Journal of Cellular Biochemistry 101:873–886 (2007)

The Role of Tumor Microenvironment in Prostate Cancer Bone Metastasis

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Abstract Prostate cancer (PCa) epithelial cells require a number of factors to facilitate their establishment and growth at a distant site of metastasis. Their ability to adapt to their microenvironment, proliferate and recruit an underlying stroma is integral to the survival and growth of the metastasis. PCa predominantly metastasizes to the bone, and bone metastases are the main cause of morbidity. The bone marrow provides a permissive environment for the formation of a metastasis. In some cases, the cells may remain dormant for some time, eventually proliferating in response to an unknown ''trigger.'' The marrow is rich in progenitor cells that differentiate into numerous cell types, producing new blood vessels, supporting fibroblasts, and an underlying extracellular matrix (ECM) that form the reactive stroma. By secreting a number of cytokines, growth factors and proteases they recruit auxiliary cells required to produce a functional stroma. These components are involved in a reciprocal interaction between the stroma and the PCa cells, allowing for the growth and survival of the tumor. Left unchecked, once a PCa tumor has established itself in the bone marrow it will eventually replace the marrow, interrupting bone homeostasis and typically promoting an osteoblastic response in the bone including osteoclastic events. The abundant deposition of new woven bone results in nerve compression, bone pain and an increase in fractures in patients with PCa bone metastases. This review will examine the tumor microenvironment, its role in facilitating tumor dissemination, growth and the resultant pathologies associated with PCa bone metastasis. J. Cell. Biochem. 101: 873–886, 2007.

Key words: prostate cancer; osteoblastic; osteolytic; bone; reactive stroma

The incidence of prostate cancer (PCa) has increased over the past decade and is now the most common non-cutaneous malignancy in men in Europe and North America. The American Cancer Society estimates that during 2006 about 234,460 new cases of PCa will be diagnosed in the US and 27,350 men will die of metastatic disease. Radical prostatectomy and radiation therapy are the current therapies for patients with early disease for curative intent. Androgen ablation therapy is the mainstay for progressive PCa. However, most men will eventually fail this therapy and die of recurrent androgen independent PCa. While chemotherapeutic strategies show some promise, there is no effective therapy for hormone refractory PCa that substantially prolongs survival.

Lymph nodes, bone, lung, and liver are the most frequent sites of distant PCa metastases. PCa metastasizes to the bone with skeletal involvement in approximately 90% of patients with advanced PCa [Bubendorf et al., 2000; Roudier et al., 2004]. Androgen deprivation therapy adversely affects bone metabolism, increasing markers of osteoblast and osteoclast activity, decreasing bone mineral density, and increasing fracture risk [Oefelein et al., 2001; Michaelson et al., 2004]. Thus, treatment-related osteoporosis may account for some skeletal complications experienced in men with advanced PCa [Daniell, 1997]. While there is an osteolytic component to the disease [Niell et al.,

Grant sponsor: Veterans Affair's merit review grant; Grant sponsor: Department of Defense Consortium grant; Grant number: DAMD170320033; Grant sponsor: PO1 National Institutes of Health grant; Grant number: PO1CA085859; Grant sponsor: Pacific Northwest Prostate Cancer SPORE grant; Grant number: P50CA097186; Grant sponsor: Richard M. Lucas Foundation; Grant sponsor: Prostate Cancer Foundation.

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Received 21 October 2006; Accepted 24 October 2006

DOI 10.1002/jcb.21214

1983], PCa bone metastases are usually osteoblastic in nature with characteristic deposition of unstructured woven bone [Shimazaki et al., 1992; Roudier et al., 2003]. Bone resorption and formation resulting in increased fractures is only one of the components of the disease. Dissemination of tumor cells to the bone can lead to replacement of bone marrow, spinal cord compression, severe bone pain, cachexia, and death [Lange and Vessella, 1998; Olson and Pienta, 1999].

TUMOR DISSEMINATION AND ESTABLISHMENT

The reasons for preferential establishment and growth of disseminated PCa cells in bone marrow are unclear. The process, however, requires the following to occur (a) vascular spread of cancer cells to the bone marrow, (b) adhesion of cancer cells to bone microvasculature and matrix components, (c) invasion of PCa cells into the bone marrow, (d) survival in the bone marrow, and (e) establishment of the tumor in the bone microenvironment and recruitment of a reactive stroma from cells in the marrow (Fig. 1).

Vascular Spread of Cancer Cells to the Bone Marrow

After PCa cells have intravasated into the vasculature, cells may travel as part of a fibrin clot, surrounded by platelets activated by protease (i.e., thrombin) activated receptor 1 (PAR1) on PCa cells [Walz and Fenton, 1994; Chay et al., 2002]. P-selectin is an adhesive molecule present on the surface of activated platelets. Platelet adherence to DU145 cells may be inhibited by anti-CD24 and P-selectin antibodies in vitro suggesting platelet adherence to PCa cells may be mediated by P-selectin/CD24 interactions [Chen et al., 2004].

The prevalence of vertebral metastasis in PCa has been attributed to the passage of tumor cells via Batson's venous plexus [Bubendorf et al.,

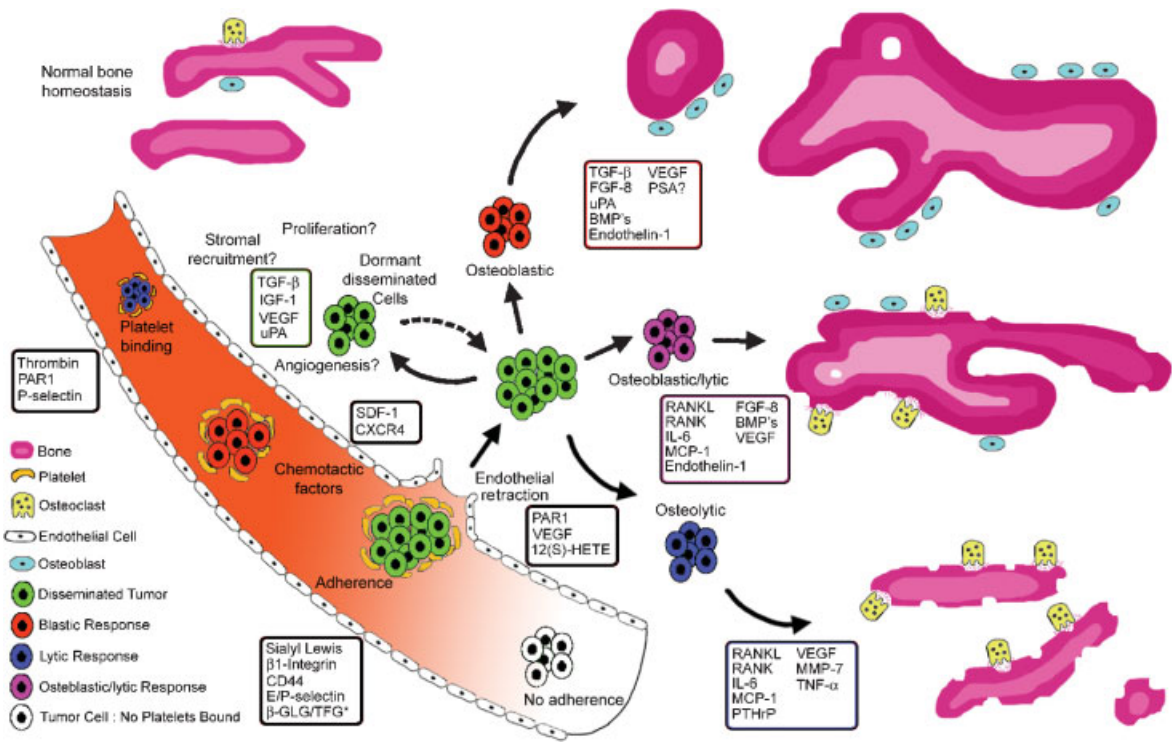

Fig. 1. Factors associated with dissemination, stromal recruitment, and bone response in PCa bone metastases once PCa cells have invaded the vasculature, they are bound by platelets and adhere to the bone marrow endothelium through platelet-tumor-endothelial interactions. The cells could be attracted by chemotactic factors and extravasate into the bone marrow. Once in the marrow the tumor cells can remain dormant or proliferate and recruit reactive stroma immediately. Depending upon the tumor phenotype and the site of bone metastasis, the cells can elicit a number of different bone reactions including: osteoblastic, osteolytic, or a mixed response. -GLG/TFG*: beta-galactoside-binding lectin galectin-3 and Thomsen–Friedenreich glycoantigen.

2000]. PCa migration into the bone marrow may be enhanced by chemo-attractants within the bone microenvironment. An example of a chemo-attractant that may participate in localizing tumors to the bone marrow in PCa is chemokine stromal-derived factor-1 (SDF-1 or CXCL12) a member of the CXC cytokines. SDF-1/CXCR4 interaction is critical for the trafficking of lymphocytes, homing and retention of hematopoietic stem cells (HSC) within the bone marrow, and more recently has been implicated in chemotaxis in cancer metastasis [Dewan et al., 2006].

The SDF-1 receptor CXCR4 is expressed at elevated levels on metastatic PCa cells, with neutralizing antibodies to SDF-1 decreasing the metastatic load and proliferation of LNCaP, C4-2B, and PC3 tumor cells in vivo [Sun et al., 2005]. In other studies, invasion of C4-2B and PC3 cells is supported by SDF-1 and inhibited by an antibody to CXCR4 in vitro [Taichman et al., 2002], and PC-3 invasion has also been blocked by the SDF-1 inhibitor T-140 in vitro [Hart et al., 2005].

Whether these chemo-attractants act solely at the local level or are influential systemically remains a subject of considerable interest and debate.

Adhesion of PCa Cells to Bone Microvasculature and Matrix Components

Metastatic cell arrest in target organ microvessels appears to be more than a consequence of mechanical trapping although this cannot be ruled out for every metastatic site. Intercellular adhesion is mediated in part by proteins like cancer-associated Thomsen-Friedenreich glycoantigen and beta-galactoside-binding lectin galectin-3 on the endothelium and integrin β1 on PCa cells [Scott et al., 2001; Khaldoyanidi et al., 2003].

PCa cells preferentially adhere to bone marrow endothelial cells (BMECs) compared with endothelium from other tissue microvessels [Scott et al., 2001]. Some of the adhesive interactions are dependent on the expression of E-selectin on BMECs and sialylated glycoconjugates (possibly a hyaluronan matrix) on PCa cells [Simpson et al., 2001; Dimitroff et al., 2004]. Cell surface expression of CD44 on PCa cells may promote the retention of a hyaluronan matrix that facilitates their initial arrest on bone marrow endothelium [Draffin et al., 2004]. Scott et al. [2001] reported that both malignant and non-malignant prostate epithelial cells bind preferentially to primary human bone marrow endothelium. However, only malignant prostate epithelia show increased invasive ability in response to bone marrow endothelium.

Invasion of PCa Cells Into the Bone Marrow

While there are limited data available specific to PCa extravasation into the bone marrow, there are data available in other systems. Sialyl Lewis(x) and/or sialyl Lewis(a) are ligands for P- and E-selectin. P- and E-selectin are involved in leukocyte binding, rolling, and adhesion to the endothelium and extravasation to sites of inflammation. Renkonen et al. [1997] reported that endothelia in primary breast lesions express more Sialyl Lewis(x) than in normal tissue and that metastatic lesions express even higher amounts of Sialyl Lewis(x) compared to primary lesions. The expression of P- and E-selectin is also greatly enhanced in tumor-bearing tissue compared with normal tissue. Their data support the hypothesis that while circulating in the blood, Sialyl Lewis(x)- and/or sialyl Lewis(a)-expressing carcinoma cells have a higher probability for extravasation at sites where the endothelium expresses E- and P-selectin.

Once attached to the endothelial cells, the PCa cells invade through the vessel wall into the marrow. Cooper et al. [2003] have demonstrated that the binding of PCa cells to endothelial cells causes endothelial retraction. Activation of PAR1 on the tumor and endothelial cell by thrombin, in concert with the fibrin clot surrounded by platelets, may increase tumor cell motility and cause endothelial retraction [Nieuw Amerongen et al., 2004]. The tumor cell-platelet-endothelial cell interaction may also allow movement of the tumor cells through the endothelial layer via the enhanced biosynthesis of 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) resulting in endothelial cell retraction [Honn et al., 1994].

Survival and Dormancy

PCa tumor cell dissemination may occur early in disease progression with tumor cells preferentially concentrating in the bone marrow, with a subset of cells surviving and evolving into clinically apparent disease. However, early dissemination of PCa cells to the

marrow may be insufficient by itself for the development of metastases [Melchior et al., 1997; Ellis et al., 2003]. This insufficiency may be due to a number of causes including immune response, genomic instability of the disseminated cells, or failure to establish a functional reactive stroma [Klein et al., 2002]. Defining the phenotype of PCa cells that have disseminated to the bone marrow associated with clinically significant disease progression is an ongoing focus of research in our laboratory and others [Melchior et al., 1997; Klein et al., 2002; Ellis et al., 2003; Kraus et al., 2003]. In some instances, the time from dissemination to evidence of metastasis can be prolonged often exceeding 10 years. This suggests that the disseminated tumor cells have entered a period of dormancy in which the cells have stopped proliferating or proliferate at a much-reduced rate. This is not a unique feature of PCa as it occurs in several other tumor types, including some of the hematological malignancies. In the discussions that follow we highlight various aspects of the interactions that occur between the PCa cells and the microenvironment. However, keep in mind that in certain situations, there is at least one additional and critical event, that is, the trigger that reactivates tumor cell dormancy. Unfortunately, the mechanism remains unknown.

Establishment of Tumor in the Bone Microenvironment and the Recruitment of a Reactive Stroma From Cells in the Bone Marrow

Bone marrow is comprised of hematopoietic cells and adherent stromal cells of nonhematopoietic origin that, together with the extracellular matrix (ECM), form the bone marrow environment. The cellular components of bone marrow include endothelial cells, macrophage, adipocytes, fibroblasts, and osteogenic precursors.

This platform provides for the generation, self-renewal and differentiation of HSC. Typically $CD45^+$, $CD34^+$, and $CD133^+$, HSC are capable of self-renewal and differentiate into all types of mature blood cells [Dawn and Bolli, 2005]. In humans, $CD34^+CD38^-$ and side population (SP) cells are enriched for HSC.

Bone marrow stromal cells (BMSC) or mesenchymal stem cells support the generation of hematopoietic lineages from HSC. BMSC are a $CD45^-$ subpopulation of non-hematopoietic pluripotent cells within the marrow that have the ability to differentiate into osteogenic, chondrogenic, adipogenic, myogenic, and other lines. BMSC also have potential to modulate growth and differentiation of blood vessel precursors to endothelial cells, secreting sufficient quantities of vascular endothelial growth factor (VEGF) to enhance survival and differentiation of endothelial cells in vitro. This suggests that they may be capable of directly orchestrating angiogenesis in vivo [Kaigler et al., 2003]. BMSC are typically Stro-1^+, $CD90^+$, $CD106^+$, $CD13^+$, and $CD45^-$ and are separated from hematopoietic cells due to their adherence to tissue culture surfaces binding preferentially to collagen 1, collagen IV, and fibronectin [Conget and Minguell, 1999]. The BMSC clearly has many of the elements required to form a tumor reactive stroma and may be recruited to become part of a PCa reactive stroma in the bone marrow.

Importance of Reactive Stroma in PCa

The growth of primary malignant and nonmalignant PCa cultures is significantly greater on bone-marrow stroma than on control stroma [Lang et al., 1998]. While reactive stroma facilitates tumor growth, the interaction between reactive stroma and epithelial cells in the tumor is bidirectional. Epithelial-stromal interactions have been well documented in the development and maintenance of the prostate gland [Chung and Cunha, 1983]. The osteomimetic response of PCa cells in bone marrow is an example of this interactivity and interdependence. Koeneman et al. [1999] have described PCa cells as osteomimetic when present in bone marrow, expressing bone-related proteins like bone sialoprotein, osteopontin, and osteocalcin. We have also observed differential expression of bone sialoprotein in PCa bone metastases when compared to soft tissue metastases in our rapid autopsy series of patients with PCa.

The importance of reactive stroma in tumor development cannot be emphasized enough. The reactive stroma can confer an androgen insensitive state to androgen sensitive PCa cells, and alter the phenotype of normal prostate epithelial cells to a tumorigenic phenotype underpinning the importance of the reactive stroma in tumorigenesis [Chung et al., 1989; Wu et al., 1994]. Understanding the differences between normal stroma and cancer-associated

stroma may allow us to exploit stroma as a target for therapy.

Stromal Components of Marrow and the Recruitment of the Reactive Stroma

Each of the components that comprise the reactive stroma, including endothelial cells, fibroblasts and the underlying ECM are recruited by the tumor. In primary PCa, Tuxhorn et al. [2002a] describe a primary tumor stromal microenvironment that is different from normal prostate stroma. Smooth muscle cells are predominant in normal prostate stroma. Reactive stroma in PCa, however, is enriched with myofibroblasts and fibroblasts and shows a significant decrease in differentiated smooth muscle cell content. Transforming growth factor beta (TGF-β) may regulate prostate fibroblast-to-myofibroblast transdifferentiation [Tuxhorn et al., 2002a; Untergasser et al., 2005]. However, there is very little published on the fibroblast-like portion of the reactive stroma in PCa bone metastasis.

Osteoblasts and osteoclasts are essential to the bone reaction that occurs in patients with PCa bone metastasis. Factors secreted by PCa cells in marrow disrupt bone homeostasis by altering the recruitment and activity of osteoclasts and osteoblasts. Osteoblasts and osteoclasts in turn, express factors that may influence tumor-stromal interactions in the marrow microenvironment [Sugihara et al., 1998].

The Osteoblastic Response

Osteoblasts are derived from osteoprogenitor cells (mesenchymal cells), and produce osteoid which is primarily composed of type 1 collagen. They mineralize the collagen matrix by depositing calcium phosphate in the form of hydroxyapatite. They are also responsible for regulating the activity of osteoclasts via soluble mediators.

PCa metastases promote the formation of woven bone that results from rapid, relatively disorganized growth in which the osteoid is laid down in a haphazard, disorganized arrangement, as in immature bone, leading to fractures in areas of excessive bone remodeling. The osteoblastic response to PCa metastasis increases bone volume due to replacement of the existing trabecular tissue with abnormal woven bone, which on occasion may be associated with eroded surfaces [Clarke et al., 1991]. As the osteoblastic response results in the production of type I collagen, procollagen (I) carboxyterminal propeptide (PICP) a metabolite of procollagen, can be used as a marker of osteoblastic activity.

Bone is constantly being remodeled, balancing osteoblastic and osteoclastic activity. This balance is disrupted by the presence of PCa cells and is tilted towards bone deposition. This disruption may be due to factors or ECM components secreted by the PCa cells, the tumor reactive stroma, the loss of marrow or a combination of all of these factors. The bone reaction may be due to over-activity of the osteoblasts, the inhibition of osteoclastic activity or a combination of both.

A number of growth factors and cytokines are implicated in the osteoblastic reaction. Insulin-like growth factor (IGF-1) has been implicated, however, Rubin et al. [2006] could find no effect of IGF-1 on the osteoblastic response in an intratibial model of PCa in vivo or in vitro.

Endothelin-1 is secreted by the prostate glandular epithelium and PCa cells. Endothelin actions are mediated via the endothelin-A and -B receptors. Nelson et al. [1995] have shown that endothelin-1 concentrations are significantly elevated in men with metastatic PCa. Endothelin-1 is also a PCa mitogen in vitro increasing alkaline phosphatase activity and mitogenesis in osteoblasts, and inhibits osteoclastic bone resorption [Takuwa et al., 1990; Chiao et al., 2000].

Atrasentan (ABT-627), a selective endothelin-A receptor antagonist, has demonstrated some clinical activity. In a randomized phase II placebo-controlled trial, Atrasentan showed a significant difference in time to disease progression in patients with hormone-refractory PCa [Carducci et al., 2003]. In another trial (M00-211), the results were disappointing as the treatment failed to achieve significance at the primary and most secondary end points. However, a reanalysis of the data using the hazard ratio test did reach significance ($P < 0.016$) and showed that men with bone metastasis on Atrasentan had a 19% delay in progression (personal communication: Joel Nelson M.D., University of Pittsburgh).

Other growth factors like fibroblast growth factor-8 (FGF-8), bone morphogenetic proteins (BMPs), and VEGF are all expressed by PCa and stimulate osteoblast activity. Proteases also secreted by tumor cells including

urokinase-type plasminogen activator (uPA) and prostate specific antigen (PSA) have also been implicated in activating an osteoblastic response.

While PCa metastases are usually osteoblastic in nature [Shimazaki et al., 1992; Roudier et al., 2003], there is an osteolytic component to the disease, displaying a mixed blastic/lytic response, and sometimes an osteolytic response only [Shimazaki et al., 1992; Roudier et al., 2004]. In a small number of bone cores from our rapid autopsy series of patients with PCa we have observed no local bone response to the PCa metastasis in situ [Roudier et al., 2003].

PCa cells implanted into the mouse intratibial model can generally be categorized as osteoblastic (LuCaP 23.1 and LAPC-9), mixed (MDA PCa2b, CWR22, LuCaP 35, C4-2B, LNCaP, and C4-2) or osteolytic (PC-3 and DU145) lesions.

The Osteolytic Response

Osteoclasts are multinucleated cells that degrade and reabsorb bone. Osteoclasts arise from hematopoietic cells of the monocyte/neutrophil lineage. Once activated, they digest bone by releasing hydrogen ions, acidifying and dissolving the mineralized bone matrix.

The osteolytic response is manifested by increases in osteolytic markers in the serum and urine of patients with advanced PCa [Revilla et al., 1998; Sugihara et al., 1998]. Increased bone resorption is also a prognostic factor for skeletal-related events in metastatic PCa [Berruti et al., 2000]. Due to the predominantly lytic effect of melanoma, breast and colon cancer bone metastases and discoveries in the field of osteoporosis, there have been major advances in understanding the osteolytic response to solid tumors in bone. However, while a number of factors have been identified, how PCa cells induce an increased osteolytic reaction is not fully understood.

The RANKL/RANK/OPG system is critical in regulating osteoclastogenesis, and therefore is involved in bone remodeling. Osteoclastogenesis is regulated by the interaction between receptor activator of NFκB ligand (RANKL) and its receptor, receptor activator NF-kappaB (RANK). RANK is expressed on bone marrow-derived osteoclast progenitors, and its activation upon binding of RANKL is required for differentiation of these progenitors into osteoclasts. Osteoprotegerin (OPG), a soluble decoy receptor for RANKL inhibits osteoclastogenesis by interfering with RANKL–RANK interactions. Recently, RANKL has been identified as a potential mediator of cancer-induced bone destruction in humans [Grimaud et al., 2003].

While the specificity of the RANKL antibodies currently available continues to be questioned, we have shown that normal prostate and primary PCa express RANKL and OPG, and the levels of RANKL and OPG are increased in PCa bone metastases versus those in primary tumors and soft-tissue metastases [Brown et al., 2001]. PCa cell lines also express RANK and RANKL [Brown et al., 2001; Zhang et al., 2001], and RANKL is reported to be instrumental in induction of osteoclastogenesis by PCa cells in vitro [Zhang et al., 2001]. Soluble RANKL released from PCa cells by matrix metalloproteinase-7 (MMP-7) may also have a role in the establishment of PCa bone metastases and osteolysis associated with PCa bone lesions [Lynch et al., 2005].

Results of preclinical studies have also shown that inhibition of RANK/RANKL signaling decreases tumor growth and/or establishment and prevented osteolysis in PCa [Zhang et al., 2001] in the bone environment. The development of AMG 162 a monoclonal antibody targeting human RANKL may also represent a novel therapy for osteolytic bone metastases [Dougall and Chaisson, 2006].

Other growth factors and cytokines that may promote osteoclast differentiation and activity include interleukin (IL)-1α, IL-1β, IL-3, IL-6, macrophage colony stimulating factor (M-CSF) tumor necrosis factor-α (TNF-α), and parathyroid hormone-related protein (PTHrP). IL-1α, IL-1β, IL-3, IL-6, M-CSF, VEGF-A, TNF-α, and PTHrP are all expressed by PCa. Monocyte chemo-attractant protein-1 (CCL2/MCP-1) is another chemokine induced by RANKL in human osteoclasts; it can also be expressed by osteoblasts, human PCa tissues and PCa cell lines in vitro, promoting PCa migration and proliferation [Lu et al., 2006].

Endothelial Cells

A solid tumor metastasis requires vasculature to survive and grow in size making endothelial cell recruitment a vital aspect of reactive stroma. PCa bone metastases initially recruit a functional reactive stroma. This tumor stroma then promotes angiogenesis and growth of PCa by inducing the proliferation and

migration of blood vessels (Fig. 2). Tumor blood vessels, like normal blood vessels are composed of endothelial cells, pericytes, smooth muscle cells, and basement membrane. All of these are considered to be abnormal in tumor blood vessels when compared to normal tissue types [Baluk et al., 2005]. Although no molecule specific to tumor endothelial cells has been identified, several show higher expression in tumor vessels, including: αvβ3, αvβ5, α5β1, CD105, VEGFR-2, CD36, and prostate specific membrane antigen (PSMA) [McDonald and Choyke, 2003].

αvβ3 and αvβ5 are mainly expressed on the endothelial cells of new vessels and have been used as new vessel markers [Friedlander et al., 1996]. CD31, CD171, and developmental endothelial locus-1 (Del-1) adhesion molecules are known to be ligands of αvβ3 and αvβ5 as well as components of the ECM [Penta et al., 1999; Voura et al., 2001; Aoka et al., 2002]. Del-1 was recently cloned and characterized as a unique protein transiently expressed by endothelial cells in the embryo as well as some tumor cells [Hidai et al., 1998; Aoka et al., 2002]. Recent studies indicate that CD171 overexpression on malignant tumor cells is associated with tumor metastasis [Thies et al., 2002].

The growth of tumor vessels exhibits many features of sprouting angiogenesis. The tumor blood supply can also expand by co-opting existing vessels, or incorporating bone marrow endothelial progenitor cells into existing blood vessels. Tumor vessels can lack a tight endothelial layer resulting in leakiness, this together with a lack of lymphatic vessels increases interstitial fluid pressure and compromises blood flow interfering with drug delivery [Hashizume et al., 2000; Jain et al., 2002; Padera et al., 2004]. These data suggest that co-targeting both tumor and its stroma as described by Hsieh et al. [2004] could be more efficacious than targeting the tumor alone.

Endothelial progenitor cells can be derived from the bone marrow. However, increasing evidence suggests that there are additional bone marrow-derived cell populations in adults (e.g., myeloid cells and mesenchymal cells), which can also give rise to endothelial cells [Urbich and Dimmeler, 2004].

Endothelial progenitor cells are present in the bone marrow and are $CD34^{+}/CD45^{-}$, express

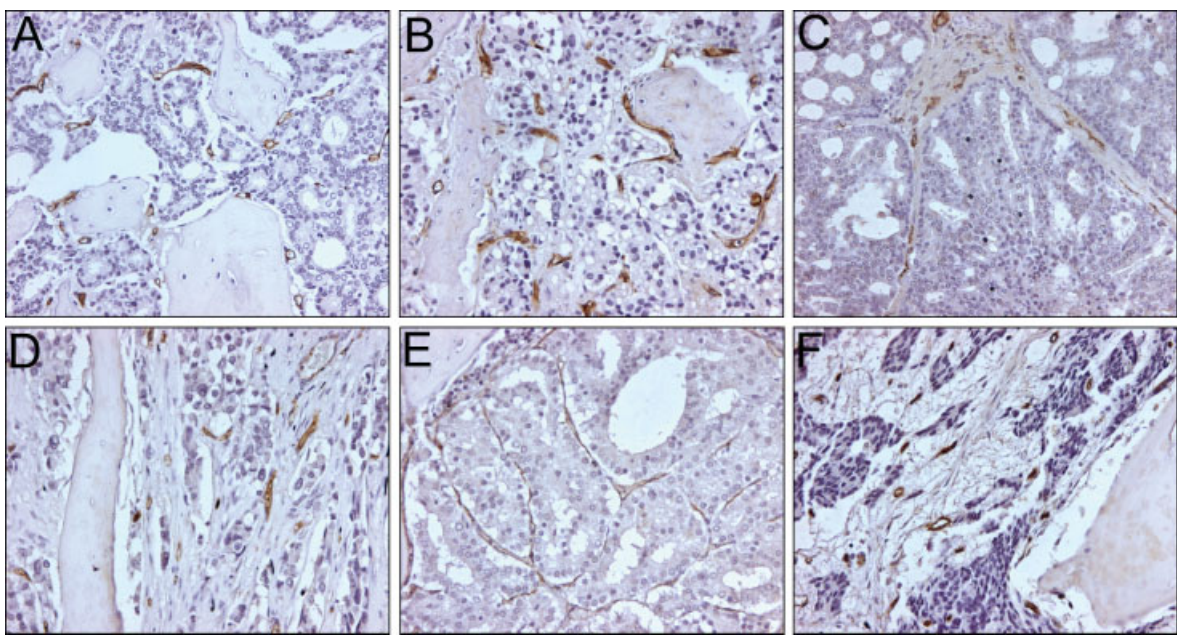

Fig. 2. Variation in the reactive stroma and vasculature of PCa bone metastases the recruitment and type of reactive stroma may differ considerably from patient to patient. PCa bone metastases were stained for CD34. The distribution of CD34 positive vascular endothelial cells (brown) varies between different tumor phenotypes (**Panels A–F**). The differences in endothelial cell distribution are related to the differences in stromal formation in the tumors. For example, small, well formation CD34 positive vessels are observed on occasion in a thick stroma supporting tumor cells (Panel D), compared to the elongated vascular endothelial cells surrounding tumor foci embedded in a very thin stroma (Panel E).

VEGFR2, and are released to regenerate vasculature in adults. CD133 is present on HSC, but absent on mature endothelial and monocytic cells. $CD133^+$/VEGFR2 cells represent a population with endothelial progenitor capacity. $CD34^+$/VEGFR2 cells also represent an endothelial progenitor cell. However, CD34 is also expressed on mature endothelial cells, so CD133 is a marker of a more immature phenotype [Gehling et al., 2000; Urbich and Dimmeler, 2004].

Interestingly, Al Khaldi et al. [2003] have shown that BMSC $CD31^-$/$CD45^-$/VEGF-$R2^-$ form CD 31^+ capillary-like networks ex vivo on Matrigel with the addition of VEGF or bFGF, and contribute to neo-vascularization of tumors in vivo. Similarly, Annabi et al. [2004] have shown that following addition of VEGF, $CD31^-$ BMSC cultured ex vivo in Matrigel undergo endothelial transdifferentiation to become $CD31^+$. The same Matrigel-embedded BMSC implanted subcutaneously in mice elicit an angiogenic response, leading to a significant increase in vessel density. These studies suggest there may be an endothelial progenitor cell involved in angiogenesis in the BMSC portion of the bone marrow.

Growth Factors, Proteases and ECM Underlying the Reactive Stroma

Tumor growth is supported by growth factors and ECM components. TGF-β is pivotal in regulating epithelial-stromal interactions in PCa, controlling the expression of growth factors and ECM components.

TGF-β expressed by PCa cells alters gene expression in the reactive stroma promoting angiogenesis [Tuxhorn et al., 2002b]. Connective tissue growth factor (CTGF) is a downstream mediator of TGF-β action in cancer-associated reactive stroma and is likely to be one of the key regulators of angiogenesis in the tumor-reactive stromal microenvironment regulating fibronectin expression in prostate stromal cells [Suzuki et al., 2006]. It also induces significant increases in microvessel density and growth in PCa xenografts [Tuxhorn et al., 2002a; Yang et al., 2005].

TGF-β may also increase platelet-derived growth factor (PDGF) expression in PCa cells [Sintich et al., 1999]. PDGF activates PDGFR and is implicated in angiogenesis. In an osseous model of PCa, Uehara et al. [2003] observed endothelial cells expressing phosphorylated PDGF-R when exposed to PDGF expressing PCa cells. Inhibiting PDGFR interactions using STI571, they observed a decrease in microvessel density in STI571 treated animals suggesting PDGF signaling can regulate angiogenesis and tumor growth in PCa. The same group went on to further substantiate these findings using an inhibitor of the epidermal growth factor receptor (EGFR) (PKI166) in combination with STI571 and paclitaxel, and showed significant suppression of PC-3MM2 cell growth in the tibiae of nude mice [Kim et al., 2004].

PDGF is activated by uPA in PCa cells [Ustach and Kim, 2005]. The generation of the serine protease plasmin from plasminogen is regulated by uPA, the uPA inhibitor plasminogen activator inhibitor-1 (PAI-1), uPAR and the plasmin inhibitor α(2)-antiplasmin. Latent TGF-β beta secreted by osteoblasts in vitro is activated by plasmin-mediated proteolysis [Yee et al., 1993]. The local concentration of TGF-β in bone may be controlled by the PCa associated plasminogen activator/plasmin system. This protease which also promotes the proliferation of PCa cells in vitro, is significantly higher in patients with a higher degree of PCa dissemination. uPA and its receptor uPAR may be predictors of progression and prognosis in patients with PCa [Kirchheimer et al., 1987; Hienert et al., 1988; Miyake et al., 1999].

Achbarou et al. [1994] inoculated inbred male Copenhagen rats with Dunning R-3227, MatLyLu rat prostate carcinoma cells overexpressing uPA. Animals developed earlier and more widespread skeletal metastases, had elevated alkaline phosphatase levels and markedly increased osteoblastic activity in bone metastases, suggesting uPA is involved in mediating an osteoblastic skeletal response. In patients with PCa we have observed higher expression of uPA and PAI-1 in bone when compared to visceral metastases.

Activated stromal cells exhibit increased production of specific ECM components and matrix remodeling enzymes, type 1 collagen, hyaluronic acid, tenascin, and matrix metalloproteinases MMP-2 and MMP-9. Elevated expression of versican in the stroma may be regulated by TGF-β [Sakko et al., 2001]. Cross et al. [2005] have shown that in prostatic stromal cell cultures TGF-β decreases ADAMTS protease expression, possibly aiding the accumulation of versican.

Bone sialoprotein is an acidic glycoprotein that is a major constituent of the noncollagenous proteins in human bone. It is overexpressed in PCa metastasis, more so in bone compared to visceral metastases [Waltregny et al., 2000]. TGF-β can regulate bone sialoprotein gene transcription [Ogata et al., 1997], and bone sialoprotein may mediate TGF-β effects on invasion and collagen degradation [Nam et al., 2006]. Bone sialoprotein may also enhance osteogenic cell migration by localizing MMP-2 on the cell surface through the αvβ3 integrin during the invasion process [Karadag and Fisher, 2006].

DISCUSSION

The propensity of PCa to metastasize and the tumor-related effects associated with PCa metastases to the bone make this aspect of PCa clinically significant. Disseminated metastatic disease progression may be broken down into stages: extravasation, dormancy/proliferation, reactive stroma recruitment, and bone remodeling. Each stage presents us with possible targets for treatment. The tumor microenvironment is necessary for the survival of disseminated PCa cells at each stage of metastasis, whether bound by platelets, interacting with endothelial cells, recruiting reactive stroma or disrupting bone homeostasis. If these interactions are necessary for survival, then they are all potential targets for therapy.

Extravasation

As discussed, platelets, specific ECM proteins, membrane bound receptors, proteases, and growth factors are required by disseminated PCa cells in the vasculature to facilitate preferential binding to and extravasation through the bone marrow vessel wall. This requirement for specific proteins like P- and E-selectins, sialylated glycoconjugates, etc for preferential binding and extravasation present us with specific targets required for extravasation of PCa disseminated cells into the bone marrow. Inhibiting these events would decrease tumor burden and skeletal related events in patients.

However, targeting these events may not be solely sufficient to prevent metastatic spread in newly diagnosed patients, as our studies suggest ~70% of patients pre-radical prostatectomy have detectable disseminated tumor cells in the bone marrow. This high frequency is above the historic recurrence rate of 10–25% in patients with presumed organ confined disease, suggesting that in some patients these early detectable disseminated tumor cells fail to adapt to the bone microenvironment, enter a prolonged phase of dormancy or are eliminated over time by immune mechanisms.

Dormancy/Proliferation

As the presence of disseminated PCa cells in the bone marrow is insufficient to predict clinically significant disease in the bone, the field has moved towards identifying technologies (e.g., telomerase activity, comparative genomic hybridization (CGH) arrays, and gene arrays) that characterize the cells [Klein et al., 2002; Pfitzenmaier et al., 2006]. The molecular phenotype might be useful in predicting clinical outcome. However, the characterization of disseminated tumor cells is complicated by the heterogeneity of the cellular population. Thus, unless one is interrogating single cells, rather than pools of cells, the most revealing molecular/genetic profile may not be readily observed. Furthermore, the molecular/genomic profile is likely to evolve over time and it is possible that profiles acquired from the earliest disseminated cells may not be as predictive as those that have somewhat evolved [Klein et al., 2002; Roudier et al., 2004].

Reactive Stroma Recruitment

Analogous to this approach is targeting the recruitment of vasculature and reactive stromal cells that facilitate disseminated cell proliferation and early tumor growth in the bone marrow. While there is heterogeneity in disseminated cells and established tumors in the marrow, the tumor cells must secrete requisite levels of cytokines, growth factors, proteases, and ECM components that facilitate the formation of a reactive stroma and angiogenesis; these factors may be predictors of disease progression and therapeutic targets.

Tumor angiogenesis clearly has been and continues to be an important target for therapy, and PCa in the bone marrow may provide unique opportunities. An unusual feature of the marrow is the plethora of endothelial and reactive stromal precursors available, making

the marrow a more favorable microenvironment for tumor growth. This coupled with a cytokine and growth factor rich bone microenvironment would suggest there could be differences in angiogenic factors produced by PCa tumor cells in the marrow when compared to visceral metastasis. These differences allied with the abnormalities associated with tumor vasculature, make the vasculature in the bone marrow another possible target for treatment.

TGF-β is an important factor that may control the stromal production of PDGF and CTGF, reactive stroma proliferation, differentiation and ECM deposition facilitating angiogenesis in PCa bone metastasis. In addition, these factors along with ECM components secreted by the tumor cells may also stimulate disorganized new bone formation and destruction at sites of metastases.

Bone Remodeling

The symptoms associated with PCa bone metastasis, that is, nerve compression, bone pain, an increase in fractures and cachexia are associated with late stage disease.

Current therapies associated with the effect of tumor on bone currently focus on palliative rather than curative intent. Bisphosphonates and future treatment modalities including inhibitors of the RANK/RANKL/OPG pathway may decrease the osteolytic reaction of the bone; however, PCa is typically much more osteoblastic in character, a characteristic not shared by other solid tumor types in the bone. Much less is known about the osteoblastic response and other than bisphosphonates and radiotherapy as palliative care there is no effective treatment for osteoblastic PCa bone metastases.

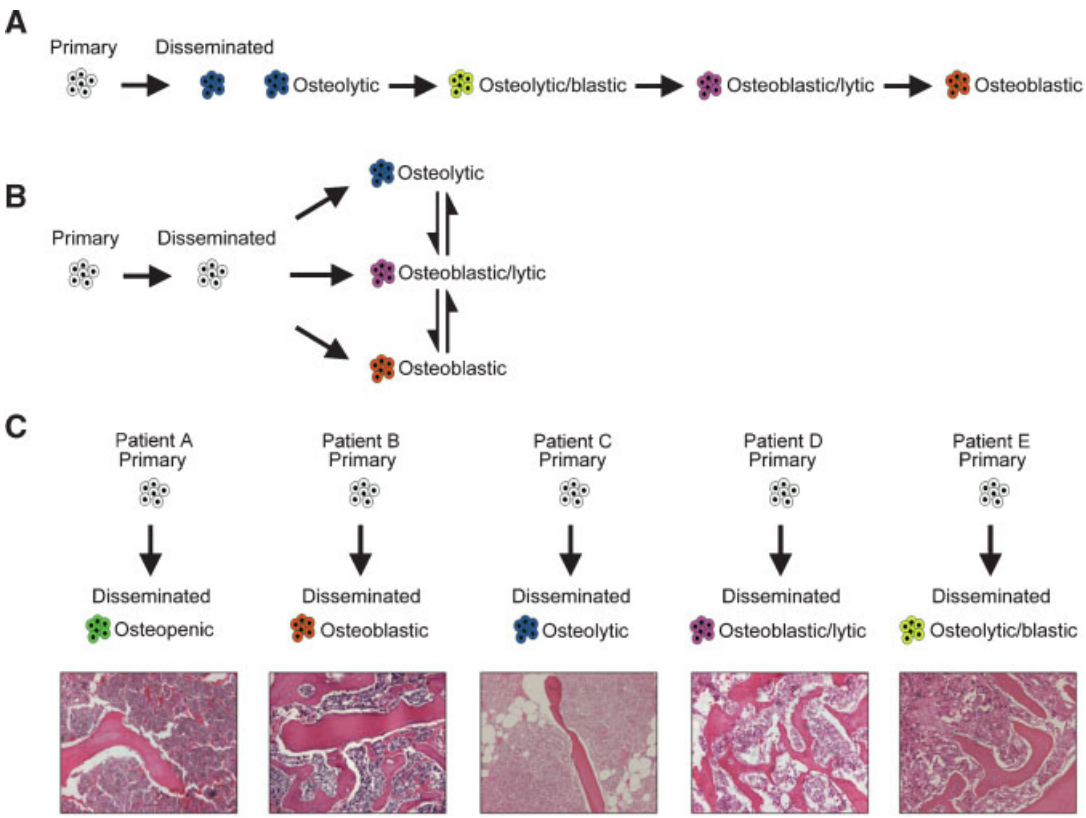

Fig. 3. Alternative hypotheses for the variable bone reaction in PCa Bone metastases. **A**: Hypothesis 1. Once disseminated to the bone marrow, PCa cells elicit an osteolytic response, releasing factors from the bone matrix, eventually resulting in an osteoblastic response. Different patterns of bone response may also reflect site-to-site variability in bone activity within a patient. **B**: Hypothesis 2. Once disseminated to the bone marrow, PCa cells may elicit an osteolytic, osteoblastic or mixed response. These responses will vary over time depending upon the interaction between osteoblasts, osteoclasts and the tumor at the site of metastasis. **C**: Hypothesis 3. Once disseminated to the bone marrow, each PCa tumor has acquired a specific phenotype. Each phenotype may elicit a specific bone reaction. The bone reaction, however, may be altered due to site-to-site variability in bone activity. Thus, we may observe different patterns of bone response depending on the balance of the tumor phenotype and localized bone activity within a patient.

While PCa bone metastases are constantly described as osteoblastic in character, in truth there is a variety of bone responses, osteolytic, osteoblastic, mixed, and in some cases no bone reaction. Despite the immense value of rapid autopsy programs in revealing the vast heterogeneity of bone metastases, these specimens only represent a snapshot in time. Because of this we can only hypothesize about the events leading to variable bone reactions in the patient.

One hypothesis is that the osteoblastic reaction follows an osteolytic response, while others hypothesize that they are concurrent (Fig. 3A,B). An alternative hypothesis is that the disseminated tumor cells which successfully seed the bone marrow have acquired a phenotype that results in a specific bone reaction governed/tempered in part by features of the bone environment (Fig. 3C).

In conclusion, the complexities in mechanisms and microenvironmental issues associated with PCa bone metastases are nearly overwhelming, encompassing tumor cell dissemination, adaptation, and proliferation. Each of these is influenced by host factors such as the reactive stroma, angiogenesis, and bone remodeling. Additional complexities arise from behaviors such as tumor cell dormancy where the malignant cell is "inactive" for prolonged periods of time following which undefined events may trigger activation. Nevertheless, the optimist looks at these scenarios as a plethora of opportunities for novel therapeutic strategies. From our perspective we share this optimism and suggest that a combination of therapies targeting the tumor cells and the microenvironment will ultimately prevail in the control of PCa bone metastases.

ACKNOWLEDGMENTS

We would like to thank Dr. Martine Roudier, Dr. Eva Corey, Dr. Lawrence True, and Dr. Paul Lange for their contributions to our studies referred to in this review.

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