Suppression of Prostate Carcinogenesis by Dietary Supplementation of Celecoxib in Transgenic Adenocarcinoma of the Mouse Prostate Model

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ABSTRACT

Epidemiological studies and clinical observations suggest that nonsteroidal anti-inflammatory drugs and certain selective cyclooxygenase (COX)-2 inhibitors may reduce the relative risk of clinically evident prostate cancer. This prompted us to investigate the chemopreventive potential of celecoxib, a selective COX-2 inhibitor, against prostate carcinogenesis in a transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Similar to prostate cancer in humans, prostate malignancies in TRAMP mice progress from precursor intraepithelial lesions, to invasive carcinoma that metastasizes to lymph nodes, liver, lungs, and occasionally to bone. The basal enzyme activity and protein expression of COX-2 is significantly higher (4-fold) in the dorsolateral prostate of TRAMP mice up to 24 weeks of age compared with their nontransgenic littersmates. Eight-week-old TRAMP mice were randomly divided and fed either control diet (AIN 76A) or a custom prepared AIN 76A diet containing 1500-ppm celecoxib. These animals exhibited substantial reduction in tumor development (50% of 20 mice on test, showed that the cumulative incidence of prostate cancer was lower in animals fed with AIN 76A diet containing 1500-ppm celecoxib compared with the AIN 76A-fed group. Histopathological examination of celecoxib-fed animals showed reduced proliferation, and down-modulation of COX-2 and prostaglandin E2 receptor levels in the dorsolateral prostate and plasma, respectively. These results correlated with retention of antitumorigenic markers, viz E-cadherin, and α- and β-catenin, along with a significant decrease in vascular endothelial growth factor protein expression. Celecoxib supplementation also resulted in enhanced in vivo apoptosis in the prostate as monitored by several techniques including a recently perfected technique of 32P-dCTP-labeled annexin V in live animals followed by phosphor imaging. One striking observation in an additional study was that celecoxib feeding to mice with established tumors (16 weeks of age) significantly improved their overall survival (P = 0.014), compared with AIN 76A-fed group. Our findings suggest that celecoxib may be useful in chemoprevention of prostate cancer.

INTRODUCTION

In the search for agents that will prevent or delay the onset of cancer, it has become apparent that cyclooxygenase (COX)-2 is a promising therapeutic target (1–5). COX-2 is an inducible isoform of COX, an enzyme that catalyzes the rate-limiting step in prostaglandin synthesis from arachidonic acid (6). Arachidonic acid and its derivatives are involved in inflammation; COX-2 is rapidly induced by cytokines, growth factors, tumor promoters, oncogenes, and carcinogens (7). In cell culture studies and animal models, induction of COX-2 has been shown to promote cell growth, inhibit apoptosis, and enhance cell motility and adhesion (7, 8). Aberrant expression of COX-2 has been implicated in the pathogenesis of many cancer types and has been shown to be up-regulated in transformed and cancer cells (7–10). Previous studies by our group and other investigators have shown that COX-2 is overexpressed in human prostate adenocarcinoma (11–16). Recent molecular studies on prostate tumors indicate that COX-2 is inappropriately induced in prostate cancer and is prominently expressed in proliferative inflammatory atrophy, a putative precursor lesion of prostate cancer (17). Although there has not been a common consensus on the association of COX-2 with disease stage and/or type of COX-2-expressing cells, it is generally agreed that COX-2 overexpression is associated with prostate carcinogenesis, a concept supported by a recent in vivo study showing that introduction of full-length COX-2 cDNA in prostate carcinoma cells promotes cancer progression (18). On the basis of these studies it has been suggested that selective inhibition of COX-2 may be useful for prevention and/or therapy of prostate cancer (19). This approach is most logical for prostate cancer, because it is relatively indolent in the great majority of cases and even a modest delay in its development achieved through pharmacological or nutritional intervention could result in a substantial reduction in the incidence of clinically evident disease.

Epidemiological studies and clinical observations suggest that regular consumption of nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of certain types of human cancer (19–22). Conventional NSAIDs block the activity of both forms of COX. Unfortunately, NSAID usage is associated with gastrointestinal toxicity and altered platelet function (23), side effects that have prompted the search for selective COX-2 inhibitors, such as celecoxib (24). Studies have shown that selective inhibition of COX-2 by celecoxib reduces inflammation and side effects associated with traditional NSAIDs (25). In addition to its usefulness in the management of osteoarthritis, celecoxib has shown promise in the field of human cancer prevention. Several studies have demonstrated that celecoxib possesses anticancer properties in both carcinogen-induced and genetically manipulated murine carcinogenesis models (26–33). Celecoxib has been approved by the United States Food and Drug Administration for adjuvant treatment of familial adenomatous polyposis, an inherited syndrome that predisposes individuals to colon cancer (34). In addition, the chemopreventive effects of celecoxib have been tested in numerous clinical trials addressing a variety of carcinomas including bladder, colon, esophagus, and skin cancer (19–22). Because earlier studies have verified that COX-2 is overexpressed in human prostate malignancies, we hypothesized that inhibition of COX-2 may prevent development and progression of prostate cancer.

In recent years, genetically engineered mouse models have emerged as powerful tools in understanding many aspects of cancer and have provided opportunities for conducting studies on cancer prevention.
and therapy at various stages of disease progression (35, 36). Transgenic adenocarcinoma of the mouse prostate (TRAMP) has emerged as an appropriate animal model for evaluation of chemopreventive and therapeutic agents against prostate cancer, as it exhibits molecular pathways of multistage prostate tumorigenesis (37–43). Male TRAMP mice express a PB-Tag transgene consisting of the minimal −426/+28 bp regulatory element of the rat probasin promoter directing prostate-specific epithelial expression of the SV40 early genes (T and t; Tag; Ref. 37). Prostate cancer progresses in this model in a typical fashion, from a neoplastic intraepithelial lesion (prostatic intraepithelial neoplasia; PIN), to invasive carcinoma that metastasizes to lymph nodes, liver, lungs, and bone (43, 44). During our initial studies, we established that the basal level of COX-2 enzyme activity and protein expression is higher in the dorsolateral prostate of TRAMP mice up to 24 weeks of age compared with their nontransgenic littermates. Therefore, we tested the efficacy of celecoxib in altering the onset and progression of prostate cancer in these animals by measuring the growth of primary tumor, and effects on distant site metastases, intermediate, and end point markers of prostate cancer progression.

MATERIALS AND METHODS

Animals. Male and female heterozygous C57BL/TGN TRAMP mice, Line PB Tag 8247NG, were purchased as breeding pairs from The Jackson Laboratory (Ann Arbor, MI). The animals were bred and maintained at the Assocation for Assessment and Accreditation of Laboratory Animal Care-accredited Animal Resource Facility of Case Western Reserve University. Housing and care of the animals was in accordance with the guidelines established by the University’s Animal Research Committee and with the NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic males for these studies were routinely obtained as [TRAMP X C57BL/6]F1 or as [TRAMP X C57BL/6]F2 offspring. Identity of transgenic mice was established by PCR-based DNA-screening as described previously (39, 40).

Study Design and Celecoxib Supplementation. Custom-prepared AIN 76A diet pellets containing 1500-ppm celecoxib, a selective COX-2 inhibitor, and regular AIN 76A diet pellets were obtained from ICN Biochemicals (Cleveland, OH). This dietary preparation of celecoxib has been successfully used in murine cancer models in many prior chemoprevention studies and is based on the recommended human dose of celecoxib (26–32). Celecoxib-supplemented AIN 76A diet was provided to TRAMP and nontransgenic littermates beginning at 8 weeks of age, and was continued until the animals were 32 weeks old, at which time the experiment was terminated. Throughout the experiment the animals had access to drinking water and food ad libitum. For each experiment, 20 male TRAMP mice of 8 weeks of age were divided into two equal groups of 10 mice. The experimental group of animals was fed with the custom-prepared AIN 76A diet supplemented with 1500 ppm celecoxib, whereas the control group of animals was fed regular AIN 76A diet pellets. This feeding regimen was well tolerated by TRAMP and nontransgenic littermates. At the termination of the experiment, blood was collected from the retro-orbital plexus under anesthesia from both experimental and control groups. The animals were then sacrificed by cervical dislocation and examined for distant metastases. The genito-urinary (GU) apparatus consisting of bladder, urethra, seminal vesicles, ampullary gland, and the prostate was excised, weighed, and a small portion was fixed overnight in (10%) zinc-formalin. Sections (4 μm) were stained with H&E as described previously (39, 40). Histological sections were reviewed by light microscopy for the presence of prostate cancer and classified as PIN (epithelial stratification with occasional mitotic figures or cribriform pattern), well differentiated (multiple epithelial mitotic figures and apoptotic bodies, invasive glands with stromal hypercellularity), moderately differentiated (many acini completely filled with tumor yet still forming some glandular structures), or poorly differentiated (sheets of malignant cells with little or no gland formation) prostate cancer, or atrophic glands only (no identifiable tumor).

Metastases Examination. Metastasis to lymph nodes and liver was observed under the microscope. The India ink method was used to examine cancer metastasis to lungs as described previously (39, 40). Briefly, animals from control and treated groups were sacrificed, and the respiratory system was excised. India ink was injected through the trachea into the lungs until completely filled. The trachea was then tied with a thread. The ink fills normal respiratory structures and metastases stand out as unstained regions.

COX-2 Enzyme Activity. COX-2 activity was assayed using the chemiluminescent COX activity kit from Assay Designs, Inc. (Ann Arbor, MI) by following the manufacturer’s protocol. COX activity kit uses a chemiluminescent COX activity kit from Assay Designs, Inc. (Ann Arbor, MI) by following the manufacturer’s protocol. COX activity kit uses a chemiluminescent substrate to detect the peroxidative activity of COX enzymes. After inhibition by nonsteroidal anti-inflammatory drug, NS-398 (specific COX-2 inhibitor), the direct residual activity of COX was measured by addition of arachidonate and a proprietary luminescent substrate. Light emission, directly proportional to the COX activity in the sample, was measured over 5 s. COX-2 activity was calculated by subtracting COX-1 activity from total COX activity and expressed as relative light units/ml.

Immunoblot Analysis. The dorsolateral prostate was removed from both treated and control groups, homogenized in lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM NaVO4, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (pH 7.4)] at 4°C to prepare cell lysates. The protein concentration was determined by DC Bio-Rad assay using the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA). Appropriate amount of protein (25–50 μg) was resolved over 8–14% Tris-glycine polyacrylamide gel electrophoresis.
gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% nonfat dry milk and probed using appropriate primary antibody of COX-2 (mouse monoclonal, BD Transduction Laboratories), E-cadherin (mouse monoclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), proliferation cell nuclear antigen (PCNA; mouse monoclonal, Santa Cruz Biotechnology, Inc.), α-catenin (rabbit polyclonal, Santa Cruz Biotechnology, Inc.), β-catenin (goat polyclonal, Santa Cruz Biotechnology, Inc.), vascular endothelial growth factor (VEGF; mouse monoclonal, Santa Cruz Biotechnology, Inc.), and PAR-4 (mouse monoclonal, Santa Cruz Biotechnology, Inc.) in blocking buffer overnight at 4°C. The membrane was then incubated with antimouse or antirabbit secondary antibody horseradish peroxidase conjugate (Amersham Life Sciences Inc., Arlington Heights, IL) followed by detection using chemiluminescence ECL kit (Amersham Life Sciences Inc.). Equal loading of protein was confirmed by stripping the membrane and reprobing it with α-tubulin primary antibody (Santa Cruz Biotechnology) and appropriate secondary horseradish peroxidase conjugate.

**Immunohistochemical Analysis.** Sections 1 μm in thickness were cut from paraffin-embedded prostate tissues. Immunostaining was performed using antibody against COX-2 (1:200 dilution) as described previously (39). The stained slides were visualized on a Zeiss-Axioptix DM HT microscope (Zeiss-Axioptix, Oberkochen, Germany). Images were captured with an attached camera linked to a computer.

**Apoptosis by ELISA.** Apoptosis was assessed by Cell Death Detection ELISAPLUS® kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol. Briefly, a portion of dorsolateral prostate tissue was incubated on ice for 30 min in Tris lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 0.5% NP40, and 1% Triton-X-100] containing fresh protease inhibitors (5 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml sodium vanadate), homogenized in microfuge tube and then centrifuged at 12,000 rpm for 10 min at 4°C. The total tissue lysate was used for protein determination by the DC Bio-Rad protein assay. The lysates (30 μg of total protein) were added to lysis buffer provided with the kit and pipetted on a streptavidin-coated 96-well microtiter plate to which immunoreagent mix was added and incubated for 2 h at room temperature with continuous shaking at 500 rpm. The wells were then washed with washing buffer, and color was developed by addition of substrate solution, which was read at 405 nm against the blank, reference wavelength of 490 nm after 10–15 min. The enrichment factor (total amount of apoptosis) was calculated by dividing the absorbance of the sample (A405 nm) by the absorbance of the controls without treatment (A400 nm).

**In Vivo Apoptosis Determination by 99mTc-Annexin V Labeling.** In vivo apoptosis was serially determined in real time on live animals by using 99mTc-annexin V-labeled product as described recently (45). Briefly, the control and treated groups of animals were injected with 50 μCi (5.55 MBq) of 99mTc-annexin V under anesthesia. The animals were visualized on a storage phosphor screen MP (Packard Instruments Co., Meriden, CT) and left there in dark for 10 min. In addition, a small 137Cs point source as a geometric marker was placed on the phosphor screen, and an optical photograph of the animals was taken. The autoradiograph was developed using a cyclone Phosphor Imager (Packard Instruments Co.) and analyzed using Optiquant software.

**RNA Isolation and Reverse Transcription.** Total RNA was isolated from the prostate of control and treated group of animals using the RNAeasy Total RNA isolation kit (Qiagen, Valencia, CA), and was checked for its purity and concentration as described previously (11, 46). Two μg of total RNA was reverse transcribed using murine leukemia virus-reverse transcriptase and oligo(dT)12–18 primer from Life Technologies, Inc. (Gaithersburg, MD) for cDNA synthesis.

**Semiquantitative PCR for COX-2.** Two μl of the reverse transcription product was subjected to PCR in 25-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each deoxynucleoside triphosphate, 2 μg of AmpliTaq polymerase, and 40 pmol of each primer for COX-2 (sense primer, 5′-GGTGCTGTGGCTGCTGTAG3′; antisense primer, 5′-GTCCTTCAGAGGAATGTCG-3′) obtained from Oxford Biomedical Research, Inc. (Rochester Hills, MI). About 10-μl aliquots (having equal RNA) of the reverse-transcribed cDNA samples were added to the reaction mix and amplified for 35 cycles, denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 10 min. A constitutively expressed gene, β2-microglobulin, was used as an internal control, generating a 266-bp PCR product. Ten μl of PCR products were loaded on 1.5% agarose gels and visualized by ethidium bromide staining. Negative control of COX-2, which contained no reverse transcriptase, showed no PCR products.

**Statistical Analysis.** All of the statistical analyses were carried out with Statistical Analysis System software (SAS Institute, Cary, NC) and Ps < 0.05 were considered significant. For measurement of prostate and GI weight, Kruskal-Wallis test, a nonparametric test based on Wilcoxon scores, was used to examine the mean/median difference between two groups by using box plots. For other continuous measurements, the difference between two groups was examined by Student’s t test after checking normality. Overall survival between the two groups was measured from the start of treatment date to date of death, and censored at the date of last follow-up (60 weeks for survivors). Survival distribution was estimated using Kaplan-Meier method, and the difference between groups was examined using log rank test (47).

**RESULTS**

**COX-2 Enzyme Activity and Protein Expression in TRAMP Mice.** COX-2 is an inducible isofrom of COX (the enzyme that catalyzes the rate-limiting step in prostaglandin synthesis from arachidonic acid) associated with inflammation and carcinogenesis, and is found to be up-regulated in most forms of human cancer (Refs. 6–16 and references therein). In the first experiment, we compared the levels of COX-2 enzyme activity and protein expression in the dorsal and lateral lobes of the prostate between male TRAMP mice and their age- and sex-matched nontransgenic littersmates. We chose to evaluate these prostate lobes because of the previous findings demonstrating frequent transformation from normal to high-grade prostate intraepithelial neoplasia in these lobes at 8 weeks of age that subsequently progress to carcinoma at 24 weeks of age (44). As shown in Fig. 1A, COX-2 enzyme activity was significantly higher in TRAMP mice at 8, 16, and 24 weeks (2.0-fold, 3.5-fold, and 6.3-fold, respectively) compared with that in nontransgenic littersmates. Similarly, COX-2 protein expression in 8-, 16-, and 24-week-old TRAMP mice was 3.2-, 4.5-, and 6.8-fold higher than in nontransgenic littersmates (Fig. 1B). After 24 weeks of age a progressive loss in COX-2 protein expression was observed in the prostate of TRAMP mice (data not shown).

**Immunohistochemical Studies of COX-2 Expression in TRAMP Mice.** Prostate cancer in TRAMP mice progresses from a precursor lesion, PIN, evident as early as 8 weeks, to well-differentiated cancer (10–18 weeks), moderately differentiated cancer (18–24 weeks), and later to poorly differentiated cancer (after 24 weeks). The histopathological findings in the prostates of TRAMP mice of various ages have been well documented (43, 44). We performed immunohistochemical staining for COX-2 in the prostates of TRAMP mice and age-matched nontransgenic littersmates (Fig. 1C). COX-2 immunostaining was not detected in prostates of nontransgenic littersmates of various age groups (8–24 weeks; n = 12; Fig. 1C, panels a–c), whereas increased COX-2 immunostaining was noted in the prostates of TRAMP mice from 8–24 weeks of age (Fig. 1C, panels d–g). Focal positive immunostaining for COX-2 in PIN lesions was noted in 50% of TRAMP mice at 8–10 weeks of age (n = 6); no significant immunostaining was observed in the rest of the animals. Focal COX-2 immunopositivity was evident in 66% (n = 9) of prostates from 15–16-week-old TRAMP mice with insignificant staining in the remainder. Staining was present in multifocal well-differentiated cancers that had developed in TRAMP mice of this age (Fig. 1C, panel d). Prostates of TRAMP mice 22–24 weeks of age also contained multiple foci of adenocarcinoma, which demonstrated prominent COX-2 immunostaining in 6 of 9 animals in this group, whereas remaining animals exhibited moderate to weak COX-2 immunostaining (Fig. 1C, panels e and f). In sharp contrast, COX-2 immu-
nostaining was absent in all of the TRAMP mice with adenocarcinoma at 32–36 weeks (n = 5) and metastatic carcinoma at 40–44 weeks of age (n = 3; Fig. 1C, panels h and i). Because COX-2 appears to be overexpressed during selective stages of prostate carcinogenesis in TRAMP mice, this observation offered a unique opportunity to study the efficacy of a specific COX-2 inhibitor, celecoxib, in preventing the development and progression of prostatic cancer in these animals.

Effect of Celecoxib Feeding on Prostate Tumorigenesis in TRAMP Mice. Celecoxib feeding for 24 weeks did not significantly affect the body weight gain profile of TRAMP mice compared with their nontransgenic littermates. Celecoxib-fed TRAMP mice weighed less than TRAMP mice on the regular AIN 76A diet, perhaps because of the differences in GU apparatus weight between these groups (Fig. 2).

To investigate the effect of celecoxib feeding on growth and progression of prostate cancer in TRAMP mice, groups of these animals were fed either a standard AIN 76A diet or else an AIN 76A diet supplemented with celecoxib. Feeding with these diets commenced at 8 weeks of age, continued for 24 weeks, and was terminated at 32 weeks. As summarized in Table 1, in the first experiment, 10 of the 10 TRAMP mice receiving AIN 76A diet developed palpable tumors by age 32 weeks. Autopsy in these animals revealed tumor masses involving prostate and seminal vesicles, as well as metastases to lymph nodes (7 of 10), lungs (4 of 10), and liver (3 of 10). In contrast, only 2 of 10 celecoxib-fed TRAMP mice developed palpable tumors. Only moderate enlargement of prostate and seminal vesicles was
Fig. 3. Effect of celecoxib feeding on prostate volume in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice evaluated by longitudinal magnetic resonance imaging analysis. A, magnetic resonance imaging was used to assess neoplastic growth of dorsolateral prostate in TRAMP mice followed longitudinally in individual animal. A marked reduction in prostate growth was observed in TRAMP mice fed with celecoxib between 8–32 weeks, compared with TRAMP fed with AIN 76A diet. Representative image of TRAMP control (a–c) at 16, 24, and 32 weeks of age and after celecoxib feeding after 8, 16, and 24 weeks (d–f) are shown. Arrow indicates prostate. B, volumetric analysis of TRAMP prostate after celecoxib feeding. The data are represented as total pixels observed at 8, 16, and 24 weeks of celecoxib feeding. Values represent mean of six animals: bars, +SE. *P < 0.001, Student’s t test. TRAMP celecoxib-fed versus TRAMP control.

noted in 8 of 10 of these animals, and none exhibited metastatic cancer. In the repeat experiment, all 10 of the mice in the control group exhibited invasive prostate tumors with frequent metastases to lymph nodes (6 of 10), lungs (3 of 10), and liver (1 of 10), respectively. In the celecoxib-fed group, none of the mice exhibited any distant metastases.

During the course of these experiments we also monitored prostate growth in these animals using a recently perfected technique of MRI. Prostate volumes measured by MRI at 16, 24, and 32 weeks of age on the regular AIN 76A diet were greater than prostate volumes of male nontransgenic littermates (data not shown), consistent with development and progression of prostate cancer. Prostate volumes in celecoxib-fed male TRAMP mice were substantially less than those on a regular AIN 76A diet (Fig. 3A). Celecoxib-fed TRAMP mice exhibited a significant reduction in the development of prostate cancer measured at 8 weeks on test (16-week-old animals; ~40% inhibition), 16 weeks on test (24-week-old animals; ~42% inhibition), and 24 weeks on test (32-week-old animals; ~35% inhibition), respectively as observed by volumetric analysis of the prostate (Fig. 3B). This inhibitory effect of celecoxib feeding on prostate carcinogenesis was also evident from abdominal pelvic palpation, and from assessment of the weights of the GU-apparatus and prostates of these groups of mice. As shown by the box plots, celecoxib feeding to TRAMP mice resulted in a significant decrease in average prostate weight (~56% reduction; P < 0.0003) and GU-weight (~48% reduction; P < 0.008) compared with AIN 76A fed TRAMP mice (Fig. 4, A and B). In addition, celecoxib feeding led to a significant decrease in seminal vesicle size (Fig. 5, A and B).

Effect of Celecoxib Feeding on Prostate Histology in TRAMP Mice. As noted previously, the histological findings in TRAMP mice of various ages have been well documented, and it is known that TRAMP mice older than 24 weeks typically have poorly differentiated prostate adenocarcinoma (43, 44). We elected to evaluate the dorsolateral prostates of TRAMP mice in control and experimental groups of animals ~32 weeks old. Prostates of AIN 76A-fed TRAMP mice, as expected, exhibited cancers of variable size, predominantly poorly differentiated carcinomas (~60%) composed of sheets of anaplastic cells with scant cytoplasm and marked nuclear pleomorphism. Occasional scattered apoptotic cells were noted (Fig. 5C). The histological findings in prostates of celecoxib-fed TRAMP mice at 32 weeks were significantly different. Prostates of 12 of 20 celecoxib-fed TRAMP mice contained well-differentiated adenocarcinoma that occupied ~10% at surface area, admixed with lesser amounts of moderately differentiated adenocarcinoma (~3% of surface area) and poorly differentiated adenocarcinoma (~1% of surface area). The well-differentiated cancers exhibited abundant apoptotic cells (Fig. 5D). The remaining celecoxib-fed animals (8 of 20) had multiple foci of moderately differentiated carcinoma (10–20% of surface area) and poorly differentiated carcinoma (~5% of surface area; data not shown).

Fig. 4. Effect of celecoxib feeding on prostate and genito-urinary (GU) weight in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice. A, box plot for prostate weight, and (B) GU weight of TRAMP mice after celecoxib feeding. A marked reduction in prostate and GU weight was observed after celecoxib feeding. I represent minimum-maximum value; represent 25–75% value; and represents median value. Kruskal-Wallis test, TRAMP celecoxib-fed versus TRAMP control.

Fig. 5. Effect of celecoxib feeding on genito-urinary (GU) apparatus and prostate histology in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice. A, photographs of typical GU apparatus of TRAMP mice exhibiting hyper-proliferation and increased vascularization. B, GU apparatus of TRAMP mice after celecoxib feeding for 24 weeks. A marked decrease in GU weight and volume was observed in TRAMP mice after celecoxib feeding. C, histological examination of a typical TRAMP mouse prostate at 32 weeks of age revealed poorly differentiated prostate cancer characterized by pronounced proliferation of papillary structures lined by pseudo-stratified neoplastic cells with marked hyperchromatia and scattered apoptotic cells. (Magnification: ×400). D, celecoxib feeding to TRAMP mice exhibited acini with many epithelial nuclei elongated and hyperchromatic with clumping of chromatin, and sporadically dark stained cells. (Magnification: ×400). Representative figures are shown.
Effect of Celecoxib Feeding on COX-2 Enzyme Activity, mRNA, and Protein Expression in TRAMP Mice. TRAMP males exhibit high basal levels of COX-2 enzyme activity, mRNA, and protein expression that significantly increases with disease progression at selective stages during prostate carcinogenesis (Fig. 1, A and B). As shown in Fig. 6A, celecoxib feeding for 16 weeks to TRAMP mice resulted in a significant decrease in COX-2 enzyme activity (~45%) in the dorsolateral prostate as compared with TRAMP mice fed with AIN 76A diet. Furthermore, celecoxib-fed TRAMP also showed a significant reduction in COX-2 mRNA and protein expression in the dorsolateral prostate compared with TRAMP mice fed with AIN 76A diet (Fig. 6, B and C). These results were supported by immunohistochemical studies. Immunostaining for COX-2 was conducted in randomly selected TRAMP mice (n = 8) from the AIN 76A group. Strong diffuse COX-2 immunostaining was seen in >30% of the carcinoma cells in prostates from 24-week-old AIN 76A-fed TRAMP mice. In the group of TRAMP mice fed with celecoxib-enhanced AIN 76A diet, prostate cancers exhibited less immunoreactivity to antibodies against COX-2. In 7 of 10 animals, <5% of carcinoma cells showed immunoreactivity, whereas in the remaining 3 animals, 5–15% of carcinoma cells showed immunoreactivity to antibodies against COX-2 (Fig. 6D).

Effect of Celecoxib Feeding on PGE2 Levels in Plasma of TRAMP Mice. We evaluated the effect of celecoxib feeding on prostaglandin levels. Prostaglandins (PGE2, PGD2, and prostacyclin) are derivatives of prostaglandin H2 (PGH2), formed through both enzymatic and nonenzymatic pathways, which promote inflammation and cellular proliferation (6–8, 48). Overexpression of COX-2 has been shown to be associated with increased prostaglandin production (6–8). Alterations in plasma PGE2 levels have been observed in cancer patients, which relates with disease progression (49–51). Therefore, we measured the levels of PGE2 in the plasma of control and experimental TRAMP mice, which could reflect prostate cancer progression. We found a significant decrease (~60%; P < 0.0001) in the plasma PGE2 levels of 24-week-old TRAMP mice fed a celecoxib-enhanced diet as compared with TRAMP mice on regular AIN 76A diet for the same time period (Fig. 7). This reduction in plasma PGE2 was consistent with reduction in the COX-2 enzyme in the tumors of celecoxib-fed TRAMP mice.

Effect of Celecoxib Feeding on Surrogate Marker(s) of Proliferation, Metastases, and Angiogenesis in TRAMP Mice. We evaluated the effect of celecoxib feeding to TRAMP mice on cellular proliferation in prostate by assessing the expression of a proliferation-related protein PCNA. PCNA is a requisite auxiliary protein for DNA polymerase δ-driven DNA synthesis, and is cell cycle regulated (52). TRAMP mice fed a celecoxib-enhanced diet had markedly reduced PCNA protein expression in their prostates, compared with prostatic expression of this protein in TRAMP mice fed with regular AIN 76A diet (Fig. 8A). These results were additionally confirmed by densitometric analysis of the blots, where a significant decrease (77%) of PCNA protein was observed in the celecoxib-fed TRAMP mice (Fig. 8B).

Loss of expression of E-cadherin and cadherin-catenin complex in epithelial malignancies is associated with increased invasiveness and the development of metastases (53). Studies have shown that the normal expression of these markers is lost during prostate cancer progression in TRAMP mice in a fashion that parallels human disease (43). In the present study, celecoxib feeding to TRAMP mice for 24 weeks was effective in significant retention of the protein expression of E-cadherin, α-catenin, and β-catenin in the dorsolateral prostate (Fig. 8A). These results were additionally confirmed by densitometric analysis of the blots where a significant retention of E-cadherin (~58%), α-catenin (~61%), and β-catenin (~25%) protein was observed in celecoxib-fed TRAMP mice (Fig. 8B).
We evaluated the effect of celecoxib feeding on the angiogenesis marker, VEGF. VEGF plays an important role in tumor growth, progression, and metastases (54). Celecoxib feeding for 24 weeks resulted in a marked reduction in VEGF protein expression in the prostate of TRAMP mice compared with TRAMP mice fed with AIN 76A diet (Fig. 8A). These results were additionally confirmed by densitometric analysis of the blot where a significant decrease (~33%) of VEGF protein was observed in the celecoxib-fed TRAMP (Fig. 8B).

**Effect of Celecoxib Feeding on SV40 Transgene Expression in TRAMP Mice.** We evaluated the effect of celecoxib feeding on SV40 transgene expression in TRAMP mice. Because the transgene is expressed during the early stages of cancer development in TRAMP mice, with loss of expression in the later stages of cancer progression, we assessed SV40 protein expression in the dorsolateral prostate of TRAMP after 8 weeks of celecoxib feeding (16 weeks of age). Celecoxib feeding to TRAMP mice did not alter the protein expression of SV40 and was detectable in both celecoxib-fed and control group (data not shown). This observation indicates that inhibition of prostate cancer in TRAMP mice by celecoxib is not due to alteration in the transgene expression.

**Effect of Celecoxib Feeding on Extent of Apoptosis in TRAMP Mice.** Because celecoxib has been shown to induce apoptosis in several types of human carcinoma cells in vitro (55–59), we hypothesized that the observed inhibition of prostate tumorigenesis by celecoxib feeding may be mediated by increased apoptosis of cancer cells. To test our hypothesis, we used multiple approaches for determination of apoptosis in vitro and in vivo in live animals. In the first approach, we determined the effect of celecoxib by our recently perfected technique of in vivo apoptosis determination after administration of 99mTechnicium-labeled annexin V followed by autoradiography. Annexin V relies on the early externalization of phosphatidyl serine to the outer leaflet of the bilayer lipid cellular membrane. 99mTechnicium-labeled annexin V has successfully been used for the detection of apoptosis in various animal models (45, 60). This technique is highly selective and provides excellent resolution for the detection of apoptosis (45, 60). As shown in Fig. 9A, the autoradiograph of animals fed with AIN 76A diet after 99mTechnicium-labeled annexin V administration exhibited clear and specific regions of apoptotic cell death in the neoplastic prostate and seminal vesicle, mostly concentrated in the lower abdominal region. A significant increase in apoptosis was observed in celecoxib-fed TRAMP mice, concentrated in the lower abdomen (Fig. 9A).

In the second approach, we performed ELISA assay for detection of apoptosis. As shown in Fig. 9B, celecoxib feeding for 24 weeks resulted in a significant increase in apoptosis in the prostate of TRAMP mice compared with TRAMP mice fed with AIN 76A diet.

We also determined the protein expression of prostate apoptosis response-4 (PAR-4), which is selective for prostate apoptosis. PAR-4 is specifically expressed by cells entering apoptosis, and is not inducible by growth factor stimulation, oxidative stress, necrosis, or growth arrest (61). Celecoxib feeding for 24 weeks to TRAMP mice resulted in a significant increase (~3.6-fold) in the PAR-4 protein expression compared with TRAMP mice fed with AIN 76A diet (Fig. 9C). Together, these results confirm that celecoxib is capable of inducing selective apoptosis in prostate cancer cells; this may be one of the mechanism(s) of suppression of prostate carcinogenesis in celecoxib-fed TRAMP mice.

**Effect of Celecoxib Feeding on Survival in TRAMP Mice.** Survival is one of the most desirable effects of any chemoprevention.
A regimen (40). Therefore, we evaluated whether or not celecoxib feeding leads to increased survival of TRAMP mice. We studied 24 TRAMP males of ~16 weeks of age that exhibited palpable tumors. Twelve of these animals (the control group) were fed with AIN 76A diet, whereas the experimental group of 12 animals received a celecoxib-supplemented AIN 76A diet throughout the experiment, which was carried out until the mice died or reached 60 weeks of age. As shown in Table 2, the survival for TRAMP mice fed with AIN 76A diet was 91.7% at 30 weeks, 75% at 40 weeks, and 33.3% at 50 weeks. All 12 of the animals in this group died with advanced prostate cancer between 50 and 60 weeks of age. In contrast, survival of TRAMP mice fed with celecoxib-enhanced diet was significantly increased (\( P = 0.014 \)), 100% at 30 weeks, 91.7% at 40 weeks, 66.7% at 50 weeks, and 33.3% at 60 weeks. Continuous celecoxib feeding to TRAMP mice resulted in prolongation of the life span of these mice (Fig. 10A). Furthermore, histological evaluation of the prostate revealed poorly differentiated prostate cancer in 52-week-old TRAMP mice fed a regular AIN 76 A diet, whereas prostate cancers in celecoxib-fed mice of similar age appeared moderately differentiated (Fig. 10B).

**DISCUSSION**

Epidemiological observations and experimental studies have suggested that NSAIDs may have considerable promise as cancer chemopreventive as well as cancer chemotherapeutic agents (21, 22). The anticancer and anti-inflammatory properties of NSAIDs stem from blockade of prostaglandins by inhibiting the activity of the rate-limiting enzyme, COX (62, 63). It is hypothesized that NSAIDs exert an anticancer effect by sensitizing cancer cells to apoptosis by blocking COX (I and II) activity and decreasing prostaglandin levels (5–8, 61–63). Regular use of NSAIDs has been shown to decrease the risk of developing several types of human cancer, especially of the gastrointestinal tract (21). Unfortunately, the safety of long-term NSAID use remains a matter of concern (23). A new class of selective COX-2 inhibitors has been synthesized recently (24). It seems pertinent to evaluate whether these selective COX-2 inhibitors are capable of preventing and/or controlling cancer without the side effects associated with NSAIDs use.

Numerous investigators have implicated aberrant or increased COX-2 expression in the pathogenesis of many diseases, including cancer (7–16). COX-2 has been shown to be up-regulated in transformed human cells, and cancer of the breast, colon, rectum, esophagus, lung, pancreas, and upper aerodigestive tract (Refs. 7–10 and references therein). These observations have been additionally supported by genetic studies on murine transgenic and knockout models of COX-2 (64–66). Studies from our laboratory (11) and elsewhere (12–16) have suggested that COX-2 expression in normal and/or benign prostate tissue is either weak or nonexistent, with a marked COX-2 up-regulation observed in prostate cancer. Expression of COX-2 has been observed in both androgen-sensitive LNCaP and androgen-insensitive PC-3 cell lines (17–19), and treatment with COX-2 inhibitors has been shown to induce apoptosis in these tumor cells (59). COX-2 inhibitors are capable of suppressing the proliferation of LNCaP and PC-3 cells, whereas PsSC cells (stromal cells) were not affected by COX-2 inhibition (67). Studies by Zha et al. (17) have shown that COX-2 is up-regulated in proliferative inflammatory atrophy of the prostate, a putative precursor lesion for prostate cancer, but not in human prostate carcinoma. Because of these conflicting findings, the role of COX-2 up-regulation in the pathogenesis of prostate cancer remains controversial. However, studies where possible involvement of COX-2 has been shown at initiation and promotion stages of prostate carcinogenesis suggest that COX-2 may be a rational target for chemoprevention (Ref. 10 and references therein).

It has been shown that enhanced levels of PGE2 and its precursor arachidonic acid stimulate prostate cancer growth, and COX-2 inhibitors suppress prostate tumor growth in murine models of prostate carcinogenesis.

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**Table 2. Effect of celecoxib feeding on survival probability in TRAMP mice**

<table>
<thead>
<tr>
<th>Groupb</th>
<th>Number of animals</th>
<th>Number of death</th>
<th>Survival 30 week</th>
<th>Survival 40 week</th>
<th>Survival 50 week</th>
<th>Survival 60 week</th>
<th>Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAMP control</td>
<td>12</td>
<td>12</td>
<td>91.7</td>
<td>75</td>
<td>33.3</td>
<td>—</td>
<td>0.014</td>
</tr>
<tr>
<td>TRAMP celecoxib-fed</td>
<td>12</td>
<td>8</td>
<td>100</td>
<td>91.7</td>
<td>66.7</td>
<td>33.3</td>
<td></td>
</tr>
</tbody>
</table>

b TRAMP, transgenic adenocarcinoma of the mouse prostate.
Mice 16 weeks of age received AIN 76A diet (control group) and 1500 ppm celecoxib in AIN 76A diet for 60 weeks. Overall survival was measured from the start of celecoxib feeding (16 weeks of age) to date of death, and censored at the date of last follow up (60 weeks) for survivors.

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**Fig. 10. Effect of celecoxib feeding on survival probability in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice.** A, a significant increase in survival probability (\( P < 0.014 \); log-rank test) in celecoxib fed TRAMP mice was observed. B, effect of celecoxib feeding on prostate histology in TRAMP. Representative figure. H&E staining of histology of dorsal prostate of 52-week-old TRAMP representing poorly differentiated tumor. A, exibit regression of prostate cancer after celecoxib feeding representing moderately differentiated tumor at similar age.
COX-2 expression has been shown in HGPIN lesions in LPB-tag mice with complete loss of expression in poorly differentiated cancer that developed later in these mice (69). Our studies are in agreement with these observations. We found that COX-2 expression was present in prostate cancers developing in TRAMP mice up to 24 weeks of age; however, this expression was completely lost in the poorly differentiated cancers that had developed by 40 weeks of age. Furthermore, we found that celecoxib feeding significantly reduced COX-2 expression in TRAMP mouse prostate tissue, and also suppressed prostate carcinogenesis in these mice. It is important to note that prostate cancer development and progression in TRAMP mice is under the regulation of PB-Tag transgene. Under the influence of PB-Tag transgene, normal cells undergo malignant transformation forming a cancer that mimics human prostate cancer. In this study, celecoxib feeding to TRAMP mice inhibited progression of prostate cancer without alteration in transgene expression (data not shown). This observation suggests that the mechanism of celecoxib chemoprevention against prostate carcinogenesis in TRAMP mice is not due to inhibition of the transgene but rather to direct suppression of carcinogenesis.

Because COX-2 inhibitors are known to inhibit cell proliferation, we studied the protein expression of PCNA, a ubiquitous proliferation marker, in the prostate of TRAMP mice. We found that dietary supplementation with celecoxib significantly reduced prostate cancer growth in these mice. These results correlated with decrease in COX-2 activity. These findings were supported by the results of the MRI studies that we conducted in these mice. As measured by MRI, prostate growth was significantly diminished in celecoxib-fed mice; these findings correlated well with the gross and microscopic findings noted in the prostate and GU apparatus after sacrifice. Studies have shown that COX-2 plays a significant role in the progression of established prostate cancer in mice (18). We observed a progressive increase in COX-2 activity and protein expression in the prostates of TRAMP mice up to 24 weeks of age. Feeding celecoxib to TRAMP mice beginning at 8 weeks of age resulted in suppression of prostate carcinogenesis and complete absence of metastasis, along with increased long-term survival. These tumor-inhibitory effects correlated positively with significantly diminished COX-2 activity and protein expression in the prostate and plasma PGE2 levels in TRAMP mice.

The high incidence of prostate cancer in older men suggests that agents that inhibit or delay disease development might significantly reduce cancer morbidity and mortality (70). Pneumonolastic prostate lesions such as HGPIN are commonly observed in asymptomatic men during the fourth and fifth decade of life, and it is believed that such precursors require 2 to 3 decades to develop into clinically relevant prostate cancers (70). We found that feeding celecoxib to TRAMP mice significantly delayed the development of prostate cancer, and effectively eliminated the occurrence of metastasis in these animals.

The development of metastatic cancer occurs via complex pathways, which include decreased cell-substrate attachment and cell-cell adhesion, and increased cell motility. Prostate cancer invasion and metastasis in TRAMP model is accompanied by loss of expression of E-cadherin (43). Loss of the cadherin-catenin complex correlates with loss of cellular differentiation, and acquisition of invasive and metastatic properties in several types of human cancer (53). In this study, we have demonstrated that celecoxib feeding to TRAMP mice caused a significant retention of E-cadherin, and α- and β-catenin proteins. This may account for the complete absence of metastases in celecoxib-fed mice.

Another mechanisms by which COX-2 and prostaglandin support tumor growth is by inducing angiogenesis, a process required for establishment and progression of prostate cancer (54). Studies have shown that COX-2 expression significantly induces the production of VEGF, a growth factor that plays important roles in tumor growth, progression, and metastases (54). We observed that celecoxib feeding to TRAMP mice significantly reduced VEGF protein expression, suggesting that tumor progression in this model is slowed at least in part by suppression of angiogenesis. These findings are consistent with previous studies in which COX-2 inhibited VEGF production (71).

Progression of prostate cancer is associated with loss of apoptotic mechanisms. Agents that up-regulate apoptosis may be effective in cancer chemoprevention (61–63). Recent studies have shown that selective COX-2 inhibitors are capable of inducing apoptosis in a variety of cancer cell lines and some animal models of human cancer (27–29, 55–59). Studies have shown that COX-2 inhibition and induction of apoptosis are separate pharmacological effects of coxibs (72). We found that celecoxib feeding to TRAMP mice resulted in massive apoptosis of prostate cancer cells. The signaling events by which celecoxib mediate apoptotic death of cancer cells involve different signaling pathways, which has been shown recently to be mediated by COX-2-independent mechanisms. In studies of prostate cancer cell lines, inhibition of COX-2 and decreased PGE2 production has been shown to be associated with the modulation of various pro- and antiapoptotic factors, such as bcl2, PAR-4, and caspase-3 (61, 73). In our in vivo study, we found that celecoxib feeding to TRAMP mice increased PAR-4 protein expression in the prostate. However, the precise molecular mechanisms underlying celecoxib-induced apoptosis need additional investigation.

The TRAMP mouse model is important because it mimics in many ways the onset and progression of prostate cancer in humans, allowing observations of the effects of chemopreventive or chemotherapeutic agents on mouse prostate cancer in various stages of its evolution (37–42). Administration of such agents during various stages of carcinogenesis could be used to inhibit, reverse, or stabilize lesions before their progression into more lethal forms. We found that a 44-week celecoxib feeding regimen administered to 16-week-old TRAMP mice with established prostate cancer effectively delayed the progression of existing lesions, without weight loss or any apparent toxicity (data not shown). However, more detailed studies are needed to verify these findings. The survival advantage conferred on celecoxib-fed TRAMP mice with established prostate cancer has clear implications for clinical applications in humans.

Clinical studies of the associations between prostate cancer and the use of aspirin or NSAIDs are limited (74–76). No association between prostate cancer risk and NSAIDs use has been documented; some studies have suggested that frequent aspirin use diminishes the risk of developing metastatic prostate cancer (76). Our studies on the TRAMP mouse prostate cancer model have shown that celecoxib is capable of suppressing prostate carcinogenesis. Our findings strongly support the development of clinical trials to determine whether selective COX-2 inhibitors such as celecoxib can be useful as chemopreventive or chemotherapeutic agents in the management of prostate cancer.

REFERENCES


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Suppression of Prostate Carcinogenesis by Dietary Supplementation of Celecoxib in Transgenic Adenocarcinoma of the Mouse Prostate Model

Sanjay Gupta, Vaqar M. Adhami, Murugesan Subbarayan, et al.


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