Celecoxib Inhibits Prostate Cancer Growth: Evidence of a Cyclooxygenase-2-Independent Mechanism

Manish I. Patel,1,2,5 Kotha Subbaramaiah,1 Baoheng Du,1 Mindy Chang,1 Peiying Yang,6 Robert A. Newman,6 Carlos Cordon-Cardo,2,4 Howard T. Thaler,3 and Andrew J. Dannenberg1

1Department of Medicine, Weill Medical College of Cornell University; Departments of 2Urology, 3Epidemiology and Biostatistics, and 4Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York; 5Department of Surgery, University of Sydney, Sydney, Australia; and 6Department of Experimental Therapeutics, University of Texas M.D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Purpose: Selective cyclooxygenase-2 (COX-2) inhibitors may suppress carcinogenesis by both COX-2-dependent and COX-2-independent mechanisms. The primary purpose of this study was to evaluate whether celecoxib or rofecoxib, two widely used selective COX-2 inhibitors, possess COX-2-independent antitumor activity.

Experimental Design: PC3 and LNCaP human prostate cancer cell lines were used to investigate the growth inhibitory effects of selective COX-2 inhibitors in vitro. To complement these studies, we evaluated the effect of celecoxib on the growth of PC3 xenografts.

Results: COX-1 but not COX-2 was detected in PC3 and LNCaP cells. Clinically achievable concentrations (2.5-5.0 μmol/L) of celecoxib inhibited the growth of both cell lines in vitro, whereas rofecoxib had no effect over the same concentration range. Celecoxib inhibited cell growth by inducing a G1 cell cycle block and reducing DNA synthesis. Treatment with celecoxib also led to dose-dependent inhibition of PC3 xenograft growth without causing a reduction in intratumor prostaglandin E2. Inhibition of tumor growth occurred at concentrations (2.37-5.70 μmol/L) of celecoxib in plasma that were comparable with the concentrations required to inhibit cell growth in vitro. The highest dose of celecoxib led to a 52% reduction in tumor volume and an ~50% decrease in both cell proliferation and microvessel density. Treatment with celecoxib caused a marked decrease in amounts of cyclin D1 both in vitro and in vivo.

Conclusions: Two clinically available selective COX-2 inhibitors possess different COX-2-independent anticancer properties. The antitumor activity of celecoxib may reflect COX-2-independent inhibition in addition to COX-2-dependent effects.

INTRODUCTION

There are two isoforms of cyclooxygenase (COX), designated COX-1 and COX-2. These enzymes catalyze the first step in the synthesis of prostaglandins (PG) from arachidonic acid. COX-1 is expressed constitutively in most tissues and seems to be responsible for the synthesis of PGs that control normal physiologic functions (1). In contrast, COX-2 is not detected in most normal tissues. However, it is induced by a variety of mitogenic and inflammatory stimuli (2–7), which results in enhanced synthesis of PGs in neoplastic and inflamed tissues (8–10).

Multiple lines of evidence suggest that COX-2 is a promising target for the prevention and treatment of cancer. COX-2 is commonly overexpressed in premalignant and malignant tissues (11–17). The most specific data supporting a cause-and-effect relationship between COX-2 and carcinogenesis come from genetic studies. Multiparous female transgenic mice that were engineered to overexpress human COX-2 in mammary glands developed mammary gland hyperplasia, dysplasia, and metastatic tumors (18). In a related study, transgenic mice that overexpress COX-2 in skin developed epidermal hyperplasia and dysplasia (19). Consistent with these findings, knocking out COX-2 reduced the incidence of skin papillomas and intestinal tumors (20, 21). In addition to the genetic evidence implicating COX-2 in carcinogenesis, numerous pharmacologic studies suggest that COX-2 is a therapeutic target. Selective inhibitors of COX-2 reduce the formation, growth, and metastases of experimental tumors (22–29), and decrease the number of intestinal tumors in familial adenomatous polyposis patients (30). It should be stressed, however, that the antitumor effects of selective COX-2 inhibitors might reflect mechanisms in addition to inhibition of COX-2 (31–37). Hence, both COX-2-dependent and COX-2-independent mechanisms may contribute to the antitumor activity of selective COX-2 inhibitors.

Numerous investigators have evaluated the expression of COX-2 in human prostate cancer with conflicting results. Several studies have suggested that COX-2 is commonly overexpressed in prostate cancer (38–41). In contrast, others have found that levels of COX-2 are extremely low or absent in the majority of prostate cancers (42, 43). Importantly, even if COX-2 overexpression proves to be uncommon in prostate cancer, selective COX-2 inhibitors could still be active agents if the COX-2-independent effects are clinically important.
The main purpose of the present study was to investigate whether selective COX-2 inhibitors exhibit COX-2-independent antitumor properties. Androgen-insensitive (PC3) and androgen-responsive (LNCaP) human prostate cancer cell lines were evaluated because neither cell line expressed COX-2. Clinically achievable concentrations (2.5-5.0 μmol/L) of celecoxib, a selective COX-2 inhibitor, suppressed the growth of both cell lines (44, 45). In contrast, treatment with rofecoxib, a more potent selective COX-2 inhibitor, had no effect on cell growth over the same concentration range. Notably, celecoxib also caused dose-dependent inhibition of PC3 xenograft growth. In both *in vitro* and *in vivo* studies, celecoxib suppressed cyclin D1 levels. Taken together, these findings strongly suggest that celecoxib possesses significant COX-2-independent antitumor properties and could be active even in malignancies that do not express significant amounts of COX-2 in tumor cells.

MATERIALS AND METHODS

**Materials.** PC3 and LNCaP cell lines were from American Type Culture Collection (Rockville, MD). RPMI 1640 and fetal bovine serum were from Invitrogen (Green Island, NY). Celecoxib and rofecoxib were from LKT Laboratories, Inc. (St. Paul, MN). Enzyme immunoassay reagents for PGE2 assays were from Cayman Co. (Ann Arbor, MI). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue), DNase-free RNase A, butylated hydroxytoluene, EDTA, and lactate dehydrogenase diagnostic kits were from Sigma Chemical Co. (St. Louis, MO). Vector stain was from Vector Laboratories (Burlingame, CA). Anti-mouse CD34 antiserum was from PharMingen (La Jolla, CA). Anti-human Ki-67 (MIB-1) was obtained from Immunotech (Marseilles, France). Enhanced chemiluminescence Western blotting detection systems were from Perkin-Elmer Life Sciences, Inc. (Boston, MA). Male immunodeficient *nu/nu* mice were from Charles River Laboratories, Inc. (Wilmington, MA). Matrigel was from BD Biosciences (San Diego, CA). PGE2 and PGE2-d4 were purchased from Cayman Chemical.

**Cell Culture.** PC3 and LNCaP prostate cancer cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO2 incubator. All treatments with celecoxib, rofecoxib, or vehicle (0.1% Me2SO/DMSO) were done in the presence of 10% fetal bovine serum. Cellular cytotoxicity was assessed by measurements of lactate dehydrogenase release and trypan blue exclusion. There was no evidence of toxicity in any of our experiments.

**Western Blotting.** Cell lysates were prepared by treating cells with lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL trypsin inhibitor, and 10 μg/mL leupeptin. Lysates were sonicated for 20 seconds on ice and centrifuged at 10,000 × g for 10 minutes to sediment particulate material. The protein concentration of the supernatant was measured by the method of Lowry et al. (46). SDS-PAGE was done under reducing conditions on 10% polyacrylamide gels as described by Laemmli (47). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (48). The nitrocellulose membrane was then incubated with primary antibodies. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with enhanced chemiluminescence Western blot detection system according to the manufacturer’s instructions.

**Prostaglandin E2 Production in Cell Culture.** One thousand cells per well were plated in 96-well dishes and allowed to attach before treatment. Amounts of PGE2 released by cells were measured by enzyme immunoassay according to the manufacturer’s instructions. Production of PGE2 was normalized to cellular protein concentrations.

**Cell Growth.** PC3 cells (1 × 10^5^ cells/well) and LNCaP cells (2 × 10^5^ cells/well) were plated in 96-well plates. Cells were allowed to adhere and then treated with vehicle or selective COX-2 inhibitor. After treatment, the culture medium was removed and replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/mL). The reaction was stopped 4 hours later by removing medium and the formazan salt was allowed to dry. It was solubilized by adding 100 μL Me2SO and the absorbance measured at 560 nm in a 96-well plate reader.

**Flow Cytometry for Cell Cycle Analysis.** PC3 cells were treated with vehicle or 5.0 μmol/L celecoxib for 48 hours. Subsequently, the medium was replaced with fresh medium containing vehicle or celecoxib. After a total of 96 hours of treatment, the cells were released by treatment with trypsin-EDTA, washed and suspended in serum-free medium, counted, and then fixed overnight in 50% ethanol at 4°C. The cells (10^5^ cells/mL) were then washed and resuspended in PBS (pH 7.4), 0.1% Triton X-100, 0.05 mg/mL DNase-free RNase A, and 50 μg/mL propidium iodide before being maintained in the dark for 30 minutes at room temperature. The red fluorescence of single events was recorded using an argon ion laser at 488 nm excitation wavelength and 610 nm emission wavelength to measure DNA index on a flow cytometer (Coulter Epics XL flow cytometer, Hialeah, FL). The percentage of cells present in each phase of the cell cycle was determined using ModFIT software (Verity Software House, Topsham, ME).

**DNA Synthesis Assay.** Incorporation of [3H]thymidine was used to measure DNA synthesis. Cells were plated at 1 × 10^5^ cells/well in a 96-well plate and allowed to adhere overnight before being treated with vehicle or celecoxib for 24 hours at 37°C. Subsequently, the medium was replaced with fresh medium containing vehicle or celecoxib supplemented with [3H]thymidine (0.1 μCi/well) for 6 hours. Cells were then washed thrice with PBS. Radioactivity was then measured with a Beckman LS6800 liquid scintillation counter (Beckman, Fullerton, CA).

**PC3 Xenografts.** PC3 cells (1 × 10^6^) suspended in 50% Matrigel were implanted s.c. in the flanks of 8-week-old athymic male nude mice. Six days after tumor cell inoculation, small tumors were identified. The mice were randomly distributed to each of four groups (*n* = 12 mice/group): control diet (AIN-76A; Research Diets, Inc., New Brunswick, NJ) or control diet supplemented with three different doses of celecoxib (150, 450, and 750 ppm). Mice were treated with these different diets...
for the remainder of the experiment. Tumor growth was assessed twice a week by caliper measurement of tumor diameter in the longest dimension ($L$) and at right angles to that axis ($W$). Tumor volume was estimated by the formula $V = \frac{4}{3} \pi (W/2)^2 L$. At the time of sacrifice, plasma was prepared from blood collected by cardiac puncture. Tumors were excised and a portion fixed in 10% buffered formalin for 24 hours for immunohistochemical studies. A second portion of tumor was snap frozen and stored at $-80^\circ$C for measurements of PGE$_2$ and immunoblot analysis. The animal protocol was approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University.

**Determination of Prostaglandin E$_2$ in Tumor Tissues.** Approximately 50 mg of frozen tissue was ground to a fine powder using a liquid-nitrogen-cooled mortar (Fisher Scientific Co., Fair Lawn, NJ). The samples were then transferred to microcentrifuge tubes and three volumes of ice-cold PBS buffer containing 0.1% butylated hydroxytoluene and 1 mmol/L EDTA were added before further homogenization of the sample on ice using a Knotes pellet pestle (Fisher). An aliquot (100 $\mu$L) of homogenate was transferred to a glass tube (13 $\times$ 100 mm) and subjected to extraction of PGs using a method modified after Kempen et al. (49). Briefly, 20 $\mu$L citric acid (1 N) and 2.5 $\mu$L butylated hydroxytoluene (10%) were added to samples to prevent free radical peroxidation. Before the extraction, 10 $\mu$L deuterated PGE$_2$ (100 ng/mL) were added to each sample as an internal standard. PGs were then extracted with 1 mL of hexane/ethyl acetate (1:1, v/v) and vortexed for 2 minutes. Samples were centrifuged at 1,800 $g$ for 10 minutes at 4$^\circ$C. The upper organic layer was collected and the organic phases from three extractions were pooled and then evaporated to dryness under a stream of nitrogen at room temperature. All extraction procedures were done at minimum light levels and under cold ($4^\circ$C) conditions. Samples were then reconstituted in 100 $\mu$L methanol:10 mmol/L ammonium acetate buffer (pH 8.5, 70:30, v/v/v) before liquid chromatography-tandem mass spectrometry analysis. The protein concentration was determined by the method of Bradford (Bio-Rad, Hercules, CA).

Liquid chromatography-tandem mass spectrometry analyses were done using a Quattro Ultima tandem mass spectrometer (Micromass, Beverly, MA) equipped with an Agilent HP 1100 binary pump high-performance liquid chromatograph inlet. PGs were separated using a Luna 3 $\mu$m phenyl-hexyl 2 $\times$ 150 mm column (Phenomenex). Celecoxib was detected and quantified by operating the mass spectrometer in electrospray negative ion mode and monitoring the transition $m/z$ 380.2 > 316.1. Quantification was done by comparing the sample peak areas to a standard curve constructed from peak areas of extracted plasma sample added to known amounts of celecoxib.

**Immunohistochemistry.** Proliferation and angiogenesis were evaluated by staining for Ki-67 and CD34 as described previously (50, 51). Neutral buffered formalin-fixed tissue was embedded in paraffin. Tissue sections (5 $\mu$m) were prepared using a microtome and mounted on slides. Immunohistochemical analysis was done within 24 hours of the sections being cut. Sections were deparaffinized in xylene, rehydrated in graded alcohols, and washed in distilled water. Endogenous peroxidase activity was quenched with 0.01% H$_2$O$_2$. Antigen retrieval was done by microwaving the sections in 10 mmol/L citric acid (pH 6.0) for 30 minutes. In addition, sections for Ki-67 analysis were treated with 0.05% trypsin, 0.05% CaCl$_2$ in Tris-HCl (pH 7.6) for 5 minutes at 37$^\circ$C before microwave treatment. The slides were washed thrice in PBS and blocked for 30 minutes with 10% normal rabbit serum (CD34) or 10% normal horse serum (Ki-67). Tissue sections were then incubated with antisera to mouse CD34 at 25 $\mu$g/mL and antisera to Ki-67 at 1:5,000 dilution (2% bovine serum albumin in PBS) and incubated overnight at 4$^\circ$C. After being washed thrice with PBS, the sections were incubated with biotinylated anti-mouse immunoglobulins at 1:100 (CD34) or 1:500 (Ki-67; Vector Laboratories) dilution for 30 minutes at room temperature. The slides were then washed thrice in PBS and labeled using 1:25 avidin-biotin peroxidase complexes (Vector Stain) for 30 minutes at room temperature. The reaction was visualized using 3,3-diaminobenzidine. Subsequently, the slides were rinsed in tap water and counterstained with hematoxylin. The slides were then dehydrated with ethanol, rinsed with xylene, and mounted.

Proliferation was assessed by counting the number of Ki-67 positively staining nuclei and total number of cancer cells at $\times400$ magnification, in three representative regions of the tumor. Results are expressed as the proportion of positively staining cells over the total number of cells. Microvessel density was assessed by counting the number of microvessels at $\times200$ magnification in three fields that had the highest vascularization. The results are expressed as an average number of microvessels per field.

**Statistics.** Xenograft tumor growth was compared between dosage groups by fitting a quadratic curve to the log tumor volume from days 9 to 30 for each mouse. The linear and
RESULTS

COX-2 Is Not Expressed in Human Prostate Cancer Cell Lines. Levels of COX enzymes and PGE2 biosynthesis were evaluated in androgen-independent PC3 and androgen-dependent LNCaP prostate cancer cell lines. COX-2 was not detected by immunoblot (Fig. 1A) or Northern blot (data not shown) analysis in either cell line. In contrast, COX-1 was detected in both cell lines (Fig. 1B). We also used a pharmacologic approach to confirm the absence of COX-2 in these cell lines. Extremely low levels of basal production of PGE2 were detected in both PC3 and LNCaP cells (Fig. 1C). Consistent with the absence of COX-2 expression, neither treatment with 5.0 μmol/L celecoxib (Fig. 1C) nor 5.0 μmol/L NS398 (data not shown), another selective COX-2 inhibitor, suppressed PGE2 biosynthesis in PC3 or LNCaP cells.

Celecoxib Inhibited Prostate Cancer Cell Growth. As detailed above, it has been suggested that the anticancer properties of selective COX-2 inhibitors may be due, in part, to COX-2-independent effects. It was important, therefore, to investigate whether celecoxib or rofecoxib modulated the growth of either PC3 or LNCaP cells. As shown in Fig. 2A, treatment with celecoxib caused dose-dependent inhibition of PC3 cell growth. Treatment with 2.5 and 5.0 μmol/L celecoxib for 4 days inhibited cell growth by 17% and 37%, respectively (Fig. 2A). In contrast, rofecoxib, a selective COX-2 inhibitor that is more potent than celecoxib, did not suppress the growth of PC3 cells until concentrations of 10.0 μmol/L or more were used (Fig. 2A).

Flux cytometry was done to further define the mechanism by which celecoxib inhibited the growth of PC3 cells. Treatment with 5.0 μmol/L celecoxib led to an increase in the percentage of cells in G0-G1 (62-80%) and a corresponding decrease in the percentage of cells in S phase (23-9%; Fig. 3A). Celecoxib also caused a dose-dependent reduction in [3H] thymidine incorporation (Fig. 3B).

Changes in amounts of cyclin D1 can alter progression to S phase. To begin to elucidate the mechanism by which celecoxib caused a G0-G1 arrest, levels of cyclin D1 were measured. Treatment with 5.0 μmol/L celecoxib led to a profound decrease in the amounts of cyclin D1 (Fig. 4).

Celecoxib Inhibited PC3 Xenograft Growth. Based on the in vitro findings described above, it was important to investigate whether celecoxib had comparable growth inhibitory effects in vivo. The PC3 xenograft model was used to assess whether celecoxib inhibited tumor growth. Treatment with vehicle or celecoxib (150, 450, and 750 ppm) was initiated once small tumors had formed (Fig. 5). Celecoxib caused dose-dependent inhibition of tumor growth. At sacrifice, tumor volumes were reduced by 26%, 41%, and 52% in mice treated with 150, 450, and 750 ppm celecoxib, respectively. Notably, at the time of sacrifice, levels of celecoxib in plasma ranged from 2.37 μmol/L in the group treated with 150 ppm celecoxib to 5.7 μmol/L in the group that received 750 ppm celecoxib (Fig. 5).

These plasma levels of celecoxib are comparable with the concentrations used to suppress PC3 cell growth in vitro (Fig. 2). We also investigated whether treatment with celecoxib...
suppressed intratumor PGE2 levels. A small statistically insignificant decrease in amounts of PGE2 was detected in tumors from mice in each of the groups treated with celecoxib (Fig. 5). Thus, whereas celecoxib caused dose-dependent inhibition of tumor growth, levels of PGE2 were essentially identical in tumor tissues from the different treatment groups.

**Treatment with Celecoxib Inhibited Angiogenesis and Cell Proliferation in PC3 Xenografts.** The inhibition of tumor growth by celecoxib could reflect reduced cell proliferation or increased apoptosis. Examination of H&E-stained tumor sections from mice that received control or celecoxib supplemented diets revealed very few apoptotic bodies in either group and further evaluation of apoptosis was not pursued (data not shown). Nuclear staining of Ki-67 was used to assess cell proliferation. Treatment with celecoxib caused nearly a 50% decrease in cell proliferation ($P < 0.001$; Fig. 6A, B, and E). Because inhibition of angiogenesis can contribute to reduced cell proliferation, we also determined the effects of celecoxib on microvessel density. Angiogenesis was evaluated by staining for CD34, an antigen present in endothelial cells. Microvessel density was measured using an image analysis system. The results showed a significant decrease in microvessel density in the celecoxib-treated group compared to the control group ($P < 0.05$; Fig. 7A and B).
density decreased by almost 50% in tumors from mice treated with celecoxib \((P = 0.02\); Fig. 6C, D, and E). Reduced cell proliferation occurred in regions of the tumor in which significant reductions in angiogenesis were detected, suggesting that decreased cell proliferation may be a consequence of reduced angiogenesis. In an attempt to define the mechanism underlying celecoxib-mediated inhibition of tumor cell proliferation in xenografts, immunoblot analysis was done. As shown in Fig. 7, treatment with celecoxib led to a profound decrease in amounts of cyclin D1. These findings are similar to the results in PC3 cells treated with celecoxib in vitro (Fig. 4).

**DISCUSSION**

Several investigators have suggested that selective COX-2 inhibitors possess anticancer activity because of both COX-2-dependent and COX-2-independent effects (11, 31–37). This raises the intriguing possibility that selective COX-2 inhibitors may be active agents even when COX-2 is not detected in a target tissue. Two studies found that COX-2 was expressed in prostate cancer much less frequently than in many other epithelial malignancies (42, 43). With this in mind, we elected to focus on prostate cancer to determine whether selective COX-2 inhibitors possess important COX-2-independent antitumor properties.

Initially, we found that COX-2 was not expressed in either PC3 or LNCaP cells. This conclusion was based on the results of immunoblotting, Northern blot analysis, and the inability to suppress PGE2 biosynthesis with two different selective COX-2 inhibitors. Although the absence of COX-2 in these cell lines agrees with some reports (42, 52), other investigators have detected COX-2 in PC3 cells (34, 53). This apparent difference is likely to reflect the use of different clones by different laboratories. It is noteworthy that clinically achievable concentrations of celecoxib (44, 45) suppressed the growth of both PC3 and LNCaP cell lines whereas rofecoxib had no effect over the same concentration range. Because COX-2 is not expressed in these two cell lines, the growth inhibitory effect of celecoxib must reflect a COX-2-independent mechanism. Although other studies have suggested that selective COX-2 inhibitors including celecoxib possess COX-2-independent activity (31–37), the significance of this work has been questioned because extremely high concentrations of drug were used (31, 54, 55). To our knowledge, this is the first study to show that a selective COX-2 inhibitor has COX-2-independent antitumor properties using a concentration of drug that is clinically achievable (44, 45).

To further investigate the mechanism underlying the growth inhibitory effects of celecoxib, flow cytometry was done. Celecoxib caused an accumulation of cells in G0-G1 and

![Fig. 4](image-url)

*Fig. 4* Celecoxib inhibits cyclin D1 expression in PC3 cells. PC3 cells were treated with vehicle (C) or 5.0 \(\mu\)mol/L celecoxib (T) for 24 or 96 hours. Cellular lysate protein (100 \(\mu\)g/lane) was loaded onto a 10\% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibodies to cyclin D1 and \(\beta\)-actin, respectively. Reduced levels of cyclin D1 were detected following 96 hours of treatment with celecoxib.

![Fig. 5](image-url)

*Fig. 5* Celecoxib causes dose-dependent inhibition of PC3 xenograft growth. PC3 cells were inoculated into the flanks of nude mice. Six days after tumor cell inoculation, mice were randomized to control diet (vehicle) or control diet supplemented with 150, 450, or 750 ppm celecoxib (12 mice/group). Tumor volume was assessed at multiple time points for all mice. At the time of sacrifice, plasma was obtained and levels of celecoxib determined. Intratumoral amounts of PGE2 were also measured. Points, means; bars, SE. Statistically significant inhibition of tumor growth was achieved at all celecoxib doses compared with vehicle fed mice (\(aP < 0.05\), \(bP < 0.01\)). Treatment with 750 ppm led to greater inhibition of tumor growth than treatment with either 150 ppm (\(cP < 0.01\)) or 450 ppm (\(dP < 0.05\)) celecoxib. Treatment with celecoxib did not cause a statistically significant decrease in levels of intratumoral PGE2.
a corresponding decrease in S phase. Similar effects of celecoxib have been observed in rat prostate cancer cells (56). Consistent with these results, a dose-dependent decrease in [3H]thymidine incorporation was observed in our study. Changes in cyclin D1 expression impact on the progression of cells from G1 to S. Hence, the discovery that treatment with celecoxib caused a significant reduction in amounts of cyclin D1 is significant.

We next used an experimental model of prostate cancer to attempt to translate these in vitro findings. Treatment with celecoxib caused a dose-dependent decrease in the growth of PC3 xenografts. To better understand the growth inhibitory effects of celecoxib, measurements of microvessel density and cell proliferation were done. Treatment with celecoxib caused nearly a 50% decrease in both microvessel density and cell proliferation. These findings are consistent with prior evidence that selective COX-2 inhibitors can suppress the growth of transplantable tumors by inhibiting angiogenesis and cell proliferation (26, 28, 57–60). Although celecoxib inhibited the growth of cultured PC3 cells by a COX-2-independent mechanism, we acknowledge that our in vivo findings do not allow us to exclude a COX-2-dependent growth inhibitory effect. Because COX-2 is expressed in neovascularature (58), celecoxib may have inhibited tumor growth at least, in part, by a COX-2-dependent antiangiogenic mechanism. Because the vasculature comprises a relatively small proportion of the xenograft, celecoxib may have suppressed tumor growth by inhibiting angiogenesis without causing a decrease in intra-tumor PGE2. Based on our in vitro findings, levels of cyclin D1 were measured in tumor samples. Similar to what was observed in cultured PC3 cells, celecoxib caused a reduction in amounts of cyclin D1 in PC3 xenografts. This finding is consistent with previous evidence that a selective COX-2 inhibitor suppressed levels of cyclin D1 in an experimental model of colorectal carcinoma (29). Importantly, the suppressive effects that we observed occurred at plasma concentrations of celecoxib that were essentially identical to the doses required to induce COX-2-independent changes in vitro. Thus, it remains uncertain if the observed decrease in tumor cell proliferation in PC3 xenografts is a consequence of reduced angiogenesis, a COX-2-independent growth inhibitory effect of celecoxib as observed in vitro, or a combination of both mechanisms.

In summary, our in vitro studies show that clinically achievable concentrations of celecoxib inhibit the growth of prostate cancer cells by a COX-2-independent mechanism. Moreover, the discovery that celecoxib and rofecoxib have different COX-2-independent effects raises the possibility that the antitumor activity of selective COX-2 inhibitors could differ. Currently, the dose of selective COX-2 inhibitor used in chemoprevention and cancer treatment studies is based on the amount of drug required to inhibit COX-2 activity (61). Whether different doses will be required to optimize the COX-2-independent effects and therefore the antitumor activity of these drugs should be explored. We emphasize that the results of this study do not detract from the substantial body of evidence indicating that COX-2 is a rational target for anticancer therapy. Our study does suggest, however, that COX-2-independent effects may also be important for understanding the overall antitumor activity of selective COX-2 inhibitors.

### Table 1

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<th>Proliferative Index</th>
<th>Microvessel Density</th>
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<td>Control</td>
<td>71.2±8.8%</td>
<td>10.5±3.2</td>
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<tr>
<td>Treated (750 ppm celecoxib)</td>
<td>38.7±10.6%*</td>
<td>5.7±2.6**</td>
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**Fig. 6** Celecoxib inhibits cell proliferation and angiogenesis in PC3 xenografts. PC3 cells were inoculated into the flanks of nude mice. Six days after tumor cell inoculation, mice were randomized to control diet (vehicle) or control diet supplemented with 750 ppm celecoxib. After 25 days of treatment with control or celecoxib-containing diet, mice were sacrificed. Tumor tissue was formalin fixed and paraffin embedded. Immunohistochemistry was done to assess cell proliferation (Ki-67) and vascular density (CD34). Representative tumor sections stained for Ki-67 are shown (×400) from mice fed control diet (A) or 750 ppm celecoxib (B). Representative tumor sections stained for CD34 are shown (×400) from mice fed control diet (C) or 750 ppm celecoxib (D). E, summary of the findings for proliferative index and microvessel density in tumors from mice fed control diet versus control diet supplemented with 750 ppm celecoxib. Data are means ± SD (n = 6 per group; *, P < 0.001 and **, P < 0.02).

**Fig. 7** Celecoxib inhibits cyclin D1 expression in PC3 xenografts. PC3 cells were inoculated into the flanks of nude mice. Six days after tumor cell inoculation, mice were randomized to control diet (vehicle) or control diet supplemented with 750 ppm celecoxib. After 25 days of treatment with control or celecoxib-containing diet, mice were sacrificed and tumor tissue procured. Tumor cell lysate protein (100 g/lane) from six control mice (lanes 1–6) and six celecoxib-treated mice (lanes 7–12) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibodies to cyclin D1 and β-actin, respectively. Xenografts from celecoxib-treated mice showed a marked reduction in amounts of cyclin D1.
REFERENCES


