Prostate Cancer Cells-Osteoblast Interaction Shifts Expression of Growth/Survival-related Genes in Prostate Cancer and Reduces Expression of Osteoprotegerin in Osteoblasts

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ABSTRACT

Purpose: Prostate cancer specifically metastasizes to bone where it leads to bone formation. We previously reported that coculturing MDA PCa 2b prostate cancer cells with primary mouse osteoblasts (PMOs) induced PMO proliferation and differentiation. An osteoblastic reaction was also observed in vivo after injection of MDA PCa 2b cells into the bones of severe combined immunodeficient disease mice. The aim of this study was to identify the sequence of events that leads to these osteoblastic lesions in vivo and, using this in vitro model, to define the contributions of various genes and cellular pathways in the pathophysiology of osteoblastic bone metastases of prostate cancer.

Experimental Design and Results: We show histological evidence of de novo bone formation as early as 2 weeks after injection of MDA PCa 2b cells in the bone of severe combined immunodeficient disease mice. In vitro, we show that PMOs induce MDA PCa 2b proliferation, suggesting a synergistic paracrine loop between these cells and PMOs. Endothelin (ET)-1, which is a mitogen for several cell types, is produced by all prostate cancer cell lines tested, and Atrasentan, an antagonist of ET-1 receptor A, partially reversed PMO proliferation induced by MDA PCa 2b cells. ET-1 is known to be comitogenic with a number of growth factors, including insulin-like growth factor (IGF)-I. In this study, we report that IGF-binding protein (IGFBP)-3 transcripts (that regulate levels of free IGF) are down-regulated in prostate cancer cells cocultured with PMO, whereas prostate-specific antigen (a protease known to cleave IGFBP-3) is detected in the 150–400 ng/ml range. Accordingly, IGFBP-3 has antiproliferative effects in PMOs, which were attenuated in our in vitro system. Taken together, our studies also implicate the IGF axis to play a role in this model of bone metastases. Secondly, the transcript level of mouse double minute 2 (a protein that regulate p53) was increased in prostate cancer cells grown with PMOs. The p53-dependent and -independent oncogenic activities of mouse double minute 2 suggest that osteoblasts induce a survival advantage in prostate cancer cells. Lastly, we show that expression of osteoprotegerin is decreased and of receptor activator of nuclear factor-κB ligand is increased in PMOs cultured in the presence of MDA PCa 2b cells, two events associated with osteoclast activation and bone resorption.

Conclusions: Our results provide evidence that multiple and distinct molecular events affecting both bone formation and bone resorption concur to the increase bone mass in prostate cancer bone metastases. These data also provide a rationale for developing therapeutic strategies designed to target these molecular changes.

INTRODUCTION

Prostate cancer is the most frequent form of cancer in men in the United States (1). It is associated with high morbidity and mortality rates as a result of its tendency to metastasize to the bone (2). Bone is usually the first and exclusive site of progression of the disease to the androgen-independent stage, and the bone metastases are typically osteoblastic (i.e., involving excessive bone formation by osteoblasts; Refs. 2–4). These observations suggest that prostate cancer cells stimulate cells of osteoblast lineage at this site of metastasis and that the prostate cancer cell-osteoblast interaction contributes to the lethal progression of prostate cancer. The molecular bases for this interaction are poorly understood, partially because few in vivo models mimic the natural progression and dissemination of prostate cancer (5, 6). Moreover, even fewer systems lead to osteoblastic lesions, the most frequent phenotype of bone metastases from prostate cancer in humans. A number of soluble factors,
including PSA, urokinase, and bone morphogenetic proteins, have been implicated as direct or indirect osteoblast-stimulating factors expressed by prostate cancer cells (4, 7, 8).

ET-1, a potent vasoconstrictor peptide has also been implicated in the pathophysiology of osteoblastic bone metastases from prostate cancer (8–10): ET-1 is produced by prostate cancer cells; expression of the ET-1 clearance receptor B is lost during prostate cancer progression; and ET-1 levels are higher in plasma of prostate cancer patients with bone metastasis (9–11). In vitro studies have demonstrated that ET-1 increases osteoblast proliferation and osteoblast-specific gene expression (10–12). Furthermore, overexpression of ET-1 in murine bone was shown to induce an increase of bone formation that could be blocked by administration of an antagonist of ET(A) (11). However, to date, no study has formally demonstrated a direct link between the ET-1 production or inhibition in prostate cancer cells and the occurrence of osteoblastic lesions in bone.

Although ET-1 is mitogenic for a variety of cell types, it has been shown that its effect is more potent as comitogenic of several growth factors, including IGFs (11). IGFs are abundant in human bones, and they have mitogenic, chemotactic, and antiapoptotic effects on a wide variety of cells, including both prostate cancer cells (13, 14) and osteoblasts (15). IGF-1 exerts its action through the IGF-IR, a tyrosine kinase receptor. Levels of IGFs available to interact with the receptor are modulated by six IGFBPs; these IGFBPs are, in turn, hydrolyzed by a number of proteases (16). More than 90% of the circulating IGFs are bound to IGFBP-3. IGFBP-3 also have IGF-independent growth inhibitory effects on several cell types (16). Prostate cancer cells have been shown to actively regulate bioavailable IGFs levels by urokinase- and PSA-mediated IGFBP-3 proteolysis (4, 7, 8, 16). IGFBP-3 inhibits the osteoblastic growth effect of IGFs in osteoblasts (15), whereas its expression in prostate cancer cells inversely correlates with progression from benign to malignant disease (16).

As it was discussed, bone metastases of human prostate cancer possess osteoblastic features, however, an underlying osteolytic component is present in most cases (8, 17, 18). In support of this concept, preliminary evidence of a Phase III trial suggested that oral clodronate (an inhibitor of bone resorption) delays symptomatic progression of bone metastases and may improve survival (19). Major molecules implicated in cancer-induced osteolysis include PTHrP, RANKL, OPG, and MMPs. The PTHrP protein stimulates osteoclastic activity (20). RANKL, a tumor necrosis factor, induces the differentiation and activation of osteoclasts. OPG, a soluble tumor necrosis factor receptor, inhibits the formation and activity of osteoclasts by sequestering RANKL (21–23). Treatment with OPG abolishes cancer-induced bone destruction in vivo (24). MMPs are a group of neutral proteinases thought to be involved in bone formation and remodeling. Osteoblasts secrete several proteases including MMPs such as collagenases, thus remodeling their own ECM (25).

In an effort to understand the molecular basis of prostate cancer bone metastases, we established two human prostate cancer cell lines, MDA PCa 2a and MDA PCa 2b, that express PSA and the androgen receptor, and their growth is androgen regulated (26, 27). We then established an in vitro model of bone metastases, consisting of cocultured MDA PCa 2a and MDA PCa 2b cells with PMOs and showed that these prostate cancer cells induced a specific increase in osteoblast growth and differentiation (28). In the current study, we used this model to define the contribution of various genes and cellular pathways in the pathogenesis of prostate cancer bone metastases.

MATERIALS AND METHODS

Cell Cultures. MDA PCa 2a and MDA PCa 2b cell lines were routinely propagated in BRFF-HPC1 medium (Biological Research Faculty and Facility, Inc., Jamsville, MD) with 20% FBS (Life Technologies, Inc., Gaithersburg, MD). The prostate cancer cells LNCaP and PC3 were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented with 10% FBS. LNCaP is derived from a lymph node metastasis, not from a bone metastasis. PC3 is derived from a bone metastasis but does not produce PSA and is not androgen regulated.

Intrabone Injections. Male SCID mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA), and housed in a facility with constant humidity and temperature and a 12-h light-dark cycle. They had ad libitum access to standard mouse feed and water. All animal experimentation was conducted in accordance with accepted standards of humane animal care and was approved by the Animal Care and Use Committee of M. D. Anderson Cancer Center. Animals were anesthetized with intramuscular injections of ketamine (100 mg/kg) plus acepromazine (2.5 mg/kg). Aliquots of 0.5 × 10⁶ of MDA PCa 2b cells were diluted in 5 μl of growth medium and then injected into the distal epiphysis of the right femur using a 28-gauge Hamilton needle. The contralateral femur was used as an internal control, and four control mice were injected with saline only. Six additional mice served as a second control group; they were injected with 0.5 × 10⁶ of PC3 cells. Mice were monitored twice weekly for tumor bulk. Serum PSA levels were measured, and X-rays of the legs were obtained at 2, 3, 4, 6, 10, and 16 weeks after injection. Blood was obtained from a small incision in the main tail vein, and the serum was separated for PSA testing. A set of five mice was killed at each point (or earlier in the case of a bulky tumor), and pathologic examination of the subject bones was performed.

Tissue Samples and PSA Assay. Formalin-fixed, parafin-embedded tissue samples from the tumors were prepared by the Department of Veterinary Medicine at M. D. Anderson Cancer Center. The subject bones were dissected free of muscle, fixed in 10% buffered formalin, decalcified in 5% formic acid, and then embedded in paraffin. Longitudinal 3-μm thick sections were obtained from each sample and stained with H&E. PSA concentration in the culture medium and PSA serum of
mice were measured with a microparticle enzyme immunoassay (IMx PSA assay; Abbott Laboratories, Abbott Park, IL).

**Primary Cultures of Mouse Calvaria Osteoblasts and Coculture with Prostate Cancer Cells.** Primary cultures of osteoblasts were obtained from CD1 mice as reported previously (28). PMOs were then plated in α-MEM plus 10% FBS for 48 h. Trypsin was subsequently added, and the cells were replated in culture dishes for experimentation. An in vitro biocompartmental culture system was used (28). Briefly, PMOs were seeded in tissue culture plates, whereas prostate cancer cells were seeded in cell culture inserts (0.4-μm pore size; Falcon/Becton Dickinson Labware, Franklin Lakes, NJ) so that the two different cell types shared the culture medium but were not in physical contact.

**Mitogenic Assays.** [3H]Thymidine (NEN Life Science Products, Boston, MA) incorporation into DNA was evaluated as described previously (29).

**Reagents.** ET-1 was purchased from Sigma Chemical (St. Louis, MO) and IGFBP-3 from R&D Systems, Inc. Atrasentan, was provided by Abbott Laboratories.

**Protein Analysis.** Radioimmunoassay was used to assess ET-1 production by prostate cancer cells (Biotrak; Amer sham) and IGF-I concentration in the culture medium (Nichols Institute Diagnostics, San Juan Capistrano, CA). Western blot analysis was performed as described previously (26).

**Bone Differentiation Studies.** Alkaline phosphatase activity in PMOs was determined as described previously (28). Mineralization was monitored using a Von Kossa’ s staining kit (Sigma, St. Louis, MO). GAG was determined by the dimethylmethylene blue assay (28).

**RNA Extraction and Northern Blot Analysis.** Northern blot analysis was performed as reported previously (28). Human IGF-I probe containing the entire coding region was a generous gift from Dr. Rodrigo Bravo (Bristol Myers Squib, Princeton, NJ). Human PTHrP probe was prepared from a 306-bp coding region cDNA fragment of a human thyroid carcinoma. The forward primer was 5’-GGTCAGAGATGGAGGACGCT-3’ starting at exon 3 of the human gene, and the reverse primer was 5’-CCTAGGTAGTATCTCTGCC-3’ from exon 4 of the human gene. IGFBP-3 probe (clone ID no. 898218) was a generous gift from Millennium Pharmaceuticals, Inc. (Cambridge, MA), and the mdm2 probe was kindly provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). OPG probe (5130-bp coding region cDNA) was kindly provided by Dr. Christopher Wood (M. D. Anderson Cancer Center). Mouse and human RANKL probes were kindly provided by Amgen, Inc. (Thousand Oaks, CA). cDNA probe for 36b4 was used as a loading control (30).

**Gene Array Analysis.** Differential expression of multiple genes by MDA Pca 2b cells growing alone or in coculture with PMOs was analyzed using the Human PathwayFinder-1 GEArray Kit (SuperArray, Inc., Bethesda, MD). The studied genes include egfr, c-fos, c-myc, activating transcription factor-2, p53, hsf1, heat shock protein 27, heat shock protein 90, iNos, nuclear factor-κB, inhibitor of nuclear factor-κB α, interleukin 2, Fas (Apo-1), CD5, p16, p21, p57Kip2, gadd45, pig7, pig8, mdm2, bax, and CYP19 (aromatase P450).

**Statistical Analysis.** Numerical data were expressed as means ± SE. Statistical differences between means for the different groups were evaluated with Sigma Plot 4 using one-way ANOVA and the t test. Ps of <0.05 were considered statistically significant.

## RESULTS

**MDA Pca 2b Cells Result in Osteoblastic Lesions in Vivo.** Table 1 summarizes tumor size of SCID mice after intramammary injection of MDA Pca 2b cells as assessed by serum PSA levels and X-ray imaging of the injected limb. Typical results of X-ray analysis are presented in Fig. 1. Pathologic examinations were performed after the mice had been killed; results are summarized in Fig. 2.

Lesions become visible radiologically 3–4 weeks after intramammary injection of MDA Pca 2b cells (Fig. 1, Table 1). A slight increase in serum PSA was already found at this early stage (Table 1). Histopathological examination of the injected limbs 2 weeks postinjection revealed colonies of tumor cells in the medullar cavity always surrounded by bone matrix, which suggested that these cells induce bone remodeling (Fig. 2, A and B). Four weeks after cell injection, tumor cells replaced the bone marrow in the metaphysis or the epiphysis; changes at this time included destruction of trabecular bone in some cases without destruction of cortical bone (Fig. 2C).

Two months later, pathologic examination revealed the presence of tumor cells located in the metaphysis, epiphysis, and diaphysis. Tumor cells in the diaphysis were often necrotic, which was likely related to insufficient vascularization in the diaphysis. In some cases, the tumor was growing through holes from the metaphysis into the surrounding soft tissues. At this point, there was evidence of new bone formation in the diaphysis (Fig. 2D) and bone thickening in the diaphysis. Proliferation of active osteoblasts surrounding the tumor cells and in areas once occupied by tumor cells were also detectable (Fig. 2, inset). Osteoclasts were not usually seen in these lesions.

Ten to 16 weeks after injection, serum PSA had reached levels of up to 232 ng/ml (Table 1). Histopathologically, bulky tumors invading the entire bone marrow cavity of the metaphysis (and usually of the diaphysis) were seen. Tumor cells were mostly necrotic in the diaphysis, although they were viable in...
the distal metaphysis and often in the proximal metaphysis as well (data not shown). In most cases, tumor cells were found in the soft tissue surrounding the distal metaphysis. New bone formation was obvious in the distal epiphysis, the metaphysis and the diaphysis, and active osteoblasts were frequently seen in the proximal metaphysis (Fig. 2E). Necropsy showed no macroscopic evidence of distant metastases, although in some cases, tumor emboli were found in blood vessels of the lung.

To determine whether this sequence of events was specific to this cell line or could be observed with any prostate cancer cell line, we used PC3 cells. PC3 cells are also derived from a bone metastasis of prostate cancer but do not produce PSA and are not regulated by androgens. The injection of the prostate cancer cell line PC3 resulted in intrabone tumors in five of six mice 4 weeks after intrafemoral injections. In contrast to the bone lesions induced by MDA PCa 2b cells, the lesions induced by PC3 cells were all osteolytic (Fig. 2F), and the femur of one mouse was broken (Fig. 2F, inset). Viable PC3 cells were found in the bone medulla of the distal metaphysis and in the surrounding soft tissue. The cortical bone of the metaphysis was completely destroyed in most cases, and many osteoclasts were found surrounding the PC3 tumor cells (Fig. 2F). No evidence of new bone formation was found.

Increased Proliferation Rate of MDA PCa 2a and MDA PCa 2b Prostate Cancer Cells in an in Vitro Model of Bone Metastasis. We have previously reported that bone-derived MDA PCa 2a and MDA PCa 2b prostate cancer cells induce osteoblast growth and differentiation when the cells share medium during coculturing (28). In this study, we tested the effect of PMOs cocultured with prostate cancer cells in the proliferation rate of prostate cancer cells using [3H]thymidine incorporation into DNA as a direct measure of DNA synthesis. We found that DNA synthesis was increased in prostate cancer cells MDA PCa 2a and MDA Ca 2b but not PC3 at 72 h of coculture with PMOs (Fig. 3A). These results suggest that soluble factors secreted by PMO and by bone-forming prostate cancer cells are involved in a paracrine loop that results in new bone formation and prostate cancer growth.

Role of ET-1 in the Osteoblastic Phenotype of Bone Metastasis. Multiple studies have identified ET-1 as one molecule involved in the progression of osteoblastic bone metastases of prostate cancer (9, 10). We therefore studied ET-1 production and the effect of Atrasentan, an antagonist of its receptor A, on the proliferation and differentiation response of PMOs to MDA PCa 2b cells in this in vitro model of bone metastasis. ET-1 secretion was detected in all human prostate cancer cell lines tested (Table 2). In initial experiments, we determined that ET-1 stimulated the growth of PMOs with an equivalent effect in the range of concentration tested (10^{-11}-10^{-8} M; data not shown). ET-1 (10^{-9} M) induced PMO growth, whereas Atrasentan (10^{-5} M) reversed the ET-1 induction of PMO cell growth (Fig. 3B). When Atrasentan was used as a single agent, there was no significant inhibitory effect on the growth of PMOs (Fig. 3B). Furthermore, the stimulating effect on the growth of PMOs induced by MDA PCa 2b cells in the coculture system was partially inhibited by Atrasentan (Fig. 3B). Similar results were obtained when using the osteoblastic 2T3 cell line instead of PMOs (data not shown). Results were consistent with the previous experiment and supported the inhibitory effect of Atrasentan on MDA PCa 2b-induced growth of osteoblasts. That the inhibitory effect was not complete suggests that other mitogenic factors are produced by prostate cancer cells. The coculture with MDA PCa 2b cells increased alkaline phosphatase activity and calcified matrix formation by PMOs (Table 3 and Fig. 4, respectively). Although Atrasentan strongly inhibited mineralization by PMOs grown alone (Fig. 4), it did not inhibit mineralization or alkaline phosphatase activity induced by MDA PCa 2b cells. This suggests that MDA PCa 2b cells produce other soluble factors besides ET-1, which promote osteoblast differentiation. ET-1 can act alone as a mitogen, but its effect is more potent as a comitogen with a variety of growth factors (11). Among them is IGF-I, which plays a key role in bone formation, is an important regulator of the activity of mature osteoblasts (15), has strong mitogenic and antiapoptotic effects on various cancer cells, including prostate cancer, and has been implicated in the pathogenesis of osteoblastic bone metastases of prostate cancer (4, 8, 31–34).

Involvement of the IGF Axis in this in Vitro Model of Bone Metastasis. We studied IGF-I expression levels in this in vitro model of bone metastasis. Regulation of IGF-I expression is complex because this gene contains two promoters and both promoters regulate transcription at multiple initiation sites.
In addition, posttranscriptional events also regulate IGF-I gene expression (15). The result of these differential transcriptional and posttranscriptional modifications is the production of many mRNA ranging in length from ~1 to 7.5 kb. Yet, these multiple transcripts encode one IGF-I protein. Northern blot analysis demonstrated that both PMOs and prostate cancer cells express IGF-I transcripts. The major transcript expressed by PMOs is ~2.7 kb long, whereas the major transcript expressed by prostate cancer cells is ~1.1 kb long (data not shown). We then studied the concentration of IGF-I in the cultured medium conditioned by prostate cancer cells and PMOs growing alone and in coculture. IGF-I is secreted by both prostate cancer cells and PMOs (Table 4). Moreover, culture medium conditioned by MDA PCa 2b and PMOs in coculture has IGF-I levels between 32 and 42 ng/ml (4.2 and 5.6 nM), which are well within the range reported to induce DNA synthesis and collagen production in rodent calvaria cells, as well as proliferation of prostate cancer cells.

We next examined the expression of IGFBP-3 because IGFBP-3 modulates the amount of bioavailable free IGFs and inhibits their transfer from the circulation to tissue sites of action (15). IGFBP-3 has also been implicated in the osteoblastic tropism of prostate cancer cells (31, 32). We found that IGFBP-3 expression was decreased in MDA PCa 2a and MDA PCa 2b cells cocultured with osteoblasts compared with controls (Fig. 5A).

![Fig. 2](image-url)

Fig. 2  Pathologic examination of lesions induced by intrafemoral injection of prostate cancer cells into SCID mice. A and B, evidence of bone remodeling in the diaphysis and distal metaphysis 2 weeks after intrafemoral injection of MDA PCa 2b cells (arrow). Inset: control femur injected with saline (×2.5). C, a colony of MDA PCa 2b cells (arrow) growing into the distal metaphysis and the diaphysis 4 weeks after intrafemoral injection (×2.5). Inset: control (femur injected with saline); trabecular bone and normal bone marrow in the distal metaphysis (×2.5). D, evidence of new bone formation in the epiphysis (arrow) 6 weeks after injection of MDA PCa 2b cells. The bone marrow is almost no longer visible. Viable MDA PCa 2b cells in the metaphysis (×10). Inset: osteoblast proliferation (arrow) in the distal epiphysis 6 weeks after injection (×40). E, evidence of MDA PCa 2b cell proliferation (asterisk), osteoblast activation (arrow), and new bone formation in the proximal epiphysis 10 weeks after injection (×40). Inset: control: proximal epiphysis of a left leg 10 weeks after intrafemoral injection into the right leg (×40). F, PC3 tumor growing into the metaphysis 4 weeks after injection; bone destruction (arrow; ×2.5). Inset: osteoclasts (arrow) surrounded the PC3 cells (asterisk; ×40).
Previous reports, including studies of clinical samples, have implicated PSA-dependent proteolysis of IGFBP-3 in the osteoblastic phenotype of PCa bone metastases (31, 32, 35). PSA is the best characterized downstream target of the androgen receptor and a widely used marker of prostate cancer progression (31, 32). We thus examined the expression of PSA in the medium conditioned by MDA PCa 2a and MDA PCa 2b cells growing alone or as controls and given a value of 100%; prostate cancer cells grown in coculture were expressed as a percentage of respective controls. Each value represents the mean ± SD of 6 duplicated wells. *, P < 0.01. Similar results were obtained in an independent experiment.

Table 2 ET-1 production by prostate cancer cells

<table>
<thead>
<tr>
<th>Prostate cancer cell line</th>
<th>ET-1 production (fmol/10^6 cells)</th>
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<tr>
<td>DU145</td>
<td>6.4 ± 0.19</td>
</tr>
<tr>
<td>PC3</td>
<td>3.29 ± 0.23</td>
</tr>
<tr>
<td>MDA PCa 2b</td>
<td>3.17 ± 0.09</td>
</tr>
<tr>
<td>MDA PCa 2a</td>
<td>2.55 ± 0.18</td>
</tr>
<tr>
<td>LNCaP</td>
<td>0.95 ± 0.38</td>
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* Cells were cultured in duplicate for 48 h after reaching 90% confluence in α-MEM plus 10% FBS. Culture media were then assayed for ET-1 by using a radioimmunoassay kit. Values represent mean ± SD.

Table 3 Alkaline phosphatase activity in PMOs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Alkaline phosphatase activity (unit/µg of protein)</th>
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<tbody>
<tr>
<td>PMOs</td>
<td>63</td>
</tr>
<tr>
<td>PMOs + Atrasentan 10^{-5} M</td>
<td>61</td>
</tr>
<tr>
<td>PMOs + MDA PCa 2b</td>
<td>118</td>
</tr>
<tr>
<td>PMOs + MDA PCa 2b + Atrasentan 10^{-5} M</td>
<td>168</td>
</tr>
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</table>

* PMOs were seeded, grown for 24 h, and then cocultured with or without MDA PCa 2b cells and with or without Atrasentan (10^{-5} M). Medium was changed after 2 days of coculture, and fresh Atrasentan was also added. After 4 days, inserts containing prostate cancer cells were removed, and PMOs were allowed to grow to confluence. Differentiation medium was then added to PMOs and changed every 2 days. Alkaline phosphatase activity was measured after 4 days.

The effect of Atrasentan on calcified matrix deposition. PMOs were seeded, grown for 24 h, and then cocultured with or without MDA PCa 2b cells and with or without Atrasentan (10^{-5} M). Medium was changed after 2 days of coculture, and fresh Atrasentan was also added. After 4 days, culture medium and inserts containing prostate cancer cells were removed, and PMOs were allowed to grow to confluence. Differentiation medium was then added to PMOs and changed every 2 days. Bone matrix deposition was assessed by von Kossa’s staining at day 12. PMOs control (A); PMOs + Atrasentan (B); PMOs in coculture with MDA PCa 2b cells (C); and PMOs in coculture with MDA PCa 2b cells + Atrasentan (D). Similar results were obtained in an independent experiment.

As an additional attempt to understand the implications of
the decreased IGFBP-3 expression in this model system, we added IGFBP-3 to PMOs grown alone and in coculture with MDA PCa 2b cells. We found that IGFBP-3 inhibited PMOs cell growth at all concentrations used (Fig. 5B). When PMOs are grown in coculture with MDA PCa 2b cells, the inhibitory effect of IGFBP-3 is abolished at doses of 10 and 50 ng/ml. An inhibitory effect on PMOs cocultured with MDA PCa 2b cells was still detected (though of less intensity) at 100 and 1000 ng/ml IGFBP-3; these findings were confirmed in an independent experiment.

Taken together, these results implicate the IGF axis in the osteoblastic reaction of prostate cancer bone metastases and suggest that paracrine regulatory loops between prostate cancer cells and osteoblasts exist and may result in enhanced IGF bioavailability locally in bone by decreasing IGFBP-3 expression. This may in turn potentiate the mitogenic effect of ET-1.

**Molecular Analysis of Prostate Cancer Cells Cultured in this in Vitro Model of Bone Metastasis.** We have shown that PMO provides a growth advantage to MDA PCa 2b cells in the in vitro model of bone metastases (Fig. 3A). We therefore studied gene expression of MDA PCa 2b cells grown alone and after 4 days of coculture with PMOs by a human gene expression profiling system (Human PathwayFinder-1 GEArray Kit; SuperArray, Inc.) and found a 2-fold increase in OPG mRNA expression in cocultured cells, compared with controls. The increased expression of MDM2 in MDA PCa 2b cells cocultured with PMOs was confirmed by Northern blot analysis (Fig. 5C).

p53 and bcl-2 alterations are involved in the regulation of apoptosis and have been implicated in the progression of prostate cancer to the androgen-independent metastatic phenotype (36, 37). MDA PCa 2a and MDA PCa 2b express wild-type p53 and do not overexpress bcl-2 as with 50 and 70% of bone metastasis from prostate cancers, respectively (27). We thus examined whether MDA PCa 2a and MDA PCa 2b cells grown with PMO would change the pattern of p53 or bcl-2 expression. No difference was found in p53 and bcl-2 expression as assessed by Western blot analysis (Fig. 5D).

**Expression of PTHrP, OPG, and RANKL in this in Vitro Model of Bone Metastasis.** To elucidate whether a decrease in osteoblastic bone resorption may contribute to the increased bone mass observed in prostate cancer bone metastases, we analyzed the expression of three genes involved in the regulation of bone resorption: PTHrP, OPG, and RANKL. As Fig. 6A shows, the expression of PTHrP was higher in PC3 cells than it was in all other prostate cancer cell lines tested, which is consistent with the fact that PC3 cells induce osteolytic lesions. The expression of OPG was also much higher in PC3 cells than it was in MDA PCa 2a, MDA PCa 2b, and LNCaP cells, which all had undetectable levels of OPG (Fig. 6B). RANKL was undetectable in all cell lines tested (data not shown). In vivo data showed that bone lesions induced by MDA PCa 2b cells are associated with increased proliferation and activation of osteoblasts (Fig. 2), whereas in vitro, this cell line does not express any significant amount of OPG, an inhibitor of osteoclastic bone resorption. Taken together, these findings suggest that osteoblastic lesions induced by MDA PCa 2b are related to stimulation of osteoblastic activity rather than to an inhibition of osteoclast activation. We then studied whether the expression of these genes is modulated in PMO or MDA PCa 2b cells during coculture. MDA PCa 2b cells and PMOs were grown in this in vitro system of bone metastases, and RNA was harvested after 4 days of coculturing. Results showed that prostate cancer cells induce a decreased expression of OPG and an increased expression of RANKL in PMOs, which may lead to osteoclast activation (Fig. 6C). In contrast, OPG and PTHrP expression by prostate cancer cells was not changed when these cells were cocultured with PMOs (data not shown).
We have previously shown that MDA PCa 2b cells injected in the femur of SCID mice produce osteoblastic lesions as assessed by radiological and histological examination. In this study, we have established and characterized the temporal changes of tumor growth in bone and the osteoblastic reaction. We also show that the interaction of osteoblasts and prostate cancer cells leads to several important changes at the cellular and molecular levels. An increased proliferation was demonstrated in prostate cancer cells and osteoblasts in coculture, suggesting a synergistic paracrine loop. We provide evidence that ET-1-dependent pathways partially mediate this proliferative response and that molecular changes involving the IGF axis occur during coculture and may have a role in this paracrine loop cooperating with the mitogenic effect of ET-1. These changes may result in both an increase in bone mass and prostate cancer cell growth. We also found that the expression of OPG is down-regulated, whereas the expression of RANKL is up-regulated in osteoblasts cocultured with prostate cancer cell lines. These data thus provide evidence that multiple and distinct molecular events affecting both bone formation and bone resorption concur to the increased bone mass and bone resorption in prostate cancer bone metastases.

Much progress has been made in the establishment of rodent models of prostate cancer, and although these are very useful for the study of prostate cancer progression, the models do not develop osteoblastic bone metastases (5, 38). In this report we describe an in vivo model of bone metastases from prostate cancer that recapitulates typical features of prostate cancer. After intrabone injection into immunodeficient mice, MDA PCa 2b cells produce lesions with osteoblastic features, whereas PC-3 cells typically produce osteolytic lesions (39). MDA PCa 2b cells are androgen regulated, and therefore, the model could also be useful to assess the impact of androgen ablation on the growth of prostate cancer cells in bone and the response to therapy. Moreover, with the availability of a parallel in vitro model of cocultured prostate cancer cells with osteoblasts, the system can be used to elucidate the molecular events underlying the process of prostate cancer bone metastases.

Previous studies using an osteoblastic breast cancer cell line suggested that the new bone formation induced by these cells was ET-1 mediated. Moreover, the new bone formation was inhibited by an ET_A receptor antagonist (8). In preclinical studies, Atrasentan, an antagonist selective for the ET_A receptor, blocked ET-1-induced biological responses that were mediated through the ET_A receptor. In in vitro studies, Atrasentan is 1800-fold more selective for ET_A than the ET receptor B with a Ki of 0.034 nM for the ET_A receptor (40). The results reported here showed that ET-1 partially mediates osteoblast proliferation in this in vitro model of bone metastasis; moreover, Atrasentan decreased mineralization by PMOs. It is therefore expected that systemic administration of Atrasentan would result in a reduction of bone formation. Thus, our results are in agreement with preliminary findings of Phase I/II studies (41–43). This underlines the value of this in vitro model of bone metastasis to study the interaction between prostate cancer cells and osteoblasts at the molecular level.

We have shown in this study that the transcript levels of IGFBP-3 produced by MDA PCa 2a and MDA PCa 2b decrease when the cells are cocultured with PMOs. Furthermore, MDA PCa 2a and MDA PCa 2b, as with most human prostate cancers, express PSA, which is a known protease of IGFBP-3. It is therefore likely that alternative and/or additive mechanisms decrease IGFBP-3 expression in prostate cancer, thus increasing the bioavailable levels of IGFs. Numerous in vivo studies have documented the ability of IGF-1 to influence osteoblast growth and differentiation (15). Moreover, targeted overexpression of IGF-1 in the osteoblasts of transgenic mice demonstrated an increase in bone formation rate and mineral density (44).

We also found that IGFBP-3 has antiproliferative effects in PMOs. This effect was attenuated in this in vitro model of bone metastasis, probably attributable to PSA-mediated proteolysis of IGFBP-3. Previous studies have implicated PSA-dependent proteolysis of IGFBP-3 in the osteoblastic phenotype of prostate cancer bone metastases (31, 32, 35). A decrease in serum...
IGFBP-3 in patients with metastatic prostate cancer and a significant negative correlation between serum PSA and IGFBP-3 were reported (32). In support of those findings, tissue and serum concentrations of PSA vary inversely with IGFBP-3 levels in patients with metastatic prostate cancer (31, 35), and it was recently reported that plasma IGF-I and IGFBP-3 levels are predictors of biochemical progression and advanced stages of prostate cancer (33, 34). IGFBP-3 modulation may thus be an essential event in prostate cancer bone metastases. Despite the important role of IGFs in bone, there is a paucity of studies on the effect of IGFBPs on osteoblast growth and differentiation. Our laboratory is currently studying the specific mechanism of IGFBP-3 growth inhibitory effect on osteoblasts.

We have shown that transcript levels of MDM2 are increased in prostate cancer cells grown with POMO (Fig. 5C). MDM2 is one of the main negative regulators of the p53 tumor suppressor gene and 50% of bone metastases from prostate cancer are thought to have wild-type p53 (36). MDM2 and p53 form an autoregulation loop. The binding between MDM2 and p53 can negatively regulate the transrepression function of p53, inhibit p53-induced apoptosis, and target p53 for degradation (45). MDM2 has also non-p53 partners (e.g., Rb, ADP-ribosylation factor, E2F1/DP1) that might balance-out its effect on p53 function (45). This indicates that the outcome of MDM2-p53 interaction in cancer cells will likely depend on the molecular alterations present in a given tumor cell. The cells that were used in this study (MDA PCa 2a and MDA PCa 2b) have a wild-type p53. Rb and p16INK4A were considered to be normal based on the pattern of protein expression (27). On the basis of our current understanding of MDM2-p53-Rb interaction, it is difficult to predict the specific effect of MDM2 overexpression on p53 expression/function. Results presented in Fig. 5D show that p53 expression levels remain similar to the control cells. However, the oncogenic activities of MDM2 go beyond p53 to increase E2F1-mediated transactivation, thus leading to uncontrolled growth (45).

Bone metastases of prostate cancer are recognized for their osteoblastic features, yet osteolytic features are also present in most cases. The mechanisms of prostate cancer-induced osteolysis at the bone site have not been completely defined, however (8). MDA PCa 2b cells growing in the bone produce osteoblastic reaction, and although osteolysis was also observed, few osteoclasts or scalloped bone surfaces were detected. We therefore analyzed the expression of three major genes involved in bone-resorption, namely PTHrP, OPG, and RANKL. Guise et al. (20) provided strong evidence that PTHrP mediates the osteolytic metastases of breast cancer cells in vivo. We found that PTHrP expression was higher in PC-3 than in MDA PCa 2a and MDA PCa 2b cells. These findings and those of previous studies (8) suggest that the level of expression of PTHrP in prostate cancer cells inversely correlates with the propensity of those cells to form osteoblastic lesions when injected into mouse bone or into human bone implants. Moreover, evidence has been reported that PSA can cleave PTHrP and completely abolish its ability to stimulate cyclic AMP production in vitro (8). MDA PCa 2a and MDA PCa 2b cells, unlike PC-3 cells, produce and secrete PSA (26). Taken together, our results suggest that PTHrP does not play a major role in the resorptive component of prostate cancer.

Expression of OPG, a factor that inhibits the formation and activity of osteoclasts by sequestering RANKL, was not detected in MDA PCa 2b cells. Moreover, coculturing osteoblasts with prostate cancer cells resulted in a decreased expression of OPG by the osteoblasts. This dramatic decrease of OPG in the bone microenvironment might initially induce activation of osteoclasts, which is thought to be a prerequisite for tumor growth in bone. These results suggest that OPG may be a candidate chemopreventive drug for bone metastases from prostate cancer. Evidence that OPG may inhibit tumor growth in the bone was previously provided in vivo (46), and recent publications have also shown that OPG prevented the establishment of prostate cancer tumors in bone (47). Expression of OPG and RANKL in human prostate cancer was recently reported to be significantly increased in bone metastases when compared with primary prostate cancer or nonskeletal metastases (48). The OPG/RANKL ratio may therefore be an important factor influencing the degree of osteolysis in a given bone metastasis.

The results of this study demonstrate that reproducible molecular events occur in this model of bone metastases from prostate cancer. Our data thus provide a rationale for developing therapeutic strategies designed to target these molecular changes in an effort to improve the morbidity and mortality rates associated with bone metastases of prostate cancer. Indeed, strategies to inhibit the main targets identified in this study (namely IGF-I and mdm2) and to use OPG as a therapeutic agent have been developed at the bench, and some early clinical trials are currently under way (24, 49–52).

REFERENCES


Prostate Cancer Cells-Osteoblast Interaction Shifts Expression of Growth/Survival-related Genes in Prostate Cancer and Reduces Expression of Osteoprotegerin in Osteoblasts

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