RANKL Acts Directly on RANK-Expressing Prostate Tumor Cells and Mediates Migration and Expression of Tumor Metastasis Genes

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BACKGROUND. Metastases to bone are a frequent complication of human prostate cancer and result in the development of osteoblastic lesions that include an underlying osteoclastic component. Previous studies in rodent models of breast and prostate cancer have established that receptor activator of NF-κB ligand (RANKL) inhibition decreases bone lesion development and tumor growth in bone. RANK is essential for osteoclast differentiation, activation, and survival via its expression on osteoclasts and their precursors. RANK expression has also been observed in some tumor cell types such as breast and colon, suggesting that RANKL may play a direct role on tumor cells.

METHODS. Male CB17 severe combined immunodeficient (SCID) mice were injected with PC3 cells intratibially and treated with either PBS or human osteoprotegerin (OPG)-Fc, a RANKL antagonist. The formation of osteolytic lesions was analyzed by X-ray, and local and systemic levels of RANKL and OPG were analyzed. RANK mRNA and protein expression were assessed on multiple prostate cancer cell lines, and events downstream of RANK activation were studied in PC3 cells in vitro.

RESULTS. OPG-Fc treatment of PC3 tumor-bearing mice decreased lesion formation and tumor burden. Systemic and local levels of RANKL expression were increased in PC3 tumor bearing mice. PC3 cells responded to RANKL by activating multiple signaling pathways which resulted in significant changes in expression of genes involved in osteolysis and migration. RANK activation via RANKL resulted in increased invasion of PC3 cells through a collagen matrix.

CONCLUSION. These data demonstrate that host stromal RANKL is induced systemically and locally as a result of PC3 prostate tumor growth within the skeleton. RANK is expressed on prostate cancer cells and promotes invasion in a RANKL-dependent manner. Prostate 68: 92–104, 2008. © 2007 Wiley-Liss, Inc.

KEY WORDS: RANK; RANKL; OPG; PC3; osteolytic lesions; prostate cancer; bone metastases

INTRODUCTION

The majority of patients with advanced prostate cancer will develop bone metastases, and these metastases are associated with significant morbidity including pain, bone loss, fracture, hypercalcemia, and bone marrow replacement [1,2]. Prostate cancer bony metastases induce osteoblastic lesions while osteolytic

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lesions are typically observed with multiple myeloma and breast cancer. The osteoblastic or osteolytic character of bone metastases actually represents two extremes of a spectrum in which dysregulation of the normal bone remodeling process occurs [2–5]. The notion that total bone remodeling [6] is increased in patients with prostate cancer bone metastases is supported by histological evidence of osteoclast action [7] and the increases in markers of bone resorption (e.g., N-telopeptide of type I collagen, or NTX) [8]. The mechanisms by which tumor cells co-opt the normal processes of bone remodeling and the reciprocal feedback of the bone microenvironment to the bone are central to the pathophysiology of metastatic bone disease.

The RANK/RANKL/OPG axis governs osteoclastogenesis and bone resorption [9]. The TNF ligand superfamily member RANKL is critical for formation, function, and survival of osteoclasts [10,11] and exerts its action through binding and activation of its receptor RANK [12,13], which is expressed on the surface of myeloid osteoclast precursors [14]. Osteoprotegerin (OPG) is a soluble member of the TNF receptor superfamily secreted by osteoblasts which, by competing with RANK for RANKL, acts as a decoy receptor of RANKL, thereby inhibiting osteoclastogenesis. The stimulation of osteoclastogenesis observed in bone metastases is frequently a result of increases in RANKL and/or decreases in OPG levels [15]. The complete block of tumor-induced osteolytic lesions by RANKL inhibitors (e.g., RANK-Fc or OPG-Fc) in animal models of bone metastases demonstrates the vital role of this factor in cancer-induced bone disease [16,17], including that mediated by prostate cancer [18].

In addition to the expression of RANK on hematopoietic osteoclast precursors, this receptor has also been observed in some tumor cell types [19,20] and RANKL may therefore play a role in the migration and metastatic behavior of cancer in the skeleton. In this study we analyzed the expression of RANK mRNA and protein on prostate cancer cells and demonstrated that activation of RANK by RANKL results in multiple downstream signaling events and changes in global gene expression which may ultimately affect tumor metastasis.

**MATERIALS AND METHODS**

**Tumor Cell Lines**

PC3, LNCaP, DU145, MDAPCa2b, and 22Rv1 prostate cancer lines were obtained from ATCC. PC3, LNCaP, 22Rv1, and DU145 cells were cultured in RPMI with 10% fetal bovine serum (FBS). MDAPCa2b were grown in BRFF HPCI with 20% FBS (Athena Enzyme Systems, Baltimore, MD). C42B cells were obtained from UroCor, Inc. (Oklahoma City, OK) and grown in T-media [21].

**PC3 Xenograft Experiments**

At 8 weeks of age, male CB17 severe combined immunodeficient (SCID) mice (Charles River Laboratories, Wilmington, MA) were injected with $1 \times 10^7$ PC3 cells intratibially. Mice were treated with either PBS or 3 mg/kg human OPG-Fc (Amgen, Inc., Thousand Oaks, CA) weekly for 3 weeks beginning either 7 or 20 days after inoculation. The presence of osteolytic lesions was assessed by digital X-ray (Faxitron, Wheeling, IL). Tumors were allowed to progress until day 24 or 27 at which time mice were sacrificed. Lesion area was calculated from digital images using MetaMorph® Imaging software (Molecular Devices Corporation, Downingtown, PA).

**Histopathology**

Femurs and tibias were harvested and fixed in 10% neutral-buffered formalin. Bones were decalcified in 10% formic acid for 4 days and embedded in paraffin. Formalin-fixed paraffin-embedded sections were cut at 0.4 μm and stained with hematoxylin and eosin. Tumor burden was quantified on hematoxylin stained sections using MetaMorph® Imaging software (Molecular Devices Corporation). Additional sections were stained for tartrate-resistant acid phosphatase (TRAP) activity (Sigma-Aldrich, St. Louis, MO). Murine RANK and murine RANKL expression were detected using antibodies AF692 and AF462, respectively, from R&D Systems (Minneapolis, MN).

**mRNA Expression Analysis**

Total RNA was isolated from tumor cell lines or mouse tibias using the RNAeasy™ Kit (Qiagen, Valencia, CA) and transcribed into cDNA using random hexamer priming and TaqMan™ Reverse Transcription Reagents (Applied Biosystems, Inc., Foster City, CA). Tumor cell or tibia cDNA (10 ng or 25 ng, respectively) was analyzed for gene of interest expression using Assay on Demand™ primer probe sets (Applied Biosystems, Inc.). Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Inc.). Gene expression was calculated relative to β-actin expression. Reactions were performed in triplicate.

**Human RANK Protein Expression**

Surface RANK expression was determined by flow cytometry after incubation with either 1 μg/ml mouse
anti-RANK antibody (Amgen) or isotype control (BD Biosciences, San Jose, CA) in 2% FBS followed by allophycocyanin (APC)-conjugated anti-mouse secondary antibody. Fluorescence was assessed using a FAScan sorter (BD Biosciences).

**Immunoblot Analysis and Nuclear Translocation Assays**

PC3 cells were grown in serum-free RPMI overnight prior to stimulation with 1 μg/ml recombinant human RANKL (Amgen) for the times indicated. For immunoblot analysis, cells were stimulated with RANKL and harvested in 1× cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium fluoride. The soluble fraction of whole-cell lysate (15 μg) was separated on 8–16% Tris–Glycine gels and electrophobted onto nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk and subsequently incubated with antibodies to phosphorylated or total ERK, JNK, or MAP kinase p38 (Cell Signaling at 1:1,000 overnight at 4°C). Blots were incubated in anti-rabbit horseradish peroxidase (HRP; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:2,000, washed, and developed using electrochemiluminescent (ECL) imaging (GE Healthcare, Buckinghamshire, UK). To measure nuclear translocation of phosphorylated forms of p38, c-Jun, or ATF-2, cells were grown overnight in serum-free RPMI and stimulated with 1 μg/ml RANKL (Amgen) for the times indicated. Indirect immunofluorescence reagents from “Hit Kits” (Cellomics, Inc., Pittsburgh, PA) were used for cell staining. Nuclear and cytoplasmic expression was quantified using software from Cellomics, Inc.

**Gene Expression Analysis**

Cells were grown in RPMI containing 10% FBS and stimulated with 1 μg/ml human recombinant RANKL (Amgen). mRNA was isolated with the RNeasy Kit (Qiagen). For microarray expression analysis, 5 μg of total mRNA was labeled using standard protocols (Affymetrix, Santa Clara, CA) and 10 μg biotinylated cRNA was hybridized to Human Affymetrix U133A chip consisting of approximately 22,000 probe sets representing 14,500 genes. Hybridized chips were stained and washed on an Affymetrix Fluidics Station using the antibody amplification protocol and scanned using an Affymetrix GeneArray 2500 scanner. Scanned images were loaded into the Resolver™ system (Rosetta Biosoftware, Seattle, WA) for analysis.

**Cytokine Analysis**

Cells were grown in RPMI containing 10% FBS and stimulated with 1 μg/ml human recombinant RANKL (Amgen). Supernatant was harvested after 48 hr. Cytokine levels were measured using ELISAs from R&D Systems and or the Human Cytokine/Chemokine panel from LinCo (Billerica, MA).

**Cell Invasion Assay**

Recombinant human RANKL and recombinant human OPG were obtained from PeproTech, Inc. (Rocky Hill, NJ). The cell invasion assay was performed as described previously [22]. Briefly, 2.5 × 10⁴ PC3 cells in 0.5 ml RPMI-1640 medium containing 1% FBS were seeded into the upper compartment of two-layer chamber invasion plates that were separated by an uncoated membrane (for baseline control) or a membrane coated with collagen matrix (BD Transduction Laboratory, Bedford, MA). The cells were cultured for 48 hr followed by staining of the membrane with a Diff-Quik stain kit (Dade Behring, Inc, Newark, DE). Cells were quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at 200× magnification) per filter. Invasive ability was defined as the proportion of cells that invaded the matrix-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). The results are reported as the mean of duplicate assays.

**Statistical Analysis**

Statistical analysis of data was performed using Prism Software (GraphPad Software, San Diego, CA). For comparison of two groups, Student’s t-test was used. Welch’s correction of variance was used when necessary. For data comparisons with three or more groups, one-way analysis of variance (ANOVA) with Tukey’s post-test was used.

**RESULTS**

**PC3 Prostate Cancer Cells Induce Osteolysis Via Increases in RANKL**

It is well established that the prostate cancer cell line PC3 promotes osteolysis in murine experimental metastases models [23,24]. We hypothesized that PC3 cells may alter RANKL and/or OPG levels, subsequently leading to increased osteoclastogenesis. We verified the extent of PC3-induced osteolytic lesions by radiographic and histologic analysis following intratibial inoculation of PC3 cells into CB17 SCID mice (Fig. 1A,B). Osteolysis was evident by radiographic analysis at 14 days post-inoculation (Fig. 1A). The progression of these lesions over time was significant (Fig. 1A; P < 0.01 for day 24 vs. day 7 and P < 0.05 for day 24 vs. day 14). Osteolysis was associated with tumor burden and increased numbers of osteoclasts at the
tumor/bone interface compared with naïve mice (Fig. 1B,C). Osteoclasts were also observed within the tumor mass (data not shown).

To determine the relative contribution of murine host stromal RANKL versus human PC3 tumor cell RANKL expressed at tumor sites, we analyzed RANKL mRNA expression using mouse- and human-specific RT-PCR primers. There was a significant increase in mouse RANKL expression in tibias with tumor compared with contralateral non-tumor-bearing tibias at day 21 post-injection (Fig. 2A, \( P < 0.01 \)). Human RANKL mRNA was expressed at low levels in PC3 cells in vitro, but was not detectable in tumor-bearing tibias of mice at day 21 (data not shown). Murine OPG mRNA was also significantly increased in tumor-bearing tibias (Fig. 2A, \( P < 0.05 \)). However, the increase in endogenous OPG was clearly not sufficient to block RANKL, as indicated by the dramatic increase in the tumor-induced osteoclastogenesis that was observed (Fig. 1C; naïve vs. PBS). Murine RANK mRNA levels were increased approximately twofold (\( P = 0.06 \)) in tumor-bearing tibias as compared with
controls, presumably reflecting the increases in OCL numbers observed (Fig. 2A). We observed similar increases in murine RANKL and RANK protein in histological sections of naïve and tumor bearing bone (Fig. 2B). In naïve mice, low levels of murine RANKL expression were detected using immunohistochemistry in stromal cells and preosteoblasts. Tumor bearing mice demonstrated an increase in RANKL positive stromal cells located at the tumor/bone interface and within the tumor (Fig. 2B). Using IHC and antibodies against RANK, a small increase in the number of RANK positive cells, specifically mature osteoclasts and osteoclast precursors, was observed in tumor bearing mice.

We also determined the effect of PC3 tumor cell growth in vivo on systemic levels of murine RANKL and OPG. Serum murine RANKL in PC3 tumor-bearing mice was increased approximately sixfold over that of naïve mice (Fig. 2C, P < 0.0001). Serum murine OPG levels were increased less than twofold (Fig. 2C, P < 0.05). Although PC3 cells secrete OPG in vitro, human OPG was undetectable in the serum (data not shown). These alterations in murine RANKL and OPG levels resulted in a fivefold increase in the RANKL/OPG molar ratio in serum of tumor-bearing versus control animals (data not shown, P < 0.01).

To test the hypothesis that RANKL is a critical mediator of the tumor-induced osteoclastogenesis, we evaluated the ability of a RANKL inhibitor, recombinant human OPG-Fc, to block the progression of osteolytic lesions in bone. In contrast to the increase in skeletal lesions observed in PBS treated, tumor-bearing animals, mice that were treated with OPG-Fc (3 mg/kg, 3 times/week) beginning at day 7 showed no evidence of osteolytic lesions (Fig. 1A,B). Treatment with OPG-Fc eliminated tumor-induced osteoclasts (Fig. 1C) and significantly decreased tumor burden (Fig. 1D, P = 0.01). These data support a model in which tumor-associated factors converge on the mouse stroma to cause local increases in RANKL, in turn leading to the dramatic increases in osteoclasts.

**RANK Is Expressed on Prostate Cancer Cell Lines**

Our results indicate a role for the tumor-induced stromal RANKL in osteolytic lesion development after PC3 tumor establishment in the skeleton. The functional role of RANK on mammary epithelial cells [25] and tumors of epithelial origin [19,26,20] suggests...
that stromal RANKL could also contribute to skeletal metastasis via direct activation of RANK expressed by tumor cells. We examined several prostate cancer cell lines established from primary tumor (22Rv1) or metastases to lymph node (LNCaP), brain (DU145), or bone (PC3, MDAPCa2b, C42B) for RANK expression. These six lines represent androgen-dependent (LNCaP, 22Rv1, and MDAPCa2b) and androgen-insensitive (PC3, DU145, C42B) prostate cancers [27,28]. RANK mRNA was detected by quantitative real-time RT-PCR in LNCaP, DU145, C42B, and PC3 cells (Fig. 3A). We did not observe RANK mRNA expression in 22Rv1 and MDAPCa2b cell lines (data not shown). Since a functional response to RANKL requires expression of RANK on the cell surface we used flow cytometry to examine these prostate lines for RANK. LNCaP, DU145, C42B, and PC3 cells all expressed RANK on the cell surface, with PC3 cells showing particularly high levels (Fig. 3B). These data demonstrate RANK expression in prostate cancer cell lines derived from a variety of stages of disease.

**RANKL Induces Multiple Signaling Pathways in PC3 Cells**

RANK activation has been shown to lead to downstream signaling events including mitogen-activated protein (MAP) kinase p38, extracellular signal-regulated kinase (ERK), c-Jun NH2 kinase (JNK), and NF-xB pathways [20,29–31]. Treatment of PC3 cells with RANKL resulted in rapid (within 5 min) stimulation of MAP kinase p38 and ERK phosphorylation (Fig. 4A). RANKL-dependent JNK phosphorylation occurred 15 min after treatment (Fig. 4A). We expanded upon these findings by investigating the ability of RANKL to induce signaling events downstream of MAP kinase p38 and JNK. Within 15 min of RANKL exposure, phosphorylated forms of MAP kinase p38, c-Jun, and ATF-2 translocated to the nucleus (Fig. 4B), confirming the activation of MAP kinase p38 and JNK signaling pathways by RANKL.

**RANK Signaling Results in Changes in Global Gene Expression in PC3 Cells**

To further characterize the consequences of RANK activation in PC3 cells, we performed gene expression analysis using the Affymetrix U133A chip on PC3 cells treated with 1 µg/ml RANKL for 6, 12, or 24 hr. We identified genes that were regulated by RANKL activation of RANK at each timepoint, with the greatest number and magnitude of changes in expression observed at 24 hr (Table I and data not shown). Analysis of bioduplicate samples of control and RANKL-treated cells at 24 hr resulted in identification of 52 probe sets (representing 42 genes) regulated greater than twofold with $P < 0.05$. The expression of 35 probe sets (representing 28 genes) was increased in the presence of RANKL while 17 probe sets representing 14 genes reflected a decrease in expression.

We chose to focus on genes that had previously been identified as either regulated by RANKL in other cell systems (e.g., osteoclasts), increased in disease-associated osteolysis, or capable of stimulating cell migration. We looked at genes upregulated in response to RANKL during osteoclastogenesis (identified in Chaissen et al. [6]), and studied the regulation of a subset of these genes in PC3 cells. Using RT-PCR we confirmed that MMP-9 was upregulated approximately 25-fold in PC3 cells at 12 hr post-RANKL stimulation (Fig. 5A). Amongst the factors which have been implicated in stimulation of osteoclasts and involvement in osteolysis in disease states (reviewed in Ref. [15]), we observed the upregulation of CSF-1, IL-1β, TNF-α, IL-8, and IL-6 by array analysis and confirmed these gene expression changes by RT-PCR. The greatest fold induction of IL-6 was 30-fold at 12 hr, whereas maximal induction of CSF-1, IL-1β, TNF-α,
and IL-8 was approximately three- to fourfold at 24 hr after RANKL treatment (Fig. 5A). Array analysis revealed upregulation of CXCL-1, CXCL-2, CXCL-3, CXCL-5, CXCL-6, and IL-8, members of the C-X-C chemokine family which can stimulate migration and chemotaxis of tumor and immune cells [32,33]. Using RT-PCR analysis, we confirmed that CXCL-1, CXCL-2, CXCL-3, CXCL-5, CXCL-6, and IL-8 were upregulated 3- to 12-fold in PC3 cells stimulated for 24 hr with RANKL (Fig. 5A).

We expanded upon the observed gene expression changes by confirming the regulation of protein levels using cytokine specific ELISAs. Analysis of the conditioned media of PC3 cells 24 hr post-stimulation with RANKL demonstrated that CXCL-1, CXCL-2, CXCL-3, CXCL-5, CXCL-6, and IL-8 were upregulated 3- to 12-fold in PC3 cells stimulated for 24 hr with RANKL (Fig. 5A).

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Previous work has shown that RANKL increases the survival of osteoclasts [11] and is important for the proliferation and survival of mammary epithelial cells during pregnancy [25]. To investigate the pro-metastatic effects of RANK stimulation on prostate cancer cells, we studied the effect of RANKL treatment on PC3 cell proliferation, viability, and cell invasion. Under a variety of conditions, RANKL did not detectably alter PC3 cell proliferation in vitro (data not shown). Additionally, RANKL treatment did not affect the sensitivity of PC3 cells to a variety of
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</table>

Genes regulated by RANKL with greater than twofold expression differences relative to untreated cells and their associated P-values are shown. Affymetrix probe set identifiers, gene descriptions, and gene accession numbers are included.
chemotherapeutics, including CPT-11, doxorubicin, 5-Florouracil, etoposide, or cisplatin (data not shown). RANKL has been shown to stimulate chemotaxis of osteoclast-like cells and RANK-expressing tumor cells [19,34,35]. Given that RANKL is expressed in the bone microenvironment and PC3 tumor cells express RANK, we hypothesized that RANKL might serve as a “soil” factor that stimulates migration of PC3 cells to bone. We studied the effect of RANKL on invasion of PC3 cells through a collagen matrix and found that invasion was increased significantly in the presence of RANKL (Fig. 6, *P < 0.05 RANKL treated versus no treatment).

**Fig. 5.** RANKL induced changes in gene expression were confirmed at the mRNA and protein levels. **A:** mRNA levels of genes identified by array were increased in response to RANKL. mRNA was isolated from PC3 cells 12 hr (MMP-9, IL-6) or 24 hr (all other genes) after RANKL treatment. Gene expression was determined by quantitative real-time RT-PCR, normalized to β-actin and expressed relative to untreated cells. **B:** Protein levels of genes identified by array were increased in response to RANKL. Supernatant was collected 48 hr after RANKL treatment and cytokine levels determined by ELISA.

**Fig. 6.** RANKL stimulated migration of PC3 cells through collagen matrix. PC3 cells were seeded into the upper compartment of two-layer chamber invasion plates that were separated by an uncoated membrane (for baseline control) or a membrane coated with collagen matrix. The cells were cultured for 48 hr followed by staining of the membrane with a Diff-Quik stain kit. The number of cells that penetrated the membrane was counted in five microscopic fields (at 200× magnification) per filter. Invasive ability was defined as the proportion of cells that invaded the matrix-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). The results are reported as the mean of duplicate assays. *P < 0.05 for 100 ng/ml RANKL versus control and OPG treated samples.
control). RANKL induced invasion occurred in a dose-dependent manner (data not shown). Invasion of PC3 cells through collagen was blocked by the addition of OPG, indicating that RANKL activation of RANK is sufficient to lead to invasion (Fig. 6, *P* < 0.05 RANKL treated vs. OPG treated). This data demonstrates that RANKL stimulated invasion of PC3 cells in vitro and supports the hypothesis that RANKL may serve as a chemoattractant factor for RANK-expressing tumor cells in vivo.

**DISCUSSION**

A universal attribute that distinguishes cancer cells that have a high propensity to metastasize to the bone is their ability to initiate or accelerate bone remodeling. There is considerable experimental evidence that the co-opted bone microenvironment provides reciprocal feedback to support tumor growth and occurs in both osteolytic and osteoblastic bone metastases (reviewed in Ref. [15]).

In this study, we observed that PC3 prostate tumor co-opt the critical processes that govern osteoclastogenesis. We observed that interaction of PC3 cells with the bone microenvironment results in excessive RANKL levels within the bone stroma and an increased ratio of RANKL to OPG at local and systemic levels. The operative role of RANKL in PC3 tumor-induced osteoclastogenesis and bone lesions was proven by blocking RANKL in vivo with OPG-Fc which eliminated tumor-induced osteoclastogenesis and reduced skeletal lesions. Importantly, we also demonstrate that RANK is expressed in prostate tumor cell lines derived from different metastatic sites and from both androgen-dependent and androgen-independent tumors. RANKL can directly activate RANK on tumor cells as was shown by the stimulation of several biochemical signaling pathways in PC3 cells. Moreover, we demonstrate that RANKL stimulation alters the expression of genes involved in chemotaxis, migration, and invasion of cancer cells. These effects at 24 hr may reflect the direct activation of RANK or be a consequence of a cascade of downstream factors initiated by RANKL stimulation. The data suggest that RANKL may stimulate tumor cell metastases. This hypothesis is supported by our observation that RANKL can activate the invasive/metastatic behavior of PC3 cells in vitro.

The critical involvement of RANKL in bone lesion formation and progression of prostate tumor growth within the skeleton has been well established in animal models of prostate cancer bone metastasis. Pharmacological inhibition of RANKL using recombinant constructs of either OPG-Fc or RANK-Fc in these models reduces tumor induced osteoclast numbers [18,36–38]. The reduction in osteoclastic activity presumably also blocks the release of growth factors [e.g., including transforming growth factor β (TGF-β), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), bone morphogenetic proteins (BMPs), and insulin-like growth factor (IGF-1)] from the bone which normally would stimulate tumor growth. Thus, one mechanism by which RANKL inhibition reduces skeletal tumor burden is indirect, via inhibition of osteoclast activity and reduction of bone-derived growth factors [37–39]. The dependency of this effect on the bone microenvironment is supported by the observation that RANKL inhibition with OPG-Fc or RANK-Fc does not reduce the tumor growth of subcutaneously implanted prostate cancer cells [21,37,38].

While RANK expression has been primarily observed on osteoclasts, recent studies have demonstrated RANK expression on tumor cells, including breast, prostate, and melanoma cancer cell lines [19,20,26,40,41]. The ability of RANKL to act directly on RANK-expressing tumor cells that we observe in this study is consistent with a recently described role for RANKL in migration of prostate tumor cells [20] or other tumor cells [19]. Given the stimulation of tumor cell migration by RANKL, the authors of this study suggest that the rich source of RANKL within the bone may serve as a soil factor which attracts RANK-expressing tumor cells. To test this hypothesis, Jones et al., inhibited RANKL in a bone metastasis model in which the RANK-expressing melanoma does not lead to osteoclastic activation. The observed decrease in tumor growth in long bones with RANKL inhibition (using OPG-Fc) but not bisphosphonates, which suppress osteoclast activation, suggests that RANKL may act directly on tumor cells presumably via activation of cell migration [19].

In our own study we observed that RANKL inhibition with OPG-Fc reduced not only tumor-induced osteoclastogenesis and osteolytic lesions but also reduced PC3 skeletal tumor burden in a setting in which skeletal tumor and lesions were already established prior to treatment. Based on our observations of direct activation of RANK-expressing tumor cells by RANKL and the increased stromal levels of RANKL observed after PC3 interaction with the bone, there is potential for OPG-Fc to have a direct inhibitory effect on tumor cells. However, it is difficult to distinguish between direct and indirect effects of RANKL inhibition in vivo because of the degree of osteolysis induced by the tumor cells. One way in which OPG-Fc probably acts to reduce PC3 skeletal tumor burden is via indirect action on the bone microenvironment and interruption of the skeletal support of tumor growth. The identification in this current study of specific molecular markers of RANKL response in the PC3 cells (e.g.,
biochemical pathways, mRNA) will facilitate the development of pharmacodynamic markers of RANKL inhibition that can be used to demonstrate a direct effect of RANKL inhibition on the tumor cell. These experiments are currently underway.

Prostate tumors will give rise to a heterogeneous mix of blastic and lytic bone lesions in patients suggesting the activation of both osteoblast and osteoclasts. However, analysis of the critical osteoclastic regulatory proteins, RANKL and OPG, has not shown a correlation between expression and severity of bone disease. For instance, serum OPG is increased in patients with bone metastases relative to patients with non-osseous metastases and primary tumors [42–45]. Given that the majority of prostate cancer patients with bone metastases have elevated bone remodeling [7,8], the increase in OPG may be a compensatory response to skeletal remodeling or a consequence of the increased tumor burden. In patients with prostate cancer bone metastases, there is considerable evidence of tumor-induced osteoclastogenesis [7,8], however whether the main source of RANKL is from the tumor itself or the bone stroma is somewhat controversial. There are now three studies of clinical prostate bone metastasis using immunohistochemical staining for RANKL and OPG that have revealed expression in prostate tumor cells, but not the bone stroma surrounding the tumor [26,46,47]. In our own observations in this animal model of prostate cancer-induced lesions we clearly demonstrate an excessive RANKL/OPG ratio that can be observed at the local and systemic level. Through the use of species-specific PCR primers and antibodies, we show that the host contributes to the majority of RANKL and OPG changes, including a significant increase in stromal RANKL levels, while the tumor cell production of these factors is not significantly altered at the end of study (day 21). We report an increase in the mouse RANKL/OPG ratio, which is consistent with data from other observations in which osteolysis is present in disease settings [48]. The discrepancy between these clinical data [26,46,47] and our own presented here may be due in part to the PC3 model representing an extreme osteolytic response to prostate cancer while in patients there is a greater contribution of osteoblastic lesions. Alternatively, the poor sensitivity and technical challenges of immunohistochemical analysis of RANKL expression in decalcified clinical bone sections that provide obstacles to specific RANKL detection are not problematic in the PC3 bone metastases model. In fact these earlier findings of RANKL-positive prostate cancer cells within bone metastases have not been confirmed and more recent studies in clinical samples indicate that RANKL protein is elevated in the bone stroma [49]. It is formally possible that the PC3 cells did express RANKL at an earlier point prior to day 21 that was not addressed in our own study. Either human or mouse RANKL (or both) must be operating at an early point to cause osteolysis, since the RANKL inhibitor OPG-Fc blocked the progression of PC3-induced x-ray lesions from days 7 to 14 (Fig. 1A). Irrespective of the source of RANKL, RANKL inhibitors should reduce the tumor-induced osteoclastogenesis, as was clearly demonstrated in the current study.

Altogether, these data underscore the functional importance of a dysregulated RANK/RANKL/OPG axis in the pathophysiology of prostate cancer bone metastasis. Moreover, these data substantiate the newly established direct role for RANKL in the metastatic/invasive activity of tumor cells and support further studies in vivo on the role of RANKL and RANK in bone metastases.

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