Cyclooxygenase-2 dependent and independent antitumor effects induced by celecoxib in urinary bladder cancer cells

Deepika Dhawan,[1] Antonella Borgatti Jeffreys,[2] Rong Zheng,[2] Jane C. Stewart,[1] and Deborah W. Knapp[2,3,4]

Departments of [1]Basic Medical Sciences and [2]Veterinary Clinical Sciences, [3]Purdue Cancer Center, and [4]Purdue Oncological Sciences Center, Purdue University, West Lafayette, Indiana

Abstract

Transitional cell carcinoma of the urinary bladder is the second most common genitourinary malignancy in people in the United States. Cyclooxygenase-2 (COX-2) is overexpressed in bladder cancer. COX-2 inhibitors have had antitumor activity against bladder cancer, but the mechanisms of action are unclear. Clinically relevant concentrations of COX-2 inhibitors fail to inhibit proliferation in standard *in vitro* assays. In pilot experiments, different culture conditions [standard monolayer, modified monolayer, soft agar, collagen, and poly(2-hydroxyethyl methacrylate)–coated plates] were assessed to determine conditions suitable for the study of COX inhibitor growth-inhibitory effects. This was followed by studies of the effects of clinically relevant concentrations of a selective COX-2 inhibitor (celecoxib) on urinary bladder cancer cell lines (HT1376, TCCSUP, and UMUC3). Celecoxib (≤5 μmol/L) inhibited proliferation of COX-2–expressing HT1376 cells in soft agar and modified monolayer cell culture conditions in a COX-2–dependent manner. COX-2 expression, however, did not always correlate with response to celecoxib. TCCSUP cells that express COX-2 were minimally affected by celecoxib, and UMUC3 cells that lack COX-2 expression were modestly inhibited by the drug. When UMUC3$^{Cox\text{-}2/Tet}$ cells overexpressing COX-2 under the control of tetracycline-inducible promoter were treated with celecoxib in modified monolayer cell culture, growth inhibition was found to be associated with changes in the expression of pRb. Not surprisingly, the proliferation of all cell lines was inhibited by excessively high concentrations of celecoxib. In conclusion, the modified culture conditions allowed detection of COX-2–dependent and COX-2–independent growth-inhibitory activity of celecoxib in urinary bladder cancer cells. [Mol Cancer Ther 2008;7(4):897–904]

Introduction

Urinary bladder cancer is a common malignancy, afflicting more than 2 million people worldwide. The highest incidence of reported bladder cancer occurs in industrialized countries such as the United States, Canada, and France. In the United States, it ranks as the fifth most frequent form of cancer with more than 67,000 new cases diagnosed annually. Approximately 75% of bladder cancer occurs in men and 25% occurs in women (1). More than 90% of bladder cancers originate in the transitional epithelial cells [forming transitional cell carcinoma (TCC); ref. 1]. Although low-grade TCC is usually controlled successfully by intravesical therapy, intermediate- to high-grade TCC is more difficult to treat and is lethal in ~50% of patients. Better treatment for TCC, especially high-grade TCC, is greatly needed.

A promising target for TCC treatment is cyclooxygenase-2 (COX-2). COX-2 is an inducible isoform of COX, the enzyme that catalyzes the rate-limiting step in the synthesis of prostaglandins from arachidonic acid. COX-2 is associated with inflammation and carcinogenesis and is found to be up-regulated in many forms of human cancers including TCC (2–9). It has been reported that normal human urinary bladder epithelium does not express COX-2, but that COX-2 is overexpressed in ~85% of invasive TCCs and 75% of specimens from carcinoma *in situ* of the urinary bladder (8). Prostaglandin E_2 (PGE_2) is the predominant type of prostaglandin produced by COX-2 activity in cancer cells (10). PGE_2 is derived from cell membrane arachidonic acid, and its release is controlled by phospholipases (11).

In addition to reports of overexpression of COX-2 in various cancers, there is also evidence that COX-2 actively participates in the process of carcinogenesis and cancer progression (12, 13); that is, COX-2 is more than a bystander. Targeting COX-2 provides an intriguing opportunity to fight cancer, as COX-2 expression has been linked to imparting resistance to induction of apoptosis, suppressing the host immune system, enhancing cancer cell growth and invasion, and promoting angiogenesis (14–17). The antitumor effects of nonselective COX inhibitors (which block the enzyme activity of COX-1 and COX-2) and of selective COX-2 inhibitors *in vivo* have been reported (18, 19). Our laboratory has observed antitumor activity of these drugs against experimentally induced bladder

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Requests for reprints: Deborah W. Knapp, Department of Veterinary Clinical Sciences, Purdue University, 625 Harrison Street, West Lafayette, IN 47907-2026. Phone: 765-494-9900. E-mail: knappd@purdue.edu

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tumors in rodents[5] and against naturally occurring invasive TCC in pet dogs, where the disease very closely mimics human invasive bladder cancer (2, 19, 20). In the pet dogs with invasive TCC, complete and partial remission (overall remission rate 18%) and cancer stabilization (in 55% of dogs) has occurred with COX inhibitor treatment. In a study of pet dogs with TCC receiving the nonselective COX inhibitor, piroxicam, there was a significant association between decreased PGE_2 and increased apoptotic index with treatment (20). A correlation between high concentration of PGE_2 and elevated levels of urine basic fibroblast growth factor was also noted (20). Although enthusiasm for the long-term use of COX-2 inhibitors for cancer prevention has lessened somewhat with the reported cardiovascular risk associated with the use of these drugs (21–23), enthusiasm remains high for the use of COX-2 inhibitors for the treatment of cancer.

To optimally apply COX-2 inhibitors, an understanding of the mechanisms of the antitumor activity is needed. In spite of numerous reports concerning the possible effects of COX-2 inhibitors in different cancers, the mechanisms or pathways involved remain ambiguous. It is not even clear if COX inhibitors work through COX-2–dependent or COX-2–independent effects. One of the major limitations in defining the antitumor mechanisms of COX-2 inhibitors has been that these drugs do not have the same antitumor effects in standard *in vitro* assays as they have *in vivo* (19). *In vitro* assays have been used for the study of most anticancer agents because of the controlled, reproducible settings; ease of study; and relatively low expense compared with *in vivo* study. However, standard *in vitro* assays do not seem useful for the study of inhibitory effects of COX-2 inhibitors, at clinically relevant drug concentrations (concentrations reached safely *in vivo*; ref. 19). To inhibit proliferation in standard *in vitro* assays, COX-2 inhibitors must be applied in very high concentrations; that is, concentrations far greater than those safely reached in serum *in vivo*. Therefore, the relevance of these *in vitro* studies to the human condition is questionable. In this study, several pilot experiments were done to assess the utility of different culture conditions in detecting COX-2 inhibitor antiproliferative activity *in vitro*. Then, studies were done to further define the effects of one such COX-2 inhibitor, celecoxib. In addition to standard commercially available cell lines, COX-2–overexpressing UMUC3 cells, $UMUC3^{Cox\text{-}2/Tet}$ (developed from parental COX-2–negative bladder cancer cells), under the control of a tetracycline-inducible promoter, were studied.

Materials and Methods

Study Design

In this study, the effects of a COX-2 inhibitor, celecoxib (Celebrex, Pfizer), in human bladder cancer cell lines, which have different levels of COX-2 expression, were determined. The cell lines included HT1376 (highest COX-2 expression), UMUC3 (undetectable amounts of COX-2), and TCCSUP (moderate expression of COX-2). In addition, COX-2 was expressed in UMUC3 cells under the control of a tetracycline-inducible promoter ($UMUC3^{Cox\text{-}2/Tet}$). Pilot experiments were conducted with cells grown in different culture conditions. These included soft agar, collagen, poly(2-hydroxyethyl methacrylate) (poly-HEMA)–coated plates, standard monolayer, and modified monolayer cultures. The effects of celecoxib on proliferation were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and confirmed by manual cell count in monolayer studies and by colony count in soft agar. Expression of COX-2, phosphorylated retinoblastoma (pRB), and phosphorylated AKT was determined by Western blot, and PGE_2 concentrations were measured by EIA.

Reagents and Cell Lines

Celecoxib was provided by Pharmacia/Pfizer. Urinary bladder cancer cell lines, HT1376, TCCSUP, and UMUC3, were obtained from American Type Culture Collection. Cells were cultured in modified Eagle's medium with 10% fetal bovine serum and 1% L-glutamine at 37°C in 5% CO_2. Antibodies purchased included those for COX-2 (Cayman), pAKT (Santa Cruz Biotechnology), and pRB (BD Biosciences).

Soft Agar Clonogenic Assays

Soft agar assay was done as previously described (24). Briefly, 2×10^3 cells were suspended in 0.3% Bacto-agar (Life Technologies) containing 10% fetal bovine serum in MEM medium and 5, 10, and 25 μmol/L celecoxib on a 0.6% Bacto-agar layer containing 10% fetal bovine serum in MEM medium using 60-mm cell culture plates. The plates were incubated at 37°C for 10 d. The numbers of colonies containing 6 or more cells were counted.

Poly-HEMA – Coated Plate Assay

The poly-HEMA–coated plates were prepared as instructed by the manufacturer (Sigma). Briefly, poly-HEMA (20 mg/mL) in 95% ethanol was shaken vigorously for 5 to 6 h until it dissolved. The solution was then plated in six-well plates and allowed to air dry on a shaker overnight. Cells were then plated, and treated with 0, 5, 10, 25, μmol/L of celecoxib for a period of 96 h and counted manually using a hemocytometer.

Collagen

A solution of collagen (Vitrogen, Cohesion Technologies) was made by adding 8 mL of collagen to 2 mL of 5× MEM/0.05 mol/L NaOH and applied to 96-well cell culture plates. The plates were covered in saran wrap and incubated at 37°C to allow the collagen to set. Cells (3,000 per well) were suspended in complete medium; added to each well; and treated with 0, 5, 10, and 25 μmol/L celecoxib for 7 d. The *in vitro* MTT cell proliferation assay described below was followed with the exception that DMF/SDS buffer [50% DMF/20% SDS (pH 4.7)] was added to the collagen-containing cells instead of DMSO to solubilize the end product.

Modified Monolayer Cell Proliferation Assay

Proliferation of cells in monolayer posttreatment with celecoxib was estimated using the MTT assay as described by Mosmann (25) with some modifications. Briefly, cells

[5] S.I. Mohammed, D.W. Knapp, unpublished data.

were grown in 96-well flat-bottomed cell culture–treated plates and treated with 0, 5, 10, and 25 μmol/L of celecoxib. At least triplicate wells were used for each experimental condition. The medium and celecoxib were replaced every 48 h. MTT (Sigma) was dissolved in PBS (pH 7.2) to obtain a concentration of 5 mg/mL and filtered. MTT solution (20 μL) was then added to each culture well, and the plates were incubated for 1 h at 37°C. The medium was removed, plates were dried, and the cells were lysed with 100 μL of DMSO. Absorbance was measured at a wavelength of 570 nm. The data are represented as percent growth inhibition.

Western Blotting

Protein (20–30 μg) from lysates of cultured cells (cells grown in modified monolayer culture conditions) was separated by SDS-PAGE and transferred to a nitrocellulose membrane overnight (19). Membranes were blocked with 5% nonfat milk in TBS with Tween 20 and 0.1% bovine serum albumin for 1 h and incubated with antibodies as indicated for 1 h at room temperature. Membranes were subsequently incubated with secondary antibody (either goat anti-mouse or goat anti-rabbit conjugated with horseradish peroxidase; 1:10,000 dilution) for 1 h. Protein was detected on BioMax MR film (Kodak) using chemiluminescence (Super Signal, Pierce Biotechnology). Equal protein loading was confirmed by detection of β-ACTIN using mouse primary antibody (1:5,000 dilution) and goat anti-mouse secondary antibody (1:10,000 dilution). Selected blots were quantified using Kodak ImageStation 440CF, Eastman Kodak.

PGE_2 Quantitation Assay to Determine the Concentration of PGE_2

HT1376 and UMUC3 cells (2×10^5) were plated in 60-mm cell culture dishes. Culture medium (500 μL) was collected from each dish to determine the concentration of PGE_2 released into the medium. An EIA monoclonal antibody kit (PGE_2 EIA Kit, Cayman Chemical Co.) was used according to the manufacturer's protocol to determine the concentrations.

Overexpression of COX-2 in UMUC3 Cells

To generate the expression construct of human COX-2 (pcDNA4/*cox-2*), the plasmid pcDNA3.1-*cox-2* (a kind gift from Dr. R.J. Kulmacz, University of Texas, Houston, TX) was digested with *Hin*dIII/*Apa*I to isolate the full-length human *cox-2* and cloned at the *Hin*dIII/*Apa*I sites in the mammalian expression vector pcDNA4/TO (Invitrogen). Tet repressor protein was encoded by the pcDNA6/TR regulatory vector (Invitrogen). This was transfected in UMUC3 cells with selection using blasticidin and zeocin (Invitrogen, 4 and 800 μg/mL, respectively) for 14 to 18 wk. Individual clones were identified by treating with tetracycline (1 μg/mL Sigma) for 24 h and determining expression of COX-2 protein and confirmed by resulting PGE_2 production. This transfected cell line is called $UMUC3^{Cox\text{-}2/Tet}$, and it was characterized by confirming expression of COX-2 when treated with tetracycline.

Characterization of $UMUC3^{Cox\text{-}2/Tet}$ Cells

The rate of proliferation of $UMUC3^{Cox\text{-}2/Tet}$ was compared with that of the rate of proliferation of UMUC3 cells. Equal numbers of cells (both cell lines) were plated in varying percentage of serum with and without the presence of tetracycline for a period of 72 h and the rate of proliferation was measured by MTT assay. PGE_2 expression was also quantified when the $UMUC3^{Cox\text{-}2/Tet}$ cell line was treated with tetracycline as compared with UMUC3 cells.

Statistical Analysis

All data are reported as the mean ± SE. Data were analyzed by *t* test (two samples assuming unequal variance). Differences with $P < 0.01$ were considered significant.

Results

Selection of Culture Conditions from Pilot Studies

Two cell culture conditions (modified monolayer, soft agar) were found to be useful for detection of the antiproliferative effects of celecoxib. Modified monolayer assays had an advantage over soft agar assays in that cells could be recovered from the plate for further study (e.g., Western blot). COX-2 expression in the cell lines was confirmed using cells grown in monolayer culture conditions (Fig. 1A). HT1376 cells had the highest expression of COX-2. UMUC3 cells did not express COX-2, and TCCSUP

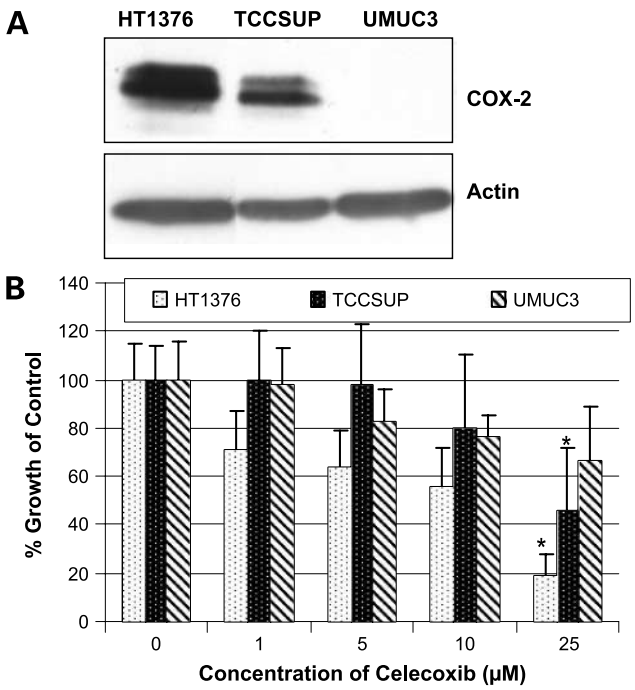

Figure 1. **A,** expression of COX-2 protein in urinary bladder cancer cell lines (HT1376, TCCSUP, and UMUC3 cells grown in monolayer) as detected by Western blot. Actin confirms equal loading of protein. **B,** effects of celecoxib on colony formation of urinary bladder cancer cells (HT1376, TCCSUP, and UMUC3) in soft agar. Celecoxib (10 d treatment) reduced the number of colonies of HT1376 and UMUC3 cells at clinically relevant concentrations (e.g., 5 μmol/L) in soft agar, yet did not inhibit TCCSUP cells at concentrations below 10 μmol/L. Data shown are representative of three different experiments. Statistical analysis was done using *t* test assuming unequal variance. *, $P < 0.01$.

cells expressed COX-2 at moderate levels. Poly-HEMA assays were not selected for further experiments because some of the cells (especially HT1376 cells) displayed abnormal morphology and prolonged doubling time in poly-HEMA assays. There was no appreciable change in proliferation of the bladder cancer cells when grown in poly-HEMA–coated plates, collagen, or in standard monolayer cell culture conditions with different concentrations of celecoxib for up to 96 hours compared with untreated controls.

Effect of Celecoxib on Proliferation of Bladder Cancer Cells in Soft Agar and Modified Monolayer Conditions

The growth-inhibitory effects of celecoxib in soft agar assays are summarized in Fig. 1B. The effects of celecoxib on proliferation of TCC cells in monolayer culture conditions were evaluated at different time points. The medium was changed every 48 hours in an attempt to maintain the concentration of the drug and replenish medium components. Proliferation was inhibited by 31%, 42%, and 58% when HT1376 cells were treated with 5, 10, and 25 μmol/L celecoxib, respectively, compared with control cells ($P < 0.001$), for 5 days (Fig. 2A). In TCCSUP cells that have lower COX-2 expression, a slight increase in the rate of proliferation was observed with ≤10 μmol/L celecoxib even when the treatment time was increased to 7 days, but this was not statistically significant. Proliferation was inhibited by 9% in TCCSUP cells with 25 μmol/L celecoxib at 7 days but this was not statistically significant (Fig. 2A).

In UMUC3 cells that lack COX-2 expression, there was a slight reduction in proliferation (11% and 20% at 5 and 10 μmol/L celecoxib, respectively; $P < 0.001$). A larger antiproliferative effect, similar to that of HT1376 cells (65% reduction in proliferation; $P < 0.001$), was noted when UMUC3 cells were treated with 25 μmol/L celecoxib (Fig. 2A).

UMUC3$^{Cox\text{-}2/Tet}$ cells were tested for expression of COX-2 under the control of tetracycline (Fig. 2B) by Western blot analyses. As compared with UMUC-3 cells, the rate of proliferation of the UMUC3$^{Cox\text{-}2/Tet}$ cell lines was different when both cell lines were grown for a period of 96 hours without the addition of tetracycline in the presence of varying amounts of serum (data not shown). The presence of COX-2 was confirmed in the presence of tetracycline, when UMUC3$^{Cox\text{-}2/Tet}$ cells were found to synthesize substantial amounts of PGE_2 compared with UMUC3 cells (Fig. 2C) by PGE_2 ELISA Assay. When UMUC3$^{Cox\text{-}2/Tet}$ cells grown in the presence of tetracycline and overexpressing COX-2 were treated with 5, 10, and 25 μmol/L celecoxib, there was 9% ($P < 0.01$), 19%, and 40% growth inhibition, respectively, as measured by MTT proliferation assay ($P < 0.001$). When UMUC3$^{Cox\text{-}2/Tet}$ cells were cultured in the absence of tetracycline and treated with the same concentrations of celecoxib, there was 6% and 33% growth inhibition observed with 10 and 25 μmol/L ($P < 0.001$ at 25 μmol/L) celecoxib, and there was no appreciable growth inhibition when the cells were treated with 5 μmol/L celecoxib (Fig. 2D). Celecoxib at 5 μmol/L was found to be sufficient to inhibit the synthesis of PGE_2 by UMUC3$^{Cox\text{-}2/Tet}$ cells (under the control of tetracycline; Fig. 2C).

Effect of PGE_2 on Growth Inhibition of Bladder Cancer Cells When Treated with Celecoxib

The concentration of celecoxib necessary and sufficient to block the enzymatic activity of COX-2 was also evaluated. Celecoxib at 5 μmol/L was sufficient to inhibit the enzymatic activity of COX-2 in HT1376 cells, as measured by the quenching of the concentrations of PGE_2 in the medium (Fig. 3A). UMUC3 cells, which are COX-2 negative, had minimally detectable levels of PGE_2.

The concentration of PGE_2 in the medium as a function of time was evaluated. HT1376 cells were cultured for a period of 4 days, and the medium with 10% serum was replaced every 48 hours. Concentrations of PGE_2 were elevated in the presence of fresh medium, followed by gradual decline at 24 to 48 hours (Fig. 3B). Similarly, when exogenous PGE_2 was added to the cell culture, the concentration of PGE_2 initially increased, but then decreased over time (data not shown).

The effect of the exogenous addition of PGE_2 on the antiproliferative effects of celecoxib was evaluated in HT1376 cells treated with celecoxib for 5 days (Fig. 4A). The addition of 5 ng/mL PGE_2 to HT1376 cells rescued the cells from the antiproliferative effects of 5 and 10 μmol/L celecoxib ($P < 0.001$). It is interesting to note that there was no significant difference in inhibition of proliferation of HT1376 cells when comparing HT1376 cells treated with 5 or 10 μmol/L celecoxib in the presence of 5 ng/mL PGE_2. PGE_2 also increased the rate of proliferation of HT1376 cells (without 5 μmol/L celecoxib) compared with control cells without PGE_2 ($P < 0.001$). Cells growing in the presence of 25 μmol/L celecoxib along with 5 ng/mL PGE_2 only grew to a level of 65% to 75% of control cells ($P < 0.001$; Fig. 4A). It is also important to note that there was a significant increase in rate of proliferation of HT1376 treated either with 5 or 500 ng/mL PGE_2 compared with their corresponding PGE_2 untreated controls, with 5, 10, and 25 μmol/L celecoxib ($P < 0.001$).

When HT1376 cells were treated with 5, 10, and 25 μmol/L celecoxib, there was an induction in the expression of COX-2 and an accompanying minor decrease in the expression of pAKT. The addition of exogenous PGE_2 abrogated the increase in the expression of COX-2 and the decrease in expression of pAKT (Fig. 4B).

Effect of Celecoxib on Cellular Proteins Involved in Proliferation

When UMUC3$^{Cox\text{-}2/Tet}$ cells grown (modified monolayer culture conditions) in the absence of tetracycline were treated with 5, 10, and 25 μmol/L celecoxib, there was an increase in pRb (Fig. 5). Conversely, when UMUC3$^{Cox\text{-}2/Tet}$ cells grown in the presence of tetracycline (and therefore overexpressing COX-2) were treated with 5, 10, and 25 μmol/L celecoxib in modified monolayer culture conditions, there was a marked decrease in the expression of pRB (Fig. 5). No change in COX-2 or pAKT was observed in UMUC3$^{Cox\text{-}2/Tet}$ cells with celecoxib treatment.

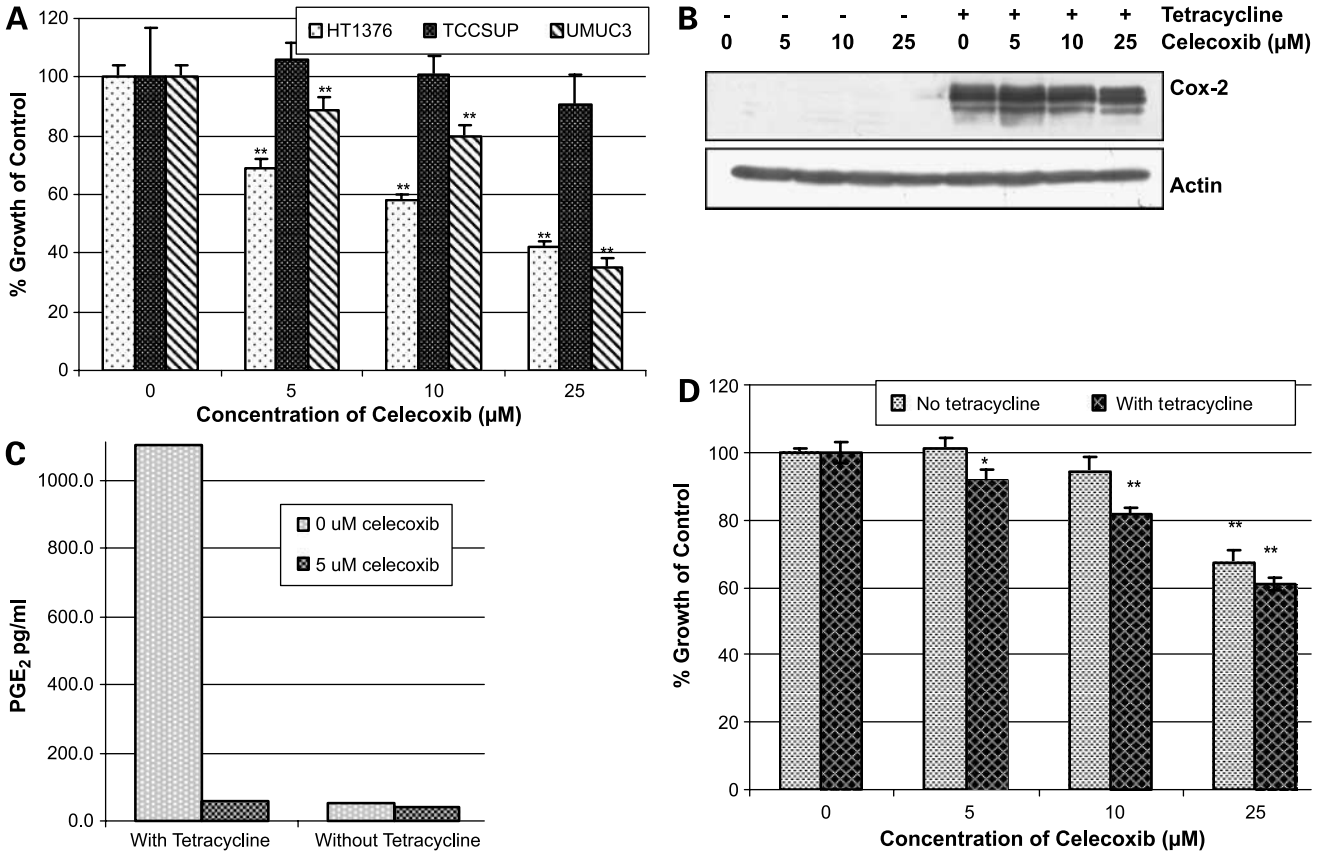

Figure 2. **A,** modified monolayer cell proliferation assay. Effects of celecoxib on the growth of bladder cancer cells (HT1376, TCCSUP, and UMUC3) when grown under modified monolayer cell culture conditions for a period of 5 d (7 d for TCCSUP). Medium containing celecoxib was replaced every 48 h. Celecoxib inhibited the growth of HT1376 cells at clinically relevant concentrations, but had minimal effect on UMUC3 and TCCSUP cells at concentrations below 10 and 25 μmol/L, respectively. Statistical analysis was done using *t* test assuming unequal variance. *, $P < 0.01$; **, $P < 0.001$. **B,** expression of COX-2 in UMUC3$^{Cox\text{-}2/Tet}$ cells in the presence of tetracycline. UMUC3$^{Cox\text{-}2/Tet}$ cells grown in the absence of tetracycline did not express COX-2, whereas expression of COX-2 was induced in the presence of tetracycline. When UMUC3$^{Cox\text{-}2/Tet}$ cells expressing COX-2 were treated with different concentrations of celecoxib, there was no appreciable change in the expression of COX-2. Actin confirms equal loading of protein. **C,** quantification of PGE_2 levels in UMUC3$^{Cox\text{-}2/Tet}$ cells. PGE_2 was assayed in UMUC3$^{Cox\text{-}2/Tet}$ cells in the presence and absence of tetracycline. Celecoxib (5 μmol/L) was found to be sufficient to block the synthesis of PGE_2 in UMUC3$^{Cox\text{-}2/Tet}$ cells expressing COX-2 in the presence of tetracycline. **D,** effects of celecoxib on the growth of UMUC3$^{Cox\text{-}2/Tet}$ cells grown in the presence of tetracycline and overexpressing COX-2. Celecoxib (5 d) had minimal effect on UMUC3$^{Cox\text{-}2/Tet}$ cells at concentrations below 10 μmol/L when the cells did not express COX-2. When COX-2 expression was induced, the growth inhibition by celecoxib was slightly augmented. Statistical analysis was done using *t* test assuming unequal variance. *, $P < 0.01$; **, $P < 0.001$.

Discussion

Antitumor activity of COX inhibitors has been observed in rodents with experimentally induced bladder tumors and in pet dogs with naturally occurring invasive TCC where the cancer very closely mimics human invasive TCC (19, 20). However, the reported mechanisms underlying the antiproliferative effects of COX inhibitors (*in vitro*) have largely been those that occur with drug concentrations much greater than what can be achieved *in vivo* in humans. It has previously been noted that under standard cell culture conditions using clinically relevant concentrations of COX inhibitors (those attainable *in vivo*), no inhibition of proliferation of bladder cancer cells occurred *in vitro*, despite the reported antitumor activity of COX inhibitors in rodents and in pet dogs *in vivo* (19, 20).

Pilot studies were initiated in this work to define appropriate conditions to study the antitumor effects of relevant concentrations of COX-2 inhibitors *in vitro* using bladder cancer cells. Soft agar culture is a traditional method for assessing anchorage-independent growth, and, in our studies, the colony formation of bladder cancer cells in soft agar was reduced in the presence of low doses of celecoxib (concentrations that are more relevant *in vivo*; Fig. 1B). However, a limitation to soft agar assays is the inability to remove cells from the agar posttreatment. This limits analyses of signaling and other mechanistic events. Growing the cells with poly-HEMA and collagen (26–28) allows the study of anchorage-independent growth (as do soft agar assays). In our study, however, the bladder cancer cells grown in poly-HEMA–coated plates looked unhealthy and had different doubling times compared with cells grown in monolayer. Successful recovery of bladder cancer cells grown in collagen as in soft agar was found to be a serious limitation of this assay.

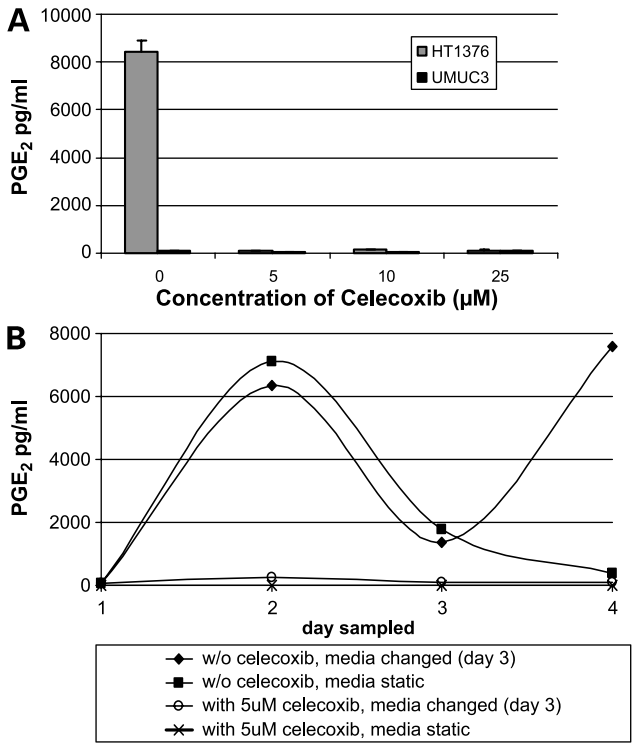

Figure 3. **A,** effect of celecoxib on PGE_2 concentration. PGE_2 was assayed in HT1376 and UMUC3 cells pretreatment and posttreatment with celecoxib (24 h). Celecoxib (5 μmol/L) was found to be sufficient to block the synthesis of PGE_2. UMUC3 cells, which do not express COX-2, have minimally detectable PGE_2. **B,** effect of renewing the medium upon the concentrations of PGE_2 found in HT1376 cell cultures grown in standard monolayer. Medium was either changed on day 3 or was not renewed (*media static*). Celecoxib (5 μmol/L) was added to the cultures as indicated and was continued in the changed medium. Celecoxib at 5 μmol/L was sufficient to inhibit PGE_2 synthesis. Cultures receiving renewed medium reflect an increase in PGE_2 concentration compared with the cultures where medium was unchanged.

Monolayer cell culture conditions were modified to replace medium and drug every 48 hours to avoid build up of metabolites and to replenish nutrients to the cells. Other scientists have observed differences in cell growth and response to celecoxib when the medium is replenished regularly.[6] Our studies showed that COX-2–positive cells synthesize PGE_2 in response to fresh fatty acid available in the medium. Within a day or two, depending on cell density, the PGE_2 precursors seem to be exhausted as reflected by a drop in PGE_2 concentrations. Replenishing the medium enabled the cells to maintain synthesis of PGE_2. This allowed differences in PGE_2 to be observed between untreated and celecoxib-treated cells. Untreated cell cultures had higher concentrations of PGE_2, and COX-2 inhibitor–treated cell cultures had minimal concentrations of PGE_2. It is possible that the inability to observe subtle antiproliferative effects at low concentrations of celecoxib in traditional monolayer is related to exhausting of the available substrate, arachidonic acid over 24 to 48 hours, leading to low PGE_2 concentration in the medium of COX-2–positive cells. Therefore, the concentrations of PGE_2 in both COX-2–positive and COX-2–negative cell cultures after 24 to 48 hours of incubation would be similarly low, and the effect of COX-2 inhibition on proliferation would be negligible. Studies done in our laboratory showed that under monolayer culture conditions, the antiproliferative effects of COX-2 inhibitors can be observed at low doses, if medium is replenished, and cells are treated with celecoxib every 48 hours.

Using modified monolayer cell culture conditions, COX-2–dependent antiproliferative effects in HT1376 cells

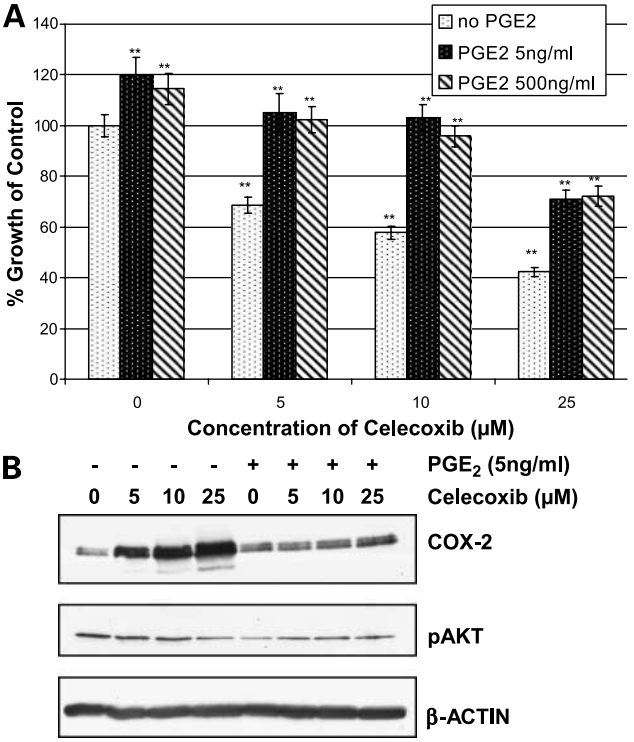

Figure 4. **A,** effect of exogenous PGE_2 addition on HT1376 cell proliferation 5 d posttreatment with 0, 5, 10, and 25 μmol/L celecoxib. PGE_2 at 5 ng/mL is sufficient to rescue the antiproliferative effects of 5, 10, and 25 μmol/L celecoxib ($P < 0.001$). There is no significant difference in the rescue when using either 5 or 500 ng/mL PGE_2. Statistical analysis was done using *t* test assuming unequal variance. Statistical significance was calculated using the respective treatment groups (without exogenous PGE_2) and by comparing them with the treatment groups with exogenous PGE_2. For instance, HT1376 cells treated with 5 μmol/L celecoxib were compared with HT1376 cells treated with 5 μmol/L celecoxib and PGE_2 (5 or 500 ng/mL) and so on. *, $P < 0.01$; **, $P < 0.001$. **B,** effect of exogenous PGE_2 addition on the expression of COX-2 and pAKT, 5 d posttreatment with 0, 5, 10, and 25 μmol/L celecoxib. When 5 ng/mL PGE_2 was added to HT1376 cells treated with 0, 5, 10, and 25 μmol/L celecoxib, an increase in the expression of COX-2 (and a slight, although not appreciable, decrease in the expression of pAKT) was observed when cells were treated with celecoxib in the absence of exogenous PGE_2. There was no change in the expression of COX-2 (and no appreciable change in the expression of pAKT) when cells were treated with celecoxib in the presence of exogenous PGE_2. Actin confirms equal loading of protein.

[6] A.J. Dannenberg, personal communication.

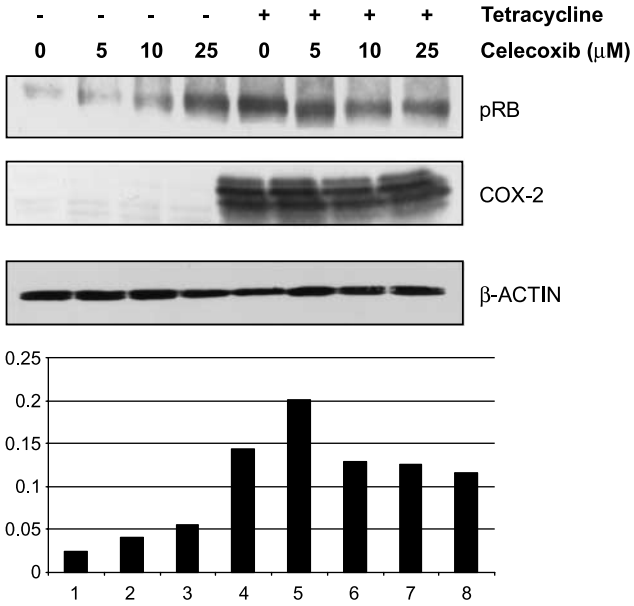

Figure 5. Effect of celecoxib on the expression of pRB in UMUC3$^{Cox\text{-}2/Tet}$ cells. When UMUC3$^{Cox\text{-}2/Tet}$ cultures are grown in the presence of tetracycline and COX-2 is expressed, pRB decreases as celecoxib treatment increases. On the other hand, when UMUC3$^{Cox\text{-}2/Tet}$ cells are not grown in the presence of tetracycline and COX-2 is not expressed, there is a slight increase in the expression of pRB when treated with celecoxib. Actin shows equal protein loading. Expression of pRB was quantified by densitometry analyses using the Kodak ImageStation. Graph of pRB expression depicts the expression of pRB as normalized with respect to actin.

were observed with ≤5 μmol/L celecoxib, concentrations of celecoxib that can safely be achieved in serum *in vivo* in humans (29). Celecoxib at 5 μmol/L effectively prevented the production and release of PGE_2 in the medium. In addition, exogenous PGE_2 rescued the antiproliferative effects exerted by 5 or 10 μmol/L celecoxib thereby providing evidence for a COX-2–dependent antiproliferative effect at low doses of celecoxib. [It is important to point out that 5 ng/mL PGE_2 was found to be sufficient to rescue the antiproliferative effect of 5 or 10 μmol/L celecoxib, and no significant advantage was gained by addition of a 100-fold higher amount of PGE_2.] It was interesting to note a significant increase in proliferation of HT1376 cells in the presence of exogenous PGE_2 (5 or 500 ng/mL). The role of PGE_2 in regulating cancer cell proliferation in an autocrine and/or paracrine manner has been documented (30, 31). Others have also reported that celecoxib inhibits proliferation and induces apoptosis via PGE_2-related effects in human cholangiocarcinoma cell lines (32).

In contrast to the COX-dependent effects of low concentrations of celecoxib (as seen in HT1376 cells), high concentrations of celecoxib seemed to inhibit proliferation of all three cell lines in a COX-independent manner. The antiproliferative effects of 25 μmol/L celecoxib on HT1376 cells could not be completely rescued by the addition of exogenous PGE_2, implying COX-2–independent effects when higher concentrations of celecoxib were used. The relevance of inhibition by high concentrations of celecoxib (≥10 μmol/L) to therapy in humans, however, is questionable.

Another important finding of this study was that different bladder cancer cell lines responded differently to celecoxib. Although TCCSUP has moderate expression of COX-2, proliferation of these cells was not inhibited by celecoxib, whereas celecoxib had antiproliferative effects in UMUC3 cells, a COX-2–negative cell line. It seems that COX-2 activity is not always necessary for cell survival as inhibiting COX-2 does not result in growth inhibition in all COX-2–expressing cancer (33). Therefore, it is not possible to predict the antitumor effects of COX-2 inhibitors merely by the presence or absence of COX-2. This has been observed in studies of dogs with naturally occurring invasive TCC, in that the antitumor effects of COX inhibitors were not associated with the level of COX-2 expression in the cancer (33). Similarly, in another type of cancer, relevant concentrations of celecoxib have been reported to inhibit the proliferation of COX-2–negative prostate cancer cell lines (34) via suppression of cyclin D1. This reinforces the need to explore mechanisms involved in the antiproliferative effects of COX-2 inhibitors, and also the potential need to explore the doses of the inhibitors needed to induce antitumor effects in COX-2–negative tumors *in vivo*.

Previous studies have shown that cell cycle arrest associated with apoptosis is clearly one of the mechanisms by which celecoxib blocks cell cycle progression (19, 35, 36), and that this occurs in a dose-dependent manner. To understand the mechanism of growth inhibition of HT1376 cells by COX-2 inhibition, the expression of proteins involved in cell cycle regulation and cellular survival pathway was determined. There was an increase in the expression of COX-2 in HT1376 cells when treated with celecoxib, which was abolished by the addition of exogenous PGE_2, supporting the presence of the feedback mechanism of the regulation of COX-2 and PGE_2. Minimal reduction in pAKT was observed in HT1376 cells treated with celecoxib, which was then rescued by exogenous PGE_2, implicating a COX-2–dependent pathway in HT1376 cells. No appreciable change in COX-2 or pAKT was observed in UMUC3$^{Cox\text{-}2/Tet}$ cells with celecoxib treatment. It was most interesting that treating UMUC3$^{Cox\text{-}2/Tet}$ cells (without tetracycline) with celecoxib was associated with up-regulation of pRB. It is well known that the dephosphorylation of pRB in G_1 phase of the cell cycle is a key event for the transition of G_1-S phase, thereby leading to the induction of apoptotic pathway, and hyperphosphorylation of pRB leads to increase in cellular proliferation (37). Despite the fact that there are differences in UMUC-3 and UMUC3$^{Cox\text{-}2/Tet}$ cell lines, the increased phosphorylation of RB could explain why UMUC3 cells when treated with celecoxib showed a slight increase in cell proliferation when treated with low doses of celecoxib. When COX-2 was expressed in UMUC3$^{Cox\text{-}2/Tet}$, there was a dramatic reduction in the expression of pRB following treatment with 5 μmol/L celecoxib, which was associated with the growth inhibition when these cells were treated in modified monolayer conditions with celecoxib. It is important to

mention that HT1376 and TCCSUP cells have undetectable levels of pRB (38).

It seems that the mechanism of action of celecoxib varies according to the capacity of the cell type being treated. Celecoxib effects depend not only on the conditions under which it is administered to the cells and to the cell type, but also on key players like COX-2, pRB, and pAKT. This finding highlights the need to study closely the mechanisms underlying the action of celecoxib within and between different tumor types.

In conclusion, celecoxib inhibited the proliferation of human urinary bladder cancer cell lines by COX-2–dependent and COX-2–independent effects. As also observed in pet dogs with naturally occurring invasive TCC, COX-2 expression in the human bladder cancer cell lines studied was not found to be predictive of response to celecoxib *in vitro*.

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