ANTICANCER RESEARCH *25*: 107-116 (2005)

The Effect of Cyclooxygenase-2 (COX-2) Inhibition on Human Prostate Cancer Induced Osteoblastic and Osteolytic Lesions in Bone

SETH C. GAMRADT[1], BRIAN T. FEELEY[1], NANCY Q. LIU[1], JASON ROOSTAEIAN[1], YING Q. LIN[2], LI X. ZHU[2], SHERVEN SHARMA[2], STEVEN M. DUBINETT[2] and JAY R. LIEBERMAN[1]

[1]*Department of Orthopaedic Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA;*
[2]*University of California Los Angeles Lung Cancer Research Program, Jonsson Comprehensive Cancer Center, Los Angeles, CA, U.S.A.*

Abstract. *Background: The mechanism of bone formation by osteoblastic prostate cancer metastases is not well defined. Using knockout mice, it has been demonstrated that prostaglandins produced by COX-2 are critical for fracture repair. Therefore, our aim was to determine if COX-2 plays a role in the bone formation in osteoblastic prostate cancer metastases in bone. Materials and Methods: We assessed the influence of pharmacologic COX-2 inhibition in a SCID mouse intratibial injection model of bone metastasis using two human prostate cancer cell lines that produce either osteoblastic lesions (LAPC-9) or osteolytic lesions (PC-3, negative control). SC-58236, a COX-2 specific inhibitor, was used at a dose of 3 mg/Kg intraperitoneally 3 times per week in the Treatment groups for 8 weeks until sacrifice. Results: Western blot for COX-2 demonstrated that LAPC-9 cells expressed high levels of COX-2 while PC-3 cells did not. Treatment with SC-58236 significantly reduced the size of osteoblastic lesions after LAPC-9 injection based on both radiographic and histomorphometric criteria compared to the control group. In contrast, large osteolytic lesions were seen in both control and SC-58236 treated animals after PC-3 cell injections. The results of this study indicate that COX-2 inhibition can decrease the size of osteoblastic lesions produced by LAPC-9, a human prostate cancer cell line that expresses high levels of COX-2. This treatment had no effect on the osteolytic activity of PC-3 cells. Conclusion: These findings suggest that the progression of osteoblastic metastases induced by human prostate cancer cells may be limited by COX-2 inhibitors.*

Correspondence to: Jay R. Lieberman, MD, David Geffen School of Medicine at UCLA, Department of Orthopaedic Surgery, Center for Health Sciences 76-134, Los Angeles, CA 90095, U.S.A. Tel: 310.825.7687, Fax: 310.206.0063, e-mail: jlieberman@mednet.ucla.edu

Key Words: Prostate cancer, COX-2, osteolytic lessions, osteoblastic, bone metastases.

Skeletal metastasis is common in prostate cancer, affecting up to 70% of men afflicted with the disease. The resulting bony metastases are associated with significant morbidity and mortality (1-3). Prostate cancer, when metastatic to bone, produces predominately an osteoblastic (bone forming) lesion (1, 3, 4). The mechanism of bone formation by prostate cancer metastases has not yet been elucidated, but it is believed that reciprocal cellular interactions between tumor cells and the bone microenvironment result in osteoblast proliferation and differentiation (5). A variety of osteogenic factors and cytokines produced by tumor cells appear to contribute to the formation of osteoblastic lesions *in vivo*, such as bone morphogenetic proteins (BMP), endothelin-1 (ET-1), insulin like growth factors (IGF), urokinase plasminogen activator (uPA), and transforming growth factor-B (TGF-B) (6-8). However, the relative importance of these growth factors and the sequence of biologic events that leads to formation of an osteoblastic lesion are still unclear.

Cyclooxygenase (COX) is the key enzyme in the pathway responsible for converting arachadonic acid to prostaglandins. Two isoforms of this enzyme have been described. COX-1 is commonly thought of as a housekeeping gene and is constitutively active, while COX-2 activity is detectable in few normal tissues (9). In contrast to the ubiquitous nature of COX-1 in normal tissues, COX-2 is inducible in response to inflammatory cytokines or growth factors (10). In addition, COX-2 is known to be upregulated in many types of neoplasia including cancer of lung (11), breast (12), cervix (12), stomach (11), colorectal (13), skin (14) and prostate (15, 16). Elevated COX-2 expression in neoplastic tissue is associated with increased angiogenesis, decreased apoptosis, and increased invasiveness of the tumor (11, 15, 17).

In addition to its role in inflammation and cancer, recent studies have implicated COX-2 as a critical enzyme for fracture healing and bone formation in response to mechanical loading (18-22). PGE-2, a product of COX-2, can stimulate both osteoblasts and osteoclasts (23), and PGE-2 activity is also associated with recruitment and differentiation of osteoblasts from their precursors (24, 25). In mice, pharmacologic inhibition or gene knockout of COX-2, but not COX-1, resulted in fracture nonunion, thus delineating the critical role of COX-2 in fracture healing (19, 22). In one knockout mouse model of fracture healing, COX-2 -/- mice had decreased activity of *cbfa1* and *osterix*, which are osteoblast specific genes, and the authors concluded that COX-2 activity was the key regulator of these two genes (19). Prostaglandins produced by COX-2 gene expression induced by mechanical loading are critical for bone formation (21), and selective inhibition of COX-2 blocks bone formation induced by mechanical loading (20). Since COX-2 plays a critical role in the inducible bone formation of fracture healing and mechanical loading, we attempted to determine if there is a link between COX-2 expression and the osteoblastic nature of prostate cancer metastases to bone. COX-2 is know to be upregulated in prostate cancers, but it is not known if elevated COX-2 contributes to the bone formation associated with prostate cancer metastasis.

Our laboratory has developed a reliable human prostate cancer metastasis model using injection of human prostate cancer cells into the tibias of severe combined immunodeficient (SCID) mice (26-28). Using this model, we have characterized the nature of the cytokine profile of prostate cancer metastases. PC3 cells, a highly undifferentiated, bone derived prostate cancer line produces osteolytic lesions *in vivo* and high levels of RANK-L, TNF-α, and IL-1 (28). LAPC9 produces an osteoblastic lesion *in vivo* and high levels of BMP-2, -4, -6, and IL-6, which are associated with bone formation (28). In addition, treatment with either a bisphosphonate or receptor activator of NF-KB: Fc fusion protein (RANK-Fc), which are potent osteoclast inhibitors, blocked the formation of osteolytic lesions after intratibial injection of PC-3 (27, 29), but the formation of osteoblastic lesions was not influenced by osteoclast inhibiting treatment regimens.

The molecular pathway that is responsible for the development of osteoblastic bone metastases has not been delineated. Since COX-2 plays a role in the bone formation in fractures, it may also influence the formation of osteoblastic bone metastases. Our goal was to determine if pharmacologic inhibition of COX-2 would decrease bone formation in osteoblastic lesions based on the radiographic and histologic results. In addition, both cell lines were also injected subcutaneously to delineate between a direct antitumor effect versus the ability of a COX-2 inhibitor to act in the bone microenvironment.

Materials and Methods

Cell culture. All of the reagents were purchased from Life Technologies, Inc. (Rockville, MD) unless otherwise mentioned. Human prostate cancer cell line PC-3 was cultured in Iscove's medium (Irvine Scientific, Irvine, CA) supplemented with 15% fetal bovine serum and 1% glutamine and maintained at 37˚C in a humidified atmosphere with 5% CO_2.

Cell lines. The human prostate cancer cell lines PC-3 and LAPC-9 were used in this study. PC-3 (American Type Culture Collection) cells were chosen for this study because our prior experience with this cell line demonstrated that it produces an osteolytic lesion when implanted into bone (27, 28, 30). LAPC-9 was derived from a metastatic lesion to bone in a human patient (31). In prior studies, LAPC-9 produced pure osteoblastic lesions when implanted into bone (27, 28, 32). PC-3 cells can be maintained in tissue culture while LAPC-9 cells survive in tissue culture only 48 hours. For this reason, both LAPC-9 and PC-3 cells were obtained from tumors propogated in the subcutaneous tissue of male SCID mice one day prior to use.

Western blot. A Western blot for COX-2 was performed on PC-3 and LAPC-9 cells after treatment with protein lysis buffer. B-Actin level was used as a control. For Western blotting, 20 μg protein samples were resolved on a 12% SDS-PAGE gel and transferred to Hybond-ECL membranes (Amersham Life Sciences, Arlington Heights, IL). The Western analysis was performed using anti-COX-2 and anti-actin (both from Santa Cruz Biotechnology, Santa Cruz, CA) and the Amersham Life Science enhanced chemiluminescence protocol (33).

Prostaglandin levels in tibial tumors. To quantify the level of PGE-2 produced in tibial tumors, tumor homogenates from soft tissue tumors adjacent to tibiae were analyzed for PGE-2. PGE-2 concentrations were determined according to the Cayman Chemicals EIA kit protocol as described previously (34, 35). Briefly, 96-well Costar plates were precoated overnight with 4 μg/ml of goat anti-mouse PGE-2 (BioSource International). PGE-2-acetylcholinesterase conjugate, mouse anti-PGE-2 mAb, and either standard or sample were added to each well. After an 18-h incubation at 25˚C, the plate was washed five times to remove all unbound reagents. Ellman's reagent was then added to each well, and absorbance was determined at 405 nm by a Dynatech MR5000 spectrophotometer.

Single cell suspension protocol. Both PC-3 and LAPC-9 tumor cells were isolated 24 hours before use. Mice with subcutaneous tumors approximately 1 cm in diameter were anesthetized (100 mg ketamine/kg body weight, 10 mg xylazine/kg body weight) and sacrificed. The skin overlying the tumor was then shaved and prepped with 70% ethanol and Betadine. The tumor was dissected free of soft tissue in sterile fashion and placed in a sterile 50-ml conical Falcon tube (Becton Dickinson Labware, Franklin Lakes, NJ). The tumor was then finely minced with a sterile razor blade in sterile PBS (Life Technologies, Inc.). The resulting slurry centrifuged at 1300 rpm for 5 min at room temperature. The supernatant was then aspirated. The pellet was resuspended in Iscove's medium and centrifuged again at 1300 rpm for 5 min at room temperature. The supernatant was again aspirated, and the pellet was resuspended

with a 0.1% Pronase E (EM Science, Gibbstown, NJ)/Iscove's mix. Prior to resuspension the Pronase E/Iscove mix was filtered through a Steriflip Millipore filter with a 0.22 μm membrane (Millipore Corporation, Bedford, MA). The resuspension was then gently shaken on a rotating platform for 18 min at room temperature in a sterile 50-ml conical Falcon tube. The resuspension was placed on ice for 2 min and then strained using a 70-μm Falcon cell strainer (Becton Dickinson Labware). Once strained, the mix was spun at 1300 rpm for 5 min at room temperature. The supernatant was aspirated and the pellet was resuspended in Iscove's medium with 15% fetal bovine serum and 1% glutamine and was plated in 10-ml culture dishes (Becton Dickinson Labware). After this, 1x fungizone (Life Technologies, Inc.) was added to each plate, and the plates were incubated at 37˚C with 5% CO_2 until use.

Animals. Eight-week-old male SCID mice weighing approximately 25g were used. These mice were housed under pathogen-free conditions. All research was conducted in accordance with a protocol approved by the Chancellor's Animal Research Committee at the author's institution.

Tibial implantation. Single-cell suspensions of LAPC-9 or PC-3 cells were combined with an equal amount of Matrigel (Collaborative Biomedical Products, Bedford, MA) so that 10 μl of the mixture contained 1 x 10^5 cells. Ten μl of either the PC-3-Matrigel mix or the LAPC-9-Matrigel mix was injected into the left tibia of 8-week-old SCID mice, as previously described (27). To summarize, the mice were anesthetized, shaved, and prepped. An incision was made with a no. 15 scalpel blade over the patellar tendon. A 2 mm longitudinal arthrotomy was made made along the medial border of the patellar tendon with the same scalpel blade to expose the tibial plateau. A 27 gauge needle was inserted through the tibial plateau with the knee flexed to enter the intramedullary canal of the tibia. A separate 27 gauge needle was used to inject 10 μl of cell/matrigel mixture into the intramedullary canal of the tibia. The skin was closed with 5–0 Vicryl suture (Ethicon Inc., Somerville, NJ). Animals were sacrificed at 8 weeks. Before sacrifice, the animals were anesthetized and radiographs were taken with a Faxitron (Field Emission Corporation, McMinniville, Oregon).

Osteolytic and osteoblastic lesions were scored radiographically as described previously (27). Radiographic scoring of the LAPC-9 groups was performed using a grading scale based on the anatomic location of the osteoblastic lesion within the tibia and the degree of involvement of the tibia (0 – normal; 1 -- metaphyseal involvement; 2 – diaphyseal extension; 3 – diaphyseal extension with extracortical involvement). Radiographic scoring of the PC-3 groups was performed according to a previously published grading scale for osteolytic lesions based on extent of cortical disruption (0 – normal; 1 – endosteal scalloping; 2 – 1 cortex disrupted; 3 – 2 cortices disrupted; 4 – complete destruction of the proximal tibia) (27). At sacrifice, the hind limbs were harvested for histological analysis. Tumor dimensions were also measured at sacrifice. Two bisecting diameters of each tumor were measured with calipers. The volume was calculated using the formula (0.4) x (ab^2), with a as the larger diameter and b as the smaller diameter. Twenty mice were implanted with PC-3 cells and twenty mice were implanted with LAPC-9; these mice were divided equally between a control group (no treatment) and a treatment group (COX-2 inhibition).

Table I. *PGE-2 levels in tumor homogenates from tibias implanted with LAPC-9 and PC-3 cells. (PGE-2/total protein, ng/mg/ml±SE).*

	Control	SC-58236 treated
LAPC9	2.91±0.18	0.14±0.01
PC3	2.82±0.09	0.23±0.03

Subcutaneous tumor formation. Single cell suspensions were created in the same fashion as for tibial implantation. The mice were anesthetized and the back was shaved and prepped with 70% ethanol and betadine. 1 X 10^5 cells in 10ul were implanted subcutaneously with matrigel. Injections were performed 5mm to the left of midline in the animal's back proximal to the iliac crest. Animals were sacrificed at 8 weeks, or earlier if the tumor became larger than the allowable limit of the Chancellor's Animal Research Committee (1.5 cm tumor diameter). Two bisecting diameters of each tumor were measured with calipers. The volume was calculated using the formula (0.4) x (ab^2), with a as the larger diameter and b as the smaller diameter. Ten mice were implanted with PC-3 cells and 10 mice were implanted with LAPC-9; these mice were divided equally between a control group (no treatment) and a treatment group (COX-2 inhibition).

Treatment. SC-58236, a potent COX-2 inhibitor (35), was obtained from Pharmacia (Piscataway, NJ). SC-58236 was used at a dose of 3 mg/Kg intraperitoneally 3 x per week in Treatment groups. The dosing of SC-58236 was based on previous studies that demonstrated successful COX-2 inhibition in murine models (35). Treatment with SC-236 began one week prior to cell implantation and continued through the duration of the experiment. All Control groups were injected 3 times per week with PBS (Life Technologies, Inc., Rockville, MD).

Histology. The tibias were fixed in 10% buffered formalin, followed by decalcification in 10% EDTA solution for 2 weeks at room temperature with gentle stirring. Sections were paraffin embedded, sectioned (3 μM), and stained with H&E, orange G, and TRAP. Digital photographs were taken of a representative slide of each tibia using an Olympus system (Olympus, Melville, NY). The resulting images were analyzed using the histomorphometry software Bioquant (Nashville, TN). Histomorphometry was performed on Orange G stained slides of each tibia. The Bioquant software (Nashville, TN) was used to differentiate between bone and soft tissue, thus allowing accurate quantification of the amount of bone present in each tibia. A two-tailed student's t-test assuming equal variance was used to compare control versus treated groups.

Results

Evidence of in vivo inhibition of PGE-2 activity. A baseline Western blot for COX-2 revealed that LAPC-9 had a strong band for COX-2 and PC-3 had no COX-2 band present. B-actin control bands were similar.

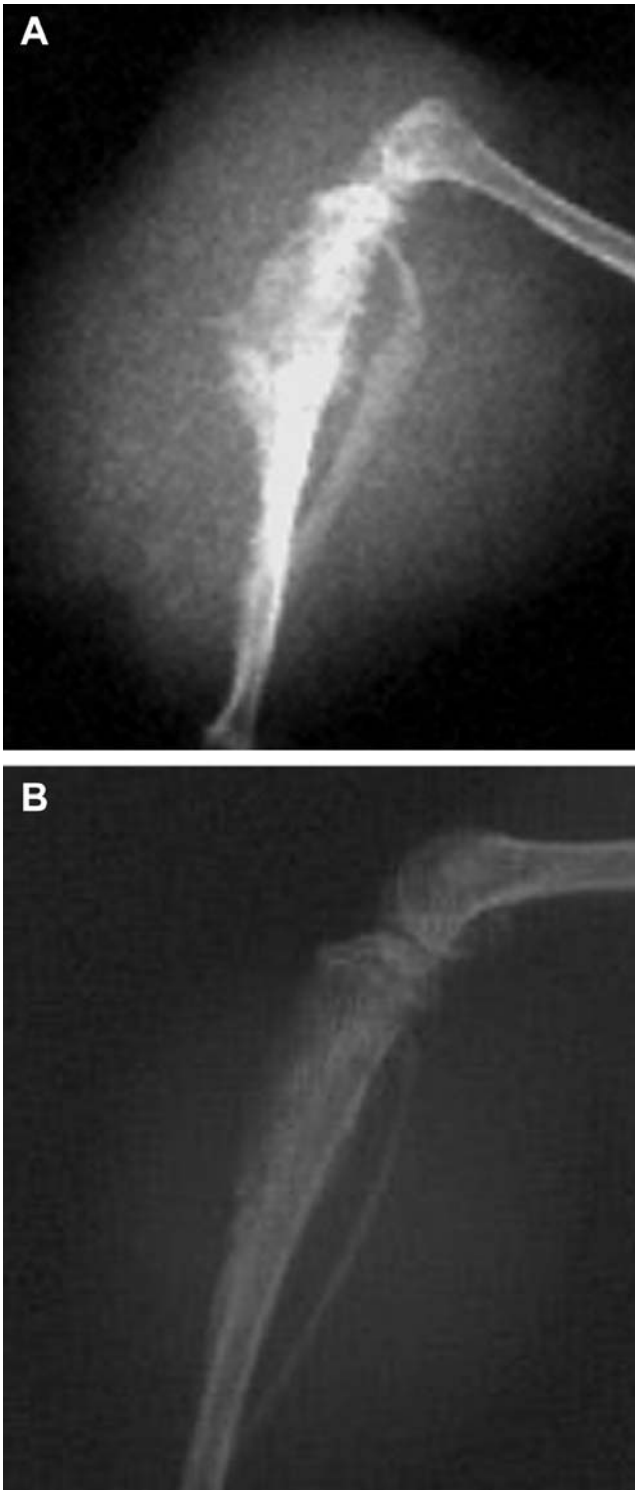

Figure 1. *Radiographic appearance of (A) control and (B) SC-58236 tibias from mouse tibias injected with LAPC-9 cells, which induce osteoblastic lesions. A dense, sclerotic osteoblastic lesion is present in the control lesion with extensive intramedullary and extramedullary new bone formation. Lesions treated with COX-2 inhibitor exhibited decreased bone formation in comparison, with a radiographic appearance showing new bone formation obscuring the intramedullary canal of the tibia.*

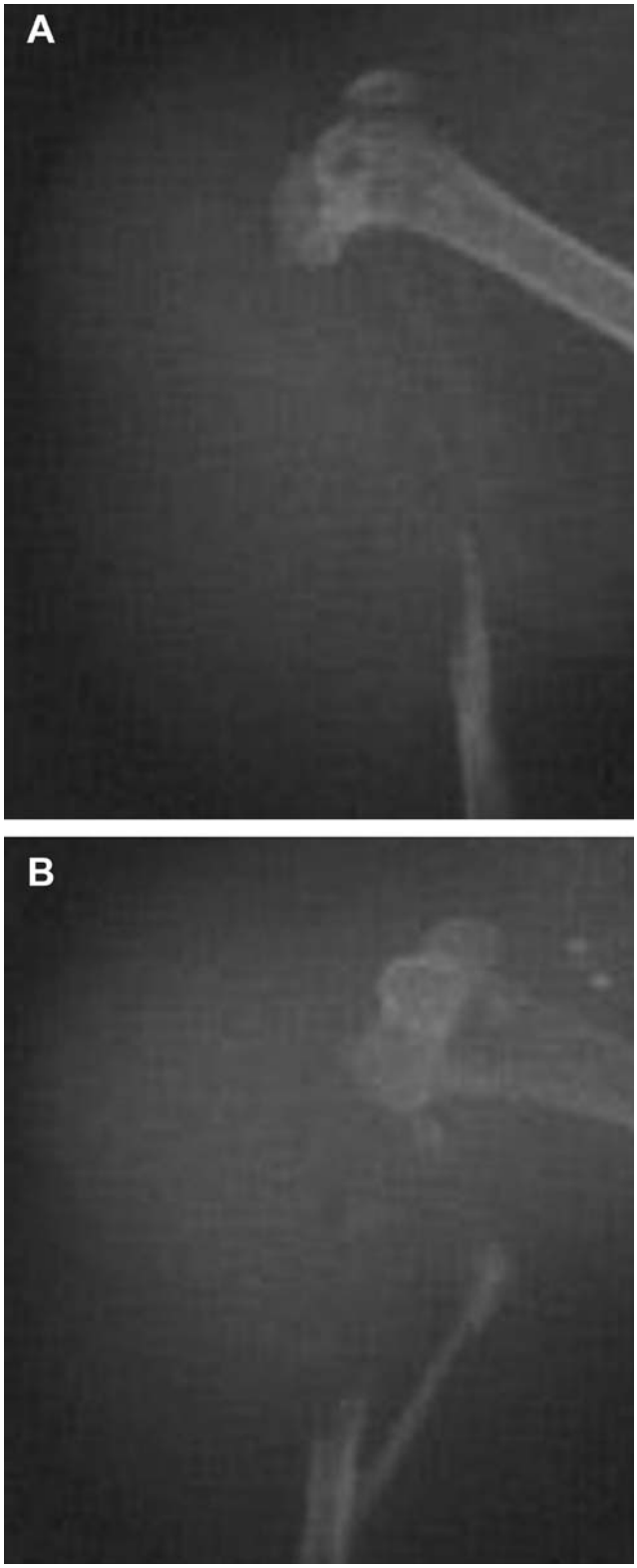

Figure 2. *Radiographic appearance of (A) control and (B) SC-58236 tibias from mouse tibias injected with PC-3 cells, which induce osteolytic lesions. Near complete destruction of the proximal tibias was observed in control and treatment mice.*

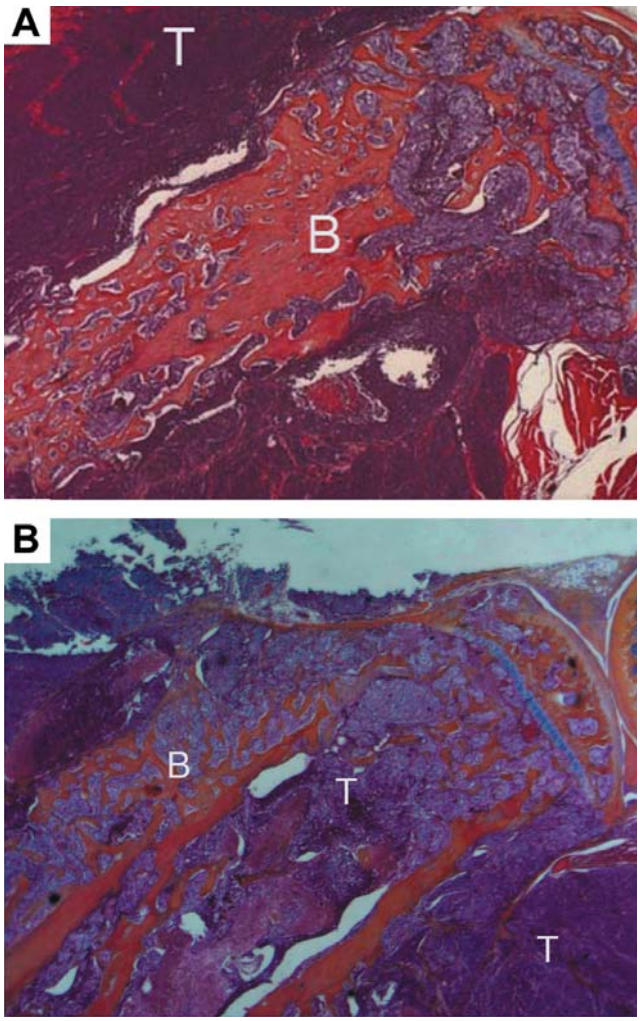

Figure 3. *Orange G Histology (4X) from LAPC9 induced osteoblastic lesions in tibias from in control (A) and SC-58236 treated (B) mice. Robust bone formation is observed in control lesions, nearly filling the intramedullary canal. Decreased bone formation is observed in the treated lesion with relative preservation of the intramedullary canal. B==New bone formation. T=Tumor cells.*

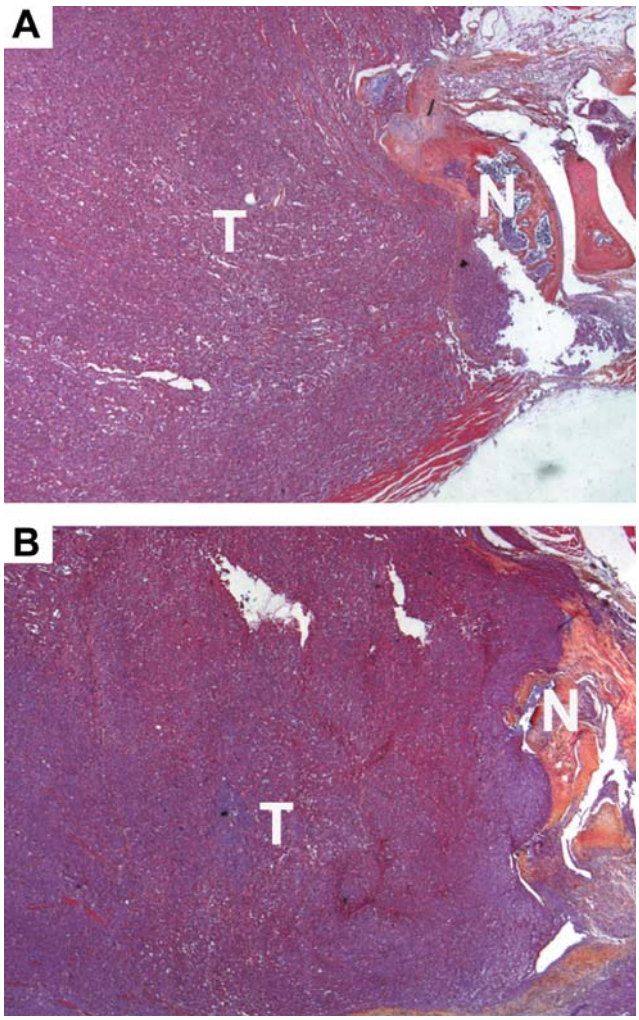

Figure 4. *Orange G histology appearance (4X) of (A) control and (B) SC-58236 tibias from mouse tibias injected with PC-3 cells, which induce osteolytic lesions. Near complete destruction of the proximal tibias was observed in control and treatment mice with replacement of the proximal tibia with a dense tumor burden of prostate cancer cells. N=Native tibia, T=Tumor.*

Tumor homogenates from soft tissue tumors surrounding tibial lesions were analyzed for COX-2 activity, quantified as PGE-2/total protein (ng/mg/ml). Tumor homogenates from LAPC-9 and PC-3 control tibial lesions both showed high COX-2 activity, while tumor homogenates from LAPC-9 and PC-3 tibial lesions in treated mice showed inhibition of COX-2 activity (Table I).

Table II. *Histomorphometry--percent bone area in proximal tibia.*

	Control	SC-58236 treated	*p* value
LAPC9	53.2±2.0	37.2±4.1	0.002*
PC3	4.9±1.8	3.1± 0.5	0.40

Radiographic evidence that SC-58236 decreases the severity of osteoblastic but not osteolytic prostate cancer lesions. 9 of 10 LAPC-9 Control animals exhibited diffuse osteoblastic lesions involving the entire proximal tibia. The one animal that did not develop an osteoblastic lesion did not have histologic evidence of tumor cells in the intramedullary canal. Most LAPC-9 induced lesions also showed extension of the bone formation outside the tibial cortex (Figure 1; mean

Table III. *Tumor volume in tibial lesions (Mean±SE, mm³).*

	Control	SC-58236 treated	*p* value
LAPC9	581.0±162.7	167.6±50.0	0.03*
PC3	277.96±33.5	272.3±60.1	0.50

Tumor volume of subcutaneous tumors (Mean±SE, mm³).

	Control	SC-58236 treated	*p* value
LAPC9	1126.1±223.9	408.4±140.7	0.03*
PC3	724.0±174.2	289.2±86.8	0.03*

radiographic score: 2.4±0.3). 7 of 10 LAPC9 animals treated with COX-2 inhibitor SC-58236 formed osteoblastic lesions, but the radiographic scores were statistically lower than control animals because there was less involvement of the proximal tibia (Figure 1; mean radiographic score: 1.1±0.3; $p=0.006$). 10 of 10 PC-3 Control animals and 9 of 10 PC-3 animals treated with SC-58236 formed osteolytic lesions in the tibia. The animal that did not form an osteoblastic lesion did not have evidence of tumor cells in the intramedullary canal. Extensive osteolytic lesions with obliteration of the entire proximal tibia and large tumor masses were seen in both Control and Treatment animals. There was no significant difference in radiographic findings between control and treated animals; (see Figure 2; mean radiographic scores: Control 4.0±0; Treatment 3.4±0.4; $p=0.43$).

Histological evidence that COX-2 inhibition with SC-58236 decreases the amount of bone formed in osteoblastic prostate cancer lesions. In concordance with radiographic data indicating that osteoblastic lesions in the tibias of mice treated with SC-58236 were less severe than control lesions, SC-58236 treatment resulted in a significant decrease in the amount of bone formed by osteoblastic prostate cancer lesions in this model (Table II). In the control tibias implanted with LAPC-9 cells, there was abundant intramedullary and extramedullary bone formation surrounding islands of tumor cells. In addition, a large extramedullary soft tissue tumor consisting of tumor cells was present (Figure 3). Bone formation was also observed in the lesions in tibias of mice treated with SC-58236, but the bone formation was more confined to the intramedullary canal, and consisted of lacelike, woven bone (Figure 3). Bone area was calculated using histomorphometry to determine the amount of new bone formation in the osteoblastic lesions of tibias implanted with LAPC-9. Bone constituted an average of 53.2% of the proximal tibias in control animals and 37.2% of the proximal tibias in SC-58236 treated animals ($p=0.002$).

Histological evidence that SC-58236 has no significant effect on the formation of osteolytic lesions. In both control and SC-58236 treated animals, the cortical bone in the proximal tibias was destroyed and PC-3 cells were observed in the resultant space (Figure 4). Treatment with SC-58236 resulted in no obvious differences in histology when compared to controls. Histomorphometric analysis confirmed that the amount of bone remaining in the proximal tibias of animals treated with SC-58236 was similar to the tibias of control animals. Bone constituted an average of less than 5% of the proximal tibial area in both control and treated animals (Table II).

SC-58236 treatment decreases LAPC-9 tumor size in both subcutaneous tumors and tibia injection model. All 10 animals injected with subcutaneous LAPC-9 cells formed subcutaneous tumors. The mean subcutaneous tumor volume of the five SC-58236-treated LAPC-9 animals was significantly decreased when compared to control animals. Similarly, the volume of tumors surrounding bone lesions induced by LAPC-9 was significantly decreased by treatment with SC-58236 (Table III).

SC-58236 treatment decreases PC-3 tumor size in subcutaneous tumors, but not in tibia injection model. All 10 animals injected with subcutaneous PC-3 cells formed subcutaneous tumors. The mean subcutaneous tumor volume of the five SC-58236-treated PC-3 animals was significantly decreased when compared to control animals. This suggests a possible direct anti-tumor effect for SC-58236 on PC-3 cells in the subcutaneous tissues of SCID mice. However, the volume of tumors surrounding bone lesions induced by PC-3 was not significantly decreased by treatment with SC-58236 (Table III)

Discussion

The results of this study indicate that COX-2 inhibition with SC-58236 can decrease the osteoblastic activity of LAPC-9, a human prostate cancer cell line that expresses high levels of COX-2. LAPC9 induced osteoblastic lesions in mice treated with SC-58236, a COX-2 inhibitor, exhibited decreased bone formation using radiographic and histomorphometric criteria. COX-2 inhibition appeared to have minimal effect on the osteolytic activity of PC-3 cells in the tibial injection model. Treatment with SC-58236 did not decrease the formation or severity of prostate cancer induced osteolytic lesions based on radiographic and histomorphometric data. Because LAPC-9 induced osteoblastic lesions did form in animals treated with a COX-2 inhibitor, it is unlikely that COX-2 is the sole mediator of osteoblastic activity in prostate cancer induced bone lesions. However, these findings suggest that the production of osteoblastic tumors in bone induced by human prostate cancer cells may be slowed by COX-2 inhibition. The disparate effects of COX-2 inhibition in osteoblastic LAPC-9

lesions and osteolytic PC-3 lesions were expected; PC-3 cells exhibited little or no COX-2 activity while LAPC-9 cells exhibited high activity. In addition, separate pathways are known to be responsible for the formation of osteolytic and osteoblastic metastatic lesions in bone (36).

COX-2 is known to be overexpressed in prostate cancer. The overexpression of COX-2 in cancer is thought to promote angiogenesis, inhibit apoptosis, and increase invasiveness (15, 37). Therefore, the antitumor effect of COX-2 inhibitors in our model is not unexpected. Nor is the effect of COX-2 blockade on LAPC9 likely limited to a blockade of osteogenesis. Therefore, the decreased osteogenic activity of lesions was probably secondary to both inhibition of a bone specific pathway and to a direct antitumor effect. The dose of COX-2 inhibitor SC-58236 used was obtained from prior studies in which this drug was effectively used to inhibit growth of subcutaneous tumors (35). It is not known how effective SC-58236 is at penetrating bone and for this reason, treatment with higher doses may provide a more complete COX-2 inhibition. A dose dependency study could eliminate incomplete pharmacologic inhibition of COX-2 as the cause of the partial response of LAPC-9 lesions to SC-58236.

In the current study, COX-2 inhibition was associated with a significant decrease in the size of the osteoblastic lesions and a decrease in severity on both plain radiographs and histomorphometric analysis. Treatment wih SC-58236 decreased the size of subcutaneous tumors in both the LAPC-9 and PC-3 cells which demonstrates that there is a direct antitumor effect on both cell lines. However, SC-58236 treatment did not affect the formation of osteolytic lesions by PC-3 cells which suggests that in contrast to the osteoblastic lesions, osteolytic lesions form *via* a COX-2 independent pathway. It is difficult to determine the relative influence of COX-2 inhibition and the direct antitumor effect of the drug on the reduction the formation of osteoblastic lesions, but these findings do suggest that COX-2 inhibitors could be used clinically to treat osteoblastic bone lesions. The LAPC-9 cells demonstrated high levels of COX-2 activity on a Western blot. Measurement of PGE-2 activity was elevated in the soft tissue tumor homogenates of LAPC-9 control animals but low in animals treated with SC-58236. This suggests that COX-2 inhibition was also responsible for limiting the progression of osteoblastic lesions.

Since osteoblastic lesions still formed despite COX-2 inhibition, this suggests that multiple cytokine pathways are associated with the progression of osteoblastic lesions. LAPC-9 cells secrete TGF-B, BMP-2, -4, and –6, which are known to be osteoinductive molecules involved in fracture healing (28), and these growth factors may be responsible for the formation of osteoblastic lesions. When compared to osteolytic metastasis pathophysiology, less agreement exists on a discrete pathway for the formation of osteoblastic bone metastases. Prostate carcinoma cells are known to produce multiple osteogenic factors, including bone morphogenetic proteins (BMPs) (28, 38, 39), endothelin-1 (ET-1) (8, 40-42), insulin-like growth factors (IGF) (43), urinary plasminogen activator (uPA) (44, 45), and transforming growth factor beta (TGF-B) (28, 46). The exact role and relative importance for each of these growth factors in establishment and maintenance of osteoblastic prostate cancer lesions has not been clearly determined. Recent research in a mouse model has demonstrated that ET-1 is a critical factor in the development of osteoblastic breast cancer metastasis. ET-1 produced by breast cancer cells induces bone formation *in vitro* and results in osteoblastic metastatic lesions in an *in vivo* intracardiac injection mouse metastasis model (40). The effect of ET-1 in this model was mediated by the endothelin-A (ETA) receptor and establishment of osteoblastic lesions was inhibited by pharmacologic ETA receptor blockade. ETA receptor blockade has also been used to decrease new bone formation in a mouse model of prostate cancer induced osteoblastic lesions (47). In addition, ET-1 levels are elevated in prostate cancer patients with bone metastases (48), and ET-1 could represent the potential pathway for the formation of osteoblastic metastases.

The mechanism of bone destruction by osteolytic metastatic lesions has been clearly delineated. Parathyroid hormone related protein (PTHrP), IL-1, and IL-6 are produced by cancer cells; these cytokines can stimulate the production of RANKL (receptor activator of nuclear factor-B ligand). Free or membrane bound RANKL can then activate osteoclasts by binding to its receptor, RANK, on the surface of osteoclasts (5, 6, 36, 49). The RANK pathway is likely responsible for osteolytic lesions and mixed osteoblastic/osteolytic lesions formed by prostate cancer cells. Inhibition of the RANK pathway with osteoprotegerin (OPG), a decoy receptor of RANKL, prevents the formation of osteolytic prostate cancer metastasis *in vivo* (50). Similarly, RANK-Fc, a fusion protein that binds RANKL inhibits the formation of osteolytic prostate cancer metastasis *in vivo* (29, 51).

On Western blot, the PC-3 cells used in our study demonstrated little or no COX-2 activity. However, measurement of PGE-2 levels of tumor homogenates from osteolytic lesion showed that PGE-2 levels were high in PC-3 control animals and low in SC-58236 treated animals. This suggests that an interplay between host cells in the bone environment and tumor cells increased COX-2 activity in the PC-3 cells *in vivo*. PGE-2 could also have been derived from non-tumor stromal cells that are present in tumor homogenates.

Ono *et al.*, in an *in vitro* model of breast cancer metastasis, have demonstrated that COX-2 inhibition could block osteoclast stimulation by breast cancer cells (52). In the same model, RANKL production and subsequent osteoclast growth in cocultures of bone marrow cells and breast cancer cells was dependent on COX-2 activity (53). Also, Ohshiba *et al.*, in a

mouse model of breast cancer metastasis, concluded that RANKL induced osteolysis was dependent on PGE2 produced by COX-2 (54). However, in the current study, the formation of osteolytic lesions did not appear to be dependent on COX-2 activity. In a prior study from our laboratory using the same SCID mouse tibial injection model, RANK-Fc was an effective inhibitor of osteolytic bone lesions normally seen with PC-3 injection (29). Therefore, it is possible that RANKL production occurs *via* a COX-2 independent pathway since SC-58236 was ineffective in blocking osteolytic lesion formation. Another potential explanation is that the bioavailability of SC-58236 in bone may be less than in subcutaneous tissue. Despite effective COX-2 blockade *in vivo*, osteolytic lesions still formed in tibias injected with PC-3 cells. The role of COX-2 in osteolytic metastases to bone deserves further study.

In this study, a SCID mouse tibial injection model of metastasis was used to determine the effect of COX-2 inhibition on human prostate cancer induced bone lesions. A partial response to treatment with a COX-2 inhibitor was observed in osteoblastic prostate cancer lesions. COX-2 is known to be critical in physiologic bone formation associated with fracture healing and mechanical loading. The current study indicates that COX-2 also plays a role in the pathophysiology of osteoblastic prostate cancer metastasis. The relative importance of COX-2 as a mediator of osteogenesis in metastatic lesions deserves further study. Clinical trials are underway to evaluate the effect of COX-2 inhibitors on prostate cancer in the clinical setting (15). COX-2 inhibition may prove to be an important component of the treatment of bone metastasis in prostate cancer.

Acknowledgements

This work was supported by a grant from the National Institutes of Health: RO1–CA103039 (JRL).

References

1 Koeneman KS, Yeung F and Chung LW: Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. Prostate *39*: 246-261, 1999.

2 Koutsilieris M: Osteoblastic metastasis in advanced prostate cancer. Anticancer Res *13*: 443-449, 1993.

3 Lange PH and Vessella RL: Mechanisms, hypotheses and questions regarding prostate cancer micrometastases to bone. Cancer Metastasis Rev *17*: 331-336, 1998.

4 Goltzman D: Mechanisms of the development of osteoblastic metastases. Cancer *80*: 1581-1587, 1997.

5 Mundy GR: Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer *2*: 584-593, 2002.

6 Keller ET, Zhang J, Cooper CR, Smith PC, McCauley LK, Pienta KJ and Taichman RS: Prostate carcinoma skeletal metastases: cross-talk between tumor and bone. Cancer Metastasis Rev *20*: 333-349, 2001.

7 Choong PF and Nadesapillai AP: Urokinase plasminogen activator system: a multifunctional role in tumor progression and metastasis. Clin Orthop S46-58, 2003.

8 Guise TA, Yin JJ and Mohammad KS: Role of endothelin-1 in osteoblastic bone metastases. Cancer *97*: 779-784, 2003.

9 Koki A, Khan NK, Woerner BM, Dannenberg AJ, Olson L, Seibert K, Edwards D, Hardy M, Isakson P and Masferrer JL: Cyclooxygenase-2 in human pathological disease. Adv Exp Med Biol *507*: 177-184, 2002.

10 Herschman HR: Primary response genes induced by growth factors and tumor promoters. Annu Rev Biochem *60*: 281-319, 1991.

11 Dubinett SM, Sharma S, Huang M, Dohadwala M, Pold M and Mao JT: Cyclooxygenase-2 in lung cancer. Prog Exp Tumor Res *37*: 138-162, 2003.

12 Dannenberg AJ and Howe LR: The role of COX-2 in breast and cervical cancer. Prog Exp Tumor Res *37*: 90-106, 2003.

13 Church RD, Fleshman JW and McLeod HL: Cyclo-oxygenase 2 inhibition in colorectal cancer therapy. Br J Surg *90*: 1055-1067, 2003.

14 Brecher AR: The role of cyclooxygenase-2 in the pathogenesis of skin cancer. J Drugs Dermatol *1*: 44-47, 2002.

15 Pruthi RS, Derksen E and Gaston K: Cyclooxygenase-2 as a potential target in the prevention and treatment of genitourinary tumors: a review. J Urol *169*: 2352-2359, 2003.

16 Sabichi AL and Lippman SM: COX-2 inhibitors and other NSAIDs in bladder and prostate cancer. Prog Exp Tumor Res *37*: 163-178, 2003.

17 Dannenberg AJ and Subbaramaiah K: Targeting cyclooxygenase-2 in human neoplasia: rationale and promise. Cancer Cell *4*: 431-436, 2003.

18 Wadhwa S, Godwin SL, Peterson DR, Epstein MA, Raisz LG and Pilbeam CC: Fluid flow induction of cyclo-oxygenase 2 gene expression in osteoblasts is dependent on an extracellular signal-regulated kinase signaling pathway. J Bone Miner Res *17*: 266-274, 2002.

19 Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN and O'Keefe RJ: Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. J Clin Invest *109*: 1405-1415, 2002.

20 Forwood MR: Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading *in vivo*. J Bone Miner Res *11*: 1688-1693, 1996.

21 Reich KM and Frangos JA: Effect of flow on prostaglandin E2 and inositol trisphosphate levels in osteoblasts. Am J Physiol *261*: C428-432, 1991.

22 Simon AM, Manigrasso MB and O'Connor JP: Cyclo-oxygenase 2 function is essential for bone fracture healing. J Bone Miner Res *17*: 963-976, 2002.

23 Kawaguchi H, Pilbeam CC, Harrison JR and Raisz LG: The role of prostaglandins in the regulation of bone metabolism. Clin Orthop 36-46, 1995.

24 Weinreb M, Suponitzky I and Keila S: Systemic administration of an anabolic dose of PGE2 in young rats increases the osteogenic capacity of bone marrow. Bone *20*: 521-526, 1997.

25 Suponitzky I and Weinreb M: Differential effects of systemic prostaglandin E2 on bone mass in rat long bones and calvariae. J Endocrinol *156*: 51-57, 1998.

26 Tsingotjidou AS, Zotalis G, Jackson KR, Sawyers C, Puzas JE, Hicks DG, Reiter R and Lieberman JR: Development of an animal model for prostate cancer cell metastasis to adult human bone. Anticancer Res *21*: 971-978, 2001.

27 Lee YP, Schwarz EM, Davies M, Jo M, Gates J, Zhang X, Wu J and Lieberman JR: Use of zoledronate to treat osteoblastic versus osteolytic lesions in a severe-combined-immunodeficient mouse model. Cancer Res *62*: 5564-5570, 2002.
28 Lee Y, Schwarz E, Davies M, Jo M, Gates J, Wu J, Zhang X and Lieberman JR: Differences in the cytokine profiles associated with prostate cancer cell induced osteoblastic and osteolytic lesions in bone. J Orthop Res *21*: 62-72, 2003.
29 Whang PGI, R C; Lieberman, J R The soluble receptor activator of NF-KB: FC fusion protein inhibits osteolytic and osteoblastic lesions induced by human prostate cancer cells. *In*: Proceedings of the Orthopaedic Research Society, New Orleans, LA, 2003.
30 Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF and Jones LW: Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol *17*: 16-23, 1979.
31 Craft N, Chhor C, Tran C, Belldegrun A, DeKernion J, Witte ON, Said J, Reiter RE and Sawyers CL: Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. Cancer Res *59*: 5030-5036, 1999.
32 Wu TT, Sikes RA, Cui Q, Thalmann GN, Kao C, Murphy CF, Yang H, Zhau HE, Balian G and Chung LW: Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. Int J Cancer *77*: 887-894, 1998.
33 Kiertscher SM, Luo J, Dubinett SM and Roth MD: Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. J Immunol *164*: 1269-1276, 2000.
34 Huang M, Stolina M, Sharma S, Mao JT, Zhu L, Miller PW, Wollman J, Herschman H and Dubinett SM: Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. Cancer Res *58*: 1208-1216, 1998.
35 Stolina M, Sharma S, Lin Y, Dohadwala M, Gardner B, Luo J, Zhu L, Kronenberg M, Miller PW, Portanova J, Lee JC and Dubinett SM: Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J Immunol *164*: 361-370, 2000.
36 Choong PF: The molecular basis of skeletal metastases. Clin Orthop S19-31, 2003.
37 Kirschenbaum A, Liu X, Yao S and Levine AC: The role of cyclooxygenase-2 in prostate cancer. Urology *58*: 127-131, 2001.
38 Autzen P, Robson CN, Bjartell A, Malcolm AJ, Johnson MI, Neal DE and Hamdy FC: Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies. Br J Cancer *78*: 1219-1223, 1998.
39 Harris SE, Harris MA, Mahy P, Wozney J, Feng JQ and Mundy GR: Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells. Prostate *24*: 204-211, 1994.
40 Yin JJ, Mohammad KS, Kakonen SM, Harris S, Wu-Wong JR, Wessale JL, Padley RJ, Garrett IR, Chirgwin JM and Guise TA: A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. Proc Natl Acad Sci USA *100*: 10954-10959, 2003.
41 Mohammad KS and Guise TA: Mechanisms of osteoblastic metastases: role of endothelin-1. Clin Orthop S67-74, 2003.
42 Chiao JW, Moonga BS, Yang YM, Kancherla R, Mittelman A, Wu-Wong JR and Ahmed T: Endothelin-1 from prostate cancer cells is enhanced by bone contact which blocks osteoclastic bone resorption. Br J Cancer *83*: 360-365, 2000.
43 Fizazi K, Yang J, Peleg S, Sikes CR, Kreimann EL, Daliani D, Olive M, Raymond KA, Janus TJ, Logothetis CJ, Karsenty G and Navone NM: Prostate cancer cells-osteoblast interaction shifts expression of growth/survival-related genes in prostate cancer and reduces expression of osteoprotegerin in osteoblasts. Clin Cancer Res *9*: 2587-2597, 2003.
44 Rabbani SA, Mazar AP, Bernier SM, Haq M, Bolivar I, Henkin J and Goltzman D: Structural requirements for the growth factor activity of the amino-terminal domain of urokinase. J Biol Chem *267*: 14151-14156, 1992.
45 Achbarou A, Kaiser S, Tremblay G, Ste-Marie LG, Brodt P, Goltzman D and Rabbani SA: Urokinase overproduction results in increased skeletal metastasis by prostate cancer cells *in vivo*. Cancer Res *54*: 2372-2377, 1994.
46 Marquardt H, Lioubin MN and Ikeda T: Complete amino acid sequence of human transforming growth factor type beta 2. J Biol Chem *262*: 12127-12131, 1987.
47 Nelson JB, Nguyen SH, Wu-Wong JR, Opgenorth TJ, Dixon DB, Chung LW and Inoue N: New bone formation in an osteoblastic tumor model is increased by endothelin-1 overexpression and decreased by endothelin A receptor blockade. Urology *53*: 1063-1069, 1999.
48 Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA and Simons JW: Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nat Med *1*: 944-949, 1995.
49 Chirgwin JM and Guise TA: Molecular mechanisms of tumor-bone interactions in osteolytic metastases. Crit Rev Eukaryot Gene Expr *10*: 159-178, 2000.
50 Zhang J, Dai J, Qi Y, Lin DL, Smith P, Strayhorn C, Mizokami A, Fu Z, Westman J and Keller ET: Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest *107*: 1235-1244, 2001.
51 Zhang J, Dai J, Yao Z, Lu Y, Dougall W and Keller ET: Soluble receptor activator of nuclear factor kappaB Fc diminishes prostate cancer progression in bone. Cancer Res *63*: 7883-7890, 2003.
52 Ono K, Akatsu T, Murakami T, Kitamura R, Yamamoto M, Shinomiya N, Rokutanda M, Sasaki T, Amizuka N, Ozawa H, Nagata N and Kugai N: Involvement of cyclo-oxygenase-2 in osteoclast formation and bone destruction in bone metastasis of mammary carcinoma cell lines. J Bone Miner Res *17*: 774-781, 2002.
53 Ono K, Akatsu T, Kugai N, Pilbeam CC and Raisz LG: The effect of deletion of cyclooxygenase-2, prostaglandin receptor EP2, or EP4 in bone marrow cells on osteoclasts induced by mouse mammary cancer cell lines. Bone *33*: 798-804, 2003.
54 Ohshiba T, Miyaura C and Ito A: Role of prostaglandin E produced by osteoblasts in osteolysis due to bone metastasis. Biochem Biophys Res Commun *300*: 957-964, 2003.

Received December 2, 2004
Accepted January 7, 2005