Molecular pathway for cancer metastasis to bone

Short title: prostate cancer – bone matrix interactions

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Summary

The molecular mechanism leading to the cancer metastasis to bone is poorly understood but yet determines prognosis and therapy. Here, we define a new molecular pathway that may account for the extraordinarily high osteotropism of prostate cancer. Using SPARC-deficient mice and recombinant SPARC, we demonstrated that SPARC selectively supports the migration of highly metastatic relative to less metastatic prostate cancer cell lines to bone. Increased migration to SPARC can be traced to activation of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on tumor cells. Such activation is induced by an autocrine VEGF/VEGFR-2 loop on the tumor cells, which also supports the growth and proliferation of the prostate cancer cells. A consequence of SPARC recognition by $\alpha_v\beta_5$ is enhanced VEGF production. Thus, prostate cancer cells expressing VEGF/VEGFR-2 will activate $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on their surface and use these integrins to migrate toward SPARC in bone. Within the bone environment, SPARC engagement of these integrins will stimulate growth of the tumor and further production of VEGF to support neoangiogenesis, thereby favoring the development of the metastatic tumor. Supporting this model, activated integrins were found to co-localize with VEGFR-2 in tissue samples of metastatic prostate tumors from patients.
INTRODUCTION

If prostate cancer is locally confined, the five-year survival rate is nearly 100%; however, if the cancer metastasizes to distant sites, the five-year survival rate is only 31%. Bone is by far the primary site (80-85%) of prostate cancer metastasis. Despite the high incidence and serious consequences of skeletal metastasis of prostate cancer, the mechanisms underlying this osteotropism are poorly understood. However, it is clear that bone-specific matrix proteins and their receptors on the surface of prostate cancer cells play a pivotal role in the process (1-3). Recent analysis of bone extracts has revealed that a key factor that mediates prostate cancer cell invasion is the protein osteonectin or SPARC (4). SPARC (secreted protein, acidic and rich in cysteine) is a component of bone matrix that modulates cellular interactions with the extracellular matrix, exerting an anti-adhesive, promigratory effect on cells (5). SPARC is expressed by certain metastatic tumors, and its expression correlates with invasive activity. Therefore, in recent publications, SPARC has also been referred to as the proinvasive protein (6). Despite the obvious importance of this particular bone matrix protein in cancer development and metastasis, the mechanisms of its recognition by prostate cancer cells remain unclear.

Cell-surface receptors on prostate tumor cells that are particularly important for bone matrix recognition and could contribute to the directed metastasis of prostate cancer cells to bone are members of the integrin family of heterodimeric adhesion receptors. In particular, the integrins that share a common $\alpha_v$ subunit, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subunit have been implicated in prostate cancer cell biology (7-11). For example, changes in $\alpha_v\beta_3$ expression correlate with the progression to androgen independence of prostate cancer (12), and with differences in the adhesive and migratory properties of different prostate cancer cell lines (13). Studies in vivo have demonstrated that tumor growth is inhibited by $\alpha_v\beta_3$ blocking antibodies (14). Central to the function of integrins is their capacity to be activated (15). Integrin activation can modulate ligand...
repertoire or can markedly enhance their apparent affinity for a particular ligand. In previous studies, we reported that the functional activity of $\alpha_v\beta_3$ on endothelial and tumor cells could be regulated by VEGF (16). VEGF has been also implicated in prostate carcinogenesis and metastasis as well as in angiogenesis. VEGF and VEGFRs are expressed by prostate carcinoma cells at a high level (17, 18), and their expression correlates with increasing grade, vascularity, and tumorigenicity (19, 20). These relationships have been observed in humans as well as in animal models of prostate cancer. High VEGF levels in prostate cancer are associated with poor prognosis (17). In addition, VEGF produced by tumor cells affects bone remodeling and might, therefore, facilitate nesting of metastatic cells in bone (21).

In the present study, we identify an interrelationship between SPARC, the $\alpha_v$ integrins and VEGF which may define the mechanism responsible for the metastasis of prostate cancer to bone: 1) SPARC attracts and subsequently anchors metastatic tumor cells within the bone; 2) Cell-surface $\alpha_v$ integrins mediate the migration of prostate tumor cells to bone and, subsequently, the retention and adaptation of the tumor cells within their new microenvironment; 3) VEGF and its receptors, regulate integrin activity to influence the recognition of the bone matrix; and 4) finally, engagement of SPARC by the integrins induces VEGF production to support bone remodeling and neovascularization to nourish the metastatic tumor.
EXPERIMENTAL PROCEDURES

Cell lines and Materials:
The LNCaP lineage-derived human prostate cancer cell line, LNCaP-C4-2, is androgen-independent, and highly tumorigenic, with a proclivity to metastasize to bone (33). All human prostate cancer cell lines LNCaP, LNCaP-C4-2, PC3 and lacZ-transfected CWR22R (H-clones), kindly provided by Dr. Lloyd A. Culp (Case Western Reserve University, Cleveland, Ohio)) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Recombinant SPARC was expressed in insect cells and purified as previously described (34). Platelet-derived purified SPARC was from Hematologic Technologies, Inc., (Essex Junction, Vermont). Fibronectin was purchased from Roche Diagnostics Corporation (Indianapolis, Indiana) and Collagen type I was obtained from Calbiochem (La Jolla, California). Antibodies against \( a_\beta_3 \) (LM609), \( a_\beta_5 \) (P1F6), \( a_5b_1 \) (JBS5) and \( a_2b_1 \) (BHA2.1) integrins were from Chemicon (Temecula, California). Anti-VEGF and anti-VEGFR-2 were from R&D Systems (Minneapolis, Minnesota). Anti-PDGF was obtained from Sigma Chemicals (Saint Louis, Missouri), WOW-1 was provided by Dr. Shattil, La Jolla, California. CRGDFv peptide was from Peninsula Laboratories Inc. (San Carlos, California)

Preparation of bone extracts. SPARC-null and control mice of the same genetic background were generated as previously described (23). The SPARC null mice do not contain detectable amounts of SPARC mRNA or protein. Bones excised from 6 week-old male wild-type and SPARC null littermates (from pelvic region and limb) (1g) were crushed and extracted for 24 h at 4°C as previously described (4). Extracts were centrifuged at 25,000 g for 30 min, and the supernatant fractions were dialyzed against PBS.

Cell migration assays. Cell migration assays on Transwell plates (8 \( \mu \)m pore size) were performed as previously described (27). Ligand (recombinant or purified SPARC or bone
extract, Fibronectin and Collagen) was diluted to a selected concentration, and 10 µl of this solution was placed on the lower surface of a polycarbonate filter and air-dried. Cell migration was performed in the presence of blocking antibodies against α3β3, α5β1 and α2β1, neutralizing antibodies to VEGF, VEGFR-2 and PDGF (20 µg/ml, each) or cRGDfK peptides (20 µM).

**Flow cytometry.** WOW-1, a Fab fragment, reacts selectively with activated αvβ3 and αvβ5 (29). Its binding to cells was assessed by flow cytometry as previously described (16). In selected experiments, cells were preincubated for 5 min in the presence of an inhibitor (anti-VEGF or anti-VEGFR-2 neutralizing antibodies or VEGFR2/Fc chimera (20 µg/ml each). WOW-1 Fab was then added at a final concentration of 30 µg/ml, followed by addition of Alexa 488-goat anti-mouse IgG (Molecular Probes, Eugene, Oregon) at 15 µg/ml. After 30 min, the cells were washed and analyzed by flow cytometry. Specific binding of WOW-1 Fab was defined as that inhibitable by 10 mM EDTA. Flow cytometry was performed using a FACSscan instrument, and the data was analyzed using the CellQuest software program (version 1.2).

**Proliferation assays.** These assays were performed as described (35). Briefly, cells were maintained in 1% serum for 20 hr prior to experiments. Trypsinized cells were distributed into 96-well microtiter plates (2x10^5 cells/well) in the presence or absence of inhibitors. The cells were labeled with 1µCi [3H]-thymidine per well. After 24-48 hr, the cells were washed, and the radioactivity was precipitated with TCA and quantified by scintillation counting.

**Soft agar assays.** LNCaP-C4-2 cells were trypsinized, washed, and resuspended in 0.4% BactoAgar (Difco, Detroit, Michigan) prepared in RPMI 1640 medium supplemented with 10% FBS. Cells were plated on the top of 0.7% agar in the presence or absence of VEGFR-2/Fc chimera or anti-VEGFR-2 blocking antibodies (10 µg/ml each). After five days, the colonies were photographed and the number of colonies per power field was quantified 7 days after plating.
**Relative Quantitative real-time PCR.** Total RNA from two prostate cancer cell lines (LNCaP and LNCaP-C4-2) was prepared with RNeasy mini kits (Qiagen, Valencia, California). Cells were plated in wells, which were left uncoated (control) or had been coated with SPARC (200 ng/well), with anti-\(\alpha_\nu\beta_5\) integrin antibodies (P1F6), or with control nonimmune IgG (10 µg/ml each). In selected experiments cRGDfv (20 µM) was added to the cells; after specific time periods of incubation at 37°C, RNA was isolated. Real-time PCR was performed using SYBR Green PCR core reagents (Perkin-Elmer Biosystems, Warrington, United Kingdom) in an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Biosystems). The forward primer was 5'-GAGGAGTCCAACATCACCATGC-3', located on exon 3; the reverse primer was 5'-CGTTTACTCAAGCTGCCTCGCC-3', located on exon 6. Relative Quantitation of VEGF mRNA expression was performed by “Comparative Ct Method” (36) using the following formula \(2^{-\Delta\Delta Ct}\), where \(\Delta\Delta Ct=[\Delta Ct \text{ VEGF (treated sample)}-\Delta Ct \text{ GAPDH (treated sample)}]-[\Delta Ct \text{ VEGF (calibrator/control sample)}-\Delta Ct \text{ GAPDH (calibrator/control sample)}}\). \(\Delta Ct\) represents the mean Ct value of each sample and GAPDH is the endogenous control used to normalize all the quantifications of VEGF gene. Alternatively, VEGF was measured by quantitative ELISA (R&D Systems).

**Immunohistochemistry.** Formalin-fixed and paraffin-embedded tissue blocks of prostate cancer and bone metastasis were cut into 6 µm sections and stained with monoclonal antibodies against SPARC (Zymed Laboratories, Inc. South San Francisco, CA), CD31 (DAKO, Carpinteria, CA), \(\alpha_\nu\beta_3\) integrin (Chemicon International, Temecula, CA), WOW-1 Fab and with antibody against VEGFR-2 (Santa Cruz Biotechnology, Santa Cruz, CA), using the antigen retrieval technique as previously described (37). Sections were counterstained with hematoxylin (Vector). A negative control was performed to ensure the specificity of peroxidase immunostaining by using nonimmune IgG. Sections were examined on an OLYMPUS
microscope, and representative areas were photographed using OLYMPUS digital camera. The quantitative data for the density of blood vessels and intensity of WOW-1 staining was obtained with the “Image-Pro Plus” program. Eight paired samples for primary prostate cancer and bone metastasis were analyzed. For each sample, five fields were quantified for the area of CD31-positive blood vessel and the density of WOW-1 staining. These two parameters were normalized to those of normal prostate tissue. The significance was calculated using the paired \( t \) test in Excel.
RESULTS

SPARC plays a critical role in prostate cancer cell migration to bone In a previous study, SPARC was isolated as a protein for bone extracts that supported the preferential migration of prostate cancer cells. The development of SPARC-null mice (22, 23) provides a means to critically address the role of SPARC in prostate tumor cell migration. Bone matrix proteins were extracted from wild-type (WT) and SPARC-null animals and were assessed for their ability to promote migration of prostate cancer cells that are known to metastasize to bone. In a transwell system, migration of PC3 cells was directly proportional to the amount of bone extract and reached a maximum at 200 ng per well (Fig.1A). Maximal migration of PC3 cells towards wild-type bone extract was about 4-fold higher than their migration toward SPARC-null bone extract. The deficient migration of PC3 cells toward the SPARC-deficient bone extracts was increased by 2.1 fold in the presence of recombinant SPARC at the concentration of 100 µM and completely restored by addition of 300 µM of SPARC (~10 fold increase) (Fig.1B). Addition of the most prominent cell matrix protein, fibronectin, also improved PC3 cell migration toward SPARC-null bone extract, however, the effect of fibronectin was relatively modest (~2.5 fold in the presence of 300 µM) compared to SPARC at the same concentration (Fig.1B). No significant improvement of PC3 cell migration was observed in the presence of collagen at the same concentrations (Fig.1B). Similar results were obtained when SPARC purified from platelets was added (data not shown).

Metastatic prostate cancer cells exhibit increased migration to SPARC We next compared the migration of LNCaP cells to that of its highly metastatic variant, LNCaP-C4-2; the maximal migration of LNCaP-C4-2 cells was 1.85-fold higher than that of LNCaP cells (Fig. 2A). This data indicates a correlation between the metastatic potential of prostate cancer cells and their migration to SPARC. We obtained similar results with other representative metastatic prostate
cancer cell lines: PC3 and CWR22Rv1-H (not shown). Using wild-type and SPARC-null bone extracts, we demonstrated that SPARC is a crucial factor for migration of highly metastatic LNCaP-C4-2 cells. In these experiments, migration towards wild-type bone extract was optimal at 100ng of bone extract per well and was 2.87-fold higher than migration towards SPARC-null bone extract (Fig.1C).

Prostate cancer cell migration to SPARC and bone matrix proteins is mediated by integrins and controlled by VEGF. Since $\alpha_v\beta_5$ and $\alpha_v\beta_3$ have been implicated in the recognition of a number of bone-specific matrix proteins (24), we assessed the effects of specific anti-integrin blocking antibodies on prostate cancer cell migration to SPARC (Fig. 2). Blockade of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ resulted in 35% and 70% inhibition of LNCaP-C4-2 cell migration to SPARC, respectively, indicating a role of both integrins in the response (Fig. 2B). In contrast, blockade of either $\alpha_5\beta_1$ or $\alpha_2\beta_1$ integrin had no effect on the migration of LNCaP-C4-2 cell to SPARC (Fig. 2B). Similar results were obtained with PC3 cells (Data not shown). Significant inhibition with cyclic RGDfv peptide (70-80%), which is a relatively specific antagonist of $\alpha_v\beta_5$, substantiates a primary role of this integrin in migration to SPARC. Direct interaction of LNCaP and PC3 prostate cancer cells with SPARC and the cooperative roles of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ were also demonstrable in adhesion assays (not shown). To our knowledge this is the first demonstration that recognition of SPARC is mediated by these integrins.

In previous studies, we and others demonstrated that the adhesive and migratory phenotypes of certain tumor cells are sustained by an autocrine loop initiated by VEGF (16, 25). Prostate cancer cells express high levels of VEGF (18), and VEGF production correlates with the metastatic potential of prostate cancer cells (26). The highly metastatic variant LNCaP-C4-2 produces higher amounts of VEGF compared to its nonmetastatic counterpart LNCaP (73.8 ±8 pg/ml vs 19.5±2.5 pg/ml after 72 hours, respectively). Since prostate cancer cells not only
produce VEGF but also express VEGF receptors (VEGFR-1 and VEGFR-2 by FACS), we postulated that the migration to SPARC could be controlled by a VEGF-dependent autocrine loop. We, therefore, assessed the effects of VEGF and VEGFR antagonists on the migration of prostate cancer cells to SPARC. As shown in Fig. 2C for LNCaP and LNCaP-C4-2, anti-VEGF and anti-VEGFR-2 neutralizing antibodies antagonized the migration of prostate cancer cells to SPARC to a level similar to that obtained with anti-integrin blocking antibodies and the cRGDfv peptide (~80% inhibition). In contrast, anti-PDGF (platelet-derived growth factor) neutralizing antibodies inhibited the migration rate by ~35%. These results suggest that αvβ5 and αvβ3, together with VEGF/VEGFR-2, play a critical role in prostate cancer cell migration to SPARC. Similar results were obtained when bone extracts were substituted for SPARC (Fig. 3A, 3B).

In the absence of SPARC, migration was significantly lower, but was still dependent on αvβ5 and αvβ3 integrins and on VEGF. We attribute this residual activity to the presence of other matrix proteins (e.g. bone sialoprotein and osteopontin), which are known to interact with αvβ5 and αvβ3 (27, 28).

VEGF supports prostate tumor growth by autocrine stimulation of VEGFR-2  As shown in Fig. 4A, proliferation of LNCaP-C4-2 cells was inhibited by a blockade of VEGFR-2 by >70%. This data indicates that a VEGF-dependent autocrine loop, initiated by engagement of VEGFR-2, influences LNCaP-C4-2 growth. Since the anchorage-independent tumor growth is one of the defining characteristics of neoplastic transformation, we determined how the blockade of VEGF affects the growth of these prostate tumor cells in soft agar. When LNCaP-C4-2 cells were grown in the presence of anti-VEGF neutralizing antibodies and VEGFR2/Fc, the number of colonies was significantly decreased (Fig. 4C). Of note, the size of colonies was also altered: in the presence of VEGF inhibitors, tumor colonies were significantly smaller (Fig. 4B).
VEGF activates integrins on metastatic prostate cancer cells by an autocrine loop in vitro and in vivo To assess the molecular basis for increased recognition of SPARC by integrins on metastatic prostate cancer cells, we analyzed the activation state of their αv integrins using WOW-1 Fab (29), which reacts selectively with activated αvβ3 and αvβ5 (29). As monitored by FACS (Fig. 5A), WOW-1 binding is 4.2 times higher to LNCaP-C4-2 cells than to LNCaP cells, despite the expression of similar levels of αvβ3 and αvβ5 by both cell lines (specific fluorescence intensity values were 15±3 and 13±5 for αvβ3 and 18±4 and 20±6 for αvβ5). This data indicates that the functional activity of αvβ3 and αvβ5 integrins is higher on the metastatic variant of prostate cells. In addition, figure 5A demonstrates that integrin activation can be inhibited by neutralization of VEGF, since anti-VEGFR-2 antibodies inhibited WOW-1 binding by >60%. We also analyzed two other metastatic prostate cancer cell lines, PC3 and CWR22Rv1. Integrin activation monitored by WOW-1 binding was inhibited by >50% when VEGF or VEGFR-2 were neutralized.

αvβ3 and αvβ5 are present in an activated state on prostate tumor cells in vivo (Fig. 5B). Tumor areas were stained with WOW-1. The pattern of WOW-1 staining was similar but more restricted than that of LM609, the first demonstration of αvβ3 activation in vivo. VEGFR-2, which is critical for induction of the VEGF-dependent autocrine loop, co-localized with activated αvβ3 and αvβ5, supporting the concept that engagement of VEGFR-2 by VEGF induces integrin activation. Interestingly, in the sites of bone metastasis, but not in the primary prostate adenocarcinoma, SPARC expression within or around invading tumor follows WOW-1 expression, indicating colocalization and possible interaction (Fig. 6 A, C).

SPARC increase VEGF levels via αvβ5 integrin ligation in prostate cancer cells: Since VEGF affects SPARC engagement by integrins, we considered whether SPARC engagement might alter VEGF production and hence the autocrine loop. Accordingly, we assessed VEGF
production in LNCaP and LNCaP-C4-2 cells plated on SPARC. After 3 or 4 hrs, VEGF mRNA level in the LNCaP-C4-2 cells was increased 1.35- or 1.75-fold, respectively, and reached a maximum value (2.8-fold) after 6 hrs (Fig. 5C). The increase in VEGF mRNA after 6 hrs detected in metastatic LNCaP-C4-2 cells was 1.65 fold greater than in non-metastatic LNCaP cells and in all these cases the Ct value of GAPDH was ~ 16. Quantitative ELISA demonstrated that VEGF expression was also altered at the protein level and that blockade of $\alpha_v\beta_5$ with cRGDfv peptide suppressed VEGF production in LNCaP-C4-2 and LNCaP cells by 3.57±0.16 and 2.1±0.1 fold, respectively. Thus, ligation of $\alpha_v\beta_5$ by SPARC amplifies VEGF production and provides metastatic prostate cancer cells with a significant growth advantage in the bone matrix.

In similar experiments, using M21 melanoma cells that do not metastasize to bone, but which express comparable amount of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to LNCaP-C4-2 cells, we did not observe any increase in VEGF production at 3, 4, 6, or 12 hours after plating onto SPARC. In fact, VEGF expression decreased by 40% compared to control (data not shown). We can tentatively conclude that the increased production of VEGF by SPARC via $\alpha_v\beta_5$ is a characteristic displayed by prostate cancer cells tested.

**Activation state of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins is increased in bone metastasis.** The intensity of WOW-1 staining in primary tumor located within the prostate and tumor that metastasized to bone (matched pairs of tissue samples obtained from the same patient) were compared. An example of such paired specimens is shown in Fig. 6B and D. Quantitative aspects of WOW-1 staining in prostate in comparison to bone metastasis are shown in Fig. 6E. This analysis reveals that the intensity of WOW-1 staining was 4-fold higher at the sites of bone metastasis. As anticipated, vascular density was significantly (19-fold) higher at these sites compared to the tumor within the prostate itself (Fig. 6F). Our results indicate that $\alpha_v\beta_3$ is activated in prostate
tumors \textit{in vivo}. Moreover, the extent of activation is significantly increased in the sites of bone metastasis compared to the localized tumor.
DISCUSSION

Previous studies have implicated SPARC, integrins and VEGF as major players in the pathogenesis of prostate cancer. Our study develops a cohesive model, which may explain the interrelationship between these molecules and may account for the high osteotropism of prostate cancer metastasis. The major elements of this model are shown in Fig. 7 and are supported by the following findings: 1) SPARC is a key protein that attracts prostate cancer cells to bone. SPARC-deficient bone extracts support minimal migration of prostate cancer cells and addition of purified SPARC restores the rate of cell migration. 2) Recognition of SPARC correlates with the metastatic potential of prostate cancer cell lines: high metastatic variants exhibit increased migration to SPARC compare to nonmetastatic ones; 3) SPARC recognition is mediated by $\alpha_\nu\beta_3$ and $\alpha_\nu\beta_5$ integrins and is controlled by an autocrine loop in which VEGF engages VEGFR-2; 4) VEGF supports prostate cancer cell proliferation and anchorage-independent growth; 5) Increased migration of metastatic cells to SPARC can be traced to activation of $\alpha_\nu\beta_3$ and $\alpha_\nu\beta_5$ as monitored by WOW-1; activation of these integrins is substantially higher on metastatic vs. nonmetastic prostate cancer cells. Moreover, activated integrins are expressed by prostate tumors in vivo and colocalize with VEGFR-2. 6) A particularly relevant pathophysiological consequence of SPARC recognition by $\alpha_\nu\beta_5$ is the upregulation of VEGF production, which provides prostate cancer cells with significant growth advantage in bone tissue. 7) At sites of metastasis characterized by abundant SPARC, high VEGF production and enhanced neovascularization, $\alpha_\nu\beta_3$ and $\alpha_\nu\beta_5$ integrin activation is substantially higher in comparison to the tumor localized in the prostate.

Metastatic prostate cancer cells exhibit low migration to SPARC-deficient bone extracts and addition of recombinant SPARC completely rescues this response in a concentration-dependent manner. Although fibronectin was ~5 fold less potent compared to SPARC, it also
provided certain increase of prostate cancer cell migration which is due to the high level of expression of $\alpha_5\beta_1$ (fibronectin receptor) on these cells. Our results strongly support the findings of Jacob et al (4) who reported that extracts from bones but not from other tissues promoted a 4-fold increase in the invasive ability of human prostate carcinoma cells, with SPARC but not any other matrix protein as the factor responsible for this activity. In our studies purified SPARC supported the enhanced migration of the metastatic prostate cancer cells LNCaP-C4-2 and PC3, compared to the non-metastatic variant LNCaP. Thus, migration to SPARC positively correlates with a characterized metastatic potential of cell lines. We demonstrated that the cell adhesion receptor responsible for migration to SPARC is integrin $\alpha_V\beta_5$, and to a lesser extent, $\alpha_V\beta_3$ but not $\alpha_5\beta_1$ and $\alpha_2\beta_1$. The differences in the migratory activity of metastatic vs. nonmetastatic cell lines can be explained by the differences in the activation state of bone matrix receptors, $\alpha_V\beta_5$ and $\alpha_V\beta_3$, as demonstrated by WOW-1 binding. Activated integrins are known to be responsible for increased cell migration and adhesion on a variety of cell types, including tumor cells. Thus, our results provide an explanation for the previously reported increased usage of $\alpha_V\beta_3$ by the metastatic subline LNCaP-C4-2 vs. LNCaP cells in the migration to laminin (30). Integrin activation state and the recognition of SPARC is controlled by a VEGF-dependent autocrine loop, since the neutralization of VEGF or the inhibition of its receptor VEGFR-2 decreases WOW-1 binding and cell migration. Furthermore, activated $\alpha_V\beta_3$ colocalizes with VEGFR-2 on tumor cells in vivo. The existence of growth factor-dependent autocrine loop, which in addition to the effect on the integrin functions, also supports tumor growth, is the most recently uncovered characteristic of malignant phenotype (16, 26).

Due to the increased VEGF production and autocrine stimulation, integrin activation is significantly higher on the metastatic prostate cancer cells compared to the nonmetastatic cells in vitro, and at sites of bone metastasis compared to the original prostate tumor, in vivo. Thus,
at least two lines of evidence demonstrate that the integrin activation state controlled by VEGF contributes to the metastatic behavior of prostate cancer. These findings link together previously published observations that increased integrin function (31) as well as increased VEGF plasma levels in patients (32) are hallmarks of metastasis development.

We found that SPARC not only attracts prostate cancer cells to bone but also, via the ligation of integrin $\alpha_v\beta_5$, further increases VEGF production by metastatic cancer cells and provides an additional enhancement of the VEGF autocrine loop. In turn, VEGF facilitates the accommodation of tumor cells in bone environment by stimulation of the growth of the tumor itself, by further increase of integrin activation on the tumor, and by the induction of neovessel formation. Thus, high levels of SPARC at sites of bone metastasis lead to increase in VEGF and in integrin activation. Our results not only suggest a model to explain the osteotropism of prostate cancer metastasis but also identify potential targets to prevent the process. For example, SPARC, activated integrins, VEGF or VEGFR-2 would be a candidate targets to interfere with metastatic process. Since prevention of bone metastasis is paramount to effective treatment of prostate cancer, these targets are particularly worthwhile pursuing.
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FIGURE LEGENDS

**Figure 1.** Migration of Prostate Cancer Cells towards Bone extracts from SPARC (+/+ ) and SPARC (-/- ) mice. **A** and **C.** Migration of PC3 and LNCaP-C4-2 cells respectively to bone extracts (50, 100, 200 ng/well) of normal and SPARC (-/-) mice. Migration was quantified by performing microscopic counts of 10-12 random fields at 200x power. **B.** SPARC restores the migration of PC3 cells towards SPARC (-/-) bone extracts. Cells were placed in transwell plates coated with bone extract (200 ng/well) and with or without rSPARC, Fibronectin or Collagen (200 and 300 µM). After 4 hrs of incubation, cell migration was quantified. The data shown are means ± SD of three separate experiments.

**Figure 2.** **A.** Migration of LNCaP & LNCaP-C4-2 cells towards rSPARC at different concentrations (50, 100, 200 ng/well). Integrins and VEGF regulate cell migration towards SPARC. **B.** Cell migration to SPARC after treatment with or without anti integrins αvβ3, anti-αvβ5, anti-α5β1, anti-α2β1 mAb (monoclonal antibody) and cRGDfv peptide. **C.** Effect of neutralizing antibodies to VEGF, VEGFR-2 and PDGF on migration to SPARC.

**Figure 3.** **A.** LNCaP-C4-2 cells were preincubated with or without anti-αvβ3, anti-αvβ5 mAb as well as anti-VEGF/VEGFR-2 and placed in bone extracts from SPARC (+/+ ) & SPARC (-/- ) mice. **B.** Migration of PC3 cells respectively to bone extracts from SPARC (+/+ ) & SPARC (-/- ) mice after treatment with or without anti-αvβ3, anti-αvβ5 mAb as well as anti-VEGF/VEGFR-2 inhibitors. Cell migration was quantitated by performing microscopic counts of 10-20 random fields at 200x power. The data shown are means ± SD of three separate experiments.
Figure 4. A. Proliferative response of LNCaP-C4-2 cells was measured by [3H]-thymidine incorporation in the presence of control or blocking anti-VEGFR-2 antibodies (10 µg/ml each). The data shown are means ± SD of quadruplicates in one experiment and are representative of three separate experiments. B and C. Growth of LNCaP-C4-2 in soft agar. Cells were plated on top of the agarose base in the presence or absence of VEGFR-2/Fc chimera or anti-VEGFR-2 blocking antibodies. After five days, the colonies were photographed (B) and the number of colonies per high power field was quantified (C). The data shown are means ± SD of quadruplicates in one experiment and are representative of three separate experiments.

Figure 5. A. $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin activation states on LNCaP and LNCaP-C4-2 cells were detected by WOW-1 binding. Cells were preincubated in the absence or presence of anti-VEGFR-2 blocking abs. WOW-1 Fab was then added and followed by addition of Alexa 488-goat anti-mouse IgG. After 30 min, the cells were washed and analyzed by flow cytometry. B. Adjacent tissue sections of metastatic prostate cancer were stained with control nonimmune abs (control), LM609 ($\alpha_v\beta_3$), WOW-1 and anti-VEGFR-2, followed by secondary abs (ABC kit from Vector). Scale bar equals 50 µM. C. SPARC induces VEGF in prostate cancer cells. VEGF mRNA was quantified relative to control (assigned a value of 1). The data shown are means ± SD of triplicates in one experiment and are representative of three separate experiments.

Figure 6. Paired tissue sections of prostate tumor (A and B) and bone metastasis (C, D) were stained with abs against SPARC (A, C) and WOW-1 (B, D), followed by secondary abs (ABC kit from Vector). E. The mean intensity of WOW-1 expression was quantified using “ImagePro Plus” software for four representative fields for each patient. The density of WOW-1 staining in normal prostate tissue was negligible and it was subtracted from the values for primary tumor
and bone metastasis. Bars represent means ± SE for four patient samples in each primary tumor and bone metastasis. F. Blood vessels were visualized by staining for CD31, an endothelial cell marker. The area of the CD31-positive blood vessels was measured using “Image-Pro Plus” software and was expressed as a percentage of the total area. Four representative fields were quantified for each patient. Bars represent means ± SE for 4 patient samples for both primary tumor and bone metastasis. Scale bar equals 50 µM.

Figure 7. Mechanism of SPARC recognition by prostate cancer cells. The expression of VEGF and VEGFR2 on prostate tumor cells creates an autocrine loop that stimulates prostate cancer cell growth and proliferation and activates integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. These activated integrins, in turn, mediate enhanced adhesion and migration to SPARC and bone matrix. Engagement of the integrins by SPARC, in turn, enhances VEGF production.
Figure 2

A

SPARC, ng
50  100  200

LNCaP  LNCaP-C4-2

cell migration, number per field

B

control anti-α_3β_3 anti-α_5β_3 cRGDFv anti-α_5β_1 anti-α_2β_1
100% 57% 38% 19% 96% 100%

LNCaP  LNCaP-C4-2

cell migration, number per field

C

control anti-VEGF anti-VEGFR-2 anti-PDGF
100% 37% 19% 65%

LNCaP  LNCaP-C4-2

cell migration, number per field
Figure 3

Panel A: Comparison of cell migration in control and in the presence of various inhibitors (anti-\(\alpha_v\beta_3\), anti-\(\alpha_v\beta_5\), anti-VEGFR-2, anti-VEGF) for SPARC (+/+). The bars represent the number of cells migrated per field.

Panel B: Similar comparison for SPARC (-/-).

The x-axis represents the cell migration, measured as the number of cells per field, ranging from 0 to 30 for Panel A and from 0 to 200 for Panel B.
Figure 4

A

Thymidine incorporation, 10^3 cpm

no inhibitors

anti-VEGFR2

B

control

VEGFR2/Fc

C

number of colonies per field

no inhibitors

VEGFR2/Fc

anti-VEGFR2

p=0.03

p=0.07
Figure 5

A

B

control

WOW-1

αvβ3

VEGFR-2

C

fold change compared to control

SPARC

αvβ3

3 4 6 12 hrs

cRGDf

3 4 hrs

Control

LNCaP

LNCaP-C4-2

no inh anti-VEGFR-2
Figure 7

Bone matrix SPARC
adhesion migration

VEGF

VEGFR-2

Prostate Cancer Cell

integrin activation
proliferation

VEGF expression
Molecular pathway for cancer metastasis to bone
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