linked to less favorable prognosis and poor response to therapy, the effects of nuclear survivin are less well defined (25, 37–43). In cervical cancer cells, nuclear survivin has actually been found to facilitate induction of apoptosis (39). Survivin in the nucleus seems to degrade more rapidly than that in the cytoplasm, and this could suggest that nuclear survivin expression would be less deleterious in cancer progression. Making

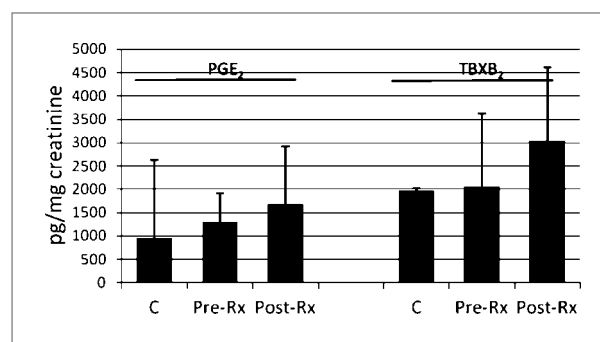


Figure 2. PGE₂ and TBXB₂ concentrations (pg/mg creatinine) in the urine from normal healthy controls (C) and from patients with InvTCC before (Pre-Rx) and after (Post-Rx) celecoxib treatment. Columns, mean; bars, SD. For PGE₂ analyses, 30 control samples and 9 samples from patients with InvTCC before and after celecoxib were included. For TBXB₂ analyses, 8 control samples and 9 samples from patients with InvTCC before and after celecoxib were included. Although reductions in PGE₂ and TBXB₂ were expected with celecoxib treatment, this was not observed, possibly due to drug withdrawal the day before collecting the posttreatment samples.

note of survivin expression and its subcellular localization in larger studies could help define the possible involvement between this protein and the Cox inhibitor effects in InvTCC.

It was of interest that in this celecoxib pilot study, urine PGE₂ and TBXB₂ concentrations did not consistently decrease with celecoxib. In fact, PGE₂ concentration increased in 5 of 9 patients and TBXB₂ increased in 8 of 9 patients with celecoxib treatment. A possible explanation for these unexpected findings relates to the timing of celecoxib dosing and the time of urine collection. Urine samples after celecoxib treatment were collected the day of cystectomy. Many patients, however, discontinued celecoxib the day before surgery. Thus, samples collected the day of surgery would not necessarily reflect celecoxib effects. It is likely that in some patients, celecoxib concentrations were not sufficient to block Cox-2 activity at the time the posttreatment urine samples were collected. In fact, these samples could reflect the "rebound" effect of higher PGE₂ and TBXB₂ concentrations that can be produced when Cox inhibitors are withdrawn (44).

Celecoxib was generally well tolerated, with only 2 patients reporting mild symptoms (fatigue, stomach ache, and back ache) that may or may not have been related to treatment. The celecoxib dose selected for the study (400 mg bid) was selected because this dose has resulted in antitumor activity in patients with other cancers, and this dose is Food and Drug Administration approved for use in patients with familial polyposis coli (45–47).

Of the 13 cases treated with celecoxib, no residual InvTCC was identified in 3 patients at the time of cystectomy (post celecoxib). One of the 3 patients had carcino-

ma *in situ*, and the other 2 patients did not have cancer identified in the cystectomy sections. In previous studies in the Urology Clinics at Indiana University where the pilot project was done, a small percentage (<7%) of patients not receiving any treatment have been noted to have no residual cancer at the time of cystectomy. In these patients, it is usually thought that the cancer was removed at the time of TURBT. It is not possible to know if the finding of 3 of 13 patients with no residual InvTCC after celecoxib treatment was due to celecoxib treatment or not, although this finding is intriguing.

The biological effects of celecoxib in inducing apoptosis in InvTCC provide justification for continued investigation of Cox-2 inhibitor treatment of this cancer. Further studies could include those to document cancer regression with Cox-2 inhibitors, studies of the chemotherapy-enhancing effects of Cox-2 inhibitors, and work to further define mechanisms involved in the antitumor activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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