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The Expression of Osteoclastogenesis-Associated Factors and Osteoblast Response to Osteolytic Prostate Cancer Cells

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Abstract

Background—Prostate cancer (PCa) has a propensity to metastasize to bone. Tumor cells replace bone marrow and can elicit an osteoblastic, osteolytic, or mixed bone response. Our objective was to elucidate the mechanisms and key factors involved in promoting osteoclastogenesis in PCa bone metastasis.

Methods—We cultured osteoblast-like MC3T3-E1 cells with conditioned medium (CM) from PC-3 and C4-2B cells. MC3T3-E1 mineralization decreased in the presence of PC-3 CM, whereas C4-2B CM had no effect on mineralization. Using oligo arrays and validating by real-time PCR, we observed a decrease in the expression of mineralization-associated genes in MC3T3-E1 cells grown in the presence of PC-3 CM. In addition, PC-3 CM induced the expression of osteoclastogenesis-associated genes IGFBP-5, IL-6, MCP-1, and RANKL while decreasing OPG expression in MC3T3-E1 cells. Furthermore, CM from MC3T3-E1 cells cultured in the presence of PC-3 CM, in association with soluble RANKL, increased osteoclastogenesis in RAW 264.7 cells. Investigation of PCa metastases and xenografts by immunohistochemistry revealed that the osteoclastic factor IL-6 was expressed in the majority of PCa bone metastases and to a lesser extent in PCa soft tissue metastases. *In vitro* it was determined that soluble IL-6R (sIL-6R) was necessary for IL-6 to inhibit mineralization in MC3T3-E1 cells.

Results—PC-3 cells inhibit osteoblast activity and induce osteoblasts to produce osteoclastic factors that promote osteoclastogenesis, and one of these factors, IL-6, is highly expressed in PCa bone metastases.

Conclusions—IL-6 may have an important role in promoting osteoclastogenesis in PCa bone metastasis through its' interaction with sIL-6R.

Keywords

Bone metastasis; MC3T3-E1; PC-3; C4-2B; IL-6

Introduction

While chemotherapeutic strategies show some promise, there is no effective therapy for advanced prostate cancer (PCa) that substantially prolongs survival. PCa metastasizes to the bone in approximately 90% of patients with advanced disease, resulting in the replacement of bone marrow, spinal cord compression, severe bone pain, cachexia, and ultimately death. Even though bone is one of the major sites of metastases for PCa, the mechanisms and key factors that promote osteoblastic and osteolytic responses are poorly understood. Our preliminary data from patients who died of PCa demonstrates that the disease, while predominantly osteoblastic, has an osteolytic component [1]. In fact, in our rapid autopsy series in an individual patient one can observe metastases that are osteoblastic at some sites, while other sites have an osteolytic or mixed bone response. Clearly, the variation in bone response in PCa is multi-factorial as factors like DKK-1, noggin, and the BMPs have been shown to be significant modulators of the bone response [2–5].

A purely osteolytic response in a PCa bone metastasis requires both the inactivation of osteoblasts and the recruitment and activation of osteoclasts within the tumor/bone microenvironment. While tumor-derived secreted factors and others may directly stimulate osteoclastogenesis, it is more likely that these tumor-derived secreted factors have an indirect effect, shutting down osteoblast activity and/or maturation, and inducing osteoblasts to produce osteoclastogenesis-associated factors including RANKL, CSF1, MCP-1, and IL-6 [6–9]. However, at this time there is insufficient evidence to conclusively determine whether the osteolytic response in PCa is direct, indirect or a combination of both direct and indirect signaling.

In the studies reported here, we investigated factors that alter mineralization *in vitro*, using two PCa cell lines: PC-3 (osteolytic) and C4-2B (osteoblastic/osteolytic). We observed alterations in calcium phosphate deposition and an increase in the expression of osteoclastogenesis-associated factors by MC3T3-E1 cells cultured in the presence of PC-3 conditioned medium (CM). Furthermore, in an effort to relate our findings to the clinical situation, we determined that IL-6 is increased in the majority of PCa bone metastases when compared to soft tissue metastases and that soluble IL-6R (sIL-6R) is required for IL-6 activity in MC3T3-E1 cells.

Based on our results, we hypothesize that, in osteolytic PCa bone metastases, tumor-derived secreted factors block osteoblast activity and induce the osteoblasts and osteoblast-like cells to produce osteoclastogenesis-associated proteins. Furthermore, we identified differences in the IL-6-type cytokine pathway that may promote the osteolytic reaction in PCa bone metastasis.

Materials and Methods

Transwell Assay

Murine MC3T3-E1 cells were cultured for 16 days in mineralization medium (MEM with 10% FBS supplemented with 10 mM β -glycerophosphate and 50 mg/mL L-ascorbate). Twenty-five hundred PC-3 or C4-2B cells were added to the top wells of transwells (Sigma Aldrich, St. Louis, MO) on day 1, 7, or 10 during the mineralization process. Recombinant mouse Noggin/Fc chimera (1 μ g/mL; R&D Systems, Minneapolis, MN) was also added on day 1, 7, or 10 in the presence or absence of PC-3 or C4-2B cells. To determine if mineralization had occurred, on day 16 cells were fixed in 10% formalin and stained with alizarin red. Absorbance was measured at 450 nm.

Mineralization and Cell Number in Wells Treated with PCa CM and IL-6-type Cytokines

Mineralization medium was added to all cultures two days after seeding the MC3T3-E1 cells. PC-3 and C4-2B CM was added 50:50 to the MC3T3-E1 cultures typically on day eight before mineralization had occurred mineralization was assessed typically on day fourteen by Von Kossa and alizarin red staining. To determine the effect of IL-6-type cytokines on MC3T3-E1 mineralization IL-6 (25 ng/mL), IL-6R (100 ng/mL), or IL-6/IL-6R (25/100 ng/mL) was added on day two during the mineralization process. On day fourteen cells were stained with alizarin red and processed as described above. Cell numbers were determined using the Quick Cell Proliferation Assay Kit (Biovision Inc., Mountain View, CA).

RNA Isolation/cDNA Synthesis, Real-time PCR

RNA was extracted from MC3T3-E1 cells and MC3T3-E1 cells treated with PC-3 CM using STAT 60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. First-strand cDNA was synthesized with the Advantage RT-for-PCR cDNA synthesis kit (BD Biosciences, Bedford, MA). Real-time PCR was performed on a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) using Platinum Quantitative PCR SuperMix-UDG reagent (Invitrogen, Carlsbad, CA) and Sybr Green 1 (Molecular Probes, Eugene, OR). PCR primers (Integrated DNA Technologies, Coralville, IA) shown below were designed to span intron-exon boundaries. The cycling conditions for all primers, except those for RANKL and OPG, were as follows: 95°C for 5 min., 45 cycles of 95°C for 30 sec., annealing temperature (see table below) for 30 sec., 72°C for 1 min., and one cycle of 72°C for 5 min. For the RANKL and OPG primers, the cycling conditions were 95°C for 5 min., 45 cycles of 95°C for 10 sec., and 69°C for 30 sec., then one cycle of 72°C for 5 min. All values were normalized to murine GAPDH.

Murine gene symbol/name	Primer sequences (5' to 3')	Annealing temperature
ANK (Progressive ankylosis)	CTAGCAGGGTTTGTGGGAGAA TTTATGAAGCAGGGCGTGAA	55
BAMBI (BMP and activin membrane-bound inhibitor)	TGCCCACTTTGGAATGCTGTCA GAAGCATTGCAAGGCCAACAT	58
Col1a2 (Procollagen type 1, alpha 2)	TGTTGGCCCATCTGGTAAAGA CAGGGAATCCGATGTTGCC	56
CTSB (Cathepsin B)	CAACGTGGAGGTGTCTGCTGAA TGTCCAGAAGTTCATGCTCCAG	60
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	TGGCAAAGTGGAGATTGTTGCC AAGATGGTGTATGGGCTTCCCG	58
IGFBP-5 (Insulin-like growth factor binding protein 5)	GGTGTGTGGACAAGTACGGAATGA ACGTTACTGCTGTCGAAGGCGT	60
IL-6 (Interleukin 6)	CTGCAAGAGACTTCCATCCAGTT GAAGTAGGGAAGCCGTGG	57
MCP-1 (Monocyte chemotactic protein 1)	TGCTACTCATTAAACCAGCAAGAT TGCTTGAGGTGGTTGTGGAA	57
OPG (Osteoprotegerin)	GGCCTTCTTCAGGTTTGCTGTTCC GCAGGTCTTCTCGTTCTCTCAATC	69
OPN (Osteopontin)	GACCACATGGACGACGATG TGGAACCTTGCTTGACTATCGA	55
PC-1 (Plasma cell membrane glycoprotein 1)	GCCAGGATCAGATGTGGAGATTG TAACCGAGCAGCAGGTCCATAC	55

Murine gene symbol/name	Primer sequences (5' to 3')	Annealing temperature
RANKL (Receptor activator for nuclear factor κ B ligand)	GATGGAAGGCTCATGGTTGGATGT CGAAAGCAAATGTTGGCGTACAGG	69
TNAP (Tissue-nonspecific alkaline phosphatase)	AGTCCGTGGGCATTGTGACTA TGCTGCTCCACTCACGTCGAT	55

Oligo Array Analysis

Gene expression in MC3T3-E1 cells cultured in the presence of PC-3 or C4-2B CM was compared to control MC3T3-E1 cells. MC3T3-E1 cells were grown for eight days in the presence of mineralization medium after which the MC3T3-E1 cells were grown in the presence of C4-2B or PC-3 conditioned/mineralization medium (50:50) for six more days. On day fourteen RNA was extracted using STAT 60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions and DNase-treated using the RNase-Free DNase Set (Qiagen Inc., Valencia, CA). Total RNA was amplified one round using the MessageAmp aRNA Kit (Applied Biosystems/Ambion, Austin, TX), incorporating amino-allyl UTP into aRNA. aRNA probe pairs were prepared by labeling 2 μ g of amplified RNA with either Cy3-dCTP (experimental) or Cy5-dCTP (matched control) fluorescent dyes (Amersham Bioscience, Piscataway, NJ) and hybridized to mouse V4.0 OpArrayTM (Operon Biotechnologies GmbH, Cologne, Germany) expression oligonucleotide microarray slides following the manufacturer's suggested protocols. Fluorescence array images were collected for both Cy3 and Cy5 using a GenePix 4000B fluorescent scanner (Molecular Devices, Sunnyvale, CA), and GenePix Pro 4.1 software was used to grid and extract image intensity data. Spots of poor quality, as determined by visual inspection, were removed from further analysis. Normalization of the Cy3 and Cy5 fluorescent signal on each array was done using Silicon Genetics GeneSpring 7.3 software (Agilent Technologies, Santa Clara, CA). A print-tip-specific lowess curve was fit to the log-intensity versus log-ratio plot and 20% of the data was used to calculate the lowess fit at each point. This curve was used to adjust the control value for each measurement. Control channel values were truncated at 10. Data were filtered to remove values from poorly hybridized cDNAs with average background subtracted intensity levels < 300.

Cytokine Arrays

Custom RayBio[®] mouse cytokine arrays (RayBiotech Inc., Norcross, GA) were incubated with MEM medium with 10% FBS, CM from MC3T3-E1 cells, MC3T3-E1 cells treated with C4-2B, or PC-3 CM (50:50). For the cytokine arrays, membranes were labeled with biotin-labeled antibodies, probed with HRP-conjugated streptavidin, developed on X-Omat film (Kodak, Rochester, NY), and densitometry assessed using the Alpha Imager 2200 (Alpha Innotech, San Leandro, CA). For densitometry, the cytokine array incubated with MEM with 10% FBS was used to determine background, densitometry values for MC3T3-E1 CM were set as 1 and densitometry values for MC3T3-E1 cells treated with C4-2B or PC-3 CM were determined relative to MC3T3-E1 CM.

RAW Cell Osteoclastogenesis Assays

Five thousand murine RAW 264.7 cells were plated in DMEM medium with 10% FBS with or without recombinant mouse RANKL (10 ng/mL, PeproTech, Rocky Hill, NJ). CM from PC-3, MC3T3-E1, or MC3T3-E1 cells grown in the presence of PC-3 CM (PC-3/MC3T3-E1) were added to RAW 264.7 cells for 6 days, with half of the media replaced on day 3. Osteoclasts were stained on day 6 using a tartrate-resistant acidic phosphatase assay (Sigma Chemical Co.) and counted. To confirm that osteoclast generation was due to RANKL

expression, experiments were repeated with recombinant mouse OPG (100 ng/mL, R&D Systems). Positive cells were TRAP positive and had 3 or more nuclei. The number of osteoclasts was normalized relative to MC3T3-E1 CM with recombinant RANKL, which was set as 1.

Immunohistochemistry

Human tissue microarrays of fixed paraffin-embedded tissues from 26 rapid autopsy patients (consisting of 3 tissue microarray blocks with 2 replicate cores per block) were used for immunohistochemical (IHC) analyses. For the IHC study of IL-6, MCP-1, and IGFBP-5 in the tibia, 4- to 6-week-old male SCID mice were injected with approximately 1×10^5 PC-3 cells into the tibiae as described previously [10]. Animals (n=2) were sacrificed after 4 weeks and the tumor-bearing legs were harvested, processed by fixation in 10% neutral buffered formalin, decalcified in 10% formic acid, and embedded in paraffin. To assess IL-6 expression in intra-tibial and subcutaneous tumors, the LuCaP xenografts, LuCaP 23.1 and 35, and the PC-3, C4-2, and C4-2B cell lines (n=2) were injected subcutaneously or intra-tibially, processed and embedded in paraffin as described previously [11,12]. Five-micron sections of the human tissue microarrays, tumored mouse tibiae, and subcutaneous tumors were deparaffinized, and antigen retrieval was performed in 10 mM citrate buffer (pH 6) for 20 min. at 120 °C, incubated with 3% H₂O₂ for 15 min., blocked with avidin/biotin blocking solution (Vector Laboratories Inc., Burlingame, CA) for 30 min. and then incubated in a 5% chicken/goat/horse serum solution for 30 min.

Antigen	Source	Clone	Dilution	Antigen Retrieval
IL-6	Ab6672; Abcam Inc.	rabbit	1:400	Yes
MCP-1	MAB2791; R&D Systems	mouse	10 µg/mL	Yes
IGFBP-5	Ab4255; Abcam Inc.	rabbit	1:300	Yes

The sections were incubated with primary antibody for 60 min. Negative control slides were incubated with rabbit IgG (Vector Laboratories Inc.) or mouse anti-MOPC21 (generated in house from a hybridoma obtained from ATCC, Manassas VA) at the same concentration as the primary antibody. All slides were then incubated with either goat anti-rabbit or horse anti-mouse biotinylated secondary antibody (1:150) (Vector Laboratories Inc.) for 30 min. and developed using the Vectastain ABC kit (Vector Laboratories Inc.) and stable DAB (Invitrogen Corp.), counterstained with hematoxylin, and dehydrated and mounted with Cytoseal XYL (Richard Allan Scientific, Kalamazoo, MI). Immunostaining was assessed using the following 4-point categorical compositional scale: 0=no staining, 1=faint/equivocal or focal staining, 2=definite staining of a minority of cells, and 3=definite staining of a majority of cells. The immunostain results were determined by consensus by CM and FVL (listed authors).

Statistical Analysis

Statistical analysis of IHC comparing bone and soft tissue metastases on tissue microarrays was described previously [13]. To quantify association with bone formation, an indicator of bone response (0=lytic to no change and 1=blastic/lytic to dense blastic) was regressed on binarized IHC staining intensities (0=faint or no staining and 1=staining of minority or majority of cells); logistic regression parameters were estimated using generalized estimating equations to account for multiple cores from the same patient.

Results

Co-Culturing PC-3 and C4-2B Cells with MC3T3-E1 Osteoblast-Like Cells Decreases Mineralization

PC-3 and C4-2B cells have been reported to stimulate an osteoclastic and osteoblastic response, respectively, *in vivo* [14,15]. To determine if PC-3 and C4-2B secreted factors block or promote osteoblast activity *in vitro*, we co-cultured PC-3 and C4-2B cells with osteoblast-like MC3T3-E1 cells. Within one day of seeding MC3T3-E1 cells, the addition of PC-3 or C4-2B cells significantly decreased mineralization as assessed by alizarin red staining (Figure 1A). When added at later time points (day 7 and 10) PC-3 cells still suppressed mineralization while C4-2B cells had little effect on mineralization. Similar results were obtained by Von Kossa staining (data not shown).

PCa cells (e.g. PC-3 and LNCaP) have been shown to express noggin, an inhibitor of the BMPs [16]. Since the BMPs promote osteoblast activity, we set out to investigate whether the loss of osteoblast activity in the presence of PC-3 cells may be due to the noggin produced by these cells. This, we cultured MC3T3-E1 cells in the presence of an excess of noggin in combination with PC-3 and C4-2B cells and determined effects on mineralization [17]. Noggin alone decreased mineralization in MC3T3-E1 cells (Figure 1A). Noggin in combination with PC-3 or C4-2B cells had an additive effect on decreasing mineralization when compared to PC-3 or C4-2B co-culture alone (Figure 1A). This suggests that factors other than noggin are responsible for the loss in osteoblast activity observed in the PC-3 co-cultures and to a lesser extent in the C4-2B co-cultures.

While MC3T3-E1 cells could mineralize the collagen matrix in the presence of PC-3 and C4-2B cells, the mineralization was disorganized with little matrix deposition when compared to control (+L-Asc) (Figure 1B). This disorganization may reflect a disruption in the differentiated phenotype of the osteoblast-like cells.

Co-culture of PCa and MC-3T3-E1 cells in transwells resulted in altered mineralization, therefore we surmised that the factors involved in decreasing matrix mineralization were secreted factors and would be present in PC-3 and C4-2B CM.

PC-3 CM Decreases Matrix Mineralization and the Expression of Matrix and Mineralization-Associated Genes in MC3T3-E1 Cells *In Vitro*

To determine the effect of PC-3 and C4-2B CM on the mineralization of an established MC3T3-E1 matrix, we allowed the MC3T3-E1 cells to establish a collagen matrix and then added PC-3 or C4-2B CM to the cells for six days. Our results show that PC-3 CM blocked mineralization, while C4-2B CM had no effect (Figure 2A).

Since this effect could be due to a decrease in MC3T3-E1 cell number, we counted MC3T3-E1 cells after culturing them for six days in the presence of PC-3 or C4-2B CM (Figure 2B). There was a significant decrease in MC3T3-E1 cells ($p=0.016$) in the presence of PC-3 CM when compared to control cells, while C4-2B CM did not significantly alter MC3T3-E1 cell count ($p=0.773$) (Figure 2B). Mineralization was assessed by both alizarin red and Von Kossa staining (Figure 2C).

To determine if the inhibition of mineralization in established MC3T3-E1 cells by PC-3 CM was at the transcriptional level, we used real-time PCR to examine the expression of five genes involved in calcium phosphate and matrix deposition: PC-1, ANK, osteopontin (OPN), tissue-nonspecific alkaline phosphatase (TNAP), and collagen type 1 α (Col 1 α) in the MC3T3-E1 cells. PC-1, OPN, and ANK mRNA expression levels were decreased in MC3T3-E1 cells cultured in the presence of PC-3 CM, while only a little change was

detected in the levels of TNAP and Col 1 α (Figure 2D). C4-2B CM increased PC-1 expression, but did not alter the expression of the other genes evaluated in the MC3T3-E1 cells (Figure 2D).

PC-3 CM Alters the Expression of IGFBP-5, IL-6, Cathepsin B, and MCP-1 in MC3T3-E1 Cells

To obtain the gene expression profile of MC3T3-E1 cells cultured in the presence and absence of PCa cell CM, RNA was isolated from MC3T3-E1 cells grown in the presence and absence of PC-3 and C4-2B CM and alterations in gene expression were identified by oligo gene array analysis. The expression of a number of genes was altered in MC3T3-E1 cells in the presence of PC-3 and C4-2B CM (Figure 3A).

However, we focused on secreted factors that promote osteoclastogenesis. Insulin-like growth factor binding protein 5 (IGFBP-5), interleukin 6 (IL-6), cathepsin B, and monocyte chemotactic protein 1 (MCP-1) were determined to be genes of interest that are secreted and expressed at elevated levels by MC3T3-E1 cells in the presence of PC-3 CM. BMP and activin membrane-bound inhibitor (Bambi) was a gene of interest that showed the highest increase in gene expression in MC3T3-E1 cells cultured in the presence of C4-2B CM (Figure 3A).

To validate the results of the oligo gene arrays, we used real-time PCR on RNA from MC3T3-E1 cells cultured in PC-3 or C4-2B CM. IGFBP-5, IL-6, cathepsin B, and MCP-1 were increased in MC3T3-E1 cells cultured in PC-3 CM (Figure 3B). Bambi was not altered in MC3T3-E1 cells cultured in the presence of C4-2B CM (Figure 3B). As IGFBP-5, IL-6, and MCP-1 have been associated with osteoclastogenesis, we also examined the effect of PC-3 and C4-2B CM on the expression of receptor activator of NF- κ B ligand (RANKL) and osteoprotegerin (OPG) in MC3T3-E1 cells in culture using real-time PCR as these two genes have been shown to be central to osteoclastogenesis. The expression of RANKL was increased and OPG was decreased in MC3T3-E1 cells cultured in PC-3 CM. This increase in RANKL may be exaggerated by its relatively low expression levels in control MC3T3-E1 cells (Figure 3C). These data show that there is an increase in osteoclastogenesis-associated gene expression in MC3T3-E1 cells in the presence of PC-3 CM.

Cytokine Arrays of IGFBP-5, IL-6, MCP-1, OPG, and RANKL Protein Expression and Secretion in the Media of MC3T3-E1 Cells Cultured in the Presence of PC-3 or C4-2B CM

To ensure the real-time PCR validated genes IGFBP-5, IL-6, MCP-1, OPG and RANKL were also altered at the protein level, we constructed mouse cytokine arrays of known osteoclastogenesis-associated proteins. Using these mouse cytokine arrays we detected a greater than four-fold increase in IL-6, a nearly 3-fold increase in MCP-1 and a 2-fold increase in IGFBP-5 in the medium of MC3T3-E1 cells grown in the presence of PC-3 CM. In agreement with our real-time PCR results, the level of OPG protein was decreased in PC-3 conditioned MC3T3-E1 medium. However, RANKL was not significantly altered for either CM (Figure 4). The discordance between the real-time PCR results and mouse cytokine arrays for RANKL may be because RANKL is membrane bound in these cells and not secreted. We saw no differences in these proteins of interest when we compared the cytokine array profile of PC-3 CM to MEM with 10% FBS. These results show that the changes detected in the cytokine arrays are produced by the murine-derived MC3T3-E1 cells (data not shown).

IL-6, MCP-1, and IGFBP-5 Are Expressed by PC-3 Cells in the Tibia of Mice

Our *in vitro* results suggest that the changes in IL-6, MCP-1, IGFBP-5, RANKL, and OPG expression in the mouse osteoblasts promote osteoclastogenesis. PC-3 cells injected into the

tibia of SCID mice result in an osteolytic lesion, and we investigated whether some of these osteoclastic factors are also expressed by the tumor cells themselves. Using IHC, we observed the expression of tumor-derived IL-6, MCP-1, and IGFBP-5 in intra-tibial PC-3 tumors (Figure 5). The expression of these factors by the tumor cells may exacerbate the inhibition of osteoblast activity and further promote the production of osteoclastogenesis-associated factors by the osteoblasts within the tumor microenvironment.

MC3T3-E1 Medium Conditioned with PC-3 Medium (PC-3/MC3T3-E1) and Soluble RANKL Increases Osteoclastogenesis in Osteoclast Precursor RAW 264.7 Cells

To determine if PC-3/MC3T3-E1 CM could drive osteoclastogenesis, we added PC-3, PC-3/MC3T3-E1, or MC3T3-E1 CMs to RAW 264.7 cells. We found that the addition of soluble RANKL was required for osteoclastogenesis in RAW 264.7 cells. Not surprisingly, OPG completely blocked osteoclastogenesis under all conditions (Table 1).

The production of osteoclasts (defined as TRAP positive cells with ≥ 3 nuclei) in the presence of MC3T3-E1 CM and soluble RANKL was used as a control and set to 1. The addition of soluble RANKL to RAW 264.7 cells in the presence of PC-3 CM slightly decreased osteoclast number (0.61 ± 0.94). PC-3/MC3T3-E1 CM slightly increased osteoclast number (1.89 ± 1.17) in RAW 264.7 cells (Table 1). However, these differences were not statistically significant ($p=0.14$).

IL-6 Has Higher Expression in PCa Cells in Intra-Tibial Tumors When Compared to Subcutaneous Tumors in Mice

To determine IL-6 expression was consistent in the bone and subcutaneous microenvironments in animal models of PCa, we stained intra-tibial and subcutaneous PC-3, C4-2, C4-2B, LuCaP 23.1, and LuCaP 35 tumors for IL-6. IL-6 was consistently observed in intra-tibial tumors with less expression observed in the subcutaneous tumors from the same cell or xenograft line (Figure 6).

The Expression of IL-6 Is Associated with Osteoblastic PCa Bone Metastasis

IL-6 is associated with osteoclastogenesis, and our results showed that it was produced in MC3T3-E1 osteoblast-like cells in response to PC-3 CM and that IL-6 was expressed by the PC-3 cells *in vivo*. To examine the expression of IL-6 in patient samples, IHC was performed on samples of patients who died from PCa using specimens from our rapid autopsy series. IL-6 was expressed in the majority of PCa bone metastases and expressed in soft tissue metastasis (including lymph nodes, liver, and lung) to a lesser extent (Figure 7A). There was very strong evidence that IL-6 staining was more intense in bone tissue than in soft tissue ($p < 0.0001$). We estimated the odds of intense staining in bone metastases to be 51 times that of soft tissue metastases (95% CI from 21 to 122). These results are concordant with previously published results showing IL-6 is increased in the serum of patients with PCa bone metastases [18, 19].

Interestingly, our results shown in Figure 7B suggest that PCa osteoblastic metastases express high levels of IL-6, and we found weak evidence that IL-6 staining intensity was associated with the osteoblastic bone response ($p=0.07$). This discordance between IL-6 as an osteoclastic factor and the association of IL-6 expression with osteoblastic metastases is addressed in the discussion.

Due to the considerable variation in bone response in each bone core, we grouped the bone response into two groups: predominantly osteolytic or predominantly osteoblastic. We estimated the odds of intense staining in predominantly osteoblastic tissue to be 3.1 times the odds in predominantly osteolytic tissue (95% CI from 1 to 10).

The Soluble IL-6 Receptor (sIL-6R) Is Required to Inhibit Osteoblast Activity in Response to IL-6 in MC3T3-E1 Cells

IL-6 is an osteoclastogenesis-associated factor that was secreted by the MC3T3-E1 cells in response to PC-3 CM. Published reports suggest that IL-6 does not influence osteoblast activity in the absence of the soluble IL-6 receptor [20]. To test this hypothesis in our *in vitro* system, we treated MC3T3-E1 cells with IL-6, sIL-6R, or a combination of both. IL-6 did not significantly decrease mineralization, but the combination of IL-6 with sIL-6R significantly decreased the mineralization of the MC3T3-E1 cells *in vitro* ($p=0.0054$) (Figure 8A and B). This suggests that the sIL-6R is necessary for IL-6 to inhibit mineralization in MC3T3-E1 cells.

Discussion

While PCa bone metastases are predominantly osteoblastic, there is an osteolytic aspect to the disease. This osteolytic behavior is observed for most PCa cell lines that are implanted in bone. PC-3 cells are extremely osteolytic when injected into the tibia of SCID mice, whereas C4-2B cells have produced mixed osteoblastic/osteolytic lesions [14,15]. In our initial *in vitro* studies both PC-3 and C4-2B cells decreased mineralization if present prior to collagen deposition by the osteoblast-like cells. At later stages, the C4-2B cells had little or no effect on mineralization whereas PC-3 cells inhibited mineralization at all stages. This suggests that PC-3 cells are more effective at blocking mineralization than C4-2B cells. Also in these studies, noggin, which is an inhibitor of the BMPs given in excess, had an additive effect on decreasing mineralization in the osteoblast-like cells, suggesting that the BMPs do not have a role in PC-3 abrogated mineralization.

PC-3 CM blocked both mineralization and the transcription of mineralization-associated genes, while promoting the production of osteoclastogenesis-associated genes. The limited effect of PC-3 CM on TNAP and collagen type 1 α mRNA expression might stem from the fact that the cells were treated with the PC-3 CM after an initial eight days of culture, during which time the MC3T3-E1 cells had already produced a sufficient collagen matrix. C4-2B CM had little to no effect on mineralization or mineralization associated genes, suggesting it was not promoting or inhibiting mineralization in these cells.

While an increase in the expression of other genes like slit2, which is an inhibitor of MC3T3-E1 differentiation, was observed in MC3T3-E1 cells grown in the presence of PC-3 CM, we concentrated on the expression of secreted factors that were known to be or might be associated with osteoclastogenesis. The mRNA of one of these factors, RANKL was increased in osteoblast-like cells in response to PC-3 CM, with a concomitant decrease in OPG mRNA expression. Of interest was the lack of soluble RANKL in the PC-3 CM as determined by the lack of RANKL binding to the cytokine array and the inability of PC-3/MC3T3-E1 CM to stimulate osteoclastogenesis without the addition of recombinant mouse RANKL. These data support the notion that soluble RANKL probably isn't central to the osteolytic activity of PC-3 tumors; rather an increase in osteoblast-derived membrane-bound RANKL may be required to promote osteoclastogenesis. This further suggests that the local expression of osteoclastic factors and receptor interactions between osteoblasts and the osteoclast precursor cells is important for the formation of osteoclasts in PCa tumors *in vivo*.

The osteoblasts also produced other known osteoclastic factors in response to PC-3 CM: IGFBP-5, MCP-1, and IL-6. Interestingly, the PC-3 cells also produce these factors, and we postulate that if produced at the required concentrations within the bone microenvironment they could directly facilitate osteoclastogenesis.

The C4-2B CM had little effect on altering the gene expression profile determined by the oligo array data. While there was an increase in RANKL mRNA expression, C4-2B CM had a limited effect on the expression of other osteoclastogenesis-associated genes, again suggesting that C4-2B cells have a limited effect on osteoblasts *in vitro*.

A novel finding from our IL-6 expression data from the PCa soft tissue and bone metastases indicates that IL-6 is highly expressed in bone metastases when compared to soft tissue metastases. It has already been reported that IL-6 is significantly elevated in the sera of patients with PCa bone metastases [18,19]. IL-6 is purported to promote the osteolytic response [21], and IL-6 increases the expression of osteoclastogenesis promoting factors in osteoblasts [22,23]. All of these data suggest that the expression of IL-6 by PCa cells in the bone could promote the osteoclastic response in PCa bone metastases.

However, in our IHC analyses of IL-6 expression in the bone we found weak evidence that IL-6 staining intensity is associated with the osteoblastic metastases. Furthermore, while the serum levels of IL-6, PICP (a marker of bone formation), and ICTP (a marker of bone resorption) are significantly higher in patients with PCa bone metastasis, the serum levels of IL-6 do not directly correlate with increased bone resorption [18]. This suggests that IL-6-regulatory factors may have a role to play in bone resorption in PCa.

A possible reason for the discordance between the high levels of IL-6 expression and bone resorption in PCa bone metastases is that IL-6-type cytokine signaling is highly regulated in the bone microenvironment. Osteoblasts express low levels of IL-6R mRNA and do not appear to respond to classical IL-6/IL-6R/gp130 signaling. They preferentially respond to the soluble form of the IL-6R (sIL-6R) which may be shed from osteoblast precursors or from other sources within the microenvironment [20]. The IL-6/sIL-6R complex then binds to the membrane-bound gp130 receptor on the surface of the osteoblast precursors, thereby blocking osteoblast activity and promoting osteoclastogenesis [24,25].

Therefore, the shedding or the bio-availability of sIL-6R determines the ability of IL-6 to induce gp130 phosphorylation through trans-signaling in osteoblast precursors [20]. We verified that sIL-6R is required to potentiate the ability of IL-6 to block MC3T3-E1 mineralization in our model system *in vitro*.

If however sIL-6R is not shed from osteoblasts in the PCa bone metastasis, IL-6 expression alone would not determine the phenotype of the osteoblast progenitor cells present in the bone marrow. This scenario is what we believe is portrayed in the IHC studies. This means that the osteolytic component of the bone response may in part be due to variable expression levels of the IL-6-type cytokines and their associated regulatory proteins such as sIL-6R in PCa bone metastasis.

In conclusion, we have shown that osteoblast-like cells stop mineralizing and produce osteoclastogenesis factors (IGFBP-5, MCP-1, RANKL and IL-6) in the presence of PC-3 cells. We have also shown that osteolytic factors including IL-6 are expressed by PC-3 cells, and that osteoblast-like cells follow the osteoblastic lineage when the balance favors decreased sIL-6R availability in the presence of IL-6. Finally, we have shown that PCa cell lines and xenografts express elevated levels of IL-6 in the bone microenvironment and that IL-6 expression is significantly increased in the majority of PCa bone metastasis when compared to soft tissue metastases in patients from our rapid autopsy program.

We hypothesize that, in osteolytic PCa bone metastases, tumor-derived secreted factors block osteoblast activity and induce osteoblast-like cells to produce osteoclastogenesis promoting proteins. We also expect that tumor or osteoblast-derived secreted factors like IL-6 stimulate osteoclast formation and activity and that the osteoclastogenesis promoting

activity of IL-6 may be regulated by sIL-6R in the bone microenvironment (Figure 9). Ongoing studies in our laboratory are currently addressing the role of sIL-6R in osteolytic PCa tumors in bone.

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Figure 1. PC-3 and C4-2B co-culture alters mineralization of MC3T3-E1 osteoblast-like cells (A) MC3T3-E1 cells were cultured for 16 days in control mineralization medium with L-ascorbate (+L-Asc). PC-3 or C4-2B cells were added in transwells on day (D) 1, 7, or 10 during the mineralization process. The BMP inhibitor noggin (1 mg/mL) was also added on day (D) 1, 7, or 10 in the presence or absence of PC-3 or C4-2B cells in transwells. Alizarin red levels (mineralization) were assayed and graphed relative to control. Results are plotted as mean $\pm$ SD of three independent experiments. * indicates significant difference from control ($p < 0.05$). No L-ascorbate (-L-Asc) was used as a negative control. (B) The mineralization pattern was more defined in control wells and more diffuse in cells grown in the presence of PC-3 or C4-2B cells from day 7 onwards.

Figure 2. PC-3 secreted factors decrease mineralization-associated gene expression and calcium phosphate deposition by MC3T3-E1 cells

(A) Mineralization of MC3T3-E1 cells in the presence of C4-2B and PC-3 CM relative to control (+L-Asc, which was set to 1) in three independent experiments. * indicates significant difference from control ($p < 0.05$). (B) Proliferation assay to determine MC3T3-E1 cell number grown in the presence of C4-2B conditioned, PC-3 conditioned, or control (+L-Asc) medium in four independent experiments. * indicates significant difference from control ($p < 0.05$). (C) Von Kossa and alizarin red staining of MC3T3-E1 cells cultured without L-ascorbate (-L-Asc), in the presence of C4-2B or PC-3 CM, or in the presence of L-ascorbate (+L-Asc). (D) Expression of matrix and mineralization-associated genes by real-time PCR in MC3T3-E1 cells grown in the presence of C4-2B (black) or PC-3 (white) CM. Values are fold differences in gene expression when compared to MC3T3-E1 cells cultured in control (+L-Asc) medium. The experiments were performed a minimum of three times.

Figure 3. Oligo gene array analysis and real-time PCR analyses of osteoclastogenesis-associated gene expression in MC3T3-E1 cells in response to PCa CM

(A) Gene arrays of genes upregulated in MC3T3-E1 cells grown in the presence of C4-2B or PC-3 CM. Genes that encode for secreted proteins of interest are highlighted. (B) Real-time PCR analyses of differentially expressed genes in MC3T3-E1 cells grown in the presence of C4-2B (black boxes) or PC-3 (white boxes) CM. Values are fold differences in gene expression compared to MC3T3-E1 cells cultured in control (+L-Asc) medium. (C) Real-time PCR analysis of RANKL and OPG in MC3T3-E1 cells grown in the presence of C4-2B (black boxes) or PC-3 (white boxes) CM. Values are fold differences in gene expression compared to MC3T3-E1 cells cultured in control (+L-Asc) medium. The experiments were repeated four times.

Figure 4. Osteoclastogenesis-associated proteins secreted by MC3T3-E1 cells cultured in the presence of C4-2B or PC-3 CM

Representative pictures of mouse cytokine arrays are presented (MC3T3-E1 CM, CM from MC3T3-E1 cells grown in the presence of C4-2B CM, or CM from MC3T3-E1 cells grown in the presence of PC-3 CM). Relative expression was assessed by densitometry. Table values are relative to control MC3T3-E1 CM (set to 1). This experiment was repeated twice.

Figure 5. Immunohistochemical localization of IGFBP5, MCP-1, and IL-6 in PC-3 tumored tibiae in SCID mice

Two hundred-fold magnification of IL-6, MCP-1, and IGFBP-5 staining in PC-3 cells injected into the tibia of SCID mice and IgG controls (n=3).

Figure 6. Immunohistochemical analysis of IL-6 in different PCa intra-tibial and subcutaneous PCa tumors in SCID mice

IL-6 had consistently higher expression in all PCa tumors examined in the bone (200-fold magnification). Control sections were stained with rabbit IgG.

Figure 7.

Immunohistochemical analysis of IL-6 expression in (A) human PCa lymph node (Panel A) and bone (Panel B) metastases (100-fold magnification). IL-6 was significantly increased in bone versus soft tissue metastases. Specific immunostaining was assessed on a four-point scale: 3 = intense, 2 = defined, 1 = faint, and 0 = absent. (B) Scatterplot of bone response by staining intensities for bone tissue samples. Bone response levels are lytic (L), lytic/blastic (LB), penic/blastic (PB), penic/lytic (PL), penic (P), no change (NO), blastic/lytic (BL), dense blastic/lytic (DBL), blastic (B), or dense blastic (DB). IL-6 expression was weakly associated with an osteoblastic bone reaction.

Figure 8. IL-6 and sIL-6R are required to significantly inhibit mineralization in MC3T3-E1 cultures *in vitro*

(A) Representative pictures of MC3T3-E1 mineralization in the presence of no L-ascorbate (-L-Asc), L-ascorbate (+L-Asc), IL-6 (25 ng/mL), an IL-6/sIL-6R (25/100 ng/mL) combination, or sIL-6R (100 ng/mL). (B) MC3T3-E1 cells were cultured for 14 days. IL-6 (25 ng/mL), a combination of IL-6/sIL-6R (25/100 ng/mL) or sIL-6R (100 ng/mL) was added on day 2 during the mineralization process. Alizarin red levels (mineralization) were assayed on day 14 and graphed relative to control (+L-Asc). Results are plotted as mean $\pm$ SD for three independent experiments. * indicates significant difference from control ($p < 0.05$).

Figure 9. Hypothetical model of the events that contribute to osteoclastogenesis in prostate cancer bone metastasis

Osteolytic secreted factors block osteoblastic activity in osteoblasts and promote the production of osteoclastic factors, including IL-6 in the bone microenvironment. IL-6 produced by the PCa cells in bone can only block osteoblast activity and promote the production of osteoclastogenesis promoting factors through interacting with the sIL-6R.

Table 1

An osteoclastogenesis assay using RAW 264.7 cells treated with PC-3, PC-3/MC3T3-E1, or MC3T3-E1 CM. RAW cells were cultured with or without RANKL (10 ng/mL) or with RANKL (10 ng/mL) plus OPG (100 ng/mL). Positive cells were TRAP positive and had ≥ 3 nuclei. Osteoclast number is defined relative to the number of osteoclasts present in MC3T3-E1 CM, which was set as 1. This is the result of four independent experiments.

MEDIA	– RANKL	+ RANKL	+ RANKL + OPG
PC-3	0.0	0.61 $\pm$ 0.94	0.0
PC-3/MC3T3-E1	0.0	1.89 $\pm$ 1.17	0.0
MC3T3-E1	0.0	1.0	0.0