Stromal Factors Involved in Prostate Carcinoma Metastasis to Bone

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BACKGROUND. Prostate carcinoma (PC) frequently metastasizes to bone, where it causes significant morbidity and mortality. Stromal elements in the primary and metastatic target organs are important mediators of tumor cell intravasation, chemotaxis, adhesion to target organ microvascular endothelium, extravasation, and growth at the metastatic site.

METHODS. The role of stromal factors in bone metastasis was determined with a cyclic DNA microarray comparison of a bone-derived cell PC cell line with a soft tissue-derived cell PC cell line and by evaluating the effects of selected stromal components on PC cell chemotaxis, cell adhesion to human bone marrow endothelium (HBME), and PC cell growth.

RESULTS. The authors demonstrate that PC cells express protease-activated receptor 1 (PAR1; thrombin receptor), and its expression is up-regulated in PC compared with normal prostate tissue. In addition, this overexpression was very pronounced in bone-derived PC cell lines (VCaP and PC-3) compared with soft tissue PC cell lines (DUCaP, DU145, and LNCaP). The authors report that bone stromal factors, including stromal cell-derived factor 1 (SDF-1) and collagen Type I peptides, are chemotactic for PC cells, and they demonstrate that some of these factors (e.g., extracellular matrix components, transforming growth factor β, bone morphophenetic proteins [BMPs], and SDF-1) significantly alter PC-HBME interaction in vitro. Finally, stromal factors, such as BMPs, can regulate the proliferation of PC cells in vitro.

CONCLUSIONS. Soluble and insoluble elements of the stroma are involved in multiple steps of PC metastasis to bone. The authors hypothesize that PAR1 may play a central role in prostate tumorigenesis. Cancer 2003;97(3 Suppl):739–47.

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Skeletal metastases occur in approximately 90% of patients with advanced prostate carcinoma (PC).¹² Typical clinical presentations include pain, spinal cord compression, and pathologic fractures.³ Pain is usually the first symptom and results from mechanical or chemical stimulation of pain receptors in the periosteum/endosteeum by the growing tumor mass. Spinal cord compression results from expanding extradural tumor growth, spinal angulation secondary to vertebral collapse, or dislocation of the vertebra after pathologic fracture. Back pain, motor weakness, sensory loss, and autonomic dysfunction all are common symptoms of spinal cord compression. Pathologic fractures occur as a result of the tumor mass weakening the bone and are associated with both osteolytic and osteoblastic bone lesions.³⁴
The clinical consequences of bone metastases are well documented; however, the molecular and cellular mechanisms involved in PC preferential metastasis to bone are not well defined. The ability of PC cells to home to the bone is the result of multiple factors, including attainment of the requisite metastatic abilities by the tumor cell; cell chemotactic response to bone factors; preferential adhesion to bone marrow endothelium; and the interaction of PC cells with the bone microenvironment, leading to growth of PC in the bone marrow (Fig. 1). Metastasis is the result of tumor cell interactions with three distinct microenvironments: those of the primary organ, the circulation, and the target organs where metastases develop. Soluble and insoluble stromal elements, as part of these microenvironments, are involved in each step of the metastatic cascade.

The Tumor Cell and Breakdown of the Primary Organ Microenvironment

In addition to the intrinsic genetic mutations that tumor cells acquire, it is well recognized that the growth of the tumor mass in the primary organ requires a complex interaction with the tumor microenvironment. The stroma provides growth factors that stimulate the tumor cells to grow. In addition, as the tumor cells grow and divide, they secrete matrix metalloproteinases (MMPs) that break down the stroma and basement membrane. At the same time, there is down-regulation of tissue inhibitors of MMP that amplify the process. We hypothesize that protease-activated receptor 1 (PAR1; thrombin receptor) plays a critical role in PC cell motility as well as the activation of the MMPs, leading to breakdown of the stromal environment and active intravasation of the tumor cells into the bone marrow (Fig. 1).

**FIGURE 1.** Proposed steps involved in prostate carcinoma metastasis to bone. During transformation of the prostate epithelium, protease-activated receptor 1 (PAR1) expression is increased (1). Tissue factor (TF) facilitates the generation of thrombin (Thr), which activates PAR1 on prostate carcinoma cells to induce their secretion of matrix metalloproteinases and to increase cell motility; ultimately promoting tumor cell intravasation (2). Prostate carcinoma cells must survive the trauma of the circulation (3) and respond to chemotactic factors (4) from target organs (i.e., prostate carcinoma cells that metastasize to bone [P_b] respond to a bone chemoattractant, whereas prostate carcinoma cells that metastasize to the dura [P_d] respond to a dura chemoattractant). The P_b cells dock on a bone endothelium specific lectin, and P_d cells may be able to do this as well (5). However, P_b cells may lock preferentially to a bone endothelium specific integrin (6) and then extravasate into the bone microenvironment through PAR1-induced endothelial retraction (7). The tumor cells replicate in response to the growth factors present in the bone marrow and communicate with osteoblasts (O_b) and osteoclasts (O_c) (8). Consequently, PAR1 expression is increased further. P_c, prostate cancer cell that homes to lymph nodes; C, non-prostatic cancer cell.
the circulation. We have demonstrated that PAR1 is overexpressed in PC tissue compared with normal tissue.9 It has been demonstrated that PAR1 is present in multiple types of tumor cells.10,11 In breast carcinoma and melanoma cells, it has been demonstrated that PAR1 activation can lead to increased MMP expression and subsequent invasion.10,11 We believe that this activation is a result of the presence of tissue factor (TF) in the microenvironment as well as thrombin, which comes into contact with tumor cell PAR1 as a result of the leaky microvasculature of tumors.12

Intravasation

The intravasation of tumor cells requires cell detachment from the expanding tumor mass, degradation of the stromal tissue and basement membrane, and increased cell motility. Examples of stromal factors that contribute to this process include factors that result in the activation of PAR1. TF, which is present in the stroma, plays a direct role in the generation of the protease thrombin from prothrombin, and it has been reported that TF contributes to the molecular events necessary for tumor cell invasion.13,14 We hypothesize that prothrombin and thrombin are present in the tumor microenvironment as a direct result of the leaky blood vessels found in tumors.12 Thrombin activates several protease-activated G protein-coupled receptors (PARs) that are expressed on the surface of tumor cells and endothelial cells.13,15 PAR activation on a cell can result in increased cell adhesion to matrix proteins, secretion of MMPs, and increased cell motility.13,14,16 To determine the role of thrombin in the progression of PC, several PC cell lines with differing metastatic phenotypes were evaluated for PAR1 expression by using microarray analysis, reverse transcriptase-polymerase chain reaction analysis, and Northern analysis.9 The data demonstrated that the LNCaP, PC-3, VCaP, and DUCaP PC cell lines overexpressed PAR1 compared with normal prostate tissue, suggesting that PAR1 may be involved in PC tumorigenesis and metastasis. It is interesting to note that PAR1 expression was increased further in the cell lines that were derived from bone metastases (the PC-3 and VCaP cell lines) compared with cell lines that were derived from soft tissue metastases (the LNCaP, DuCaP, and Du145 cell lines).9,17,18 The precise role of PAR1 expression and thrombin in PC metastasis has yet to be determined; however, these data suggest that PAR1 activation may be important in the early stages of PC metastasis.

Survival in the Circulation

The microenvironment of tumor cells in the circulation remains under appreciated and understudied. The tumor cells must evade the immune system and the mechanical stresses of blood flow.19 The microenvironment of the circulating tumor cells includes traveling through the blood as part of a fibrin clot surrounded by other tumor cells and platelets.13 In this setting, activation of PAR1 on tumor cells by thrombin allows for increased motility of the tumor cells as well as potentially up-regulating integrins, which allow the tumor cells to bind to other tumor cells and platelets to protect themselves from the shear stresses of the circulation.20

Going to a New Home: Chemotactic Factors

PC metastasis to bone may be mediated in part by chemotactic factors. The bone is constantly being remodeled and, subsequently, releases potential chemoattractants for tumor cells.21,22 Several investigations have reported that collagen Type I peptides, underscribed components of bone marrow fibroblast conditioned media, transforming growth factor β (TGF-β), insulin-like growth factor I (IGF-I), IGF-II, and osteonectin all act as bone-derived chemoattractants for PC cells in vitro (for review, see Cooper and Pienta23). In addition, it has been shown that underscribed components of bone marrow fibroblast conditioned media mediate PC-3 cell chemotaxis through the Rho/Rho-kinase pathway.24 A more recent study demonstrated that stromal cell-derived factor 1 (SDF-1) facilitated the migration of PC cells across human bone marrow endothelial (HBME) cell monolayers and their invasion into matrigel or collagen Type I, a major component of the bone matrix.25 The chemotactic response of PC cells to SDF was mediated by their expression of CXCR4 because pretreatment with a CXCR4 antibody blocked SDF-1 activity. These observations suggest that PC cells, like hematopoietic cells, may use the SDF-1/CXCR4 pathway to facilitate their movement to and around the bone microenvironment.

PC-Endothelium Interaction: Docking

Adherence of tumor cells to organ microvascular endothelial cells is a critical step in the bone metastatic cascade because it determines the site of metastasis and is necessary for tumor cell extravasation.26–28 A recent study demonstrated that the adhesion of tumor cells to their preferred endothelium initiated tumor cell proliferation within the vessel prior to extravasation.29 Studies from our laboratory demonstrated that the PC cells preferentially adhered to immortalized HBME cells compared with immortalized human umbilical vein endothelial cells (HUVECs), immortalized human aortic endothelial cells, and immortalized human dermal microvascular endothelial cells.30,31
These observations were confirmed in another investigation that demonstrated the preferential adhesion of PC-3 cells, a PC cell line derived from a bone metastasis, to a primary culture of HBME cells compared with primary cultures of HUVECs and lung microvascular endothelial cells (Hs888Lu). Although these studies suggest that preferential adhesion of circulating PC cells to bone marrow endothelium partly contributes to the metastatic pattern observed in advanced PC, it is important to consider that these adhesion studies were done in the absence of naturally occurring soluble factors. These soluble factors, which may be growth factors, cytokines, and extracellular matrix (ECM) components, may alter the expression of cell adhesion molecules (CAMs) involved; thus, their effects on PC cell-endothelial cell interaction should be determined.

Tumor cell binding to the microvascular endothelium involves two distinct steps that are described in the docking and locking hypothesis. The initial docking of the tumor cell to the endothelium is mediated by lectins. Integrins are responsible for the subsequent locking of the tumor cell to the endothelium. Both lectins and integrins have been implicated in PC adhesion to HBME cells. PC-3 cells treated with galactose-rich, modified citrus pectin (an antibody to galectin-3) and arginine-glycine-aspartic (RGD) peptides, had a reduced ability to bind HBME cell monolayers in vitro. The effects of modified citrus pectin and galectin-3 polyclonal antibody suggest the involvement of a lectin, whereas the RGD peptide effect suggests integrin involvement. Other investigations suggest that β1 integrins, hyaluronan, and a type C cell surface lectin expressed on the surface of PC-3 cells also may mediate PC-3-HBME interaction.

Previously, we demonstrated that β1 integrins expressed on HBME cells did not mediate PC-3 cell adhesion to these cells. It has been reported that the αvβ3 integrin mediates the adhesion of PC-3 and DU145 cells to cytokine-activated HUVEC monolayers. We characterized αvβ3 expression in a variety of PC cells and determined that PC-3 cells expressed the greatest amount (Table 1). To determine the role of αvβ3 in PC-3-HBME interaction, PC-3 cells were treated with LM609, a well-characterized αvβ3-blocking antibody, prior to starting HBME adhesion assays. The data demonstrated that LM609 treatment did not significantly alter the adhesion of PC-3 cells to HBME cell monolayers, suggesting that this integrin, expressed on PC-3 cells, does not mediate preferential adhesion (data not shown). Although HBME cells express αvβ3 as well (data not shown), its role in the PC-3-HBME interaction could not be tested because treatment of HBME cells with LM609 causes detachment of HBME cells from the plastic substratum. To date, CAMs involved in PC-3-HBME interaction that are specifically expressed on HBME cell surface have yet to be identified and characterized. Because it has been demonstrated that activation of PAR1 can alter CAM expression, we currently are investigating whether thrombin stimulation of PAR1 alters CAM expression.

**Table 1**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>αvβ3</th>
<th>β1</th>
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<td>MDA PCa 2a</td>
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</tr>
<tr>
<td>PC-3</td>
<td>78</td>
<td>96</td>
</tr>
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</table>

*Fluorescence analyses of αvβ3 expression on the surfaces of prostate carcinoma cell lines. The numbers represent the percentages of positive cell minus background in a population. The LM609 antibody (Chemicon) was used to detect αvβ3 expression, and 33L antibody (Chemicon) was used to detect β1 expression. The MDA PCa 2a and 2b cells were derived from bone lesions in the same patient and were kindly provided by Dr. Nora Navone (see Navone et al. [38]). The ALVA-41 cell line also was derived from a bone metastasis and was kindly provided by Dr. Rosner (see Nakhi and Rosner [39]).

**PC-Endothelium Interaction: Locking**

The expression of CAMs on endothelial cells as well as tumor cells is not static, but is dynamic and is regulated strictly by factors like growth factors, cytokines, and the composition of the ECM. Some CAMs involved in docking and locking a tumor cell to the microvascular endothelium typically are not produced by endothelial cells until stimulated by a cytokine. Therefore, the effects of these naturally occurring factors on CAM expression should be considered when trying to identify CAMs involved in tumor cell-endothelial cell interaction. This consideration is especially vital to the study of PC adhesion to HBME cells due to the plethora of growth factors and cytokines in the bone marrow.

We investigated how PC cell adhesion to HBME cell monolayers was affected by growing HBME cells on soluble ECM components extracted from kidney, bone, and placenta. The growth of HBME cells on bone, kidney, and placenta ECM proteins significantly increased their ability to bind PC-3 cells, independent of the ECM protein concentrations. It was expected that bone matrix components would enhance PC-3 cell adhesion to HBME cells selectively; however, similar results with kidney and placenta ECM compo-
ponents were demonstrated. These results suggest that the expression of CAMs involved in PC-3-HBME cell interaction also is regulated by soluble components of the bone matrix.

In another study, we determined the effects of tumor necrosis factor α (TNF-α), TGF-β, and dihydrotestosterone (DHT) on PC-3-HBME interaction. Both TNF-α and TGF-β regulate CAM expression on endothelial cells, and TGF-β also regulates CAM expression on PC-3 cells. DHT enhances the effect of TNF-α on HBME cell monolayers and may contribute to the effect of TNF-α on HBME cell monolayers. The data demonstrated that treatment of HBME cells with TGF-β prior to performing adhesion assays, significantly reduced PC-3 cell adhesion in a dose-dependent fashion. However, the treatment of PC-3 cells with TGF-β did not alter PC-3-HBME adhesion. TNF-α alone, or in combination with DHT did not demonstrate a measurable effect in these adhesion assays.

In a recent study, we determined the effect of SDF-1 on PC cell adhesion to HBME cell monolayers. C4-2B cells and PC-3 cells were pretreated with SDF-1 (at doses that ranged from 0 ng/mL to 200 ng/mL) for 30 minutes at 37 °C prior to performing adhesion assays. The data demonstrated that SDF-1 significantly increased the adhesion of both PC-3 cells and C4-2B cells to HBME cell monolayers in a concentration-dependent manner.

The effects of bone ECM components and TGF-β on HBME cell growth was not determined in the studies described above; thus, it is possible that altered HBME cell growth, mediated by both of these soluble bone factors, may alter PC-3-HBME cell interaction. To characterize the effects that bone ECM components and TGF-β have on the growth of HBME cells in our adhesion assay model system, HBME cells were grown on bone ECM components for 24 hours or were treated with TGF-β for 24 hours, and growth rates were determined. The data demonstrated that neither bone ECM components nor TGF-β altered the growth of HBME cells significantly in our adhesion assays (data not shown). These results suggest that the enhanced adhesion of PC-3 cells to HBME cell monolayers, mediated by bone ECM components, and the reduced adhesion of PC-3 cells to HBME cell monolayers, mediated by TGF-β, are due to the ability of these soluble factors to regulate CAM expression on HBME cells.

Along with TGF-β and ECM components, the bone microenvironment contains other cytokines that conceivably may modulate PC cell interaction with the bone marrow endothelium. One family of cytokines is the bone morphogenic proteins (BMPs), which are a group of proteins that belong to the extended TGF-β family. These proteins, including BMP-1–BMP-7, induce cartilage and bone formation, and it has been reported that BMPs regulate integrin expression and, subsequently, cell adhesion. Normal human prostate and neoplastic human prostate cell lines express BMPs, with BMP-4 being the most prevalent. Although it has been reported that BMP expression by PC cell lines contributes specifically to the osteoblastic nature of bone lesions mediated by PC cells, the role of BMPs in PC adhesion to bone marrow endothelium is not known. We demonstrated in a previous study that TGF-β had a significant effect on PC-3 adhesion to HBME cell monolayer. Because BMPs belong to the extended TGF-β family, it is possible that these cytokines, like TGF-β, can alter PC-3-HBME interaction as well.

The potential role of BMPs in PC-3-HBME interaction was determined by treating HBME cell monolayers with BMP-4, BMP-5, and BMP-6 or by treating PC-3 cells with the same BMPs for approximately 24 hours prior to performing the adhesion assays. The data demonstrated that treatment of HBME cell monolayers with BMP-4, BMP-5, and BMP-6 at several concentrations did not alter PC-3-HBME adhesion or the growth of HBME cells significantly (data not shown). BMP-4 treatment of PC-3 cells significantly increased their ability to bind HBME cell monolayers in a dose-dependent fashion (Fig. 2). It is interesting to note that BMP-5 and BMP-6 treatments of PC-3 cells failed to alter their interaction with HBME cell monolayers (data not shown). This study strongly suggests that distinct BMPs have varying effects on PC cell adhesion to bone endothelium and that BMP-4 spe-
specifically may increase PC cell adhesion to the bone endothelium.

**Extravasation**
The locked PC cell must break through the endothelial barrier and extravasate through to the underlying target bone microenvironment. We hypothesize that activation of PAR1, on both the tumor cell and the endothelial cell, plays an important role in this step. The adherence of the PC cell to the endothelial cell creates a mini-microenvironment at the point of adherence that includes the fibrin clot, the tumor cells, the platelets, and the endothelial cell. PAR1-activated PC cells may have increased motility, increased secretion of MMPs and vascular endothelial growth factor (VEGF), and alterations in the cell cytoskeleton that allow movement through the endothelial monolayer.\(^{13,14,16,52}\) Activation of PAR1 on endothelial cells causes endothelial cell retraction and increased expression of VEGF receptors.\(^{53,54}\) We have demonstrated that binding of PC cells to endothelial cells causes endothelial retraction (Fig. 3). Recent evidence suggests that this is mediated by apoptosis of the endothelial cells.\(^{55}\)

**Bone Stromal Elements and PC Cell Growth**
The bone microenvironment is replete with growth factors and cytokines that can regulate the proliferation of PC cells.\(^4\) Koeneman and colleagues\(^{50}\) reported that basic fibroblast growth factors (bFGF), IGF-I, IGF-II, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and TGF-\(\alpha\) are mitogens for PC cells. Some bone growth factors, such as TGF-\(\beta\), can inhibit or stimulate the growth of PC cells, depending on their phenotype.\(^4\) SDF-1 alone is not a mitogen for PC cells but may synergize the mitogenic effect of other growth factors.\(^25\)

Because advanced PC metastasizes to bone and is hormone refractory, we hypothesized that bone-associated growth factors and cytokines preferentially stimulate the growth of hormonally independent PC
cells. This hypothesis was tested by comparing the mitogenic effects of bone-associated growth factors and cytokines, including EGF, bFGF, TNF-α, IGF-I, PDGF, TGF-β1, interleukin-6 (IL-6), and IL-1, on androgen-sensitive cell lines (LNCaP, VCaP, MDA PCa 2a, PCa 2b, and DuCaP) to androgen-insensitive cell lines (PC-3 and DU145). The data demonstrated that no tested bone growth factor preferentially stimulated the growth of androgen-insensitive cells, suggesting that androgen-independent PC cells do not respond preferentially to mitogenic signals mediated by bone-associated growth factors and cytokines.

In the current investigation, we determined the effect of BMPs on PC cell growth. The growth responses of LNCaP and PC-3 cells were determined and compared. The results showed that BMP-4 preferentially inhibited LNCaP cell growth (Fig. 4A). BMP-5 and BMP-6 inhibited the growth of PC-3 and LNCaP cells in a dose-dependent fashion (Fig. 4B,C). These observations suggest that some BMPs may be growth inhibitors for PC cells, and their effect is independent of androgen receptor expression and metastatic phenotype. It is noteworthy that BMP-4, which reportedly inhibits prostate epithelial cell proliferation, preferentially may inhibit the growth of PC cells that do not metastasize to bone.

**Emerging Concepts**

PC cell metastasis is a multistep process that requires the tumor cell to interact with three distinct microenvironments; those of the primary organ, the circulation, and the target organ. The soluble and insoluble factors present in the microenvironments can both enhance and inhibit tumorigenesis at every step. We hypothesize that PAR1 plays a role in prostate tumorigenesis by increasing tumor cell motility and activating MMPs. It has been demonstrated that PC cells bind to each other and to platelets to survive in the circulation, and we have demonstrated that PC cells adhere preferentially to HBME cells independent of their respective metastatic phenotypes. For instance, the rate of lymph node-derived LNCaP cell adhesion to HBME cell monolayers was equal to or, in some instances, greater than the rate of bone-derived PC-3 cell adhesion. Based on this observation and on the docking-and-locking hypothesis, we speculate that LNCaP cells may be able to dock equally as well as PC-3 cells, but only cells with a bone-homing phenotype preferentially will lock to HBME cells. Experiments currently are being designed to test the preferential lock concept for PC cells with a bone-metastasizing phenotype (Fig. 1).

PAR1 expression is up-regulated especially in PC cells derived from bone metastases compared with PC cells derived from soft tissue metastases. Soluble factors produced by osteoblasts can alter the expression of several genes in PC cells, affecting the migration and growth of PC cells in the target organ. Thrombin activation of PAR1 may facilitate PC cell secretion of MMPs, allowing these tumor cells to liberate potential growth factors from the bone matrix and enhance PC extravasation and migration with the bone microenvironment. PAR1, therefore, may play an important role in PC tumorigenesis both in the escape of tumor cells from the primary site and in their movement into the microenvironment at the site of metastasis.

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