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Interleukin-6 and Prostate Cancer Progression

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Prostate carcinoma (PCA) initially responds to androgen deprivation. However, it usually reoccurs in a form that is unresponsive to further hormonal manipulations. This latter form of PCA, termed androgen independent cancer, inexorably progresses resulting in the demise of the patient. Interleukin-6 (IL-6) and IL-6 receptor are expressed in PCA and activates the androgen receptor (AR). In the current work we are exploring the hypothesis that IL-6 contributes to the progression of PCA that is observed post-androgen deprivation, through enhancing AR activity. In the current year's activity, we created cells that are stably transfected with an AR-green fluorescent protein (GFP) protein, we used these cells to determine that IL-6 activates the AR through several signal transduction pathways. We have also initiated several experiments to evaluate the ability of anti-IL-6 to prevent reestablishment of prostate cancer tumors in castrated mice. Finally, we have used an array technology to identify which transcription factors are activated by IL-6 in prostate cancer cells.
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INTRODUCTION:
Prostate carcinoma (PCA) initially responds to androgen deprivation. However, it usually reoccurs in a form that is unresponsive to further hormonal manipulations. This latter form of PCA, termed androgen independent cancer, inexorably progresses resulting in the demise of the patient. The mechanism responsible for development of androgen independent cancer is unknown. However, some clues may be found in the response of PCA cells to the cytokine interleukin-6 (IL-6). Specifically, IL-6 and IL-6 receptor are expressed in PCA. Furthermore, inhibition of IL-6 in prostate cell culture diminishes PCA cell proliferation demonstrating the presence of an autocrine mechanism of IL-6 activity. Finally, IL-6 has been shown to both activate the androgen receptor (AR) in the absence of androgen and sensitize the AR to androgen. These observations have important implications regarding androgen-deprivation therapy. In the current work we are exploring the hypothesis that IL-6 contributes to the progression of PCA, that is observed post-androgen deprivation, through enhancing AR activity. We will test our hypothesis by the following combination of in vitro and in vivo objectives: Objective I: Determine the mechanism through which IL-6 sensitizes AR to androgen. Objective II: Evaluate if inhibition of IL-6 diminishes PCA proliferation in a rodent model. Objective III: Determine if IL-6 contributes to PCA progression post-androgen deprivation. In summary, these experiments should help identify the extent and mechanism of IL-6’s role in PCA progression. They are designed to elucidate if IL-6 promotes androgen hyperresponsive tumors or truly androgen-independent tumors. These data should provide a rationale for target IL-6 for inhibiting PCA progression.

BODY:
Statement of Work Tasks for the Initial Funding Period:
Task 1. Determine the mechanism through which IL-6 sensitizes AR to androgen. (months 1-18)

- perform Western and PCR analyses to determine if IL-6 increases AR expression (months 1-3)
- perform transfection experiments to determine if IL-6 increases AR gene activation (months 4-9)
- perform transfection experiments to determine if IL-6 increases AR transactivation strength (months 10-12)
- perform bandshift assays to determine if IL-6 increases nuclear levels of AR (months 13-14)

These aims have all been accomplished and were presented in the previous progress report and manuscript #1 that accompanied last year’s progress report.

We have now extended these studies to evaluate the role that signal transduction plays in IL-6-mediated activation of the AR. Specifically, we have stably transfected several prostate cancer cell lines with the AR fused to green fluorescent protein. We could then visualize that IL-6 induces nuclear translocation (Fig. 1).

![Figure 1. IL-6 induces AR nuclear translocation. PC-3 cells transfected with AR-GFP fusion protein were treated with IL-6 and 3 hours later examined under a fluorescent microscope. Note nuclear localization of strong fluorescence.](untreated.png)  ![IL-6 (10 ng/ml)](treasured.png)
Task 2. Produce reagents needed for Tasks 3 and 4 (months 1-12)

- prepare anti-murine IL-6 and anti-murine isotype antibodies for Tasks 3 and 4 by inoculating mice with hybridoma, collecting ascites fluid, purifying antibodies (months 1-4)
- maintain tumor in nude mice until ready for transplantation [20 mice] (months 1-12)

We have accomplished these aims as reported in the previous progress report and used these materials as reported in manuscript #2 that accompanied last year’s progress report.

Task 3. Evaluate if inhibition of IL-6 diminishes PCA proliferation in a rodent model [80 mice] (months 10-21)

- initiate tumor model in sham operated or orchiectomized nude mice and administer IL-6 and isotype antibody (months 10-16)
- euthanize mice, analyze tumor tissue for growth, AR/IL-6 expression and androgen sensitivity (months 17-21)

We have performed an in vivo study using anti-IL-6 to inhibit development of prostate tumor growth. These results were reported in the last annual report and the appended Manuscript #2. We are now performing experiments in which we are testing the ability of blocking IL-6 (with antibody) to prevent progression of prostate cancer. We have established tumors in animals and then castrated the animals and initiated anti-IL-6 or isotype control antibody.

KEY RESEARCH ACCOMPLISHMENTS:

- Creation of several prostate cancer cell lines that are stably transfected with androgen receptor (AR)-GFP fusion protein.
- Visualization using the AR-GFP cell lines that IL-6 induces nuclear translocation.
- Identification that several kinase inhibitors can block IL-6-mediated AR nuclear translocation.
- Initiated tumor establishment/castration and anti-IL6 experiments.
- Beginning to explore the contribution of IL-6 to prostate cancer bone metastasis.
- We have identified a variety of transcription factors activated by IL-6 in prostate cancer cells using an array technology that allows us to identify transcription factors in nuclear extracts.

REPORTABLE OUTCOMES:

1. MANUSCRIPTS

2. ABSTRACTS
CONCLUSIONS:
Our in vivo results document that IL-6 mediates a role in prostate cancer progression in vivo in an animal model. Furthermore, our in vitro data show that signal transduction cascades are required for IL-6 to mediate the activation of AR. The identification of signal cascades provides potential targets to block IL-6’s contribution to prostate cancer.
Prostate carcinoma skeletal metastases: Cross-talk between tumor and bone

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Key words: prostatic neoplasms, skeletal metastases, bone morphogenetic protein, parathyroid hormone-related protein, stromal-derived factor, matrix metalloproteinase

Abstract

The majority of men with progressive prostate cancer develop metastases with the skeleton being the most prevalent metastatic site. Unlike many other tumors that metastasize to bone and form osteolytic lesions, prostate carcinomas form osteoblastic lesions. However, histological evaluation of these lesions reveals the presence of underlying osteoclastic activity. These lesions are painful, resulting in diminished quality of life of the patient. There is emerging evidence that prostate carcinomas establish and thrive in the skeleton due to cross-talk between the bone microenvironment and tumor cells. Bone provides chemotactic factors, adhesion factors, and growth factors that allow the prostate carcinoma cells to target and proliferate in the skeleton. The prostate carcinoma cells reciprocate through production of osteoblastic and osteolytic factors that modulate bone remodeling. The prostate carcinoma-induced osteolysis promotes release of the many growth factors within the bone extracellular matrix thus further enhancing the progression of the metastases. This review focuses on the interaction between the bone and the prostate carcinoma cells that allow for development and progression of prostate carcinoma skeletal metastases.

1. Introduction

Prostate carcinoma is the most frequently diagnosed cancer in men and the second leading cause of cancer death among men in the United States [1]. The most common site of prostate carcinoma metastasis is the bone with skeletal metastases identified at autopsy in up to 90% of patients dying from prostate carcinoma [2–4]. Skeletal metastasis results in significant complications that diminish the quality of life in affected patients. These complications include bone pain, impaired mobility, pathological fracture, spinal cord compression and symptomatic hypercalcemia [5–7]. Despite advances in the diagnosis and management of prostate carcinoma, advanced disease with skeletal metastasis remains incurable. Current therapeutic modalities are mostly palliative, and include hormonal therapy, pharmacological management of bone pain, radiotherapy for pain and spinal cord compression [8], various chemotherapy regimens, and the use of bisphosphonates to inhibit osteoclast activity [9]. In spite of the severe complications of prostate carcinoma skeletal metastasis, there have not been many advances in the therapeutic arena to prevent or diminish these lesions. It is critical that a solid understanding of the pathophysiology of prostate carcinoma skeletal metastatic process is developed to provide the basis for creating strategies to prevent or diminish their occurrence and associated complications. A preponderance of evidence suggests that establishment and progression of prostate carcinoma bone metastases is dependent on interaction between the bone microenvironment and the prostate carcinoma cell through both soluble and cell-membrane bound bioactive factors. In this review, we will summarize some of the cross-talk mechanisms between bone and prostate carcinoma.

2. The effects of bone on prostate carcinoma metastasis

In agreement with the ‘seed and soil’ theory of metastases espoused by Paget [10], the predilection of prostate carcinoma to establish metastases in bone as
opposed to other organs suggests that the bone microenvironment offers a fertile soil for prostate carcinoma growth. Prior to interacting on the bone cells and bone matrix, the prostate carcinoma cells must enter the bone compartment. This is accomplished by several general mechanisms that include chemotaxis from the circulation, attachment to bone endothelium, extravasation, and invasion. The bone microenvironment is a complicated mixture of mineralized and non-mineralized bone matrix and endothelial, hematopoietic, immune, and bone marrow stromal cells. Each of these components of the bone microenvironment may contribute to the establishment of prostate carcinoma metastases through provision of chemotactic, angiogenic, adhesion and growth factors.

2.1. Chemotaxis

When prostate carcinoma cells are injected adjacent to adult human bone implanted in SCID mice, the prostate carcinoma cells migrate to adult human bone [11]. This observation provides evidence that bone provides chemotactic factors for prostate carcinoma cells. This is further supported by the observation that bone undergoing active resorption facilitated adhesion [12] and chemotaxis [13,14] of tumor cells to bone compared to non-resorbing bone. Collagen products appear to be one component of bone that induces tumor chemotaxis [15]. The factors through which bone induces chemotaxis are not clear. However, low glycylated osteonecrotic was found to be an active chemotactic factor in crude bone extracts that promoted chemotaxis of human prostate epithelial cells and increased the invasive ability of human prostate carcinoma cells [16]. In contrast with this observation, purified fibronectin, but not crude bone extracts induced migration of the prostate carcinoma DU-145 cell line [17]. Cell line specificity may account for these differences. Epidermal growth factor induced migration of the TSU-prl prostate carcinoma cell line [18]. Since EGF is present in medullary bone, this observation suggests that it may act as a chemotactic factor for bone metastases. Finally, the Rho-kinase inhibitor, Y-27632, inhibited in vitro chemotactic migration to bone marrow fibroblast conditioned media and metastatic growth in immune-compromised mice of highly invasive human prostatic cancer (PC3) cells [19]. This observation suggests that modulation of kinase activity may prove fruitful in inhibition of skeletal metastasis.

In addition to the above substances, which typically are not considered chemotactic factors, prostate carcinoma cells may commandeer the normal leukocyte bone marrow homing mechanism using the chemokine pathway [20]. Chemokines are classified based upon the relative position of cysteine residues near the NH2-terminus into four major families: CC, CXC, C,CX3C (as reviewed in [21]). Chemokines activate receptors that are members of the large family of seven-transmembrane G protein-coupled proteins. In addition to the role that chemokines have in cell migration, they play significant roles in normal development, inflammation, atherosclerosis and angiogenesis. The rapidly increasing knowledge of chemokines has begun to impact many aspects of tumor biology including modulation of proliferation, angiogenesis and immune response to tumor (as reviewed in [22]).

An important role for chemokines may be to regulate metastatic behavior. Localization in tissues and migration to target organs are essential steps in the pathobiology of metastasis which strongly support the analogy to hematopoietic cell homing. In this context, the CXC chemokine stromal-derived factor (SDF-1; CXCL12) and its receptor, CXCR4 appear to be critical molecular determinants for these events [23,24]. This has been substantiated in gene knockout investigations [25,26] and by the demonstration that level of CXCR4 expression correlates with the ability of human hematopoietic progenitors to engraft into nude mice [26]. In the bone marrow, SDF-1 is constitutively produced by osteoblasts, fibroblasts and endothelial cells [27]. However, not all vascular endothelial cells express SDF-1, suggesting that organ-specific expression SDF-1 may account for the selectivity of metastases to target certain organs [28].

Several lines of evidence suggest that SDF-1 contributes to the pathogenesis of prostate carcinoma metastases. Inhibition of chemokines diminished in vitro proliferation of PC-3 cells [29] and anti-CXCR2 antibody inhibited IL-8-stimulated migration of PC-3 cells in vitro [30]. These studies suggest that chemokines contribute to prostate metastatic pathophysiology. This possibility is reinforced by the observation that CXCR4 is expressed in normal prostate tissues, albeit at low levels [31], as well as several neoplasms that invade the marrow (e.g., breast cancers, Burkitt's lymphoma, leukemias) [31–33]. Furthermore, several prostate carcinoma cell lines express CXCR4 mRNA, and SDF-1 increased migration of these cells in vitro [34]. It was recently demonstrated that normal breast tissues express little CXCR4, whereas breast neoplasms express high levels of CXCR4 [35,36], and antibody to CXCR4 blocked the
metastatic spread of the tumors to the bone in an experimental metastasis model [35]. Taken together, these data suggest that SDF-1 and CXCR4 are likely critical regulators of prostate carcinoma metastasis to bone.

2.2. Attachment to endothelium

Cell adhesion plays a vital role in cancer metastasis. In fact, the ability of cancer cells to adhere to organ-specific cells and components may be a critical regulator of their metastatic pattern. A cancer cell in the circulation initially interacts with the organ's microvascular endothelium and subsequently the organ's extracellular matrix (ECM) components [37,38]. Cell adhesion molecules (CAMs) expressed on both the cancer and endothelial cells mediate these interactions. CAMs expressed on the endothelial cells are regulated by an organ's microenvironment, which results in CAM expression specific to each organ [39].

The organ-specific composition of ECM proteins such as laminin, fibronectin, and vitronectin that are recognized by CAMs expressed on cancer cells contribute significantly to organ-specific metastasis [40,41].

It has been proposed that prostate carcinoma metastasis to bone is mediated, in part, by preferential adhesion to bone marrow endothelium as opposed to endothelium from other sites [42,43]. Two studies demonstrated that prostate carcinoma cells adhered preferentially to immortalized human bone marrow endothelial (HBME) cells as compared to human umbilical vein endothelial cells (HUVEC), immortalized human aortic endothelial cells (HAE3-1), and immortalized human dermal microvascular endothelial cells (HDMVEC) [42,44]. This observation was confirmed in another study that demonstrated that preferential adhesion of PC-3 cells to HBME cells as compared to HUVECs and lung endothelial cells, Hs888Lu [45]. Interestingly, this adhesion was enhanced when HBME cells were grown on bone ECM components [44]. The PC-3 cell line was used as a model for prostate carcinoma in these studies because it was derived from a bone metastasis. To determine the CAMs involved in prostate carcinoma (PC)-HBME interaction, galactose-rich-modified citrus pectin (MCP) and several antibodies to known CAMs expressed on HBME cell monolayers were used in adhesion assays. MCP was used because it was reported to interfere with interactions mediated by carbohydrate-binding proteins such as galectins [46]. The data demonstrated that MCP and antibodies to galectin-3, vascular cell adhesion molecule (VCAM), CD11a (alpha-L), CD18 (beta-2), and leukocyte functional antigen-1 (LFA-1) pectin, reduced PC-3 cell adhesion to HBME cell monolayers [42]. This observation suggests that carbohydrate-binding proteins, VCAM, alpha-L, beta-2, and LFA-1 may be partially involved in prostate carcinoma cell adhesion to HBME cells.

Beta-1 integrins expressed on HUVEC were demonstrated to mediate PC-3 cell adhesion to this endothelial cell line [47]. Surprisingly, the beta-1 integrins expressed on HBME cells were not involved in PC-3 cell adhesion to HBME cell monolayers [48]; however, beta-1 integrins, expressed on PC-3 cells, did mediate its interaction with HBME cell monolayers [45]. Hyaluronan and galactosyl receptor, a cell surface C-type lectin expressed on PC-3 cells, were also shown to mediate PC–HBME interaction [49,50].

The ability of metastatic prostate cells to adhere to the bone matrix may also contribute to prostate carcinoma frequent metastasis to bone matrix [51,52]. Kostenuik demonstrated that PC-3 cells adhered to the collagen type I in the bone matrix. This adhesion was mediated by a2b1 expressed on PC-3 cells and was upregulated by transforming growth factor-beta (TGF-beta), a major bone-derived cytokine [53]. Festuccia and colleagues [52] showed that osteoblast-conditioned media containing TGF-beta, modulated the PC-3 interaction with ECM proteins, including collagen type I. These results provide evidence that TGF-beta, present in the bone marrow, can influence prostate carcinoma cell adhesion to the bone matrix by modulating surface expression of selected integrins.

2.3. Growth factors

The calcified bone matrix is replete with putative prostate carcinoma growth factors including insulin-like growth factors (IGF), bone morphogenetic proteins (BMP), fibroblast growth factors (FGF) and transforming growth factor (TGF)-beta, which are released upon resorption of bone [54,55]. Furthermore, experimental evidence that resorption of calcified bone matrix promotes tumor growth was suggested by the observation that conditioned media for bone cultures undergoing resorption stimulated cancer cell growth of a variety of tumor cell lines [56]. Taken together, these data suggest that inhibiting bone resorption will diminish cancer growth by decreasing growth factors availability in the bone microenvironment.

Several purified factors from bone matrix have been demonstrated to stimulate prostate carcinoma cell growth in vitro [57–59]. For example, IGF-I
and IGF-II are important mediators of prostate carcinoma growth (as reviewed in [60,61]). Prostate carcinoma cells have IGF receptors [62] and proliferate in response to IGF [57]. Transfection of LNCaP cells with FGF-8 expression vector induced an increased growth rate, higher soft agar cloning efficiency, enhanced in vitro invasion, and increased in vivo tumorigenesis [58]. The source of these growth factors is diverse. For example, osteoblast-derived factors influence prostate carcinoma growth, adhesion, and motility [16,17,63]. Additionally, bone marrow stromal cells, as opposed to non-skeletal fibroblasts, induced prostate carcinoma cell growth in vitro and in vivo [64–66]. As research continues on the extracellular matrix of bone, it is very likely that additional prostate carcinoma growth factors will be discovered.

3. The effect of prostate carcinoma on the bone: Osteoblastic

3.1. Prostate skeletal metastases are mixed osteoblastic and osteolytic lesions

Once in the bone, prostate carcinoma tumors have pathobiology that appears to be somewhat unique to cancer skeletal metastases. Specifically, prostate carcinoma skeletal metastases are most often characterized as osteoblastic (i.e., increased mineral density at the site of the lesion) as opposed to osteolytic. Other tumors, such as breast cancer, can form osteoblastic lesions; however, these occur less frequently [67,68]. In spite of the radiographic osteoblastic appearance it is clear from histological evidence that prostate carcinoma metastases form a heterogeneous mixture of osteolytic and osteoblastic lesions although osteoblastic lesions predominate [69–72]. Sites of prostate carcinoma bone metastases are often demonstrable to have increases in osteoid surface, osteoid volume, mineralization rates [73,74]. Recent evidence shows that osteoblastic metastases form on trabecular bone at sites of previous osteoclastic resorption, and that such resorption may be required for subsequent osteoblastic bone formation [75,76]. Clinical evidence demonstrates increased systemic markers of both bone production and bone resorption in prostate carcinoma patients [77,78] in addition to bone histomorphometric findings of increased indices of bone resorption [71]. These findings suggest that prostate carcinoma induces bone production through an overall increase in bone remodeling, which in the non-pathologic state is a balance between osteoclastic resorption of bone and osteoblast-mediated replacement of resorbed bone (as reviewed in [79–81]). In the case of prostate carcinoma, it appears the induction of osteoblast-mediated mineralization outweighs the increase in osteoclast resorption resulting in overall formation of osteoblastic lesions. The osteoblastic lesions result in overall weakening of the bone for the following reasons; mature, healthy bone is formed of lamellar bone, which allows for tight packing of collagen bundles and optimum bone strength. In contrast, prostate carcinoma induces production of woven bone, which is composed of loosely packed, randomly oriented collagen bundles that produce bone with suboptimal strength [82,83]. Thus, the combination of underlying osteolysis and production of weak bone leads to a predisposition to fracture. The mechanisms through which prostate carcinoma cells promote bone mineralization remain poorly understood.

3.2. A variety of factors may contribute to prostate carcinoma-mediated bone mineralization

Prostate carcinoma produces osteoblastic factors that mediate their effect through activation of the osteoblast transcription factor Cbfa1 in the osteoblast precursor [84]. This suggests that induction of osteosclerosis occurs through normal osteoblast differentiation pathways. In addition to this observation, the prostate carcinoma cell itself demonstrates increased expression of Cbfa1 and the ability to mineralize in vitro, suggesting that it directly contributes to osteosclerosis [85]. Many factors that have direct or indirect osteogenic properties have been implicated in prostate carcinoma's osteogenic activity (Table 1) (as reviewed in [86,87–89]). Although, initially identified as a non-defined osteoblastic activity from prostate carcinoma cells in vitro [90], many specific factors have been

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<td><strong>Factor</strong></td>
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<td>Bone morphogenic proteins (BMP)</td>
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<td>Insulin-like growth factors (IGF)</td>
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<td>Parathyroid hormone-related peptide (PTHrP)</td>
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<td>Transforming growth factor-β (TGF-β)</td>
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<td>Urinary plasminogen activator (urokinase)</td>
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identified that may promote osteoblastic lesions. Some of these factors, such as bone morphogenetic proteins (BMP) [91–93] and endothelin-1 (ET-1) [94] may directly stimulate differentiation of osteoblast precursors to mature mineral-producing osteoblasts [95] or induce osteoblast protein production [93]. Other factors such as parathyroid hormone-related protein (PTHrP) may work through inhibition of osteoblast apoptosis [96,97]. Additionally, there are proteins that may work indirectly to enhance bone production, such as the serine proteases, prostate specific antigen (PSA) and urinary plasminogen activator (uPA), which can activate latent forms of osteogenic proteins, such as transforming growth factor-β (TGF-β) [98,99]. Finally, some molecules, such as osteoprotegerin (OPG) [100–102] and ET-1 (in a dual role with its osteoblast-stimulating activity) [103] can enhance osteoclastogenesis through inhibiting osteoclastogenesis. Other tumor types, such as osteosarcoma, are also known to produce a variety of osteoblastic factors [104–106]. With such a large number of factors, it is difficult to determine which the key factor is, and most likely several of these osteogenic factors work in concert to produce maximal bone production.

3.2.1. Parathyroid hormone related protein (PTHrP)

PTHrP was originally identified as a tumor-derived factor responsible for humoral hypercalcemia of malignancy (HHM). It has limited homology with the endocrine hormone, parathyroid hormone, sharing 7 of the first 13 N-terminal amino acids, but otherwise is dissimilar and immunologically distinct [107]. PTH and PTHrP bind to the same receptor (the PTH-1 receptor) and evoke the same biological activity due to similarities in their steric configurations at the region of 25–34 amino acids. Patients with solid tumors and hypercalcemia have increased serum PTHrP in 80% of the cases, emphasizing the impact of this peptide to increase bone resorption and renal tubular resorption of calcium [107]. Subsequent to its characterization in HHM, PTHrP was found to be produced by many normal tissues including, epithelium, lactating mammary gland, and cartilage where it has an autocrine, paracrine, or intracrine role [107]. PTHrP plays a critical role in the development of the skeleton as evidenced by its lethality upon gene ablation and the severe skeletal chondrodysplasia found in these animals [108]. These studies have led to the conclusion that PTHrP in cartilage functions to accelerate the growth of cartilage cells and to oppose their progression to a terminally differentiated cell [109].

Many features of PTHrP make it an attractive candidate for influencing prostate carcinoma growth. PTHrP is produced by normal prostate epithelial cells, from which prostate carcinoma arises, and PTHrP is found in the seminal fluid [87,110]. PTHrP has been immunohistochemically identified in prostate carcinoma tissue in patients with clinically localized disease [111], is found in higher levels in prostate intraepithelial neoplasia than in normal prostate epithelium, is found in higher levels in prostate carcinoma than in benign prostatic hyperplasia [112,113], and is found in human metastatic lesions in bone [114]. There is also evidence that PTHrP can regulate malignant tumor growth in an autocrine manner in human renal cell carcinoma [115], enhance breast cancer metastasis to bone [116,117], and act as an autocrine growth factor for prostate carcinoma cells in vitro [118]. Recent evidence indicates that expression of nuclear-targeted PTHrP can protect prostate and other cells from apoptosis [114,119], bind RNA [120], and act as a mitogen [121,122]. PTHrP production by primary prostatic tumors is associated with increased tumor size and rate of growth in an animal model [114] suggesting that PTHrP acts in autocrine or intracrine mechanisms to promote tumor growth. In contrast, in this same model and in an intracardiac injection model of prostate carcinoma, PTHrP was not associated with an increase in metastatic potential [83,114]. This suggests that PTHrP is not important in the process of metastasis to bone but once in the bone microenvironment where target cells with receptors are present (osteoblasts); it may play a critical role in the bone response to prostate carcinoma. Of particular interest to prostate carcinoma, PSA has been shown to cleave PTHrP leading to an inactivation of the PTHrP-stimulation of CAMP which is a key pathway for the actions of PTHrP in bone [123]. More recent studies indicate that in colon cancer cells, PTHrP enhances adhesion of cells to type I collagen but not fibronectin or laminin [124]. All these data suggest that PTHrP has a critical role in the local bone environment of metastatic prostate carcinoma; but what this precise role is has yet to be determined.

3.2.2. Endothelin-1

ET-1 is a member of the ET family which is composed of ET-1, -2, and -3. The ET family members are synthesized as a 203 amino acid precursor peptide that is cleaved to a 21 amino acid peptide with the same two characteristic disulfide bridges [125]. Initially
identified as a potent vasoconstrictor, ET-1 interacts with cell surface ET_{A} and ET_{B} receptors to induce a variety of responses including modulation of cell growth and fetal development (as reviewed in [125]). ETs are found in a variety of tissues including vascular endothelium, parathyroid gland, mammary tissue, and macrophages [125].

The role of ET-1 in bone remodeling is controversial. For example, in the murine osteoblast precursor cell, MC3T3-E1, E1 inhibits differentiation, reduces both alkaline phosphatase activity and osteocalcin expression and diminishes in vitro mineralization suggesting that ET-1 will diminish bone production [126,127]. In contrast, ET-1 has been shown to inhibit bone resorption [128], induce collagen synthesis [129] and osteopontin and alkaline phosphatase production [130,131] in a variety of osteoblastic cell lines. The conflicting results may be due to differences in cell lines, particularly with regards to ET receptor expression. Although these in vitro data are in apparent conflict, the in vivo data support that ET-1 promotes bone formation [132]. Specifically, administration of an ET_{A} receptor antagonist in mice resulted in reduced bone mass [132].

ET-1 is secreted by normal prostate epithelial cells into the ejaculate [133–135] and is now considered a putative mediator of prostate carcinoma pathophysiology (as reviewed in [136]). The ectopic expression of ET-1 in the bone metastatic site by prostate carcinoma cells may enable ET-1 to influence the bone remodeling process locally. This is supported by the report that para-tibial injection of an amniotic cell line overexpressing ET-1 induced new bone formation in the tibiae of mice, which was diminished by blockade of ET_{A} receptor [137]. Additionally, administration of an ETA receptor antagonist diminished breast cancer-induced bone production in a murine model [138]. Furthermore, co-incubating the androgen-independent prostate carcinoma cell lines DU-145 and PC-3, but not the androgen-responsive cell line LNCaP, with bone slices induced ET-1 expression from the prostate carcinoma cells [103]. The DU-145 and PC-3 cell lines also induced osteoclastogenic activity that was blocked by anti-human ET-1 antibody. Taken together, these reports suggest that ET-1 may contribute to prostate carcinoma metastases-induced osteoclastic lesions. In apparent conflict with these models, is the observation that serum ET-1 levels are elevated in people with Paget’s disease, which is characterized by low bone mineral density secondary to increased osteoclastic activity [139].

3.2.3. Bone morphogenetic proteins

BMPs are members of the transforming growth factor (TGF)-β superfamily. More than 30 BMPs have been identified to date [140]. While originally discovered because of their ability to induce new bone formation, BMPs are now recognized to perform many functions, particularly in the role of development, such as apoptosis, differentiation, proliferation and morphogenesis (as reviewed in [141–143]). BMPs are synthesized as large precursor molecules that undergo proteolytic cleavage to release the mature protein, which form active hetero- or homodimers [144,145]. BMPs bind to receptors (BMPR-IA and -IB) and a BMP type II receptor (BMPR-II), which induces Smad phosphorylation [146] resulting in modulation of gene regulation. Target genes of BMPs include osteoblast proteins such as OPG [147] and the osteoblast-specific transcription factor Cbfa-1 [148,149]. Several proteins that antagonize BMP action have been identified. For example, noggin and gremlin inhibit BMP-2, -4 and -7 by binding to them [150–152]. Furthermore, the BMPs themselves regulate their own inhibitors in an apparent negative feedback mechanism [153,154].

Many in vitro studies have demonstrated that BMPs induce osteogenic differentiation including the ability of BMP-7 (also called osteogenic protein-1; OP-1) to induce osteogenic differentiation of newborn rat calvarial cells and rat osteosarcoma cells [155–157]. The BMPs’ osteogenic properties appear to be specific to the differentiation stage of the target cells. Specifically, BMPs can induce uncommitted stem cells [155,158,159] and myoblasts [160] to express osteoblast parameters such as alkaline phosphatase or osteocalcin expression [79,161]; whereas, BMPs do not stimulate mature osteoblasts or fibroblasts [158,162–164] to increase expression of these proteins. Examination of genetically modified mice provides further evidence of the importance of BMP in bone development. The bmp7 homozygous null condition in mice is a postnatal lethal mutation and is associated with, in addition to renal and ocular abnormalities, retarded skeletal ossification [165]. In contrast, bmp6 null mice are viable and fertile, and the skeletal elements of newborn and adult mutants are indistinguishable from wildtype [166]. However, careful examination of skeletogenesis in late gestation embryos reveals a consistent delay in ossification strictly confined to the developing sternum. Finally, mice with mutations of the bmp5 gene have skeletal abnormalities and inefficient fracture repair [167]. Taken together, these data provide


evidence that BMPs are important regulators of the osteogenesis. Thus, dysregulation of their expression in the bone microenvironment would most likely impact bone remodeling.

A few studies have examined the expression of BMPs in normal and neoplastic prostate tissues. Using Northern analysis, Harris et al. [92] examined BMP-2, -3, -4 and -6 mRNA expression in human normal prostate and prostate carcinoma cell lines. They found that normal human prostate predominantly expressed BMP-4. The androgen-dependent non-metastatic LNCaP human prostate carcinoma cell line produced very low to undetectable levels of BMPs. Whereas, the aggressive androgen-independent PC-3 cell line expressed very high levels of BMP-3 and slightly lower levels of BMP-2, -4 and -6 compared to normal cells, but much higher than LNCaP cells. In support of these results, Weber et al. [168], using PCR analysis, identified 16 (73%) of 22 prostate carcinoma samples that were positive for BMP-7 mRNA compared to eight (57%) of 14 normal prostate tissue samples. In another PCR based analysis, Bentley et al. [169], found that several BMPs were expressed in both benign and malignant prostate tissue and in the PC3 and DU145 prostate carcinoma cell lines. BMP-6 expression was detected in the prostate tissue of over 50% of patients with clinically defined metastatic prostate adenocarcinoma, but was not detected in non-metastatic or benign prostate samples. In another study focused on BMP-6 mRNA and protein expression, Barnes et al. [170] observed that BMP-6 was produced by normal and neoplastic human prostate (radical prostatectomy specimens and human carcinoma cell lines DU145 and PC3). However, BMP-6 mRNA and protein expression was higher in prostate carcinoma as compared with adjacent normal prostate, with higher-grade tumors (Gleason score of 6 or more) having greater BMP-6 immunostaining than the lower-grade tumors (Gleason score of 4 or less). These results were consistent with a later study by Hamdy et al. [171], who reported that BMP-6 mRNA expression was detected exclusively in malignant epithelial cells in 20 of 21 patients (95%) with metastases, in 2 of 11 patients (18%) with localized cancer, and undetectable in 8 benign samples. In addition to BMP, there have been several reports that prostate carcinoma expresses BMP receptors. It appears that as prostate carcinoma progresses, the cells down-regulate their own expression of BMP receptors [172,173], which may be a protective mechanism as it has been demonstrated that BMP-2 can inhibit prostate carcinoma cell proliferation [174]. Taken together, these observations demonstrate that prostate carcinoma cells produce increasing levels of BMPs as they progress to a more aggressive phenotype and suggest that the up-regulation of BMP expression in prostate carcinoma cells localized in the bone is a critical component of the mechanism of development of osteoblastic lesions at prostate carcinoma metastatic sites.

4. The effect of prostate carcinoma on the bone: Osteolytic

Although the osteoblastic component of prostate carcinoma metastases has received attention, limited research has been performed on the osteoclastic aspect of prostate carcinoma. Similar to the reports for breast cancer bone metastases [175,176], several lines of evidence suggest that resorption of bone is an important mediator of prostate carcinoma bone metastases. For example, administration of bisphosphonates, inhibitors of osteoclast activity, to patients with prostate carcinoma bone metastases relieves bone pain and lowers systemic indices of bone resorption [177-179]. Furthermore, administration of osteoclast inhibitors such as OPG or bisphosphonates prevents tumor establishment or diminished tumor burden in animal models [76,180-182]. It is not clear if bisphosphonates have a direct antitumor effect [183-185] or inhibit tumor growth through its ability to diminish osteoclast activity [186,187]. In some instances, it may be a combination of activities. As described above, in addition to serum levels of bone resorption markers being elevated in men with prostate carcinoma skeletal metastases, the lesions usually are demonstrated to have histological evidence of osteoclastic activity. Thus, osteoclast activity may play an important role in development and progression of prostate carcinoma metastases. Prostate carcinoma cells secrete a variety of factors that may promote bone lysis, such as interleukin-6 (as reviewed in [188]) and PTHrP. However, it appears that these factors mediate their osteolytic effects through induction of a key pro-osteoclastogenic molecule, receptor activator of NFκB ligand (RANKL).

4.1. Receptor activator of NFκB ligand-OPG axis

A member of the tumor necrosis factor family, RANKL, is initially expressed as a membrane anchored molecule; however, a small fraction of RANKL is released
through proteolytic cleavage from the cell surface as a soluble 245 amino acid homotrimeric molecule (sRANKL) [189]. Both soluble and membrane bound RANKL promote osteoclast formation and activation by binding to RANK on the osteoclast precursor membrane [189–193].

In addition to RANKL and RANK, another key modulator of osteoclastogenesis is osteoprotegerin (OPG) (also known as osteoclastogenesis inhibitory factor-OCIF) [102,194]. OPG serves as a decoy receptor that binds RANKL and thus blocks its ability to bind to RANK and induce osteoclastogenesis. In contrast to RANKL and RANK, whose expression is mainly restricted at low levels to the skeletal and immune systems, OPG is expressed in a variety of tissues, such as liver, lung, heart, kidney, stomach, intestines, skin and calvaria in mice and lung, heart, kidney and placenta in human [102,195–201].

In bone, OPG is mainly produced by osteoblastic lineage cells and its expression increases as the cells become more differentiated [199,202,203]. Administration of recombinant OPG to normal rodents resulted in increased bone mass [102,196] and completely prevented ovariectomy-induced bone loss without apparent adverse skeletal and extraskeletal side effects [102]. In fact, based on this activity, the balance ratio of RANKL to OPG appears to be very important in controlling the overall activity (i.e., lysis vs no lysis) that will be observed [204–206].

A number of reports have shown that osteoclastic bone resorptive lesions are important to the development of bone metastases in several cancer types including breast cancer, lung cancer and prostate carcinoma [207]. These cancers may induce osteoclast activity through secretion of IL-1α, PTHrP or PGE2 [208,209]. However, tumor-mediated osteolysis occurs indirectly through expression of molecules, such as PTHrP, that induce RANKL in osteoblasts [210,211]. This contrasts with the observations that giant cell tumors directly promote osteoclast activity via RANKL [212] and our observation that prostate carcinoma cells directly induce osteoclastogenesis through RANKL [76]. Another factor that may play a role in tumor-induced osteoclastogenesis is human macrophage inflammatory protein-1α (hMIP-1α), which has been shown to be produced by myeloma cells [213]. Because of the osteoclastic activity induced by many cancers, antiresorptive approaches such as administration of bisphosphonates or anti-PTHrP neutralizing antibody have been reported in breast cancer animal models to be able to block the tumor expansion in bone [214,215].

Furthermore, OPG has been recently shown to inhibit primary bone sarcoma-induced osteolysis and tumor-induced bone pain, but not tumor burden in mice [100]. However, OPG not only blocked osteolytic bone metastasis induced by human neuroblastoma NB-19 cells [216], but also reduced tumor burden in that model. In addition to OPG, a soluble form of RANK (sRANK) has been shown to inhibit myeloma-induced lytic lesions in murine models [217].

4.2. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of enzymes whose primary function is to degrade the extracellular matrix. MMPs contribute to metastatic invasion, including destruction of bone [218]. Prostate carcinomas and their cell lines express a large number of MMPs [219–226]. The initial functional data in prostate carcinoma bone metastasis that suggested bone remodeling is modulated through MMPs was provided by in vitro studies. Specifically, blocking MMP activity with 1,10-phenanthroline, a MMP inhibitor, diminished bone matrix degradation induced by PC-3 cells in vitro [227,228]. The importance of MMPs in bone metastasis has been further confirmed in vivo. An MMP inhibitor, batimatistat, has been shown to inhibit development bone resorption in vivo and in vivo in murine models of breast [229] and prostate carcinoma [230]. The mechanism through which prostate carcinoma-produced MMPs induce bone resorption is not clear; however, it appears to involve induction of osteoclastogenesis as inhibition of MMPs reduced the number of osteoclasts associated with prostate tumor growth in human bone implants in mice [230].

5. Conclusions

A model summarizing the cross-talk between prostate carcinoma and the bone microenvironment that leads to development and progression of prostate carcinoma skeletal metastases is presented in Figure 1. The bone contributes many aspects of the metastatic cascade including chemotaxis, endothelial attachment, invasion and tumor proliferation. Once in the bone microenvironment, the prostate carcinoma cells modulate bone remodeling which favors tumor progression. The presence of many different active factors produced by both the bone and the prostate carcinoma cells that appear to contribute to the pathobiology of skeletal metastases.
Figure 1. Model of cross-talk between prostate carcinoma cells and the bone microenvironment. The bone produces chemotactic factors that attract prostate carcinoma cells to migrate (1) through the vascular system towards the skeleton. The bone marrow endothelia displays adhesion molecules that complement those expressed by the prostate carcinoma cell, resulting in attachment of the cell (2). The prostate carcinoma cell extravasates and invades into the skeletal extracellular tissue (3), at which point it releases factors that stimulate osteoclastogenesis (4). The subsequent bone resorption is accompanied by release of growth factors that stimulate prostate carcinoma proliferation (5). The progressively increasing prostate carcinoma releases factors that promote osteoblast production and inhibit osteoblast apoptosis (6) resulting in production of woven bone and the characteristic osteosclerotic lesion. This process continues in a cyclical fashion with continued induction of osteoclastic activity, carcinoma cell proliferation and bone production.

suggests that defining the mechanisms of prostate carcinoma skeletal metastases will be challenging. Continued research on how these interactions occur may lead to identification of targets to interrupt this cross-talk and prevent the establishment or progression of prostate cancer skeletal metastases.

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THE ROLE OF OSTEOCLASTIC ACTIVITY IN PROSTATE CANCER SKELETAL METASTASES

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Summary
Metastasis of prostate cancer to bone is a common complication of progressive prostate cancer. Skeletal metastases are often associated with severe pain and thus demand therapeutic interventions. Although often characterized as osteoblastic, prostate cancer skeletal metastases usually have an underlying osteoclastic component. Advances in osteoclast biology and pathophysiology have led toward defining putative therapeutic targets to attack tumor-induced osteolysis. Several factors have been found to be important in tumor-induced promotion of osteoclast activity. One key factor is the protein receptor activator of nuclear factor-xB ligand (RANKL), which is required to induce osteoclastogenesis. RANKL is produced by prostate cancer bone metastases, enabling these metastases to induce osteolysis through osteoclast activation. Another factor, osteoprotegerin, is a soluble decoy receptor for RANKL and inhibits RANKL-induced osteoclastogenesis. Osteoprotegerin has been shown in murine models to inhibit tumor-induced osteolysis. In addition to RANKL, parathyroid hormone-related protein and interleukin-6 are produced by prostate cancer cells and can promote osteoclastogenesis. Finally, matrix metalloproteinases (MMPs) are secreted by prostate cancer cells and promote osteolysis primarily through degradation of the nonmineralized bone matrix. MMP inhibitors have been...
shown to diminish tumor establishment in bone in murine models. Thus, many factors derived from prostate cancer metastases can promote osteolysis, and these factors may serve as therapeutic targets.

The importance of osteoclasts in the establishment and progression of skeletal metastases has led to clinical evaluation of therapeutic agents to target them for slowing metastatic progression. Bisphosphonates are a class of compounds that decrease osteoclast life span by promoting apoptosis. The bisphosphonate pamidronate has proven clinical efficacy for relieving bone pain associated with breast cancer metastases and has a promising outlook for prostate cancer metastases. Another bisphosphonate, zoledronic acid, appears to directly target prostate cancer cells in addition to diminishing osteoclast activity at the metastatic site. In addition to bisphosphonates, other novel therapies based on studies that delineate mechanisms of skeletal metastases establishment and progression will be developed in the near future. © 2002 Prous Science. All rights reserved.

Introduction

Prostate cancer metastasizes to bone in over 90% of men with progressive disease. Although primarily osteoblastic (i.e., induce mineralization in the skeletal metastatic site), prostate skeletal metastases always have an underlying osteoclastic component. Tumor-induced osteolysis often results in severe pain and pathologic bone fractures and thus is an important target for prostate cancer therapy. Recent advances in the biology of osteoclasts provide clues to understanding the role of osteoclasts in cancer-induced bone lesions. Some of this research has led to clinical use of inhibitors of osteoclast activity to reduce tumor-induced osteolysis and bone pain. In this review, we will summarize the biology of osteoclasts, proosteoclastic factors produced by prostate cancer and therapeutic strategies designed to inhibit this painful aspect of cancer.

Osteoclast biology

Osteoclasts are derived from the colony-forming unit-granulocyte/macrophage hematopoietic precursor cells. The colony-forming unit-granulocyte/macrophage undergoes a defined progression of maturation steps that ultimately result in fusion of the precursor cells into mature osteoclasts (Fig. 1). Several factors promote osteoclastogenesis, including growth factors and cytokines. Both colony-stimulating factor and interleukins-1 and -6 (IL-1 and IL-6) expand the osteoclast precursor pool. Tumor necrosis factor (TNF)-α promotes conversion of the monocyte to a committed osteoclast precursor (1).

Although several factors promote osteoclastogenesis, one factor that is required for production of mature osteoclasts is receptor activator of nuclear factor-κB ligand (RANKL). A member of the TNF family, RANKL is initially expressed by bone marrow stromal cells, osteoblasts and activated T cells. RANKL is most commonly a membrane-anchored molecule; however, a small fraction of RANKL is released through proteolytic cleavage from the cell surface as a soluble 245-amino-acid homotrimeric molecule (2). Both soluble and membrane-bound RANKL promote osteoclast formation and activation by binding to RANK on the osteoclast precursor membrane (Fig. 2) (2-6) that has the characteristics of a monocyte (7). RANKL binding to RANK induces NF-κB and Fos activation (8, 9). Several lines of evidence demonstrate RANKL’s importance in osteoclastogenesis. For example, RANKL has been shown to induce osteoclastogenesis in vitro from colony-forming unit-granulocyte/macrophage (10). Mice that are genetically engineered to overexpress RANKL or RANK are severely osteoporotic (11). Additionally, mice that have had their RANKL (12) or RANK (13) gene deleted have no osteoclasts and are osteopetrotic.
In addition to RANKL and RANK, another key modulator of osteoclastogenesis is osteoprotegerin (also known as osteoclastogenesis inhibitory factor) (14, 15). Osteoprotegerin serves as a decoy receptor that binds RANKL and thus blocks its ability to bind to RANK and induce osteoclastogenesis. In contrast to RANKL and RANK, whose expression is mainly restricted at low levels to the skeletal and immune systems, osteoprotegerin is expressed in a variety of tissues, such as liver, lung, heart, kidney, stomach, intestines, skin and calvaria in mice, and lung, heart, kidney and placenta in humans (14, 16-21). In bone, osteoprotegerin is mainly produced by osteoblastic lineage cells and its expression increases as the cells become more differentiated (19, 22, 23). Several factors, including 1,25-dihydroxyvitamin D3, IL-1β, TNF-α and BMP-2, induce osteoprotegerin mRNA expression in human osteoblast cell lines (19). Administration of recombinant osteoprotegerin to normal rodents resulted in increased bone mass (14, 17) and completely prevented ovarioectomy-induced bone loss without apparent adverse skeletal and extraskeletal side effects (14). Additionally, a single subcutaneous injection of osteoprotegerin is effective in rapidly and profoundly reducing bone turnover for a sustained period in women (24). In fact, based on this activity, the balance ratio of RANKL to osteoprotegerin appears to be very important in controlling the overall activity (i.e., lysis vs. no lysis) that will be observed (11, 23, 25, 26).

Once activated, osteoclasts resorb bone through secretion of a combination of proteases to resorb the nonmineralized matrix and acid to dissolve the hydroxyapatitic mineral (27). Proteases that are important mediators of osteoclastic activity include cathepsin K and metalloproteinases. Cathepsin K can cleave bone proteins such as type I collagen, osteopontin and osteonectin (28). Overexpression of cathepsin K in the mouse results in accelerated bone turnover (29), whereas knockout of cathepsin K results in retarded bone matrix degradation and osteopenosis (30). Several novel classes of cathepsin K inhibitors have been designed and may provide novel therapeutic agents to target bone resorption (31, 32). In addition to the proteases, acid is secreted from osteoclasts to resorb the mineralized matrix. Acid is believed to be secreted through vacuolar H(+)-ATPase-dependent pumps present on the osteoclast’s ruffled membranes (33). Several hormones regulate acid secretion, including parathyroid hormone, which increases acid secretion and calcitonin, which in turn decreases acid secretion. Carbonic anhydrase II appears to be an important mediator of acid production because acetazolamide, a carbonic-anhydrase-inhibitor-based diuretic, can block bone resorption (34). Another diuretic, indapamide, increased osteoblast proliferation and decreased bone resorption, at least in part, by decreasing osteoclast differentiation via a direct effect on hematopoietic precursors in vitro (35).

These findings suggest that targeting osteoclast-derived activity, in addition to targeting osteoclast production or survival, may provide therapeutic avenues to diminish tumor-induced bone resorption.

Receptor activator of nuclear factor-κB ligand

As described above, RANKL is a key osteoclastogenic factor. Several lines of evidence support the role of RANKL in prostate cancer-mediated osteolysis. Although a bone metastatic prostate cancer cell line has been shown to express osteoprotegerin (36), that same line overexpresses RANKL (37). Additionally, in normal prostate, osteoprotegerin protein was detected in luminal epithelial and stromal cells (5%-65% and 15%-70%, respectively) and RANKL immunoreactivity was observed in 15%-50% of basal epithelial cells, 40%-90% of luminal epithelial cells and 70%-100% of stromal cells (38). Osteoprotegerin was not detected in 8 of 10 primary CaP specimens but RANKL was heterogeneously expressed in 10 of 11 CaP specimens (38). Importantly, the percentage of tumor cells expressing osteoprotegerin and RANKL was significantly increased in all CaP bone metastases compared with nonosseous metastases or primary CaP. Serum osteoprotegerin levels are elevated in patients with advanced prostate cancer compared with less advanced prostate cancer (39). However, RANKL levels were not measured in that study, thus one cannot determine if the ratio of RANKL:osteoprotegerin was altered in these patients. It is possible that RANKL is only expressed locally at the skeletal metastatic site and therefore not detectable in the serum. Regardless, taken together, these observations suggest that the RANKL:osteoprotegerin axis may play an important role in prostate cancer bone metastases. Further support for this possibility was demonstrated by the observation that administration of osteoprotegerin prevented establishment of prostate cancer cells in the bones of SCID mice, although it had no effect on establishment of subcutaneous tumors in the same mice (37).
Matrix metalloproteinases

Matrix metalloproteinases (MMPs), a family of enzymes whose primary function is to degrade the extracellular matrix, play a role in bone remodeling. This activity occurs in the absence of osteoclasts (40), suggesting that MMPs have a direct resorptive effect. Several have the ability to degrade the nonmineralized matrix of bone including MMP-1, MMP-9 and MMP-13, which are collagenases. Other MMPs, such as stromelysin (MMP-3), activate MMP-1. Through their proteolytic activity MMPs contribute to metastatic invasion, including destruction of bone (41).

Prostate carcinomas and their cell lines express a large number of MMPs (42-49). Levels of MMP-9 secretion in primary prostate cancer cultures increased with Gleason histological grade (44). Active MMP-9 species were detected in 15 cultures (31%) of primary prostate cancer tissues. The presence of the mineralized matrix has been shown to induce MMP-9 expression from prostate carcinoma cells (50).

The initial functional data that suggested prostate carcinoma bone metastasis modulated bone remodeling through MMPs was provided by in vitro studies. Specifically, blocking MMP activity with 1,10-phenanthroline, an MMP inhibitor, diminished bone matrix degradation induced by PC-3 cells in vitro (51, 52). Matriksin (MMP-7) has been shown to be up-regulated in DU-145 prostate cancer cells and can enhance their invasive ability. Monoclonal antibody targeting the cytokine interleukin-6 (IL-6) has been shown to increase promatriksin expression in DU-145 cultures (53). This suggests that IL-6, which is increased in prostate cancer (reviewed in 54), enhances prostate cancer invasion through production of MMP-7.

The importance of MMPs in bone metastasis has been further confirmed in vivo. An MMP inhibitor, batimatistat, has been shown to inhibit development of bone resorption in vitro and in vivo in murine models of breast (55) and prostate carcinomas (56). The mechanism through which prostate-carcinoma-produced MMPs induce bone resorption is not clear; however, it appears to involve induction of osteoclastogenesis, as inhibition of MMPs reduced the number of osteoclasts associated with prostate tumor growth in human bone implants in mice (56). Additionally, the bisphosphonate alendronate blocked MMP production from PC-3 cells (57). This was associated with diminished establishment of bone metastasis in mice injected with PC-3 tumors (40).

Parathyroid hormone-related protein

Parathyroid hormone-related protein (PTHrP), a protein with limited homology to parathyroid hormone, was originally identified as a tumor-derived factor responsible for humoral hypercalcemia of malignancy. Parathyroid hormone and PTHrP bind to the same receptor (the parathyroid hormone-1 receptor) and evoke the same biological activity due to similarities in their steric configurations at the region of 25-34 amino acids. Patients with solid tumors and hypercalcemia have increased serum PTHrP in 80% of the cases, emphasizing the impact of this peptide to increase bone resorption and renal tubular resorption of calcium (58). Subsequent to its characterization in humoral hypercalcemia of malignancy, PTHrP was found to be produced by many normal tissues, including epithelium, lactating mammary gland and cartilage, where it has an autocrine, paracrine or intracrine role (58).

PTHrP is an attractive candidate for influencing prostate carcinoma growth. PTHrP is produced by normal prostate epithelial cells, from which prostate carcinoma arises, and PTHrP is found in the seminal fluid (59, 60). PTHrP has been immunohistochemically identified in prostate carcinoma tissue in patients with clinically localized disease (61), is found in higher levels in prostate intraepithelial neoplasia than in normal prostate epithelium, is found in higher levels in prostate carcinoma than in benign prostatic hyperplasia (62, 63) and is found in human metastatic lesions in bone (64). However, in some studies, expression of PTHrP receptor in prostate cancer appears to be more consistent than expression of PTHrP itself (65). Overexpression of ras oncogene in immortalized prostate epithelial cells has been shown to promote PTHrP expression (66). This may account for the increased expression of PTHrP as the cells progress to a malignant phenotype.

There is evidence that PTHrP can regulate malignant tumor growth in an autocrine manner in human renal cell carcinoma (67), enhance breast cancer metastasis to bone (68, 69) and act as an autocrine growth factor for prostate carcinoma cells in vitro (59), although it does not affect proliferation of normal prostate cells (70). Recent evidence indicates that expression of nuclear-targeted PTHrP can protect prostate and other cells from apoptosis (64, 71), bind RNA (72) and act as a mitogen (73, 74). PTHrP production by primary prostatic tumors is associated with increased tumor size and rate of growth in an animal model (64), suggesting that
PTHrP acts in an autocrine or intracrine mechanism to promote tumor growth. In contrast, in this same model and in an intracardiac injection model of prostate carcinoma, PTHrP was not associated with an increase in metastatic potential (64, 75). This suggests that PTHrP is not important in the process of metastasis to bone, but once in the bone microenvironment where target cells with receptors are present (osteoblasts), it may play a critical role in the bone response to prostate carcinoma. Of particular interest to prostate carcinoma, prostate specific antigen has been shown to cleave PTHrP leading to an inactivation of the PTHrP-stimulation of cAMP, which is a key pathway for the actions of PTHrP in bone (76). Overexpression of PTHrP in prostate cancer cells has been shown to induce osteolytic lesions in the bone of rats (77), although the level of expression may not directly correlate with the degree of osteolysis (75). All these data suggest that PTHrP has a critical role in the local bone microenvironment of metastatic prostate carcinoma, but this precise role is yet to be determined.

**Interleukin-6**

IL-6 belongs to the "interleukin-6-type cytokine" family that also includes leukemia inhibitory factor, interleukin-11, ciliary neurotrophic factor, cardiotrophin-1 and oncostatin M (78). Many physiologic functions are attributed to IL-6, including promotion of antibody production from B lymphocytes, modulation of hepatic acute-phase reactant synthesis, promotion of osteoclastic mediated bone resorption and induction of thrombopoiesis (79). IL-6 mediates its activity through the IL-6 receptor complex, which is composed of two components: an 80 Kd transmembrane receptor (IL-6Rα) and a soluble (σ2) subunit that specifically binds IL-6 but has no signaling capability and a 130 Kd membrane glycoprotein (gp130) that mediates signal transduction following IL-6R binding (80). In addition to the transmembrane IL-6R, a soluble form of IL-6R exists that is produced by either proteolytic cleavage of the 80 Kd subunit (81, 82) or differential splicing of mRNA (83). Although the soluble IL-6R does not possess a transmembrane component, it can still bind to IL-6, and the ligand-bound soluble IL-6R·IL-6 complex activates signal transduction and biological responses through membrane-bound gp130 (84).

Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic prostate cancer (85-87). Adler et al. (85) demonstrated that serum levels of IL-6 and transforming growth factor-β1 are elevated in patients with metastatic prostate cancer and that these levels correlate with tumor burden as assessed by serum prostate-specific antigen or clinically evident metastases. In a similar fashion, Drachenberg et al. (86) reported elevated serum IL-6 levels in men with hormone-refractory prostate cancer compared with normal controls, benign prostate hyperplasia, prostatitis and localized or recurrent disease. In an animal model, prostate tumor cells injected next to human bones implanted in the limb of mice demonstrated IL-6 expression (87). In addition to IL-6, the IL-6R has been identified in human normal prostate and prostate carcinoma tissue (90, 91).

The secretion of IL-6 by prostate cancer cells in the bone microenvironment may impact bone remodeling (reviewed in 92, 93). IL-6 promotes osteoclastogenesis (94-96) most likely through increasing osteoclastogenic precursors. IL-6-mediated osteoclastogenesis is directly related to the level of gp130 present on the precursor cells (97). It appears that IL-6-mediated osteoclastogenesis is independent of promoting RANKL expression (98). However, IL-6 has been shown to potentiate PTHrP-induced osteoclastogenesis (99, 100). Administration of anti-IL-6 antibody has been shown to diminish growth of subcutaneously injected prostate cancer cells in nude mice, thus demonstrating the potential utility of this compound in clinical prostate cancer (101). These results strongly suggest that IL-6 may serve as a therapeutic target for the osteolytic component of prostate cancer skeletal metastases.

**Therapy of cancer-associated osteolysis**

Bone metastases are associated with several clinical sequelae, including bone pain, neuralgia, pathologic bone fracture and myelophthisis. Thus, targeting these lesions has received much research effort. Bisphosphonates are a group of chemicals that inhibit osteoclast activity resulting in decreased bone resorption and thus have received much attention as inhibitors of clinical complications of bone metastases (102-104). Bisphosphonates work directly on osteoclasts to induce their apoptosis (105, 106). Animal studies have demonstrated that bisphosphonates can diminish tumor-induced osteoclastogenesis and osteolysis (107-111); although in some instances it appears to only reduce tumor-induced lysis but not tumor burden (112). Studies in breast cancer and myeloma patients have shown that these agents markedly inhibit the progression of bone disease, resulting in improved survival and decreased
morbidity from bone pain and fracture (113, 114). These results have led to their incorporation into standard treatment regimens for skeletal metastases associated with these cancers.

In addition to inhibiting osteoclast survival, bisphosphonates may have direct effects on tumor cells (115). For example, several bisphosphonates induce apoptosis in myeloma cells (116-118). However, this is not the case for all bisphosphonates (119). In addition to inducing apoptosis, bisphosphonates have been shown to inhibit breast carcinoma cell adhesion to bone (120). Furthermore, alendronate blocked collagen degradation and MMP release from prostate cancer cells (57, 121). Taken together, these findings suggest that bisphosphonate action is not limited to inhibition of osteoclasts.

Studies of bisphosphonates use in patients with prostate cancer skeletal metastases have generally shown a decrease in bone pain, although some studies have shown no benefit (122-124). A recent randomized study of the oral bisphosphate clodronate showed an encouraging decrease in the rate of progression to symptomatic bone metastases in men with prostate cancer (125). Consistent with this observation is the finding that zoledronic acid, a third generation bisphosphate, has demonstrated significantly increased activity in preclinical models when compared with early agents in this class. Exposure of prostate cancer cell lines to zoledronic acid results in marked inhibition of cell proliferation, suggesting that this agent may have a direct antitumor effect beyond its ability to inhibit osteoclast activity (126, 127). Zoledronic acid also has been shown to inhibit the invasion of prostate carcinoma cell lines in vitro (128). Clinical studies have demonstrated efficacy in treating humoral hypercalcemia of malignancy, leading to recent U.S. FDA approval for use in this clinical setting (129). Treatment with zoledronic acid results in a significant and sustained decrease in markers of bone metabolism.

Conclusions

Prostate cancer skeletal metastases promote osteolysis through several mechanisms that include both activation of osteoclast-mediated bone resorption and direct resorption on nonmineralized bone matrix (Fig. 3). Delineating the mechanisms that promote prostate cancer skeletal metastasis and the interactions between metastatic prostate cancer cells and bones should lead to development of therapies that will diminish or prevent these events. Our current understanding of the biology of prostate cancer skeletal metastases has led to identification of several putative targets and therapies aimed at these targets, some of which are currently in clinical trials at the time of this writing. Continued research into the biology of prostate cancer skeletal metastases should enable development of improved therapeutic regimens to diminish this painful aspect of prostate cancer.

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Interleukin-6 and androgen receptor cofactors in prostate cancer xenografts and cell lines

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A variety of growth factors may contribute to the progression of prostate cancer (CaP). Elevation of serum levels of one putative CaP growth factor, interleukin-6 (IL-6), has recently been associated with advanced prostate cancer in patients. IL-6 and its receptor have also been demonstrated in a number of established CaP cell lines and in CaP samples from patients. Furthermore, IL-6 has been demonstrated to activate the androgen receptor (AR) in the absence of androgen in CaP cell lines. Taken together, these data suggest that IL-6 may contribute to CaP progression through promotion of androgen independence. The goal of the current study was to determine the presence of IL-6 and its receptor components in CaP xenografts (XG). Additionally, we sought to determine if IL-6 influenced the levels of AR co-factors because of its ability to stimulate an androgen response in the absence of androgens. CaP XG were established from either primary tumor or metastases obtained within 2 hours of the patients’ death (i.e. rapid autopsy program). Homogenates were made from the XG and subjected to ELISA for determination of IL-6, soluble IL-6 receptor (sIL6R), and gp130 levels. ELISA values were normalized for total protein in the sample. To determine the influence of IL-6 on AR co-factor levels, several CaP cell lines (LNCaP, C4-2B and VCaP) were incubated with IL-6 (25 ng/ml) for 24 h, then total cell extract was subjected to Western analysis for determination of various AR cofactor levels. We evaluated a total of 9 XG from the following sites: prostate (n=1); dura (n=2); lymph node (n=2); sphenoid (n=1); femur (n=1); rib (n=1); and liver (n=1). IL-6 was detected in dura (n=1), liver, and both lymph node XG (range: 0.231-32.8 pg/ng total protein). sIL-6R was detected in all XG except the prostate and femur (range: 91-281 pg/ng total protein). gp130 was detected in all XG (range: 8.24-1762 pg/ng total protein). Addition of IL-6 to CaP cell lines did not significantly change total levels of the AR cofactors, SRC1, TIF2, or AIB1. These data further demonstrate the presence of IL-6 and its receptor in CaP. They also suggest that IL-6 may be expressed in only a subset of metastatic sites, suggesting that it may contribute to target organ specificity. The observation that IL-6 did not alter AR cofactor levels suggests that IL-6 alters association of AR cofactors with the AR (as opposed to increasing cofactors) or that IL-6 activates AR independent of modulating AR cofactors. We conclude that the presence of IL-6 in XG and its previously demonstrated ability to activate AR lend further evidence that it contributes to the progression of CaP.
Osteoblastic characteristics of a panel of xenografts derived from primary and metastatic prostate cancer lesions

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Defining molecules to target prostate cancer is dependent on cellular models of prostate cancer that reflect the pathophysiology of prostate cancer. Here we report the characterization of a panel of prostate cancer xenografts that are derived from primary tumor and metastatic sites (bone, dura and liver). All xenografts produced PSA in the serum of the host SCID mice. Tumors from bone, and dural metastatic lesions harvested from SCID mice stained with hematoxylin-eosin display adenocarcinoma histology. Because of the osteoblastic nature of prostate cancer, we evaluated the expression of bone morphogenetic protein 2 (BMP-2), which induces osteoblastogenesis. Western blot analysis revealed that BMP-2 was detected highest in a rib metastasis xenograft, but found to a lesser degree in the dural and primary prostate tumor xenografts. We have previously demonstrated that prostate cancer cells acquire an osteoblastic-phenotype, including expression of the osteoblast-specific transcription factor Cbfa1. Western analysis revealed that all xenografts express the osteoblast marker Cbfa1. The highest level was found in a xenograft derived from a metastatic lesion to the rib of a patient. These results indicate that there is variability in the expression of osteoblastic characteristics of xenografts derived from different metastatic lesions and primary tumors. This suggests that the xenografts could serve as a useful tool in the study of preferential metastasis to bone in prostate cancer, and subsequent bone remodeling.